



AU9335889

---

**(12) PATENT ABRIDGMENT (11) Document No. AU-B-35889/93**  
**(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 664075**

---

- (54) Title  
**INSECTICIDALLY EFFECTIVE PEPTIDES**
- International Patent Classification(s)  
(51)<sup>s</sup> **C12N 015/12 A01N 037/18 A01N 063/02 C07K 007/10**  
**C12Q 001/68**
- (21) Application No. : **35889/93** (22) Application Date : **19.01.93**
- (87) PCT Publication Number : **WO93/15192**
- (30) Priority Data
- (31) Number (32) Date (33) Country  
**826925 24.01.92 US UNITED STATES OF AMERICA**
- (43) Publication Date : **01.09.93**
- (44) Publication Date of Accepted Application : **02.11.95**
- (71) Applicant(s)  
**FMC CORPORATION; NPS PHARMACEUTICALS, INC.**
- (72) Inventor(s)  
**KAREN JOANNE KRAPCHO; JOHN RANDOLPH HUNTER JACKSON; JANICE HELEN JOHNSON; ERIC GEORGE DELMAR**
- (74) Attorney or Agent  
**WATERMARK PATENT & TRADEMARK ATTORNEYS , Locked Bag 5, HAWTHORN VIC 3122**
- (56) Prior Art Documents  
**AU 15844/92 A61K 37/02 C07K 7/00**  
**AU 47301/89 C07K 7/10 15/08**

- (57) It is understood that minor modifications of primary amino acid sequence may result in proteins which have substantially, <sup>the same, i.e.</sup> equivalent or enhanced activity as compared to the peptides exemplified herein. These modifications may be deliberate, as through site-directed mutagenesis and amino acid substitution during solid phase synthesis, or may be accidental such as through mutations in hosts which produce the peptide of the invention. All of these modifications are included so long as insecticidal activity is retained. A "mutation" in a protein alters its primary structure (relative to the commonly occurring or specifically described protein) due to changes in the nucleotide sequence of the DNA which encodes it. These mutations specifically include allelic variants. Mutational changes in the primary structure of a protein result from deletions, additions, or substitutions. A "deletion" is defined as a polypeptide in which one or more internal amino acid residues are absent. An "addition" is defined as a polypeptide which has one or more additional internal amino acid residues as compared to the wild type. A "substitution" results from the

This invention relates to insecticidally effective peptides. More particularly, the invention relates, *inter alia*, to a family of insecticidally effective peptides which may be isolated from *Tegenaria* spider venom, DNA encoding such insecticidally effective peptides, and methods for controlling invertebrate pests.

1. An insecticidally effective peptide derived from a spider of the genus Tegenaria and having substantially the same amino acid sequence as defined in SEQ ID NO:3 as follows:

and agriculturally or horticulturally acceptable salts thereof.

27. The peptide according to any one of claims 1 to 6 further comprising the signal peptide having the amino acid sequence in SEQ ID NO:6 as follows:

Met Lys Leu Gln Leu Met Ile Cys Leu Val Leu Leu Pro Cys Phe Phe Cys  
-15 -10 -5 1



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>C12N 15/00, 15/12, 15/63</b> <b>A01H 1/00, 5/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 93/15192</b> <b>(43) International Publication Date:</b> 5 August 1993 (05.08.93)
<b>(21) International Application Number:</b> PCT/US93/00558 <b>(22) International Filing Date:</b> 19 January 1993 (19.01.93) <b>(30) Priority data:</b> 826,925 24 January 1992 (24.01.92) US <b>(71) Applicants:</b> FMC CORPORATION [US/US]; 1735 Market Street, Philadelphia, PA 19103 (US). NPS PHARMACEUTICALS, INC. [US/US]; 420 Chipeta Way, Suite 240, Salt Lake City, UT 84108 (US). <b>(72) Inventors:</b> KRAPCHO, Karen, Joanne ; 3840 San Rafael Avenue, Salt Lake City, UT 84109 (US). JACKSON, John, Randolph, Hunter ; 3661 Gilroy Road, Salt Lake City, UT 84109 (US). JOHNSON, Janice, Helen ; DELMAR, Eric, George ; 2967 East St. Mary's Circle, Salt Lake City, UT 84108 (US).		<b>(74) Agents:</b> FELLOWS, Charles, C. et al.; FMC Corporation, 1735 Market Street, Philadelphia, PA 19103 (US). <b>(81) Designated States:</b> AU, BB, BG, BR, CA, CZ, FI, HU, JP, KP, KR, LK, MG, MN, MW, NO, PL, RO, RU, SD, SK, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>  <b>664075</b>
<b>(54) Title:</b> INSECTICIDALLY EFFECTIVE PEPTIDES  <b>(57) Abstract</b>  This invention provides a family of insecticidally effective peptides which may be isolated from <i>Tegenaria</i> spider venom, DNA encoding such insecticidally effective peptides, and methods for controlling invertebrate pests.		

## INSECTICIDALLY EFFECTIVE PEPTIDES

This invention relates to insecticidally effective peptides. More particularly, the invention relates, *inter alia*, to a family of insecticidally effective peptides which may be isolated from *Tegenaria* spider venom,  
5 DNA encoding such insecticidally effective peptides, and methods for controlling invertebrate pests.

In recent years, the public has become acutely aware of the environmental hazards and mammalian toxicity associated with the use of  
10 synthetic insecticides. As a result, the use of these insecticides has been rapidly declining. However, the need for effective insect control has not changed. This has prompted researchers to develop novel methods of insect control.

The most widely used microbial pesticides are derived from the  
15 bacterium *Bacillus thuringiensis* (hereinafter *B.t.*). This bacterial agent is used to control a variety of leaf-eating caterpillars, Japanese beetles and mosquitos. U.S. Patent No. 4,797,279 issued January 10, 1989 to Karamata et al., discloses hybrid bacterial cells comprising the gene coding for *B.t. kurstaki* delta-endotoxin and the gene coding for *B.t.*  
20 *tenebrionis* delta-endotoxin and their preparation. The *B.t.* hybrids are active against pests susceptible to *B.t. kurstaki* strains as well as against pests susceptible to *B.t. tenebrionis* strains. Generally, these hybrids have useful insecticidal properties which are superior to those observed by physical mixtures of the parent strains in terms of level of insecticidal  
25 activity, or in terms of spectrum of activity, or both. The insecticidal compositions comprising such microorganisms may be used to combat insects by applying the hybrids in an insecticidally effective amount to the insects or to their environment.

Another derivation from the bacterium *B.t.* was disclosed in  
30 European Patent Application, Publication No. 0 325 400 A1, issued to Gilroy and Wilcox. This invention relates to a hybrid toxin gene which is toxic to lepidopteran insects. Specifically, the invention comprises a hybrid delta endotoxin gene comprising part of the *B.t. var. kurstaki* HD-73 toxin gene and part of the toxin gene from *B.t. var. kurstaki* strain HD-1. The  
35 hybrid toxin gene (DNA) encoding a protein having activity against lepidopteran insects was disclosed.

The bacterium *B.t.* was also utilized for its insecticidal properties in European Patent Application, Publication No. 0 340 948, issued to Wilcox, et al. This invention concerns hybrid pesticidal toxins which are produced by the fusion of an insect gut epithelial cell recognition region of a *B.t.* gene to diphtheria toxin B chain to prepare a hybrid *B.t.* toxin which is active  
5 against lepidopteran insects. It was suggested that the hybrid *B.t.* gene may be inserted into a plant or cloned into a baculovirus to produce a toxin which can be recovered. Alternatively, the host containing the hybrid *B.t.* gene can be used as an insecticide by direct application to the  
10 environment of the targeted insect.

In the search for insecticidal compounds, scorpion venom was identified as a possible source of compounds providing insecticidal properties. Two insect selective toxins isolated from the venom of the scorpion *Leirus quinquestriatus quinquestriatus* were revealed in Zlotkin, et al., "An Excitatory and a Depressant Insect Toxin from Scorpion Venom both Affect Sodium Conductance and Possess a Common Binding Site,"  
15 *Arch Biochem and Biophysics*, 240:877-87 (1985). In a study related to their chemical and pharmacological properties, it was revealed that one toxin induced fast excitatory contractive paralysis of fly larvae and the other  
20 induced slow depressant flaccid paralysis. Both affected sodium conductance.

Canadian Patent 2,005,658 issued June 19, 1990 to Zlotkin, et al., discloses an insecticidally effective protein derived from the scorpion *Leirus quinquestriatus hebraeus buthinae*, *Buthidae*. In this invention,  
25 the venom is lyophilized and separated into fractions. The fraction with the highest toxicity to larvae and the lowest toxicity to mice was subjected to further purification and the final product is that referred to as "LqhP35".

Corresponding with the research and developments related to various compositions having insecticidal properties, researchers worked to develop methods for producing insecticidal genes and introducing these to  
30 the target to be protected. U.S. Patent No. 4,879,236 issued November 7, 1989 to Smith and Summers, relates to a method for incorporating a selected gene coupled with a baculovirus promoter into a baculovirus genome to produce a recombinant baculovirus expression vector capable  
35 of expression of the selected gene in an insect cell. The method involves cleaving baculovirus DNA to produce a DNA fragment comprising a

polyhedrin gene or portion thereof, including a polyhedrin promoter. To prepare a recombinant transfer vector, the DNA fragment is inserted into a cloning vehicle and then a selected gene is inserted into this modified cloning vehicle such that it is under the control of the polyhedrin promoter.

- 5 The recombinant transfer vector is then contacted in insect cells with a baculovirus DNA so as to effect recombination and incorporation of the selected gene into the baculovirus genome. The baculovirus *Autographa californica* (AcMNPV) and its associated polyhedrin promoter were found to be useful in producing a viral expression vector capable of extremely  
10 high levels of expression of a selected gene in an insect host cell.

- The inventors suggest that the expression vector might be used in a system for controlling insects by selecting a gene which produces a protein which is toxic to a specific insect or to a spectrum of insects and cloning that gene into the AcMNPV expression vector. They suggest that the  
15 vector could be applied to the plant or animal to be protected. The recombinant virus could invade the cells of the intestinal wall following ingestion by the insect and begin replication.

- A further method for producing insecticidal genes and introducing them to the target to be protected was disclosed in Cutler, "Electroporation  
20 Being Developed to Transform Crops: Success with Model Crop Confirmed," *AG Biotech. News* vol. 7(5):3 & 17 (1990). This article teaches that DNA may be electroporated directly into germinating pollen and that pollen may be put back on the flower to form seeds which then grow into transformed plants. This method has been employed successfully in  
25 tobacco plants and may be successful in corn and alfalfa as well. This method may be easier than the electroporation of protoplasts because the ultimate goal is to pollinate the flowers and "let the flowers do the work" rather than to regenerate the plant. The process consists of collecting pollen, germinating it in a germinating medium for 30-60 minutes after  
30 which the pollen tube will start to come out of the pollen grain, adding the desired DNA to the liquid suspension containing the pollen, administering an electric shock to open the pores of the pollen, washing the excess DNA away, and putting the altered pollen under the stigma of a plant and waiting until seeds are formed. This may be an easy method to move any  
35 gene into crop plants.

An additional delivery system was disclosed in U.S. Patent No. 4,861,595 issued August 29, 1989 to Barnes and Edwards. This invention concerns the use of treated, substantially intact, microbial cells as a delivery system of protein compounds to animals and humans. The microbial cells initially produce a protein intracellularly via a homologous gene. The protein-producing microbe is treated by chemical or physical means while the cell is substantially intact. Manipulation of the treatment process produces a nonproliferative treated microbial cell without significant loss of the activity of the intracellular compound. Since the cell will not replicate and will have a stable cell wall which may then be broken down in a desired area of the digestive system of the animal or human, it allows the timed or targeted release of the products encapsulatable by the subject invention. After suitable treatment, the protein-producing microbial cell itself is used as the delivery system so no purification of the produced compound is necessary. Any protein, polypeptide, amino acid, or compound, including insecticides, that may be produced by microbial means may be the starting material of the invention.

The possibility of using DNA technology to incorporate a synthetic gene which encodes a neurotoxin found in scorpion venom was explored in Carbonell, et al., "Synthesis of a gene coding for an insect-specific scorpion neurotoxin and attempts to express it using baculovirus vectors," *Gene* 73:409-18 (1988). This article teaches the possibility of using DNA technology to incorporate a synthetic gene which encodes a neurotoxin found in the venom of the scorpion, *Buthus eupeus*, into the baculovirus genome to improve baculovirus pesticides. Three methods of expression using the polyhedron promoter-based AcMNPV expression system to effect toxin production were studied. Expression of the 36 codon gene alone provided minuscule production of the toxin. Some success was found with the attachment of a signal peptide to the toxin. Significant levels of protein were produced when the toxin gene was fused to the N-terminus of polyhedron. However, production was ten to twenty-fold less than that observed for polyhedron itself. The limitation to expression was not believed to be at the level of transcription but at the post-transcriptional level including translation and protein stability. Paralytic activity of the toxin products was not detected.

Researchers have also been able to isolate toxins extracted from the venom of spiders. U.S. Patent No. 4,925,664 issued to Jackson and Parks on May 15, 1990, discloses methods of treating heart and neurological diseases by applying toxins derived from the spiders

5 *Agelenopsis aperta* and *Hololena curta*. The toxins are also effective as specific calcium channel or excitatory amino acid receptor blockers that may be used against insects and related pests.

Another study related to the properties of isolated spider venom toxins revealed the ability of low molecular weight factors isolated from  
10 funnel-web spider venoms to reversibly bind to calcium channels. WO 89/07608 issued August 24, 1989 to Cherksey, et al., discloses that these active low molecular weight factors reversibly bind to calcium channels with sufficient specificity and affinity to extinguish calcium conductance in neurons and to permit isolation and purification of calcium channel  
15 structures. These venoms were found to be toxic to mammals.

Other applications of spider toxins were discussed in Jackson and Parks, "Spider Toxins: Recent Applications in Neurobiology," *Ann Rev Neurosci* 12:405-14 (1989). This article teaches that there is great heterogeneity in the toxins of different taxa. It recognizes that experiments  
20 have suggested species-specific properties of calcium channels and the spider venoms might provide calcium channel antagonists. The spider venoms discussed are found to affect vertebrates. The article also identifies spider venoms as possible sources of insect-specific toxins for agricultural applications.

25 Adams, et al., "Isolation and Biological Activity of Synaptic Toxins from the Venom of the Funnel Web Spider, *Agelenopsis Aperta*," in *Insect Neurochemistry and Neurophysiology* 1986, Borkovec and Gelman eds., Humana Press, New Jersey, 1986, teaches that multiple peptide toxins which antagonize synaptic transmission in insects have been isolated from  
30 the spider *Agelenopsis aperta*.

U.S. Patent No. 4,855,405 issued August 8, 1989 to Kyoto et al., discloses a receptor inhibitor obtained from *Joro* spider venom glands, and its manufacturing method. The compound has an insecticidal effect when insects contact the compound carried in a liquid or solid.

35 U.S. Patent No. 4,918,107 issued April 17, 1990 to Nakajima et al., relates to a compound which has glutamate receptor inhibitor activity, a



process for preparing the same, and an insecticidal composition containing the same. The compound is carried in a liquid or solid carrier with a dispersing agent added and applied directly to the plant or animal to be protected. A low dosage is effective as an insecticide and has very low  
5 mammalian and fish toxicity and small adverse influence to the environment.

