Title: NOVEL mu-CONOTOXIN PEPTIDES

Abstract: Novel mu-conotoxin peptides and derivatives thereof are described together with their use as neuronally active sodium channel inhibitors (antagonists), in assays and probes and also in the treatment of conditions involving pain, cancer, epilepsy and cardio-vascular diseases.
NOVEL mu-CONOTOXIN PEPTIDES

Field of the Invention
The invention relates to novel μ-conotoxin peptides and derivatives thereof, and their use as neuronally active sodium channel inhibitors (antagonists). The invention also relates to pharmaceutical compositions comprising these peptides and nucleic acid probes useful in finding active analogues of these peptides. The invention further relates to the use of these peptides in assays, such as for finding compounds which are active sodium channel inhibitors, and the use of these peptides in the prophylaxis or treatment of conditions such as pain including neuropathic and chronic pain, gastrointestinal pain, visceral pain, post surgical and peripheral pain, tumour and cancer-related pain and tumour reduction, stroke, epilepsy and cardiovascular conditions.

Background of the invention
Ion channels are intimately linked to all neurotransmission and neurotransmitter release processes, but in disease states often contribute adversely to disease pathology. The diversity and distribution of ion channel types and sub-types being uncovered through the use of molecular biology and toxin probes presents an exciting opportunity for the discovery of new classes of therapeutics. Among these ion channels are the voltage sensitive sodium channels (“VSSC”) which open and then close (inactivate) in response to membrane depolarisation.

Based on their susceptibility to block by tetrodotoxin (TTX), VSSCs can be divided into tetrodotoxin sensitive (TTX-S) and TTX-resistant (TTX-R) classes. Members of both classes share considerable sequence homology and are closely related structurally (Goldin AL. et al. (2000) Neuron. 28, 365-368). These include the neuronal TTX-S type I/Na\textsubscript{v}1.1, II/Na\textsubscript{v}1.2, III/Na\textsubscript{v}1.3, PN1/Na\textsubscript{v}1.7 and PN4/Na\textsubscript{v}1.6, and the skeletal muscle TTX-S μ1/Na\textsubscript{v}1.4. The TTX-R sodium channels include the cardiac H1/Na\textsubscript{v}1.5 which is partially TTX-resistant, and the neuronal TTX-R SNS/PN3/Na\textsubscript{v}1.8 and NaN/PN5/Na\textsubscript{v}1.9 (Goldin et al., 2000).
It is suspected that specific subtypes of the VSSC are involved in disease states such as pain, stroke and epilepsy. A number of these VSSC subtypes have been implicated in clinical states such as pain (Akopian et al. (1999) Nat. Neurosci. 6, 541-548), stroke (Taylor et al. (1995) Trends Pharmacol Sci. 16, 309-316) and epilepsy (Ragsdale et al (1998) Brain Res. Rev. 26, 16-28). Persistent (non-inactivating) forms of the TTX-S sodium channel current that underlie repetitive firing (Smith et al. (1998) J Neurosci. 18, 6093-6102; Bevan et al (1999) J. Neurosci. 19, 7617-7628) have less well-defined origins, but appear to involve PN4 (Smith et al (1998) J. Neurosci. 18, 6093-6102) and are enhanced by hypoxia (Hammarström et al. (2000) J. Physiol. 529 Pt 1, 107-118). Oxygen deprivation can lead to VSSC-related over-excitability that may be the molecular mechanism for some neurological sequelae, such as epilepsy resulting from perinatal hypoxic encephalopathy (Xia et al (2000) Brain Res. Mol. Brain Res. 76, 211-219).

It is thought that PN3/SNS/Na\textsubscript{v}1.8, a TTX-R sodium channel located specifically in sensory neurons (Akopian et al., (1996) Nature 379, 257-62; Rabert et al., (1998) Pain 78, 107-114), plays a key role in pain (Porreca et al., (1999) PNAS 96, 7640-7644). Another TTX-R form, NaN (Na\textsubscript{v}1.9), which has defied attempts to express VSSCs in oocytes and mammalian cells, may underlie a persistent TTX-R current in DRG but appears also to play an important role in pain (Cummins et al., (1999) J Neurosci. 19, RC43 (1-6); Baker MD et al., (2003 J Physiol.,548(Pt 2):373-382.)). PN3 produces the slow TTX-R sodium current in DRG neurons (Akopian et al., 1999; Renganathan et al., (2000) J Neurophysiol. 84, 710-718) and antisense targeting of TTX-R sodium channels in sensory neurons inhibits neuropathic pain (Lai et al., (2000) Methods Enzymol. 314, 201-213). In addition, PN3-null mutant mice display pronounced analgesia to noxious mechanical stimuli, small deficits in noxious thermoreception and delayed development of inflammatory hyperalgesia (Akopian et al., 1999). This data indicates that PN3 is involved in pain pathways and suggest that blockade of SNS (and NaN) expression or function may produce analgesia without side effects.
Recently it has been demonstrated that TTX-S VSSC subtypes, PN4/Na\textsubscript{v}1.6 and type I/Na\textsubscript{v}1.1, are significantly up-regulated in response to peripheral axotomy indicating their potential role in neuropathic pain control (Yang et al, 2004).