Accordingly, due to a combination of problems associated with some synthetic insecticides including poor efficacy, there exists a continuing need for the development of novel means of invertebrate  
10 control.

There is provided by this invention novel insecticidally effective peptides derived from, for example, a spider of the genus *Tegenaria*. The peptide comprises a) about 51 amino acids in length; b) 6 cysteine residues in position 6, 22, 25, 32, 36 and 45; c) eighty percent sequence  
15 homology with the peptide defined in SEQ ID NO:3 and agriculturally or horticulturally acceptable salts thereof. The invention further provides substantially similar peptides and signal leader sequences as defined herein.

Further provided by the invention is a novel DNA sequence  
20 comprising a DNA sequence encoding an insecticidally effective peptide of this invention.

Further provided by the invention are recombinant expression vectors comprising a DNA sequence encoding an insecticidally effective peptide of this invention, wherein the vector is capable of effecting the  
25 expression of said coding sequence in transformed cells.

Further provided by the invention are novel transgenic plants comprising a DNA sequence encoding an insecticidally effective peptide of this invention, wherein said DNA is introduced into the germ line of said plant, or an ancestor of said plant, such that the trait of expression of said  
30 DNA sequence is inherited by subsequent generations of said plant through sexual propagation or asexual propagation.

Further provided by this invention is a novel recombinant baculovirus expression vector, capable of expressing a DNA sequence encoding an insecticidally effective peptide of this invention.

Further provided by this invention is a novel method for producing an insecticidally effective peptide of this invention, which method comprises:

- 5 (a) culturing recombinant host cells wherein a recombinant expression vector transformed or transfected in said host cells has a DNA sequence encoding said peptide, wherein the vector is capable of effecting the expression of said coding sequence in transformed cells; and
- (b) recovering said insecticidally effective peptide from the recombinant host cell culture.

- 10 Further provided by the invention is a novel method of controlling invertebrate pests comprising contacting said pests with an effective amount of a peptide of this invention. Further provided by this invention is a novel method of controlling invertebrate pests comprising contacting said pests with a recombinant baculovirus capable of expressing an
- 15 effective amount of an insecticidally effective peptide of this invention in said pests

- Further provided by this invention is a novel insecticidal composition comprising an insecticidally effective amount of a peptide of this invention and agriculturally or horticulturally acceptable salts thereof in an
- 20 agriculturally or horticulturally acceptable carrier therefor.

Further provided by this invention are novel antibodies substantially immunoreactive with a peptide of this invention.

- Further provided by this invention is a novel DNA probe derived from a DNA sequence encoding an insecticidally effective peptide of this
- 25 invention.

FIG. 1: Design of the Primer for NPS-326.

- FIG. 2: The complete DNA sequence corresponding to the mRNA sequence encoding NPS-326 from the spider *Tegenaria agrestis*. The signal sequence which is cleaved from the mature toxin is underlined.
- 30 Boxed regions indicate the positions of variability in the translated sequences amongst the family of related toxins.

- FIG. 3: The complete DNA sequence corresponding to the mRNA sequence encoding NPS-331 from the spider *Tegenaria agrestis*. The signal sequence which is cleaved from the mature toxin is underlined.
- 35 Boxed regions indicate the positions of variability in the translated sequences amongst the family of related toxins.

FIG. 4: The complete DNA sequence corresponding to the mRNA sequence encoding NPS-373 from the spider *Tegenaria agrestis*. The signal sequence which is cleaved from the mature toxin is underlined. Boxed regions indicate the positions of variability in the translated sequences amongst the family of related toxins.

FIG. 5: Chromatogram of fractionation of *Tegenaria agrestis* whole venom on a Vydac C<sub>18</sub> reversed-phase column eluted with a linear gradient of 0.1% TFA (aq) to 0.1% TFA in CH<sub>3</sub>CN/H<sub>2</sub>O, 1:1.

#### 10 A. Definitions

As used herein, "expression vector" includes vectors which are capable of expressing DNA sequences contained therein, where such sequences are operably linked to other sequences capable of effecting their expression. It is implied, although not always explicitly stated, that these expression vectors must be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. Clearly a lack of replicability would render them effectively inoperable. In sum, "expression vector" is given a functional definition, and any DNA sequence which is capable of effecting expression of a specified DNA code disposed therein is included in this term as it is applied to the specified sequence. In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer to circular double stranded DNA which, in their vector form are not bound to the chromosome. "Plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

"Recombinant host cells" refers to cells which have been transformed with vectors constructed using recombinant DNA techniques.

30 Spiders in the genus *Tegenaria* are members of the family Agelenidae, commonly known as the funnel-web spiders. *Tegenaria* is a large and widely distributed genus; many species live in close association with humans. Most United States species of *Tegenaria*, including *T. agrestis*, are thought to have been accidentally introduced from other continents (Gertsch, Willis J. 1979. American Spiders. Van Nostrand Reinhold, New York.). *Tegenaria agrestis* is a typical funnel-web spider,

forming its web in tall grass or in the crevices of walls, wood piles, etc. At present its distribution is limited to Oregon, Washington, and parts of Idaho (Roth, Vincent D. 1968. The Spider Genus *Tegenaria* in the Western Hemisphere (Agelenidae). American Museum *Novitates* 2323: 1-33.),  
5 although there are indications of incidental transport to nearby states such as Utah. *T. agrestis* has been implicated in several serious human envenomations (Vest, Darwin K. 1987. Necrotic arachnidism in the northwest United States and its probable relationship to *Tegenaria agrestis* (Walckenaer) spiders. *Toxicon* 25 (2): 175-184.). All available  
10 data, however (largely derived from NPS internal research), indicate that the insecticidal components of this venom are distinct from those responsible for mammalian toxicity.

The mechanism of action of the insecticidally effective peptides of this invention is unknown. These toxins produce a unique set of symptoms  
15 in *Heliothis*, *Spodoptera*, and *Trichoplusia* larvae. There is a pronounced delay, sometimes more than 24 hours, between administration of the toxins or venom and the full development of neurological symptoms. *Tegenaria* venom, and the toxins purified from it, cause a distinctive spastic paralysis which is characterized by continuous writhing for 48 hours or more. These  
20 symptoms are described more fully in "Insecticidally effective peptides."

#### B. The Isolation of peptides from *Tegenaria* venom

One source of peptide is *Tegenaria* venom. Spider venom can be removed from *Tegenaria* by any method known such as venom gland  
25 extraction from cephalothorax. However, in order to avoid impurities within the spider venom and the isolated toxins, the spider venom preferably is obtained by electrical stimulation of the spiders to cause release of the venom and subsequent suction to collect the released venom and prevent contamination of the venom by regurgitate or hemolymph as described in  
30 U.S. 4,925,664.

Once the spider venom is obtained by electrical milking techniques, it can be fractionated into its peptide (toxin) components using a high performance liquid chromatograph (HPLC) and a variety of separation modes such as gel filtration, ion exchange and reversed phase  
35 chromatography.

Thus, using the technique of electrically milking the spider coupled with high performance liquid chromatography using reversed phase and cation exchange columns, it is possible to obtain substantially purified spider toxins. It will be appreciated, however, that other equivalent techniques may also be employed within the scope of the present invention in order to isolate spider toxins. The toxins thus isolated can be assayed for insecticidal activity and the amino acid sequence determined by methods known to those in the art.

Isolated peptides, impure fractions, or whole venom may be assayed for insecticidal activity by a number of methods, such as injection, topical application, or feeding. Injection is the preferred method, because it mimics the venom's natural route of entry, allows precise determination of doses, and generates useful data while consuming relatively small amounts of material. Testing samples in one or more major pest insects, such as *Heliothis*, provides a rigorous and commercially relevant assessment of insecticidal activity.

#### C. Insecticidally effective peptides

This invention, in one of its aspects, provides a family of insecticidally effective peptides, and insecticidally effective fragments thereof and agriculturally or horticulturally acceptable salts thereof.

Once an insecticidally effective, peptide-containing fraction has been isolated from a source and purified as described herein, amino acid sequence determination can be performed in any way known to those in the art such as N-terminal amino acid sequencing and use of an automated amino acid sequencer.

It will be understood from this disclosure that additional insecticidally effective proteins are expected to be within the scope of the invention. That is, it is believed other insecticidally effective peptides in the family exist and may be isolatable from *Tegenaria* as well as other sources in addition to the three detailed herein. The following relates to a family of insecticidally effective proteins. Members of this family of insecticidally effective peptides are believed to share the following characteristics:

- 1) size: all range between about 5500 to 6000 daltons and are about 50 amino acids in length; and

2) conserved amino terminus: NPS-326, NPS-331 and NPS-373 are identical for the first 11 residues and share greater than 90% overall sequence homology; and

3) all have an identical cysteine pattern and most likely share an identical disulfide bond arrangement:  
5 5-[Cys]-15-[Cys]-3-[Cys]-7-[Cys]-3-[Cys]-8-[Cys]-5; and

4) all the peptides are acidic: isoelectric points of the toxins are all less than 5.5; and

5) their isolated cDNA sequences all encode the same  
10 signal peptide as well as a carboxy terminal glycine residue which is frequently processed to an amide group; however, it is not yet known if this is related to activity; and

6) all are known to evoke a characteristic response in infected TBW. Upon injection into insects such as the tobacco budworm  
15 (*Heliothis virescens*) or the beet armyworm (*Spodoptera exigua*), these toxins cause a unique set of symptoms. One distinctive aspect is the delayed onset of toxicity. Even when the toxins or whole venom are applied at doses ultimately causing 100% mortality, symptoms may not appear for more than 24 hours. The symptoms of toxicity, once developed,  
20 are also unique. The initial indication of toxicity is a period of hyperactivity characterized by repeated gnashing of the mandibles and tremors in the legs and body wall. Over a period of several hours this gradually gives way to a distinctive type of convulsive or spastic paralysis, characterized by continuous writhing in which the larvae contort their bodies into a helical  
25 shape. These convulsions may persist, without interruption, for more than 48 hours. The affected insects apparently die from starvation and dehydration, exacerbated by the large energy expenditure associated with the convulsions. Cabbage looper (*Trichoplusia ni*) larvae treated with these toxins undergo the same series of symptoms in a shorter time; the  
30 writhing behavior gives way to a less distinctive paralysis within 24 hours.

More specifically, three insecticidally effective peptides and their encoding cDNA sequences have been isolated and characterized herein. First, NPS-326 has been isolated and purified to homogeneity by reversed-phase and cation exchange chromatography. It has a molecular  
35 weight of 5678.55 daltons (+/- 0.37 daltons) as determined by mass spectroscopy. Partial amino acid analysis allowed design of an

oligonucleotide used to access the cDNA sequence encoding NPS-326. As defined in Seq ID No. 2, the 51 amino acid peptide encoded by the isolated cDNA terminates with a carboxy-terminal glycine residue. Glycine residues at this position of the peptide are generally processed to an amide group (Creighton, T.E. in *Proteins: Structure and Molecular Properties*, W.H. Freeman and Company, New York. 1983). Table II. gives the amino acid composition for the peptide encoded by the isolated cDNA. If one allows for C-terminal amidation and disulfide linkages for the six encoded cysteine residues, the molecular weight of the peptide encoded by Seq ID No. 2 will decrease by 64.08 daltons to 5678.85 daltons. This is equivalent to that determined by mass spectroscopy for purified NPS-326. Thus, the processed form of insecticidally active NPS-326 as isolated from spider venom appears as defined in Seq ID No. 3.

Second, NPS-331 has been isolated and purified to homogeneity by reversed phase and cation exchange chromatography. It has a molecular weight of 5700.39 (+/- 0.29 daltons) as determined by mass spectroscopy. The cDNA was isolated by virtue of its amino terminal sequence homology to NPS-326. The cDNA sequence encoding NPS-331 is presented in Seq ID No. 8. Table III. gives the amino acid composition of this peptide. Assuming C-terminal amidation and disulfide linkages for the cysteine residues as per NPS-326, the calculated MW for the peptide encoded by Seq ID No. 8 is 5,699.86 daltons. This is equivalent to that determined by mass spectroscopy for NPS-331. Thus, the processed form of insecticidally active NPS-331 as isolated from spider venom appears as defined in Seq ID No. 9.

A third member of this family was isolated by virtue of its amino terminal sequence homology to NPS-326. It was also isolated and purified by reversed-phase and cation exchange chromatography. The cDNA sequence encoding NPS-373 is given in Seq ID No. 12, and its amino acid composition is presented in Table IV. Assuming C-terminal amidation and disulfide linkages for the cysteine residues as per NPS-326 and NPS-331, the calculated MW for the peptide encoded by Seq ID No. 12 is 5,642.81 daltons. This is equivalent to that determined by mass spectroscopy. Translation and processing of the precursor molecule thus yields the insecticidally effective peptide defined in Seq ID No. 14 which is that which is purified from spider venom.

Also provided by this invention is the novel signal or leader sequence that precedes the three mature proteins, NPS-326, NPS-331 and NPS-373. The cDNA encoding the signal peptide is given in Seq ID No. 5. It is believed this unique signal sequence can be used for targeting, production or synthesis of these, and possibly other, recombinant proteins. A signal sequence plays an important role in ensuring the proper localization of a newly synthesized protein. Generally they provide "topogenic signals" (Blobel, G., "Intracellular protein topogenesis," *Proc. Nat. Acad. Sci.* 77:1496-1500 (1980)), which target the attached protein sequence to various destinations within or external to the cell. This is particularly important for secreted proteins whose target sites are extracellular. It is also helpful for recombinant protein production as it can be easier to purify an expressed protein from the extracellular media rather than having to lyse the cells and purify from a whole cell extract. For the particular peptides claimed here one can speculate that the signal peptide may have utility in ensuring that the toxins are C-terminally amidated and folded by directing them to those locations inter- or extracellularly where this processing will occur. It is believed that this signal sequence may have utility in the expression and processing of other highly structured peptide molecules as well.

It is understood that minor modifications of primary amino acid sequence may result in proteins which have substantially, <sup>the same, i.e.</sup> equivalent or enhanced activity as compared to the peptides exemplified herein. These modifications may be deliberate, as through site-directed mutagenesis and amino acid substitution during solid phase synthesis, or may be accidental such as through mutations in hosts which produce the peptide of the invention. All of these modifications are included so long as insecticidal activity is retained. A "mutation" in a protein alters its primary structure (relative to the commonly occurring or specifically described protein) due to changes in the nucleotide sequence of the DNA which encodes it. These mutations specifically include allelic variants. Mutational changes in the primary structure of a protein result from deletions, additions, or substitutions. A "deletion" is defined as a polypeptide in which one or more internal amino acid residues are absent. An "addition" is defined as a polypeptide which has one or more additional internal amino acid residues as compared to the wild type. A "substitution" results from the





replacement of one or more amino acid residues by other residues. A protein "fragment" is a polypeptide consisting of a primary amino acid sequence which is identical to a portion of the primary sequence of the protein to which the polypeptide is related.

- 5 Preferred "substitutions" are those which are conservative, i.e., wherein a residue is replaced by another of the same general type. As is well understood, naturally-occurring amino acids can be subclassified as acidic, basic, neutral and polar, or neutral and nonpolar and/or aromatic. It is generally preferred that peptides differing from the native form contain  
10 amino acids which are from the same group as that of the amino acid replaced.

- Thus, in general, the basic amino acid Lys, Arg, and His are interchangeable; the acidic amino acids aspartic and glutamic are interchangeable; the neutral polar amino acids Ser, Thr, Cys, Gln, and Asn  
15 are interchangeable; the nonpolar aliphatic acids Gly, Ala, Val, Ile, and Leu are conservative with respect to each other (but because of size, Gly and Ala are more closely related and Val, Ile and Leu are more closely related), and the aromatic amino acids Phe, Trp, and Tyr are interchangeable.

- While proline is a nonpolar neutral amino acid, it represents  
20 difficulties because of its effects on conformation, and substitutions by or for proline are not preferred, except when the same or similar conformational results can be obtained. Polar amino acids which represent conservative changes include Ser, Thr, Gln, Asn; and to a lesser extent, Met. In addition, although classified in different categories, Ala, Gly,  
25 and Ser seem to be interchangeable, and Cys additionally fits into this group, or may be classified with the polar neutral amino acids.

#### D. Methods of Peptide Preparation

##### *Recombinant expression*

- 30 Further provided by this invention is a recombinant expression vector comprising a DNA sequence which encodes an insecticidally effective peptide of this invention. The vector is capable of effecting the expression of the coding sequence in transformed cells. Also provided by the invention are recombinant host cells transformed or transfected with a  
35 DNA sequence encoding an insecticidally effective peptide of the invention in a manner allowing the host cell to express the peptide.

Provision of a suitable DNA sequence encoding the desired protein permits the production of the protein using recombinant techniques now known in the art. The coding sequence can be obtained by retrieving a cDNA or genomic sequence from a native source of the protein or can be  
5 prepared chemically using a synthesized nucleotide sequence deduced from the amino acid sequence for the protein. When the coding DNA is prepared synthetically, advantage can be taken of known codon preferences of the intended host.

Expression systems containing the requisite control sequences,  
10 such as promoters, and preferably enhancers and termination controls, are readily available and known in the art for a variety of hosts. See e.g., Sambrook et al., *Molecular Cloning a Laboratory Manual*, Second Ed. Cold Spring Harbor Press (1989).

Thus, the desired proteins can be prepared in both procaryotic and  
15 eucaryotic systems, resulting in a spectrum of processed forms of the protein.

The most commonly used procaryotic system remains *E. coli*, although other systems such as *B. subtilis* and *Pseudomonas* are also expected to be useful. Suitable control sequences for procaryotic systems  
20 include both constitutive and inducible promoters including the lac promoter, the trp promoter, hybrid promoters such as tac promoter, the lambda phage P<sub>I</sub> promoter. In general, foreign proteins may be produced in these hosts either as fusion or mature proteins. When the desired sequences are produced as mature proteins, the sequence produced may  
25 be preceded by a methionine which is not necessarily efficiently removed. Accordingly, the peptides and proteins claimed herein may be preceded by an N-terminal Met when produced in bacteria. Moreover, constructs may be made wherein the coding sequence for the peptide is preceded by an operable signal peptide which results in the secretion of the protein.  
30 When produced in procaryotic hosts in this matter, the signal sequence is removed upon secretion.

A wide variety of eucaryotic hosts are also now available for production of recombinant foreign proteins. As in bacteria, eucaryotic hosts may be transformed with expression systems which produce the  
35 desired protein directly, but more commonly signal sequences are provided to effect the secretion of the protein. Eucaryotic systems have the

additional advantage that they are able to process introns which may occur in the genomic sequences encoding proteins of higher organisms.

Eucaryotic systems also provide a variety of processing mechanisms which result in, for example, glycosylation, carboxy-terminal amidation, oxidation or derivatization of certain amino acid residues, conformational control, and so forth.

Commonly used eucaryotic systems include yeast, fungal cells, insect cells, mammalian cells, avian cells, and cells of higher plants. The list is not exhaustive. Suitable promoters are available which are compatible and operable for use in each of these host types as well as are termination sequences and enhancers, as e.g. the baculovirus polyhedrin promoter. As above, promoters can be either constitutive or inducible. For example, in mammalian systems, the MTII promoter can be induced by the addition of heavy metal ions.

The particulars for the construction of expression systems suitable for desired hosts are known to those in the art. For recombinant production of the protein, the DNA encoding it is suitably ligated into the expression vector of choice and then used to transform the compatible host which is then cultured and maintained under conditions wherein expression of the foreign gene takes place. The insecticidally effective protein of this invention thus produced is recovered from the culture, either by lysing the cells or from the culture medium as appropriate and known to those in the art.

Because recombinant materials for the proteins of the invention are provided, these proteins can be made by recombinant techniques as well as by automated amino acid synthesizers. Because of the variety of post-translational characteristics conferred by other host cells, various modifications for the naturally-occurring proteins will also be obtained. A "modified" protein differs from the unmodified protein as a result of post-translational events which change the glycosylation or lipidation pattern, or the primary, secondary, or tertiary structure of the protein and are of course included within the scope of the invention as claimed.

It should be further noted that if the proteins herein are made synthetically, substitution by amino acids which are not encoded by the gene may also be made. Alternative residues include, for example, the  $\omega$  amino acids of the formula  $H_2N(CH_2)_nCOOH$  wherein  $n$  is 2-6. These are

neutral, nonpolar amino acids, as are sarcosine (Sar), t-butylalanine (t-BuAla), t-butylglycine (t-BuGly), N-methyl isoleucine (N-Melle), and norleucine (Nleu). Phenylglycine, for example, can be substituted for Trp, Tyr or Phe, an aromatic neutral amino acid; citrulline (Cit) and methionine sulfoxide (MSO) are polar but neutral, cyclohexyl alanine (Cha) is neutral and nonpolar, cysteic acid (Cya) is acidic, and ornithine (Orn) is basic. The conformation conferring properties of the proline residues may be obtained if one or more of these is substituted by hydroxyproline (Hyp).

1 0 E. Identification of the coding sequence of insecticidally effective peptides of this invention

In another aspect of this invention, a substantially isolated DNA sequence encoding a peptide of this invention is provided.

Employing partial amino acid sequence data, the genes responsible for the production of proteins from a source can be isolated and identified. Numerous methods are available to obtain the gene responsible for the production of a peptide. Examples include Fuqua, S. et al., "A simple PCR method for detection and cloning low abundant transcript", *Biotechnology*, Vol. 9, No. 2 (Aug 1990); Frohman, M.A., "RACE: Rapid amplification of cDNA ends", *PCR protocols*, ed. Innis et al., Academic Press, San Diego, CA, (1990) and U.S. Patent No. 4,703,008 "DNA Sequences Encoding Erythropoietin" which patent is incorporated by reference.

Briefly, a DNA molecule is synthesized which encodes the determined amino acid sequence or which represents the complementary DNA strand to such a DNA molecule which encodes the determined amino acid sequence. This synthetic DNA molecule may then be used to probe for DNA sequence homology in cell clones containing recombinant DNA molecules comprising, in part, DNA sequences derived from the genomic DNA of an organism such as a spider or derived from cDNA copies of mRNA molecules isolated from cells or tissues of an organism such as a spider. Generally, DNA molecules of fifteen (15) nucleotides or more are required for unique identification of an homologous DNA, said number requiring unique determination of at least five (5) amino acids in sequence. It will be appreciated that the number of different DNA molecules which can encode the determined amino acid sequence may be very large since

each amino acid may be encoded for by up to six (6) unique trinucleotide DNA sequences or codons. Therefore, it is impractical to test all possible synthetic DNA probes individually, and pools of several such DNA molecules are used concomitantly as probes. The production of such  
5 pools which are referred to as "degenerate" probes is well known in the art. It will also be appreciated that while only one DNA molecule in the probe mixture will have an exact sequence homology to the gene of interest, several of the synthetic DNA molecules in the pool may be capable of uniquely identifying said gene since only a high degree of homology is  
10 required. Therefore, successful isolation of the gene of interest may be accomplished with synthetic DNA probe pools which do not contain all possible DNA probe sequences. In general, codons which are infrequently utilized by the organism need not be represented in the probe pool. In fact, a single sequence DNA probe may be produced by including  
15 only the DNA codons most frequently utilized by the organism for each amino acid, although, it will be appreciated that this approach is not always successful.

One technique to isolate a gene sequence employs the Polymerase Chain Reaction (PCR). See e.g., U.S. Patents 4,683,195 and 4,683,202  
20 which patents are incorporated by reference as if fully set forth herein. Essentially PCR allows the production of a selected DNA sequence when the two terminal portions of the sequence are known. Primers, or oligonucleotide probes, are obtained which correspond to each end of the sequence of interest. Using PCR, the central portion of the DNA sequence  
25 is then synthetically produced.

In one such method of employing PCR to obtain the gene which encodes a unique spider venom gene, RNA is isolated from the spider and purified. A deoxythymidylate-tailed oligonucleotide is then used as a primer in order to reverse transcribe the spider mRNA into cDNA. A  
30 synthetic DNA molecule or mixture of synthetic DNA molecules as in the degenerate probe described above is then prepared which can encode the amino-terminal amino acid sequence of the venom protein as previously determined. This DNA mixture is used together with the deoxythymidylate-tailed oligonucleotide to prime a PCR reaction. Because  
35 the synthetic DNA mixture used to prime the PCR reaction is specific to the desired mRNA sequence, only the desired cDNA will be effectively

amplified. The resultant product represents an amplified cDNA which can be ligated to any of a number of known cloning vectors. Notwithstanding this, it will be appreciated that "families" of peptides may exist in spider venoms which will have similar amino acid sequences and that in such cases, the use of mixed oligonucleotide primer sequences may result in the amplification of one or more of the related cDNAs encoding these related peptides. Genes encoding related peptides are also within the scope of the invention as the related peptides also have useful insecticidal activities.

Finally, the produced cDNA sequence can be cloned into an appropriate vector using conventional techniques, analyzed and the nucleotide base sequence determined. Examples of DNA sequences, encoding insecticidally effective proteins, are presented in the Sequence Listing and Table VIII. A direct amino acid translation of these PCR products will reveal that they corresponded to the complete coding sequence for the mature protein. The portion of the DNA sequence which might encode amino acids corresponding to precursor and or propeptide regions may not be obtained by this approach. Such sequences may be determined by isolation of genomic or cDNA clones using the cDNA clone produced in this approach as a hybridization probe which is within the scope of the art.

**F. Cross-hybridization: DNA sequences as probes for related compounds.**

DNA probes of suitable size, generally from 20 to 150 nucleotides, can be derived from a DNA sequence of this invention. Such probes can be used to detect the presence of DNA encoding a insecticidally effective peptide of this invention by hybridization with nucleic acids from other sources. Screening with oligonucleotide probes encoding the signal sequence, fragments of the cDNA, or even the entire cDNA under conditions of reduced stringency will allow access to other active peptides with functional homology to the family of toxin molecules we have described herein. Sources of nucleic acids which would be good candidates for cross-hybridization with nucleotide probes generated from DNA sequences of this invention would include, but are not limited to; spiders of the same genera but of different species, spiders of related genera, and spiders of the same genera but different locations.

#### G. Application of the peptides as insecticides.

The insecticidally effective peptides of this invention are believed to be useful in controlling invertebrate pests such as those in the order of  
5 Lepidoptera, by contacting the pests with an effective amount of a peptide of this invention. Conveniently, insects are the preferred pest.

Methods of contacting an invertebrate pest with a peptide to control said pests are known. Examples include synthetically encapsulating the protein for oral ingestion by the pest. Recombinant hosts expressing the  
10 proteins of this invention, such as *Pseudomonas fluorescens*, can be heat killed and applied to plant or appropriate substrate for subsequent oral ingestion and control.

Of course, methods of controlling invertebrate pests using the proteins of this invention can be used in combination with other methods of  
15 controlling pests. For example, the transgenic plants and *E. coli* described herein can be engineered to express other invertebrate toxins depending on the type of pests to be controlled and other important variables present.

An insecticidal composition comprising an insecticidally effective amount of a peptide according to this invention and agriculturally or  
20 horticulturally acceptable salts thereof in an agriculturally or horticulturally acceptable carrier therefor is also provided.

#### H. Transgenic plants.

Further provided by this invention are transgenic plants comprising  
25 a DNA sequence encoding an insecticidally effective peptide of this invention introduced into the germ line of the plant, such that the trait of expression of the DNA sequence is inherited by subsequent generations of the plant through sexual propagation or asexual propagation.

Genes encoding the insecticidally effective peptides according to  
30 the present invention can be introduced into a plant by genetic engineering techniques, which upon production of the peptide in the plant cell is expected to be useful as a means for controlling insect pests. Therefore, it is possible to produce a plant that is more insect-tolerant than the naturally occurring variety.

35 The coding region for an insecticidally effective peptide gene that may be used to transform a plant may be the full-length or partial active

length of the gene. It is necessary, however, that the genetic sequence coding for the peptide be expressed, and produced, as a functional peptide in the resulting plant cell. It is believed that DNA from both genomic DNA and cDNA and synthetic DNA encoding an insecticidally effective peptide may be used to transform. Further, a gene may be constructed partially of a cDNA clone, partially of a genomic clone, and partially of a synthetic gene and various combinations thereof. In addition, the DNA coding for a peptide gene may comprise portions from various species other than from the source of the isolated peptide.

Furthermore, it is believed the insecticidally effective peptide may be combined with another compound or compounds to produce unexpected insecticidal properties in the transformed plant, containing chimeric genes, expressing the compounds. These other compounds can include protease inhibitors, for example, which have oral toxicity to insects or polypeptides from *Bacillus thuringiensis*. The *B. thuringiensis* protein causes changes in potassium permeability of the insect gut cell membrane and is postulated to generate small pores in the membrane. Other pore-forming proteins could also be used in combination with the insecticidally effective peptides. Examples of such pore-forming proteins are the magainins, the cecropins, the attacins, melittin, gramicidin S, sodium channel proteins and synthetic fragments, the  $\alpha$ -toxin of *Staphylococcus aureus*, apolipoproteins and their fragments, alamethicin and a variety of synthetic amphipathic peptides. Lectins which bind to cell membranes and enhance endocytosis are another class of proteins which could be used in combination with the insecticidally effective peptides of this invention to genetically modify plants for insect resistance.

The promoter of the peptide gene is expected to be useful in expressing the chimeric genetic sequence, however, other promoters are also expected to be useful. An efficient plant promoter that may be useful is an overproducing promoter. This promoter in operable linkage with the genetic sequence for the peptide should be capable of promoting expression of the peptide such that the transformed plant has increased tolerance to insect pests. Overproducing plant promoters that are expected to be useful in this invention are known.

The chimeric genetic sequence comprising an insecticidally effective peptide gene operably linked to a promoter can be ligated into a



suitable cloning vector to transform the desired plant. In general, plasmid or viral (bacteriophage) vectors containing replication and control sequences derived from species compatible with the host cell are used. The cloning vector will typically carry a replication origin, as well as  
5 specific genes that are capable of providing phenotypic selection markers in transformed host cells, typically resistance to antibiotics. The transforming vectors can be selected by these phenotypic markers after transformation in a host cell.

Host cells that are expected to be useful include procaryotes,  
10 including bacterial hosts such as *E. coli*, *Salmonella typhimurium*, and *Serratia marcescens*; and eucaryotic hosts such as yeast or filamentous fungi.

The cloning vector and host cell transformed with the vector are generally used to increase the copy number of the vector. With an  
15 increased copy number, the vectors containing the peptide gene can be isolated and, for example, used to introduce the genetic sequences described herein into the plant or other host cells.