VSSCs are inhibited by local anaesthetics and modulated by toxins that act at one or more inhibitory sites. Figure 1 depicts a VSSC having at least 5 sites which result in excitatory actions. TTX and saxitoxin (STX) bind to VSSCs and are frequently used in RLB assays and probes to characterise VSSCs. However, the combination of several factors limits the usefulness of TTX and STX as probes of the VSSCs. For example, although potent, neither is selective towards different VSSC subtypes. Furthermore, TTX and STX are difficult to synthesise and can be difficult to obtain. It is expected that they will become more difficult to obtain as they have been listed as potential bioterrorism agents. Also, it is not readily possible to alter the characteristics of TTX and STX in order to make analogues having different and useful characteristics. Furthermore, many VSSCs are TTX-resistant which makes TTX not particularly useful for probing the characteristics of such VSSCs. There is thus a need for alternative new compounds for use in probing VSSCs and for use in new assays for identifying potentially useful sodium channel inhibitors.

Peptides with high affinity for their target are a better choice for radioligand binding (RLB) assays as they can be easy and relatively straightforward to synthesise. They can also provide a framework that may be altered to select for aspects of potency, selectivity and specificity.

The venom of marine snails of the genus *Conus* (cone snails) have provided a number of useful leads for the preparation of novel compounds that have potential therapeutic activity relating to VSSCs or could be used in assays or probes. Cone snails use a sophisticated biochemical strategy to capture their prey. As predators of fish, worms or other molluscs, the cone snails inject their prey with venom
containing a cocktail of small bioactive peptides. The toxin molecules, which are referred to as conotoxins, act by targeting a variety of receptors and ion-channels.

The venom from any single Conus species may contain more than 100 different peptides. The conotoxins are divided into classes on the basis of their physiological targets. The ω-conotoxin class of peptides target and block voltage-sensitive Ca^{2+}-channels inhibiting neurotransmitter release. The α-conotoxins and ψ-conotoxins target and block nicotinic ACh receptors, causing ganglionic and neuromuscular blockade. Peptides of the μ-conotoxin class act to block voltage-sensitive Na^{+}-channels inhibiting muscle and nerve action potential. The δ-conotoxins target and delay the inactivation of voltage-sensitive Na^{+}-channels, enhancing neuronal excitability. The κ-conotoxin class of peptides target and block voltage-sensitive K^{+}-channels, and these also cause enhanced neuronal excitability. The conopressins are vasopressin receptor antagonists and the conantokins are NMDA receptor antagonists. The γ-conotoxin class targets a voltage-sensitive nonspecific cation channel. The α-conotoxin class antagonises the 5HT_{3} receptor and the χ-conotoxin class inhibits neuronal amine transporters.

μ-Conotoxins are characterised by function (ability to block any VSSC subtype) and their three-loop structure, the latter which is defined by a distinctive pattern of disulfide bridges between the first and fourth, the second and fifth, and the third and sixth cysteines. To be included in this class, a conopeptide must display both of these characteristics. A number of the members of the three-loop μ-conotoxin class have been identified and their sequences published. GIIIA, GIIIB and GIIIC from C. geographus venom are potent blockers of skeletal muscle, but not neuronal VSSCs (Cruz et al., (1985) J Biol. Chem. 260, 9280-8). PIIIA from C. purpureascens was found to inhibit muscle and to a lesser extent neuronal TTX-S VSSC (Shon et al., (1996) J. Neurosci. 18, 6093-6102). The μ-conotoxin peptides identified in these publications have the following sequences:
μ-PIIIA  Xaa1 Arg Leu Cys Cys Gly Phe Xaa2 Lys Ser Cys Arg Ser Arg Gin Cys Lys Xaa2 His Arg Cys Cys [SEQ ID NO 1]
μ-GIIIA  Arg Asp Cys Cys Thr Xaa2 Xaa2 Lys Cys Lys Asp Arg Gin Cys Lys Xaa2 Gln Arg Cys Cys Ala [SEQ ID NO 2]
μ-GIIIB  Arg Asp Cys Cys Thr Xaa2 Xaa2 Arg Lys Cys Lys Asp Arg Arg Cys Lys Xaa2 Met Lys Cys Cys Ala [SEQ ID NO 3]
5 μ-GIIIC  Arg Asp Cys Cys Thr Xaa2 Xaa2 Lys Lys Cys Lys Asp Arg Arg Cys Lys Xaa2 Leu Lys Cys Cys Ala [SEQ ID NO 4]

In the above sequences the Xaa1 refers to pyroglutamate and Xaa2 refers to hydroxy proline (4Hyp). In nature, these amino acid residues result from post translational modification of the encoded peptide and are not directly encoded by the nucleotide sequence.

15 It is thought that these μ-conotoxins occlude the pore of VSSCs by competing with TTX and STX for binding at site 1 in the P-loop region of the α-subunit of the VSSC as depicted in figure 1.

Unfortunately, PIIIA, GIIIA, GIIIB and GIIIC have not been significantly useful in RLB assays or as probes to characterise neuronal VSSCs. These μ-conotoxins are not particularly potent at neuronal VSSCs and are either selective for skeletal muscle VSSCs (GIIIA, GIIIB and GIIIC) or are not able to discriminate between skeletal muscle and neuronal VSSC subtypes (PIIIA). Furthermore, it has been demonstrated that these peptides lack three-dimensional (3D) structural stability and are prone to conformational exchange in solution (Nielsen et al. (2002) J. Biol. Chem 277). For example, the only identifiable conformation identified for GIIIA-C via NMR studies is not the conformation in which the peptide blocks the target ion channel.

30 WO 02/07678 (University of Utah Research Foundation and Cognetix, Inc.) describes a large number of peptides as isolated μ-conotoxins. However, this
document provides an ambiguous and at times misleading description of the peptides so that it is difficult to rely on its disclosures. For the large part, most of the peptides described therein appear to have been only identified by molecular biology techniques, by the isolation and cloning of DNA coding for \( \mu \)-conotoxin peptides, translating and determining the toxin sequence. Reliance only on such techniques can cause errors, since in nature the active amino acid residues may result from post translational modification of the encoded peptide, some which can not be directly discovered from the nucleotide sequence. Additionally, it is not possible to determine the pattern of disulfide bridges from these techniques alone.

This is important as disulfide bridges form part of a peptide's structure, and have a determining influence on the 3D fold, and hence the function. This is apparent as a significant number of the peptides listed as \( \mu \)-conotoxins are simply not \( \mu \)-conotoxin peptides, as those peptides do not inhibit VSSCs and / or do not adopt the required combination of disulfide bridges and hence the overall tertiary fold that characterise and are necessary for the activity of a \( \mu \)-conotoxin. For example, of the peptides identified as \( \mu \)-conotoxins, those classified as types 1-3 and type 5 adopt configurations that preclude their inclusion in the \( \mu \)-conotoxin family. It is also unclear whether all of those classified as type 4 and 6 adopt the canonical \( \mu \)-conotoxin fold facilitated by the 1,4; 2,5 and 3,6 pattern of disulfide bridges.

Amongst the many peptide sequences described therein are the peptides S3.2 (striatus) and T3.1 (tulipa).

S3.2  
Xaa4 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Xaa6 Cys Arg Asp  
His Ala Arg Cys Cys  
[SEQ ID NO 5]

T3.1  
His Gly Cys Cys Lys Gly Xaa5 Xaa3 Gly Cys Ser Ser Arg Xaa3 Cys  
Arg Xaa5 Gln His Cys Cys  
[SEQ ID NO 6]