Methods to produce plants expressing foreign genes are known. For example, plant tissue can be transformed by direct infection of or co-  
20 cultivation of plants, plant tissue or cells with *A. tumefaciens*; direct gene transfer of exogenous DNA to protoplasts; incubation with PEG; microinjection and microprojectile bombardment.

Transformation in tobacco by electroporation a technique described in *Ag Biotechnology News*, Vol. 7 p. 3 and 17 (Sept/Oct 1990) has been  
25 confirmed. In this technique, plant protoplasts are electroporated in the presence of plasmids containing the insecticidally effective peptide genetic construct. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and form plant callus.  
30 Selection of the transformed plant cells with the expressed insecticidally effective peptide can be accomplished using the phenotypic markers as described above. The exogenous DNA may be added to the protoplasts in any form such as, for example, naked linear, circular or supercoiled DNA, DNA encapsulated in liposomes, DNA in spheroplasts, DNA in other plant  
35 protoplasts, DNA complexed with salts, and the like.

All plant cells which can be transformed by *Agrobacterium* and whole plants regenerated from the transformed cells can also be transformed according to the invention so to produce transformed whole plants which contain the transferred insecticidally effective peptide gene.

- 5 Transformation in rice has been confirmed by D.M. Raineri et al., "Agrobacterium-mediated transformation of rice (*Oryza sativa* L.)", *Biotechnology*, Vol.8, pp 33-38 (January 1990).

- Another method of introducing the insecticidally effective peptide gene into plant cells is to infect a plant cell with *A. tumefaciens* transformed with the insecticidally effective peptide gene. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots, roots, and develop further into transformed plants. The insecticidally effective peptide genetic sequences can be introduced into appropriate plant cells, for example, by means of the Ti plasmid of *A. tumefaciens*. The Ti plasmid is transmitted to plant cells on infection by *A. tumefaciens* and is stably integrated into the plant genome.
- 1 0  
1 5

- Ti plasmids contain two regions believed essential for the production of transformed cells. One of these, named transfer DNA (T DNA), induces tumor formation. The other, termed virulent region, is essential for the formation but not maintenance of tumors. The T DNA region, which transfers to the plant genome, can be increased in size by the insertion of an enzyme's genetic sequence without its transferring ability being affected. By removing the tumor-causing genes so that they no longer interfere, the modified Ti plasmid can then be used as a vector for the transfer of the gene constructs of the invention into an appropriate plant cell.
- 2 0  
2 5

The genetic material may also be transferred into the plant cell by using polyethylene glycol (PEG) which forms a precipitation complex with the genetic material that is taken up by the cell.

- 3 0 Transfer of DNA into plant cells can also be achieved by injection into isolated protoplasts, cultured cells and tissues and injection into meristematic tissues of seedlings and plants. Transgenic plants and progeny therefrom are obtained by conventional methods known in the art.

- Another method to introduce foreign DNA sequences into plant cells comprises the attachment of the DNA to particles which are then forced into plant cells by means of a shooting device, "gene guns". Any plant
- 3 5

- tissue or plant organ may be used as the target for this procedure, including but not limited to embryos, apical and other meristems, buds, somatic and sexual tissues *in vivo* and *in vitro*. Transgenic cells and callus are selected following established procedures. Targeted tissues are
- 5 induced to form somatic embryos or regenerate shoots to give transgenic plants according to established procedures known in the art. The appropriate procedure may be chosen in accordance with the plant species used. Transgenic maize plants have been prepared by using high-velocity microprojectiles to transfer genes into embryogenic cells.
- 10 "Inheritance and expression of chimeric genes in the progeny of transgenic maize plants", *Biotechnology*, Vol. 8, pp 833-838 (September 1990).

- The regenerated plant may be chimeric with respect to the incorporated foreign DNA. If the cells containing the foreign DNA develop into either micro- or macrospores, the integrated foreign DNA will be
- 15 transmitted to sexual progeny. If the cells containing the foreign DNA are somatic cells of the plant, non-chimeric transgenic plants are produced by conventional methods of vegetative (asexual) propagation either *in vivo*, from buds or stem cuttings, or *in vitro* following established procedures known in the art. Such procedures may be chosen in accordance with the
- 20 plant species used.

- After transformation of the plant cell or plant, those plant cells or plants transformed so that the peptide is expressed, can be selected by an appropriate phenotypic marker. These phenotypic markers include, but are not limited to, antibiotic resistance. Other phenotypic markers are
- 25 known in the art and may be used in this invention.

Due to the variety of different transformation systems, all plant types can in principle be transformed so that they express an insecticidally effective peptide of the present invention.

- There is an increasing body of evidence that practically all plants
- 30 can be regenerated from cultured cells or tissues, including but not limited to all major cereal crop species, sugar cane, sugar beet, cotton, fruit and other trees, legumes and vegetables. Limited knowledge presently exists on whether all of these plants can be transformed by *Agrobacterium*. Species which are a natural plant host for *Agrobacterium* may be
- 35 transformable *in vitro*. Monocotyledonous plants, and in particular, cereals and grasses, are not natural hosts to *Agrobacterium*. Attempts to transform

them using *Agrobacterium* have been unsuccessful until recently. There is growing evidence now that certain monocots can be transformed by *Agrobacterium*. Using novel experimental approaches that have now become available, cereal and grass species may also be transformable.

- 5 Additional plant genera that may be transformed by *Agrobacterium* include *Ipomoea*, *Passiflora*, *Cyclamen*, *Malus*, *Prunus*, *Rosa*, *Rubus*, *Populus*, *Santalum*, *Allium*, *Lilium*, *Nacissus*, *Ananas*, *Arachis*, *Phaseolus*, and *Pisum*.

- 10 Regeneration varies from species to species of plants, but generally a suspension of transformed protoplasts containing multiple copies of the insecticidally effective peptide gene is first provided. Embryo formation can then be induced from the protoplast suspensions, to the stage of ripening and germination as natural embryos. The culture media will generally contain various amino acids and hormones. Shoots and roots  
15 normally develop simultaneously. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is fully reproducible and repeatable.

- The mature plants, grown from the transformed plant cells, can be  
20 selfed to produce an inbred plant. The inbred plant produces seed containing the gene for the insecticidally effective peptide. These seeds can be grown to produce plants that express the insecticidally effective peptide. The inbreds can, e.g., be used to develop insect tolerant hybrids. In this method, an insect tolerant inbred line is crossed with another inbred  
25 line to produce the hybrid.

- In diploid plants, typically one parent may be transformed by the insecticidally effective peptide (toxin) genetic sequence and the other parent is the wild type. After crossing the parents, the first generation hybrids ( $F_1$ ) will show a distribution of 1/2 toxin/wild type: 1/2 toxin/wild  
30 type. These first generation hybrids ( $F_1$ ) are selfed to produce second generation hybrids ( $F_2$ ). The genetic distribution of the  $F_2$  hybrids is 1/4 toxin/toxin: 1/2 toxin wild type: 1/4 wild type/wild type. The  $F_2$  hybrids with the genetic makeup of toxin/toxin are chosen as the insect tolerant plants.

- As used herein, variant describes phenotypic changes that are  
35 stable and heritable, including heritable variation that is sexually transmitted to progeny of plants, provided that the variant still expresses an

insecticidally effective peptide of the invention. Also, as used herein, mutant describes variation as a result of environmental conditions, such as radiation, or as a result of genetic variation in which a trait is transmitted meiotically according to well-established laws of inheritance. The mutant  
5 plant, however, must still express the peptide of the invention.

In general, the ideal insecticidally effective protein chosen to be expressed in a transgenic plant, will be one that is characterized by its safety to non-target insects and vertebrates. Expression systems will be chosen such that the level of expression affords insecticidal efficacy.  
10 Thus, this technical feasibility of obtaining such transgenic agriculturally important plants is expected to offer farmers an additional weapon to use in an integrated pest management system to reduce insect damage to crops in an environmentally responsible manner.

15 I. Genetically engineered insecticidal microbes

The insecticidally effective peptide alone or in combination with another insect toxin is expected to be useful in potentiating or enhancing the toxicity of microbes such as baculoviruses and hybrid bacteria.

Several baculoviruses including those that infect *Heliothis virescens*  
20 (tobacco budworm), *Orgyia pseudotsugata* (Douglas fir tussock moth), *Lymantria dispar* (gypsy moth), *Autographa californica* (alfalfa looper), *Neodiprion sertifer* (European pine sawfly), and *Laspeyresia pomonella* (codling moth) have been registered in some countries and used as pesticides. Introduction of at least one insect-selective toxin into the  
25 genome is expected to significantly enhance the potency of such pesticides.

A recombinant expression vector expected to be particularly suitable for use in this invention is a baculovirus expression vector such as the type disclosed in U.S. Patent 4,879,236, which patent is incorporated  
30 by reference as if fully set forth herein. See also Carbonell et al. "Synthesis of a gene coding for an insect-specific scorpion neurotoxin and attempts to express it using baculovirus vectors," *Gene*, 73:409-418 (1988). The vector is expected to be useful in a system where a DNA sequence encoding an insecticidally effective peptide substantially  
35 isolatable from *Tegenaria* spider venom can be cloned into baculovirus such as *Autographa californica* (AcMNPV) expression vector as described

in U.S. 4,879,236 and Miller et al., *Science*, 219, 715-721 (1983). The recombinant expression vector virus could then be applied to the plant or animal upon which the insect is a pest, and when the virus is ingested by the pest insect, the recombinant virus will invade the cells of the midgut wall and begin replication. During replication, the gene for the insecticidally effective protein will be expressed, resulting in the disablement or death of the insect in a shorter period than if the insect had ingested the wild type AcMNPV virus.

A hybrid virus also expected to be useful is taught in European Patent Application 0 340 948. The hybrid virus expressing the DNA of this invention is expected to yield a virus having an altered insect host range. For example, fusion proteins could be expressed as a single polypeptide product of a hybrid gene consisting of DNA of this invention and a specific insect gut cell recognition protein to direct the expressed insecticidally effective peptide to the host insect target.

Various prokaryotic and eukaryotic microbes can be transformed to express a hybrid toxin gene encoding an insecticidally effective protein by the method taught in European Patent Application 0 325 400.

Hybrid bacterial cells, comprising a plasmid with the gene coding for the protein of this invention are expected to be useful in the method of this invention. Insects would be controlled by applying the hybrids to insects. *See e.g.*, U.S. Patent 4,797,279 which patent is incorporated by reference as if fully set forth herein.

Other examples of employing baculovirus that would be suitable for use in this invention are described in Tomalski et al., "Insect paralysis by baculovirus-mediated expression of a mite neurotoxin gene", *Nature*, 352: 82-85 (1991) and Stewart et al., "Construction of an improved baculovirus insecticide containing an insect-specific toxin gene", *Nature*, 352:85-88 (1991).

#### J. Antibodies to insecticidally effective peptides

Another aspect of this invention are antibodies to the insecticidally effective peptides of this invention. In the following description, reference will be made to various methodologies known to those skilled in the art of immunology for detecting and purifying peptides reactive with the antibodies described herein.

An antibody is said to be "capable of binding" a molecule if it is capable of specifically reacting with the molecule. The term "epitope" is meant to refer to that portion of a molecule which can be recognized and bound by an antibody. An antigen may have one or more than one  
5 epitope. An "antigen" is capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. The specific reaction referred to above is meant to indicate that the antigen will immunoreact, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by  
10 other antigens.

The term "antibody" (Ab) or "monoclonal antibody" (Mab) as used herein is meant to include intact molecules as well as fragments thereof (such as, for example, Fab and F(ab')<sub>2</sub> fragments) which are capable of binding an antigen. Fab and F(ab')<sub>2</sub> fragments lack the Fc fragment of  
15 intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an insect antigen.

The antibodies of the present invention may be prepared by any of a variety of methods. Methods for the production and use of such antibodies are well known and described fully in the literature. See *e.g.*, Harlow and  
20 Lane, "Antibodies: A laboratory manual", Cold Spring Harbor Press, New York (1988). Generally, an insecticidally effective peptide is prepared and purified to render it substantially free of natural contaminants or an insecticidally effective peptide fragment is synthesized, according to means known in the art. Either the purified peptide or the synthesized  
25 fragment or a combination of purified natural fragments and/or synthesized fragment may be introduced into an animal in order to produce polyclonal antisera of greater specific activity.

Monoclonal antibodies can be prepared using known hybridoma technology. In general, such procedures involve immunizing an animal  
30 with an antigen such as an insecticidally effective peptide antigen. The splenocytes of such animals are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention. After fusion, the resulting hybridoma cells are selectively maintained in a suitable medium and then  
35 cloned by limiting dilution. The hybridoma cells obtained through such a

selection are then assayed to identify clones which secrete antibodies capable of binding the insecticidally effective peptide antigen.

If the peptide source is impure, only some of the hybridoma cells will produce antibodies capable of binding to the peptide (other hybridoma cells will produce antibody capable of binding to the peptide  
5 contaminants). Thus, it may be necessary to screen among the hybridoma cells for those which are capable of secreting an antibody which is capable of binding to the peptide. Such screening is preferably accomplished by incubating a sample of the peptide (or venom) in the presence of  
10 monoclonal antibody secreted from each of a group of particular hybridoma cells and identifying any hybridoma cell capable of secreting an antibody which is able to neutralize or attenuate the ability of the venom to paralyze an insect. Once such a hybridoma cell has been identified, it may be clonally propagated by means known in the art in order to produce the  
15 peptide-specific monoclonal antibody.

To purify an insect selective toxin, native or recombinant, using antibody affinity chromatography, it is necessary to employ an antibody capable of binding to the insecticidally effective peptide. Generally, such an antibody will be a monoclonal antibody. Once a peptide-specific  
20 antibody has been obtained, it may be immobilized by binding to a solid support and used to purify the peptide from natural venom or other sources using immunoaffinity chromatography in accordance to methods which are well known in the art. Such methods are capable of mediating a high degree of purification and of thereby producing a peptide which is  
25 substantially free of natural contaminants. As used herein, a peptide is said to be "substantially free of natural contaminants" if it is present in a form which lacks compounds with which it is naturally and normally associated (e.g. other proteins, lipids, carbohydrates, etc.).

Antibodies can also be used for detection of protein produced in a  
30 recombinant expression system (ELISA or Western); quantitation of protein expressed, in field or laboratory, persistence levels etc.; and detection of other molecules with related structure/functionality from other spider venoms (of related or non-related genera) or other venomous sources.

35 Examples



## EXAMPLES: GENERAL METHODS

The spider venom was obtained by electrical stimulation of the spiders to cause release of the venom and subsequent suction to collect the released venom and prevent contamination of the venom by regurgitate or hemolymph as described in U.S. 4,925,664.

Toxin purification - Crude venom (stored at  $-80^{\circ}\text{C}$ ) was thawed, mixed thoroughly and dissolved in the starting solvent prior to chromatography. Crude venom was fractionated with a high performance liquid chromatograph (HPLC) incorporating Beckman System Gold 126 solvent delivery and 168 photodiodearray detector modules. The following columns and conditions were used in the purifications. Semi-preparative reversed phase chromatography was performed with a Vydac 300 Angstrom  $\text{C}_{18}$  column (25 cm X 10 mm i.d.,  $5\mu\text{m}$  particle size) eluting with a 50 min linear gradient from 0.1% TFA to 0.1% TFA in  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ , 1:1, at a flow rate of 3.5 ml/min. The gradient was begun 5 min after injection of the sample. Analytical reversed phase chromatography was performed with a Vydac  $\text{C}_{18}$  (25 cm X 4.6 mm i.d.,  $5\mu\text{m}$  particle size) eluting with a 50 min linear gradient from 0.1% TFA to 0.1% TFA in  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ , 1:1, unless otherwise noted in the following examples. The flow rate was 1.0 ml/min and the gradient was begun 5 min after injection of the sample. The reversed phase columns were monitored at 220 nm and fractions collected with a Gilson model 203 fraction collector. Fractions from reversed phase chromatography were lyophilized to dryness following fractionation and stored at  $-20^{\circ}\text{C}$ . Cation exchange chromatography was performed with a HEMA-IEC BIO SB column (15 cm X 4.6 mm i.d.,  $10\mu\text{m}$  particle size) eluting with a 75 min linear gradient from 50 mM sodium acetate, pH 4.0, to 1 M NaCl in 50 mM sodium acetate, pH 4.0. The gradient was started 5 min after injection of the sample and elution was at 1 ml/min. The effluent was monitored at 280 nm and fractions collected on the Gilson model 203 fraction collector. Fractions were assayed for

insecticidal activity by injection into several species of lepidopteran larvae, as described below.

5      Example 1: Initial Fractionation and Identification of Insecticidal Peptides from *Tegenaria agrestis* Whole Venom

Five  $\mu$ l of *Tegenaria agrestis* whole venom, obtained as described in Methods, was diluted with 95  $\mu$ l of 0.1% TFA (aq) and fractionated by reversed phase HPLC on the Vydac RP C<sub>18</sub> analytical column as described in Methods. Fractions were collected by monitoring the effluent at 220 nm. A second 5  $\mu$ l portion of venom was also fractionated under the same conditions and like fractions from the two chromatographies were combined and lyophilized.

10      The lyophilized fractions were dissolved in 50  $\mu$ l of phosphate buffered saline, pH 6.5 (PBS) and tested for insecticidal activity by injection into tobacco bud worms (TBW; *Heliothis virescens*). TBW larvae, 3 individuals for each fraction, were injected with 6  $\mu$ l (1.2 wve) of test solution; insects in the control group were injected with equal volumes of saline. After treatment, the larvae were held in individual Petri dishes, with food, and observed periodically. Only fractions 7 and 8 had insecticidal activity (Table I, Figure 5).

Example 2: Further Purification of *Tegenaria agrestis* Fraction 8.

25      The major insecticidal component from *Tegenaria agrestis* Fraction 8 was purified by one additional chromatography on a cation exchange column followed by desalting of the major component by reversed phase chromatography.

30      The material remaining in Fraction 8 after TBW testing (approximately 5 wve in 25  $\mu$ l volume) was diluted to 500  $\mu$ l with 50 mM sodium acetate, pH 4.0. This was chromatographed on the HEMA-IEC BIO SB column as described in Methods. The effluent was monitored at 280 nm and the insecticidal component eluted at 41 min. This fraction was desalted by chromatography on the analytical Vydac C<sub>18</sub> column as described in Methods. The purified toxin, NPS-326, eluted as a single peak with a retention time of 30.5 min.

35

Example 3: Fractionation of 100  $\mu$ l of *Tegenaria agrestis* whole venom.

One hundred  $\mu$ l of *Tegenaria agrestis* whole venom was fractionated under conditions similar to those given in Examples 1 and 2 to give approximately 300  $\mu$ g of toxin NPS-326. Specifically, 50  $\mu$ l of *Tegenaria agrestis* crude venom was diluted into 950  $\mu$ l of 0.1% TFA (aq) and fractionated on the Vydac semi-preparative RP C<sub>18</sub> column eluting as described in Methods. The effluent was monitored at 220 nm and the insecticidal fractions collected. Fraction 7 eluted between 35.4 and 37.1 min while Fraction 8 eluted between 37.1 and 38.3 min. A second 50  $\mu$ l of venom was similarly fractionated and like fractions from the two chromatographies were combined and lyophilized.

Further purification of Fraction 8 was achieved by chromatography of the lyophilized material, dissolved in 0.2 ml of 50 mM sodium acetate, pH 4.0, on the HEMA-IEC BIO SB column. The column was eluted as described in Methods and the effluent monitored at 280 nm. The insecticidal component eluted at 46 min and was desalted by reversed phase chromatography on the Vydac C<sub>18</sub> analytical column as described in Methods. The purified toxin, NPS-326, eluted at 32 min. After lyophilization, 310  $\mu$ g of toxin were obtained.

N-terminal sequence analysis of both the native, and the reduced and alkylated (pyridyethylated), peptide gave the first 30 amino acids of NPS-326.

Electrophoresis by SDS-PAGE gave an apparent molecular weight of 6 - 8 kD for NPS-326. The actual mass was found to be  $5678.55 \pm 0.37$  D by mass spectroscopy.

Example 4: Purification of Fraction 7, the minor insecticidal component from *Tegenaria agrestis*.

Two minor insecticidal components, NPS-331 and NPS-373, were obtained by cation exchange chromatography of 150 wve of Fraction 7 from the reversed phase chromatography of whole venom (100 wve from Example 3 and 50 wve of Fraction 7 from a similar chromatography). The lyophilized powders from the three, 50  $\mu$ l chromatographies were combined in 200  $\mu$ l of 50 mM sodium acetate, pH 4.0, and fractionated on the HEMA-IEC BIO SB column eluting as described in Methods. The effluent was monitored at 280 nm and the insecticidal components co-eluted at 37 min. This fraction was desalted on the Vydac analytical C<sub>18</sub>

column eluting with 0.1% TFA (aq) (solvent A) and 0.1% TFA in CH<sub>3</sub>CN (solvent B) with the following gradient: 0% B for 3 min, 0 to 15% B over 3 min and 15-35% B over 80 min. NPS-331 elutes at 29 min while the less active NPS-373 elutes at 32 min. Lyophilization of the more active fraction gave approximately 30 µg of NPS-331. The amount of NPS-373 recovered after lyophilization was estimated (from integration of peak areas on the chromatogram) at 10 µg.

N-terminal sequence analysis of both native peptides gave the first 31 amino acids of NPS-331 and the first 20 amino acids of NPS-373.

Electrophoresis by SDS-PAGE gave an apparent molecular weight of 6 - 8 kD for both NPS-331 and NPS-373. The mass indicated by mass spectroscopy (electrospray ionization; data provided by Biotechnology Research Institute, Quebec, Canada) of NPS-331 is  $5700.39 \pm 0.29$  D and of NPS-373 is  $5643.09 \pm 0.41$  D.

Table I. Activity of *Tegenaria agrestis* reversed phase chromatography fractions in TBW. All fractions tested at 1.2 wve per larva

5	Fraction Number	24 hr TBW paralysis	48 hr TBW paralysis
	1	0/4	0/4
	2	0/4	0/4
	3	0/4	0/4
10	4	0/4	0/4
	5	0/4	0/4
	6	0/4	0/4
	7	2/4 affected	4/4 paralyzed
	8	2/4 affected	4/4 paralyzed
15	9	0/4	0/4
	10	3/4 Fi	0/4
	11	0/4	0/4
	12	0/4	0/4
	13	0/4	0/4
20	14	0/4	0/4
	15	0/4	0/4

Fi = feeding inhibition

25

### Example 5

Spiders were collected and identified at Natural Product Sciences, Inc. as *Tegenaria agrestis*. Venom glands were pulled from anesthetized spiders and quickly frozen in liquid nitrogen. RNA was extracted from the  
5 venom glands using the protocol of Chomczynski and Sacchi (Analytical Biochemistry, Vol 162, p 156 (1987)).

The oligonucleotide corresponding to residues 1 through 11 of the amino acid sequence obtained for NPS-326 is illustrated in Figure 1. It was designed using both spider codon preferences and deoxyinosine  
10 residues at positions of high degeneracy. An *Xho* I restriction site was incorporated into the 5' region of the primer. The primer used for first strand cDNA synthesis was composed of a run of 15 deoxythymidylate residues adjacent to a *Not* I restriction enzyme site. All primers were synthesized at the University of Utah, Howard Hughes Medical Institute  
15 contract facility.

From the preparation of venom gland RNA, messenger RNA was reverse transcribed to cDNA with murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, MD). A 20 µl reaction mixture contained the enzyme buffer as supplied by the manufacturer, 500  
20 ng of RNA, 2 units of RNasin (Boeringer Mannheim, Indianapolis, IN), 35 ng of *d(T)Not* I primer, 1mM each deoxynucleoside triphosphates, and 100 units of reverse transcriptase. The reaction mixture was incubated for 1 h at 37°C and continued for 10 minutes at 42°C. The reaction mixture was precipitated with ethanol and resuspended in 20 µl water.

25 Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase was initially described by Saikki et al. Science, 239:487 (1988), (patent 4,683,202). For our application, 10 µl of the venom gland cDNA was used as the template in a polymerase chain reaction containing reagents contained in the GeneAmp™ DNA amplification kit (Perkin Elmer  
30 Cetus, Norwalk, CT). The amplification reaction contained the sense and antisense primers in a 2 µM concentration, 100 µM of each deoxynucleotide triphosphate, and 4 units of the thermostable recombinant Taq polymerase. The reaction was run in a programmable heat block manufactured by Perkin Elmer Cetus (Norwalk, CT). Temperature cycling  
35 parameters included a 2 minute denaturation at 95°C, primer annealing for 2 minutes at 37°C, and enzymatic extension for 1 minute at 72°C. This

cycle was repeated twice and the program then switched to an identical profile incorporating an elevated annealing temperature of 54°C. This cycle was repeated 35 times.

Anchored PCR products were purified from a 3% NuSieve/1%  
5 SeaKem composite agarose gel (FMC, Rockland, ME) using the glassmilk resin supplied in the GeneClean™ kit (Bio 101, Vista, CA). Inserts were then doubly digested with the restriction enzymes *Not I* and *Xho I* (Boehringer Mannheim). The vector, pKS (Statagene, LaJolla, CA), was double digested with the same two enzymes to generate sites specific for  
10 directional cloning. Vector and insert were ligated in the presence of 15% PEG (polyethylene glycol, Sigma, St Louis MO) and transformed into competent *Escherichia coli* strain DH5aF' (Life Technologies, Inc., Gaithersburg, MD) and plated on LB plates (10 g/liter Tryptone (Difco), 5 g/liter Yeast extract (Difco), 10 g/liter NaCl and 15 g/liter agar (BBL)  
15 containing ampicillin (50 ug/ml) and IPTG (isopropylthio-β-galactoside) and *X-gal* (5-bromo-4-chloro-3-indoyl-β-D-galactoside) as indicators. Bacterial colonies containing recombinant plasmids were identified by their inability to synthesize β-galactosidase and turn blue on the indicator plates. They were grown up in LB media supplemented ampicillin and the  
20 plasmids purified using CsCl gradients. Purified plasmids were sequenced using commercially available external primers and Sequenase® Version 2.0 reagents and enzymes (US Biochemical, Cleveland, OH).

In order to access the upstream sequences of these cDNA  
25 molecules, an internal oligonucleotide corresponding to a homologous region of the three toxin encoding cDNA sequences was synthesized corresponding to the antisense strand of the double stranded cDNA. This oligonucleotide corresponds to nucleic acid residues 76 to 97 of Seq ID No. 2, the cDNA of NPS-326. Ten microliters of single stranded venom  
30 gland cDNA was tailed at its 3' end with deoxyguanosine residues using the enzyme, terminal deoxynucleotide transferase (Bethesda Research Laboratories). A 20 μl reaction containing 14 μM of enzyme and 500 μM of dGTP was incubated at 37°C for 15 minutes. The sample was ethanol precipitated and resuspended in 20 μl H<sub>2</sub>O.

35 DNA sequences upstream of the internal primer were amplified using an anchored PCR technique similar to that used for the

downstream/mature toxin cDNA sequences. The amplification reaction contained the sense, (a d(C) tailed primer), and antisense primers in a 2  $\mu$ M concentration, 100  $\mu$ M of each deoxynucleotide triphosphate, and 4 units of the thermostable recombinant *TaqI* polymerase. The temperature profile was as follows: 2 min at 94°C, 2 min at 37°C, 1 min at 37°C. This cycle was repeated twice and the program then switched to an identical profile incorporating an elevated annealing temperature of 54°C at the second step. This cycle was repeated 35 times.

Anchored PCR yielded at 230 bp fragment as evidenced on a 4% agarose gel in the presence of ethidium bromide. This reaction product was filled in at the ends using the large (Klenow) fragment of *E. coli* DNA Polymerase I (Molecular Biology Resources, Madison, WI), and precipitated by the addition of ethanol. The product was resuspended and digested with the restriction enzyme, *Sal I*. The digested fragment was kinased in the presence of 1mM ATP by the enzyme T4 Kinase and subsequently ligated to *Sal I* and *Eco RV* digested pKS vector. Transformants were screened by double-stranded DNA sequencing. Upstream sequences of the cDNAs encoding NPS-326, NPS-331 and NPS-373 were obtained in this manner. The complete DNA sequences for NPS-326, NPS-331 and NPS-373 are presented in Figures 2, 3 and 4 respectively.

The DNA sequence encoding NPS-326 was cloned into the *BamHI*-*EcoRI* site of the procaryotic expression vector pGEX-3X (Smith, DB, et al., *Proc. Natl. Acad. Sci. USA* 83,8703 (1986)) purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). This vector was then transformed into *E. coli* cell line W3110 (ATCC 27325) and plated on LB plates containing 50  $\mu$ g/ml ampicillin. Seed cultures were grown at 37°C and thereafter diluted 10 times into fresh media and subsequently grown until the optical density at 595 nm was 0.5. The culture was then induced with 0.5 mM IPTG and grown for three hours. Soluble fusion protein was purified by affinity chromatography using glutathione cross-linked beaded agarose (Sigma, St. Louis). Yields of expressed protein were approximately 5 mg/l. The purified fusion protein was used to raise polyclonal antibodies useful for detection of expressed *Tegenaria* toxin(s) using Western blotting or ELISA assays.



Table II

Peptide Encoded by Seq ID No. 2 i.e. NPS-326

Amino acid composition and protein characteristics of NPS-326 as encoded by pAdal7. The calculated molecular weight should be adjusted to 5,678.85 daltons if one assumes all of the cysteine residues to be involved in disulfide linkages and that the C-terminus is amidated. These changes decrease the calculated molecular weight by 64.08 daltons.

		<u>Unprocessed</u>	<u>Processed</u>
10	Calculated Molecular Weight =	5742.93	5,678.85
	Estimated pI =	4.976	5.41
15	Amino Acid Composition:		
	Non-Polar:	No.	Percent
	Ala	6	11.76
	Val	3	5.88
20	Leu	0	0.00
	Ile	1	1.96
	Pro	1	1.96
	Met	1	1.96
	Phe	2	3.92
25	Trp	0	0.00
	Polar:	No.	Percent
	Gly	3	5.88
	Ser	1	1.96
30	Thr	2	3.92
	Cys	6	11.76
	Tyr	2	3.92
	Asn	4	7.84
	Gln	2	3.92
35	Acidic:	No.	Percent
	Asp	3	5.88
	Glu	6	11.76
40	Basic:	No.	Percent
	Lys	3	5.88
	Arg	3	5.88
	His	2	3.92

45

Table III

Peptide Encoded by Seq ID No. 8 i.e. NPS-331

- 5 Amino acid composition and protein characteristics of NPS-331 as encoded by pAda1. The calculated molecular weight should be adjusted to 5,699.86 daltons if one assumes all of the cysteine residues to be involved in disulfide linkages and that the C-terminus is amidated.

10

	<u>Unprocessed</u>	<u>Processed</u>
Calculated Molecular Weight =	5763.939	5,699.86
15 Estimated pI =	4.678	4.96

## Amino Acid Composition:

20	Non-Polar:	No.	Percent
	Ala	6	11.76
	Val	3	5.88
	Leu	0	0.00
	Ile	1	1.96
25	Pro	1	1.96
	Met	1	1.96
	Phe	2	3.92
	Trp	0	0.00
30	Polar:	No.	Percent
	Gly	2	3.92
	Ser	1	1.96
	Thr	3	5.88
	Cys	6	11.76
35	Tyr	2	3.92
	Asn	5	9.80
	Gln	2	3.92
40	Acidic:	No.	Percent
	Asp	3	5.88
	Glu	6	11.76
45	Basic:	No.	Percent
	Lys	3	5.88
	Arg	3	5.88
	His	1	1.96

Table IV

Peptide Encoded by Seq ID No. 12 i.e. NPS-373

- 5 Amino acid composition and protein characteristics of NPS-373 as encoded by pAdal2. The calculated molecular weight should be adjusted to 5,642.81 daltons if one assumes all of the cysteine residues to be involved in disulfide linkages and that the C-terminus is amidated.

10		<u>Unprocessed</u>	<u>Processed</u>
	Calculated Molecular Weight =	5706.890	5,678.85
	Estimated pI =	4.678	4.96
15	Amino Acid Composition:		
	Non-Polar:	No.	Percent
	Ala	6	11.76
20	Val	3	5.77
	Leu	0	0.00
	Ile	1	1.92
	Pro	1	1.92
	Met	1	1.92
25	Phe	2	3.85
	Trp	0	0.00
	Polar:	No.	Percent
	Gly	3	5.77
30	Ser	1	1.92
	Thr	3	5.77
	Cys	6	11.54
	Tyr	2	3.85
	Asn	4	7.69
35	Gln	2	3.85
	Acidic:	No.	Percent
	Asp	3	5.77
	Glu	6	11.54
40	Basic:	No.	Percent
	Lys	3	5.77
	Arg	3	5.77
45	His	1	1.92

### Biological Activity Data

The insects tested were last instar, laboratory reared larvae of the tobacco budworm, *Heliothis virescens* (TBW); the beet armyworm, *Spodoptera exigua* (BAW); and the cabbage looper, *Trichoplusia ni* (CL).

5 All three species are in the family Noctuidae of the order Lepidoptera. All samples, whether whole venom or venom fractions, were prepared in filter-sterilized physiological saline, pH 6.5. Samples were administered by injection into the hemocoel at or near the lateral midline of the fourth abdominal segment; the needle was inserted at a shallow angle to avoid  
10 injury to internal organs. Whole venom doses were calculated in terms of whole venom equivalents (WVE). One WVE is the amount of any material which is normally present in one microliter of whole milked venom. Doses of components from early fractionations were also calculated in terms of WVE.

15 Whole venom from *Tegenaria agrestis* was tested in TBW and BAW by injection at a dose of 0.3 WVE per larva (~1.0 WVE/gm). Little effect was noted initially in TBW, but 16-24 hours after injection the larvae exhibited a distinctive spastic paralysis (see next paragraph). Four of five larvae eventually died. Several of the BAW larvae initially exhibited a  
20 flaccid paralysis, but recovered within 60 minutes. Within 24 hours, however, five of six BAW larvae were exhibiting the same spastic paralysis seen in TBW larvae. A dose of 0.03 WVE per larva caused only weak, reversible effects in TBW and BAW. In CL larvae, however, a dose of 0.03 WVE/larva was lethal to two of the six larvae tested.

25 *Tegenaria* venom, and the toxins purified from it, cause affected larvae to writhe uncontrollably in a distinctive, roughly helical pattern; these spasms may last for several days before the insects die. There is a pronounced delay, sometimes more than 24 hours, between administration of the toxin or venom and onset of visible symptoms. The  
30 length of this delay varies inversely with the amount of toxin or venom injected. The reversible, flaccid paralysis which was noted in BAW in the first few minutes after injection is thought to be the effect of arylamine toxins; several other agelenid spiders are known to possess such toxins.

By injection, NPS-326 had LD<sub>50</sub> values of 0.5-1.0 nmol/gm in TBW,  
35 BAW, and CL (6 insects per dose). NPS-331 was tested in TBW only for the purpose of guiding initial fractionations, but appeared to have

- approximately the same potency as NPS-326. Dose-response experiments in BAW and CL indicated an LD<sub>50</sub> of 0.5-1.0 nmol/gm for NPS-331. Both NPS-326 and NPS-331 caused symptoms in BAW and TBW which were similar to those caused by injection of whole *Tegenaria* venom (i.e., spastic paralysis with a delayed onset). In CL, however, the spastic paralysis appeared within 4 to 6 hours of injection and quickly gave way to less distinctive symptoms. Approximately 24 hours after injection, when BAW and TBW larvae were displaying the characteristic writhing behavior, CL larvae displayed only rapid, shallow tremors.
- 10 Mammalian toxicity tests indicate that NPS-326 and NPS-331 may possess a high degree of selectivity for insects. Injection of 30 µg of NPS-326 into the cerebral ventricles (n = 3) or peritoneum (n = 2) of male Swiss-Webster mice (~30 gm) had no effect.

TABLE V

Sequence

ID #	Description
5	1 Complete cDNA sequence encoding NPS 326
	2 Coding cDNA sequence for synthesis of NPS 326
	3 Amino acid sequence of NPS 326 (mature toxin)
	4 Amino acid sequence of NPS 326 with signal/leader sequence
10	5 Coding sequence for signal/leader sequence
	6 Amino acid sequence of the signal/leader sequence
	7 Complete cDNA sequence encoding NPS 331
	8 Coding cDNA sequence of NPS 331
	9 Amino acid sequence of NPS 331 (mature toxin)
15	10 Amino acid sequence of NPS 331 with signal/leader sequences
	11 Complete cDNA sequence encoding of NPS 373
	12 Coding cDNA sequence for synthesis of NPS 373
	13 Amino acid sequence of NPS 373 with signal/leader sequences
20	14 Amino acid sequence of NPS 373 (mature toxin)

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Karen Krapcho

(ii) TITLE OF INVENTION: INSECTICIDALLY EFFECTIVE PEPTIDES

(iii) NUMBER OF SEQUENCES: 14

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Woodcock Washburn Kurtz Mackiewicz & Norris

(B) STREET: One Liberty Place - 46th Floor

(C) CITY: Philadelphia

(D) STATE: PA

(E) COUNTRY: USA

(F) ZIP: 19103

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE

(B) COMPUTER: IBM PS/2

(C) OPERATING SYSTEM: PC-DOS

(D) SOFTWARE: WORDPERFECT 5.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: Not yet assigned

(B) FILING DATE: Herewith

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: John W. Caldwell

(B) REGISTRATION NUMBER: 28,937

(C) REFERENCE/DOCKET NUMBER: FMC-0051

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (215) 568-3100

(B) TELEFAX: (215) 568-3439

(2) INFORMATION FOR SEQ ID NO:1:



## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 394

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTTTCAGTAA ACCTTTGAGA GGAAAAAGAC GTGTTAGATG TCGAACAGCA AGATGTCACT 60

TA TAC TTC GAT GTC AAC TAC TAA ACA AAC CAA GAA GAC GGG ACG AAG AAG	110
Met Lys Leu Gln Leu Met Ile Cys Leu Val Leu Leu Pro Cys Phe Phe	
-15 -10 -5	

ACG CTT GGG CTG CTC TAG ACG TCT CGA TCT TAC TGT GTG TTC CTC AAA	158
Cys Glu Pro Asp Glu Ile Cys Arg Ala Arg Met Thr His Lys Glu Phe	
1 5 10 15	

TTA ATG TTT TCG TTA CAG ACG TTA CCA ACA CCA CTA GTT CAC CGC CGA	206
Asn Tyr Lys Ser Asn Val Cys Asn Gly Cys Gly Asp Gln Val Ala Ala	
20 25 30	

ACG CTC CGA CTT ACG AAG TCT TTG CTA CAA ATA TGT CGT ACA GTA CTT	254
Cys Glu Ala Glu Cys Phe Arg Asn Asp Val Tyr Thr Ala Cys His Glu	
35 40 45	

CGT GTT TTC CCG ATT C ATTGTCTGTA ATCTTACAAA GTGAACTTA CGAAACGATA 310  
 Ala Gln Lys Gly  
 50

TTTCGCAGTT TCAAGACAAT GAGTGGAAC TGCCATATAA AGGTACACAT TATATGAAAC 370

TTTCGATTAT TTATTTATTT TTT 394

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 153

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTT GGG CTG CTC TAG ACG TCT CGA TCT TAC TGT GTG TTC CTC AAA 45  
 Glu Pro Asp Glu Ile Cys Arg Ala Arg Met Thr His Lys Glu Phe  
 1 5 10 15

TTA ATG TTT TCG TTA CAG ACG TTA CCA ACA CCA CTA GTT CAC CGC CGA 93  
 Asn Tyr Lys Ser Asn Val Cys Asn Gly Cys Gly Asp Gln Val Ala Ala  
 20 25 30

ACG CTC CGA CTT ACG AAG TCT TTG CTA CAA ATA TGT CGT ACA GTA CTT 141  
 Cys Glu Ala Glu Cys Phe Arg Asn Asp Val Tyr Thr Ala Cys His Glu  
                   35                  40                  45

CGT GTT TTC CCG ATT 156  
 Ala Gln Lys Gly  
                   50

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 51

(B) TYPE: Amino Acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Glu Pro Asp Glu Ile Cys Arg Ala Arg Met Thr His Lys Glu Phe  
 1                  5                  10                  15  
 Asn Tyr Lys Ser Asn Val Cys Asn Gly Cys Gly Asp Gln Val Ala Ala  
                   20                  25                  30  
 Cys Glu Ala Glu Cys Phe Arg Asn Asp Val Tyr Thr Ala Cys His Glu  
                   35                  40                  45

Ala Gln Lys NH<sub>2</sub>  
50

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 68

(B) TYPE: Amino Acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Lys Leu Gln Leu Met Ile Cys Leu Val Leu Leu Pro Cys Phe Phe  
-15 -10 -5

Cys Glu Pro Asp Glu Ile Cys Arg Ala Arg Met Thr His Lys Glu Phe  
1 5 10 15

Asn Tyr Lys Ser Asn Val Cys Asn Gly Cys Gly Asp Gln Val Ala Ala  
20 25 30

Cys Glu Ala Glu Cys Phe Arg Asn Asp Val Tyr Thr Ala Cys His Glu  
35 40 45

Ala Gln Lys Gly  
50

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 51
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TAC	TTC	GAT	GTC	AAC	TAC	TAA	ACA	AAC	CAA	GAA	GAC	GGG	ACG	AAG	AAG	48
Met	Lys	Leu	Gln	Leu	Met	Ile	Cys	Leu	Val	Leu	Leu	Pro	Cys	Phe	Phe	
		-15					-10				-5					
ACG																51
Cys																
-1																

50

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17
- (B) TYPE: Amino Acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: Unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Lys Leu Gln Leu Met Ile Cys Leu Val Leu Leu Pro Cys  
 -15 -10 -5

Phe Phe Cys  
 -1

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 398

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTTTCAGTAA ACCTTTGAGA GGAAAAAGAC GTGTTAGATG TCGAACAGTG AGATGTCACT 60

CA TAC TTC GAT GTC AAC TAC TAA ACA AAC CAA GAA GAC GGG ACG AAG AAG 110

Met Lys Leu Gln Leu Met Ile Cys Leu Val Leu Leu Pro Cys Phe Phe  
 -15 -10 -5

ACG CTT GGG CTG CTT TAG ACG TCT CGA TCT TAC TGT TTG TTC CTC AAA 158

Cys Glu Pro Asp Glu Ile Cys Arg Ala Arg Met Thr Asn Lys Glu Phe  
 1 5 10 15

TGC ATG TTT TCG TTA CAG ACG TTA TTA ACA CCA CTA GTT CAC CGC CGA	206
Thr Tyr Lys Ser Asn Val Cys Asn Asn Cys Gly Asp Gln Val Ala Ala	
20 25 30	
ACG CTC CGA CTT ACG AAG GCT TTA CTA CAA ATA TGT CGT ACA GTA CTC	254
Cys Glu Ala Glu Cys Phe Arg Asn Asp Val Tyr Thr Ala Cys His Glu	
35 40 45	
CGT GTT TTC CCT ATT C ATTGTCTGTA ATCTTACAAA GTGAAACTTA CGAAAAGACA	310
Ala Gln Lys Gly	
50	
TTTCGCACTT TCAAGACAAT GAGTGGAAC TGCCATATAA AGGTACACAT TATATGAAAC	370
TTAAATTTAT TTATTTATTT TTTTTTTT	398

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 153

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTT GGG CTG CTT TAG ACG TCT CGA TCT TAC TGT TTG TTC CTC AAA	45
Glu Pro Asp Glu Ile Cys Arg Ala Arg Met Thr Asn Lys Glu Phe	
1 5 10 15	
TGC ATG TTT TCG TTA CAG ACG TTA TTA ACA CCA CTA GTT CAC CGC CGA	93
Thr Tyr Lys Ser Asn Val Cys Asn Asn Cys Gly Asp Gln Val Ala Ala	
20 25 30	
ACG CTC CGA CTT ACG AAG GCT TTA CTA CAA ATA TGT CGT ACA GTA CTC	141
Cys Glu Ala Glu Cys Phe Arg Asn Asp Val Tyr Thr Ala Cys His Glu	
35 40 45	
CGT GTT TTC CCT ATT	156
Ala Gln Lys Gly	
50	

53

- (2) INFORMATION FOR SEQ ID NO:9:
- (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 51
    - (B) TYPE: Amino Acid
    - (C) STRANDEDNESS:
    - (D) TOPOLOGY: Unknown



(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:  
 Glu Pro Asp Glu Ile Cys Arg Ala Arg Met Thr Asn Lys Glu Phe  
 1 5 10 15

Thr Tyr Lys Ser Asn Val Cys Asn Asn Cys Gly Asp Gln Val Ala Ala  
 20 25 30

Cys Glu Ala Glu Cys Phe Arg Asn Asp Val Tyr Thr Ala Cys His Glu  
 35 40 45

Ala Gln Lys NH<sub>2</sub>  
 50

54

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 68

(B) TYPE: Amino Acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:  
 Met Lys Leu Gln Leu Met Ile Cys Leu Val Leu Leu Pro Cys Phe Phe  
 -15 -10 -5

Cys Glu Pro Asp Glu Ile Cys Arg Ala Arg Met Thr Asn Lys Glu Phe  
 1 5 10 15

Thr Tyr Lys Ser Asn Val Cys Asn Asn Cys Gly Asp Gln Val Ala Ala  
20 25 30

Cys Glu Ala Glu Cys Phe Arg Asn Asp Val Tyr Thr Ala Cys His Glu  
35 40 45

Ala Gln Lys Gly  
50

**(2) INFORMATION FOR SEQ ID NO:11:**

**(i) SEQUENCE CHARACTERISTICS:**

(A) LENGTH: 252

**(B) TYPE: Nucleic Acid**

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CTGTTTAACT GTGCACCTTA GCAAGTCGGC ACTTGTCTGGT ACTTA TAC TTC GAT GTC 57  
Met Lys Leu Gln  
-15

AAC TAC TAA ACA AAC CAA GAA GAC GGG ACG AAG AAG ACG CTT GGG CTG 105  
Leu Met Ile Cys Leu Val Leu Leu Pro Cys Phe Phe Cys Glu Pro Asp  
-10 -5 1

CTC TAG ACG TCT CGA TCT TAC TGT TTG TTC CTC AAA TGA ATG TTT TCG 153  
 Glu Ile Cys Arg Ala Arg Met Thr Asn Lys Glu Phe Thr Tyr Lys Ser  
           5                          10                          15

TTA CAG ACG TTA CCA ACA CCA CTA GTT CAC CGC CGA ACG CTC CGA CTT 201  
 Asn Val Cys Asn Gly Cys Gly Asp Gln Val Ala Ala Cys Glu Ala Glu  
           20                          25                          30                          35

ACG AAG TCT TTG CTA CAA ATA TGT CGT ACA GTA CTT CGT GTT TTC CCG 249  
 Cys Phe Arg Asn Asp Val Tyr Thr Ala Cys His Glu Ala Gln Lys Gly  
                           40                          45                          50

ATT 252

56

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 204

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TAC TTC GAT GTC AAC TAC TAA ACA AAC CAA GAA GAC GGG ACG AAG AAG 48  
 Met Lys Leu Gln Leu Met Ile Cys Leu Val Leu Leu Pro Cys Phe Phe  
           -15                          -10                          -5

ACG CTT GGG CTG CTC TAG ACG TCT CGA TCT TAC TGT TTG TTC CTC AAA 96  
 Cys Glu Pro Asp Glu Ile Cys Arg Ala Arg Met Thr Asn Lys Glu Phe  
 1 5 10 15

TGA ATG TTT TCG TTA CAG ACG TTA CCA ACA CCA CTA GTT CAC CGC CGA 144  
 Thr Tyr Lys Ser Asn Val Cys Asn Gly Cys Gly Asp Gln Val Ala Ala  
 20 25 30

ACG CTC CGA CTT ACG AAG TCT TTG CTA CAA ATA TGT CGT ACA GTA CTT 192  
 Cys Glu Ala Glu Cys Phe Arg Asn Asp Val Tyr Thr Ala Cys His Glu

CGT GTT TTC CCG ATT 207  
 Ala Gln Lys Gly

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 68

(B) TYPE: Amino Acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Met Lys Leu Gln Leu Met Ile Cys Leu Val Leu Leu Pro Cys Phe Phe  
-15 -10 -5

Cys Glu Pro Asp Glu Ile Cys Arg Ala Arg Met Thr Asn Lys Glu Phe  
1 5 10 15

Thr Tyr Lys Ser Asn Val Cys Asn Gly Cys Gly Asp Gln Val Ala Ala  
20 25 30

Cys Glu Ala Glu Cys Phe Arg Asn Asp Val Tyr Thr Ala Cys His Glu  
35 40 45

Ala Gln Lys Gly  
50

**(2) INFORMATION FOR SEQ ID NO:14:****(i) SEQUENCE CHARACTERISTICS:****(A) LENGTH: 51****(B) TYPE: Amino Acid****(C) STRANDEDNESS:****(D) TOPOLOGY: Unknown****(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:**

Glu Pro Asp Glu Ile Cys Arg Ala Arg Met Thr Asn Lys Glu Phe  
1 5 10 15

Thr Tyr Lys Ser Asn Val Cys Asn Gly Cys Gly Asp Gln Val Ala  
20 25 30

Ala Cys Glu Ala Glu Cys Phe Arg Asn Asp Val Tyr Thr Ala Cys  
35 40 45

His Glu Ala Gln Lys NH<sub>2</sub>  
50

**THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:**

1. An insecticidally effective peptide derived from a spider of the genus Tegenaria and having substantially the same amino acid sequence as defined in SEQ ID NO:3 as follows:

Glu	Pro	Asp	Glu	Ile	Cys	Arg	Ala	Arg	Met	Thr	His	Lys	Glu	Phe	Asn	Tyr	Lys
1				5					10					15			
Ser	Asn	Val	Cys	Asn	Gly	Cys	Gly	Asp	Gln	Val	Ala	Ala	Cys	Glu	Ala	Glu	
	20				25				30							35	
Cys	Phe	Arg	Asn	Asp	Val	Tyr	Thr	Ala	Cys	His	Glu	Ala	Gln	Lys	NH <sub>2</sub>		
			40					45						50			

and agriculturally or horticulturally acceptable salts thereof.

2. An insecticidally effective peptide derived from a spider of the genus Tegenaria and having substantially the same amino acid sequence as defined in SEQ ID:9 as follows:

Glu	Pro	Asp	Glu	Ile	Cys	Arg	Ala	Arg	Met	Thr	Asn	Lys	Glu	Phe	Thr	Tyr	Lys
1				5					10					15			
Ser	Asn	Val	Cys	Asn	Asn	Cys	Gly	Asp	Gln	Val	Ala	Ala	Cys	Glu	Ala	Glu	
	20				25				30							35	
Cys	Phe	Arg	Asn	Asp	Val	Tyr	Thr	Ala	Cys	His	Glu	Ala	Gln	Lys	NH <sub>2</sub>		
			40					45						50			

and agriculturally or horticulturally acceptable salts thereof.

3. An insecticidally effective peptide derived from a spider of the genus Tegenaria and having substantially the same amino acid sequence as defined in SEQ ID:14 as follows:

Glu	Pro	Asp	Glu	Ile	Cys	Arg	Ala	Arg	Met	Thr	Asn	Lys	Glu	Phe	Thr	Tyr	Lys
1				5					10					15			
Ser	Asn	Val	Cys	Asn	Gly	Cys	Gly	Asp	Gln	Val	Ala	Ala	Cys	Glu	Ala		
	20				25				30						35		

Glu Cys Phe Arg Asn Asp Val Tyr Thr Ala Cys His Glu Ala Gln Lys NH<sub>2</sub>

40

45

50

and agriculturally or horticulturally acceptable salts thereof.

4. An insecticidally effective peptide derived from a spider of the genus *Tegenaria* and having substantially the same amino acid sequence as defined in SEQ ID:4 as follows:

Met Lys Leu Gln Leu Met Ile Cys Leu Val Leu Leu Pro Cys Phe Phe Cys

-15

-10

-5

Glu Pro Asp Glu Ile Cys Arg Ala Arg Met Thr His Lys Glu Phe Asn Tyr Lys

1

5

10

15

Ser Asn Val Cys Asn Gly Cys Gly Asp Gln Val Ala Ala Cys Glu Ala Glu

20

25

30

35

Cys Phe Arg Asn Asp Val Tyr Thr Ala Cys His Glu Ala Gln Lys Gly

40

45

50

and agriculturally or horticulturally acceptable salts thereof.

5. An insecticidally effective peptide derived from a spider of the genus *Tegenaria* and having substantially the same amino acid sequence as defined in SEQ ID:10 as follows:

Met Lys Leu Gln Leu Met Ile Cys Leu Val Leu Leu Pro Cys Phe Phe Cys

-15

-10

-5

Glu Pro Asp Glu Ile Cys Arg Ala Arg Met Thr Asn Lys Glu Phe Thr Tyr Lys

1

5

10

15

Ser Asn Val Cys Asn Asn Cys Gly Asp Gln Val Ala Ala Cys Glu Ala Glu

20

25

30

35

Cys Phe Arg Asn Asp Val Tyr Thr Ala Cys His Glu Ala Gln Lys Gly

40

45

50

and agriculturally or horticulturally acceptable salts thereof.



6. An insecticidally effective peptide derived from a spider of the genus *Tegenaria* and having substantially the same amino acid sequence as defined in SEQ ID:13 as follows:

Met	Lys	Leu	Gln	Leu	Met	Ile	Cys	Leu	Val	Leu	Leu	Pro	Cys	Phe	Phe	Cys	
	-15				-10					-5							
Glu	Pro	Asp	Glu	Ile	Cys	Arg	Ala	Arg	Met	Thr	Asn	Lys	Glu	Phe	Thr	Tyr	Lys
1			5						10					15			
Ser	Asn	Val	Cys	Asn	Gly	Cys	Gly	Asp	Gln	Val	Ala	Ala	Cys	Glu	Ala	Glu	
	20				25					30						35	
Cys	Phe	Arg	Asn	Asp	Val	Tyr	Thr	Ala	Cys	His	Glu	Ala	Gln	Lys	Gly		
			40					45					50				

and agriculturally or horticulturally acceptable salts thereof.

7. The peptide of any one of claims 1 to 6 characterized in that the species of spider is *Tegenaria agrestis*.

8. A DNA sequence encoding the insecticidally effective peptide according to claim 1 and substantially defined in SEQ ID NO:2 as follows:

CTT GGG CTG CTC TAG ACG TCT CGA TCT TAC TGT GTG TTC CTC	42
AAA TTA ATG TTT TCG TTA CAG ACG TTA CCA ACA CCA CTA GTT	84
CAC CGC CGA ACG CTC CGA CTT ACG AAG TCT TTG CTA CAA ATA	126
TGT CGT ACA GTA CTT CGT GTT TTC CCG ATT	156

9. A DNA sequence encoding the insecticidally effective peptide according to claim 2 and substantially defined in SEQ ID NO:8 as follows:

CTT GGG CTG CTT TAG ACG TCT CGA TCT TAC TGT TTG TTC CTC	42
AAA TGC ATG TTT TCG TTA CAG ACG TTA TTA ACA CCA CTA GTT	84
CAC CGC CGA ACG CTC CGA CTT ACG AAG GCT TTA CTA CAA ATA	126
TGT CGT ACA GTA CTC CGT GTT TTC CCT ATT	156

10. A DNA sequence encoding the insecticidally effective peptide according to claim 3 and substantially defined in SEQ ID NO:12 as follows:

TAC TTC GAT GTC AAC TAC TAA ACA AAC CAA GAA GAC GGG ACG	42
AAG AAG ACG CTT GGG CTG CTC TAG ACG TCT CGA TCT TAC TGT	84
TTG TTC CTC AAA TGA ATG TTT TCG TTA CAG ACG TTA CCA ACA CCA	129
CTA GTT CAC CGC CGA ACG CTC CGA CTT ACG AAG TCT TTG CTA	171
CAA ATA TGT CGT ACA GTA CTT CGT GTT TTC CCG ATT	207

11. A DNA sequence encoding the insecticidally effective peptide according to claim 4 and substantially defined in SEQ ID NO:1 as follows:

CTTTCAGTAA ACCTTTGAGA GGAAAAAGAC GTGTTAGATG	40
TCGAACAGCA AGATGTCACT TA TAC TTC GAT GTC AAC TAC TAA ACA	86
AAC CAA GAA GAC GGG ACG AAG AAG ACG CTT GGG CTG CTC TAG	128
ACG TCT CGA TCT TAC TGT GTG TTC CTC AAA TTA ATG TTT TCG TTA	173
CAG ACG TTA CCA ACA CCA CTA GTT CAC CGC CGA ACG CTC CGA	215
CTT ACG AAG TCT TTG CTA CAA ATA TGT CGT ACA GTA CTT CGT GTT	260
TTC CCG ATT C ATTGTCTGTA ATCTTACAAA GTGAAACTTA	300
CGAAACGATA TTTCGCAGTT TCAAGACAAT GAGTGGAAC	340
TGCCATATAA AGGTACACAT TATATGAAAC TTCGATTTAT TTATTTATTT	390
TTT	393

12. A DNA sequence encoding the insecticidally effective peptide according to claim 5 and substantially defined in SEQ ID NO:7 as follows:

CTTTCAGTAA ACCTTTGAGA GGAAAAAGAC GTGTTAGATG	40
TCGAACAGTG AGATGTCACT CA TAC TTC GAT GTC AAC TAC TAA ACA	86
AAC CAA GAA GAC GGG ACG AAG AAG ACG CTT GGG CTG CTT TAG	128
ACG TCT CGA TCT TAC TGT TTG TTC CTC AAA TGC ATG TTT TCG TTA	173
CAG ACG TTA TTA ACA CCA CTA GTT CAC CGC CGA ACG CTC CGA	215
CTT ACG AAG GCT TTA CTA CAA ATA TGT CGT ACA GTA CTC CGT GTT	260
TTC CCT ATT C ATTGTCTGTA ATCTTACAAA GTGAAACTTA	300
CGAAAAGACA TTTCGCAGTT TCAAGACAAT GAGTGGAAC	340
TGCCATATAA AGGTACACAT TATATGAAAC TTAAATTTAT TTATTTATTT	390
TTTTTTTT	398

13. A DNA sequence encoding the insecticidally effective peptide according to claim 6 and substantially defined in SEQ ID NO:11 as follows:

CTGTTTAACT GTGCACCTTA GCAAGTCGGC ACTTGTCGGT ACTTA TAC	48
TTC GAT GTC AAC TAC TAA ACA AAC CAA GAA GAC GGG ACG AAG	90
AAG ACG CTT GGG CTG CTC TAG ACG TCT CGA TCT TAC TGT TTG	132
TTC CTC AAA TGA ATG TTT TCG TTA CAG ACG TTA CCA ACA CCA CTA	177
GTT CAC CGC CGA ACG CTC CGA CTT ACG AAG TCT TTG CTA CAA	219
ATA TGT CGT ACA GTA CTT CGT GTT TTC CCG ATT	252

14. A recombinant expression vector comprising a DNA sequence encoding the insecticidally effective peptide according to any one of claims 1 to 6, wherein the vector is capable of effecting the expression of said coding sequence in transformed cells.

15. A method for cultivating a transgenic plant containing a DNA sequence encoding the insecticidally effective peptide according to any one of claims 1 to 6, the said method comprising introducing said DNA into the germ line of said plant, or an ancestor of said plant, such that the trait of expression of said DNA sequence is inherited by subsequent generations of said plant through sexual propagation or asexual propagation.

16. A transgenic plant comprising a DNA sequence encoding a insecticidally effective peptide said peptide characterized by:

- a) being about 51 amino acids in length;
- b) having 6 cysteine residues in positions 6, 22, 25, 32, 36 and 45; and
- c) having 80% homology with the peptide defined in SEQ ID NO:3, wherein said DNA is introduced into the germ line of said plant, or an ancestor of said plant, such that the trait of expression of said DNA sequence is inherited by subsequent generations of said plant through sexual propagation or asexual propagation.



17. A recombinant baculovirus expression vector, capable of expressing a DNA sequence encoding the insecticidally effective peptide according to any one of claims 1 to 6 in a host or in a host insect cell.
18. A method of producing the insecticidally effective peptide according to any one of claims 1 to 6, which method comprises:
- a) culturing recombinant host cells wherein a recombinant expression vector transformed or transfected in said host cells has a DNA sequence encoding said peptide, wherein the vector is capable of effecting the expression of said coding sequence in transformed cells; and
  - b) recovering said insecticidally effective peptide from the recombinant host cell culture.
19. A recombinantly produced peptide produced by the method of claim 18.
20. A method of controlling invertebrate pests comprising contacting said pests with an effective amount of the peptide according to any one of claims 1 to 6.
21. A method of controlling invertebrate pests comprising contacting said pests with a recombinant baculovirus capable of expressing an effective amount of the peptide according to any one of claims 1 to 6 in said pests.
22. An insecticidal composition comprising an insecticidally effective amount of the peptide according to any one of claims 1 to 6, and agriculturally or horticulturally acceptable salts thereof in an agriculturally or horticulturally acceptable carrier therefor.
23. An antibody substantially immunoreactive with the peptide according to any one of claims 1 to 6.



24. A DNA probe derived from a DNA sequence of any one of claims 8 to 13 encoding the insecticidally effective peptide according to any one of claims 1 to 6 respectively.

25. The peptide according to any one of claims 1 to 6 conjugated to a label or carrier.

26. The DNA according to any one of claim 8 to 13 conjugated to a label or carrier.

27. The peptide according to any one of claims 1 to 6 further comprising the signal peptide having the amino acid sequence in SEQ ID NO:6 as follows:

Met	Lys	Leu	Gln	Leu	Met	Ile	Cys	Leu	Val	Leu	Leu	Pro	Cys	Phe	Phe	Cys
				-15					-10						-5	1

28. The DNA according to any one of claims 8 to 13 further comprising the DNA sequence encoding the signal sequence as defined in SEQ ID NO:6.

**DATED** this 21st day of August, 1995.

**FMC CORPORATION and**  
**NPS PHARMACEUTICALS, INC.**

WATERMARK PATENT & TRADEMARK ATTORNEYS  
290 BURWOOD ROAD  
HAWTHORN VICTORIA 3122  
AUSTRALIA

(DBM/CJH/SH:KP DOC 3 AU3588993.WPC)





10			20			30			40			50			60				
*			*			*			*			*			*				
GAAAGTCATT			TGGAAACTCT			CCTTTTTTCTG			CACAATCTAC			AGCTTGTCGT			TCTACAGTGA				
CTTTCAGTAA			ACCTTTGAGA			GGAAAAAGAC			GTGTTAGATG			TCGAACAGCA			AGATGTCACT				
70				80				90				100				110			
*				*				*				*				*			
AT	ATG	AAG	CTA	CAG	TTG	ATG	ATT	TGT	TTG	GTT	CTT	CTG	CCC	TGC	TTC	TTC			
TA	TAC	TTC	GAT	GTC	AAC	TAC	TAA	ACA	AAC	CAA	GAA	GAC	GGG	ACG	AAG	AAG			
	Met	Lys	Leu	Gln	Leu	Met	Ile	Cys	Leu	Val	Leu	Leu	Pro	Cys	Phe	Phe>			

[illegible]

260		270		280		290		300		310
*		*		*		*		*		*
GCA CAA AAG	] NH <sub>2</sub>	GGC TAA G	TAACAGACAT	TAGAATGTTT	CACTTTGAAT	GCTTTGCTAT				
CGT GTT TTC		CCG ATT C	ATTGTCTGTA	ATCTTACAAA	GTGAAACTTA	CGAAACGATA				
Ala Gln Lys		Gly *** Xxx>								
320		330		340		350		360		370
*		*		*		*		*		*
AAAGCGTCAA	AGTTCTGTTA	CTCACCTTGA	ACGGTATATT	TCCATGTGTA	ATATACTTTG					
TTTCGCAGTT	TCAAGACAAT	GAGTGGAAC	TGCCATATAA	AGGTACACAT	TATATGAAAC					
380		390								
*		*								
AAGCTAAATA	AATAAATAAA	AAAA								
TTCGATTTAT	TTATTTATTT	TTTT								

3/8

FIG. 2B



10                      20                      30                      40                      50                      60  
 \*                      \*                      \*                      \*                      \*                      \*  
 GAAAGTCATT TGGAAACTCT CCTTTTCTG CACAATCTAC AGCTTGTCAC TCTACAGTGA  
 CTTTCAGTAA ACCTTTGAGA GGAAAAAGAC GTGTTAGATG TCGAACAGTG AGATGTCACT  
  
 70                      80                      90                      100                      110  
 \*                      \*                      \*                      \*                      \*  
 GT ATG AAG CTA CAG TTG ATG ATT TGT TTG GTT CTT CTG CCC TGC TTC TTC  
 CA TAC TTC GAT GTC AAC TAC TAA ACA AAC CAA GAA GAC GGG ACG AAG AAG  
Met Lys Leu Gln Leu Met Ile Cys Leu Val Leu Leu Pro Cys Phe Phe>

120                      130                      140                      150  
 \*                      \*                      \*                      \*  
 TGC GAA CCC GAC GAA ATC TGC AGA GCT AGA ATG ACA AAC AAG GAG TTT  
 ACG CTT GGG CTG CTT TAG ACG TCT CGA TCT TAC TGT TTG TTC CTC AAA  
Cys Glu Pro Asp Glu Ile Cys Arg Ala Arg Met Thr Asn Lys Glu Phe>

160                      170                      180                      190                      200  
 \*                      \*                      \*                      \*                      \*  
 ACG TAC AAA AGC AAT GTC TGC AAT AAT TGT GGT GAT CAA GTG GCG GCT  
 TGC ATG TTT TCG TTA CAG ACG TTA TTA ACA CCA CTA GTT CAC CGC CGA  
 Thr Tyr Lys Ser Asn Val Cys Asn Asn Cys Gly Asp Gln Val Ala Ala>

210                      220                      230                      240                      250  
 \*                      \*                      \*                      \*                      \*  
 TGC GAG GCT GAA TGC TTC CGA AAT GAT GTT TAT ACA GCA TGT CAT GAG  
 ACG CTC CGA CTT ACG AAG GCT TTA CTA CAA ATA TGT CGT ACA GTA CTC  
 Cys Glu Ala Glu Cys Phe Arg Asn Asp Val Tyr Thr Ala Cys His Glu>

4/8

FIG. 3A

260		270		280		290		300		310
*		*		*		*		*		*
GCA CAA AAG	] NH <sub>2</sub>	GGA TAA G	TAACAGACAT	TAGAATGTTT	CACTTTGAAT	GCTTTTCTGT				
CGT GTT TTC		CCT ATT C	ATTGTCTGTA	ATCTTACAAA	GTGAAACTTA	CGAAAAGACA				
Ala Gln Lys		Gly *** Xxx>								
320		330		340		350		360		370
*		*		*		*		*		*
AAAGCGTGAA	AGTTCTGTTA	CTCACCTTGA	ACGGTATATT	TCCATGTGTA	ATATACTTTG					
TTTCGCACTT	TCAAGACAAT	GAGTGGAAC	TGCCATATAA	AGGTACACAT	TATATGAAAC					
380		390								
*		*								
AATTTAAATA	AATAAATAAA	AAAAAAAAA								
TTAAATTTAT	TTATTTATTT	TTTTTTTTT								

5/8

FIG. 3B

10	20	30	40	50	
*	*	*	*	*	
GACAAATTGA	CACGTGGAAT	CGTTCAGCCG	TGAACAGCCA	TGAAT ATG AAG CTA CAG	
CTGTTTAACT	GTGCACCTTA	GCAAGTCGGC	ACTTGTCGGT	ACTTA TAC TTC GAT GTC	
				<u>Met Lys Leu Gln&gt;</u>	
60	70	80	90	100	
*	*	*	*	*	
TTG ATG ATT TGT TTG GTT CTT CTG CCC TGC TTC TTC TGC	GAA CCC GAC				
AAC TAC TAA ACA AAC CAA GAA GAC GGG ACG AAG AAG ACG	CTT GGG CTG				
Leu Met Ile Cys Leu Val Leu Leu Pro Cys Phe Phe Cys	Glu Pro Asp>				
110	120	130	140	150	
*	*	*	*	*	
GAG ATC TGC AGA GCT AGA ATG ACA AAC AAG GAG TTT ACT TAC AAA AGC					
CTC TAG ACG TCT CGA TCT TAC TGT TTG TTC CTC AAA TGA ATG TTT TCG					
Glu Ile Cys Arg Ala Arg Met Thr Asn Lys Glu Phe Thr Tyr Lys Ser>					

6/8

FIG. 4A

160	170	180	190	200	
*	*	*	*	*	
AAT GTC TGC AAT GGT TGT GGT GAT CAA GTG GCG GCT TGC GAG GCT GAA					
TTA CAG ACG TTA CCA ACA CCA CTA GTT CAC CGC CGA ACG CTC CGA CTT					
Asn Val Cys Asn Gly Cys Gly Asp Gln Val Ala Ala Cys Glu Ala Glu>					
210	220	230	240		
*	*	*	*		
TGC TTC AGA AAC GAT GTT TAT ACA GCA TGT CAT GAA GCA CAA AAG					
ACG AAG TCT TTG CTA CAA ATA TGT CGT ACA GTA CTT CGT GTT TTC					
Cys Phe Arg Asn Asp Val Tyr Thr Ala Cys His Glu Ala Gln Lys					
					GGC
					CCG
					Gly>
					NH <sub>2</sub>

7/8

250  
\*  
TAA  
ATT  
\*\*\*>

FIG. 4B

8/8

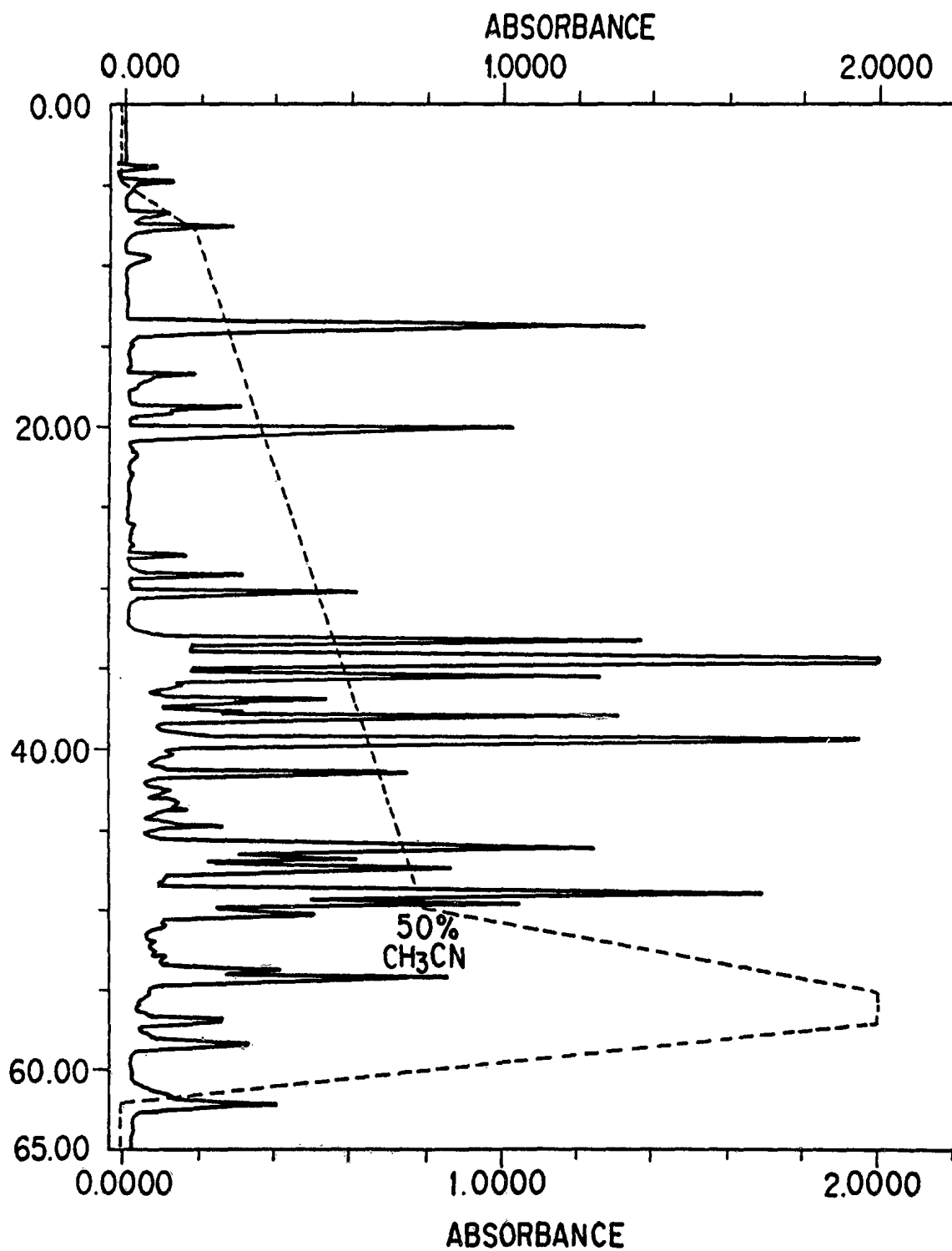


FIG. 5

## INTERNATIONAL SEARCH REPORT

 International application No.  
 PCT/US93/00558
**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : C12N 15/00, 15/12, 15/63; A01H 1/00, 5/00

US CL : Please See Extra Sheet.

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)

U.S. : 800/205; 536/27; 435/172.3, 240.2, 240.4; 935/22, 32, 55; 530/300, 345, 387.1

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	A.B. BORKOVEC et al, "INSECT NEUROCHEMISTRY AND NEUROPHYSIOLOGY" published 1986 by The Humana Press (Clifton, NJ), pages 397-400, see entire document.	1-34
Y	BioTechniques, Vol. 3, No. 4, issued 1985, Bers et al, "Protein and Nucleic Acid Blotting and Immunobiochemical Detection", pages 276-288, see page 281.	33
Y	Toxicon, Vol. 25, No. 2, issued 1987, D.K. Vest, "Necrotic Arachnidism in the Northwest United States and Its Probable Relationship to Tegenaria Agrestis (Walckenaer) Spiders", pages 175-184, see page 175.	2-3, 13-14, 18-22, 34

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*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
*A document defining the general state of the art which is not considered to be part of particular relevance	*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
*E earlier document published on or after the international filing date	*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
*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)	*Z document member of the same patent family
*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	

Date of the actual completion of the international search

17 March 1993

Date of mailing of the international search report

30 MAR 1993

 Name and mailing address of the ISA/US  
 Commissioner of Patents and Trademarks  
 Box PCT  
 Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

Authorized officer

ELIZABETH F. MCLEWAIN

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/00558

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Canada, 2,005,658 (Zlotkin et al) 19 June 1990, see pages 51-56.	12-29, 31, 34
Y	Proceedings of the National Academy of Sciences, Vol. 77, No.3, issued March 1980, G. Blobel, "Intracellular Protein Topogenesis", pages 1496-1500, see entire document.	7-11, 18-22
Y	Gene, Vol. 73, issued 1988, Carbonell et al, "Synthesis of a Gene Coding for an Insect-Specific Scorpion Neurotoxin and Attempts to Express It Using Baculovirus Vectors", pages 409-418, see page 409.	25, 29
Y	BioTechniques, Vol. 8, No. 3, issued 1990, Bronstein et al, "Rapid and Sensitive Detection of DNA in Southern Blots with Chemiluminescence", pages 310-314, see entire document.	34

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/00558

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

800/205; 536/27; 435/172.3, 240.2, 240.4; 935/22, 32, 55; 530/300, 345, 387.1