where Xaa3 is Glu or \( \gamma \)-carboxy Glu, Xaa4 is Gln or pyro-Glu, Xaa5 is Pro or hydroxy-Pro and Xaa6 is Trp or bromo-Trp.
Unlike other μ-conotoxins apparently tested therein, S3.2 is suggested to be selective for neuronal sodium channels of the CNS on the basis of the results of intrathecal (i.t.) CNS administration of the peptide into mice where paralysis followed by death ensued. In contrast, the intravenous (i.v.) injections of s3.2 into mice indicated that this peptide is not active at peripheral VSSCs, “unlike classic mu-conopeptides, s3.2 has no significant activity following intravenous administration (iv) to mice” (WO02/07678; page 89), and it was concluded that this peptide is selective for central neuronal VSSCs over muscle VSSCs. However, the experiments described in WO 02/07678 are not distinctive of VSSCs, rather, they merely demonstrate that the compounds tested are capable of affecting any number of central (but not peripheral) nervous system ion channels or receptors, including but not limited to sodium channels. The results could be interpreted to indicate that the peptide S3.2 is not active at peripheral neuronal VSSCs, including peripheral VSSCs in neurons that are critical for animal survival and, as such, teach that it has no application in the treatment of conditions mediated through such VSSC subtypes or use as a local or regional anaesthetic. In addition, this specification is wholly silent as to the extent of variation across the suite of VSSC subtypes, and the relevance of each subtype to specific disease states and conditions. For example, it is not at all clear as to which subtype of the VSCCs the disclosed sequences, particularly S3.2 are supposed to block. Example 5 describes techniques that would be used to demonstrate the effect of the μ-conotoptides as local anesthetics indicating the possibility of peripheral neuronal activity, yet no results are disclosed. The case is the same for experiments that are meant to indicate other models of pain, including neuropathic and acute pain, but yet again, no results are specifically disclosed leaving the reader confused as to the state of this particular prior art.

The article "μ-Conotoxin SmIIIA, a Potent Inhibitor of Tetrodotoxin-Resistant Sodium Channels in Amphibian Sympathetic and Sensory Neurons" (West et al., 2002 Biochemistry) describes the use of the μ-conotoxin peptide SmIIIA from the
molluscivorous *Conus stercusmuscarum* as a specific antagonist of tetrodotoxin-resistant sodium currents in frog neurons. While this does not preclude activity at mammalian VSSCs, this type of experiment by no means establishes such an activity because it well established that the VSSCs in amphibia are TTX-resistant irrespective of whether they are in sensory or other pathways (Fanzilber, M. et al (1995) *Biochemistry* 34(27), 8649-56), and there is no relationship between the inhibition of amphibian VSSCs and TTX-resistant mammalian VSSCs. The sequence for SmIIIA is as follows:

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SmIIIA    Xaa1 Arg Cys Cys Asn Gly Arg Arg Gly Cys Ser Ser Arg Trp Cys Arg
          Asp His Ser Arg Cys Cys
[SEQ ID NO 7]
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where Xaa1 is pyroglutamate

Thus there is still a need for new peptides for use in RLB assays and as probes to characterise VSSCs. The identification of new ligands displaying different binding profiles and affinities could provide new tools for further defining channel subtypes, or other receptors. Blockers of TTX-sensitive and/or resistant sodium channels can be useful in controlling seizures and pain, or may be used in the treatment diseases such as stroke, epilepsy and cardiovascular disease.

Conotoxins that selectively inhibit neuronal action potentials in the peripheral nervous system over muscle action potentials (and contractions) have utility in the treatment of acute and chronic pain, and could be administered locally near the site of injury or tumour, or on sensory nerves using cuffs or deposits to produce regional nerve block. Compounds which exhibit selectivity between neuronal and other VSSC subtypes in the peripheral nervous system and do not cross into the central nervous system following peripheral administration (i.e. oral, i.v, subcutaneous, intramuscular, rectal, nasal, lung, perineural) could be useful in the treatment of cardiovascular and neurological diseases, cancer and conditions including chronic and gut pain. It would also be preferable to provide more potent and/or more neuronally selective sodium channel blockers than that presently known, including TTX and STX, for use in assays when TTX and STX are
unavailable, or to increase the therapeutic window of existing VSSC blockers. Preferably the peptides should be relatively stable in 3D structure and should tolerate a broad range of substitutions in order to be useful as templates for the rational development of VSSC probes with altered selectivity.

5

Summary of the Invention

In an aspect of the present invention there is provided an isolated, synthetic or recombinant μ-conotoxin peptide which has the following sequence

10 Arg His Gly Cys Cys Lys Gly Xaa2 Lys Gly Cys Ser Ser Arg Glu Cys Arg Xaa2 Gln His Cys Cys [SEQ ID NO 8]

where Xaa2 is 4Hyp

and derivatives thereof.

15 The peptide of SEQ ID NO. 8 is TIIIA, a μ-conotoxin peptide that can be isolated from Conus tulipa.

Preferably the derivatives of the above conotoxin peptide exclude peptides of SEQ ID 6 above. More preferably all des Arg1-TIIIA derivatives are excluded.

In another aspect of the present invention there is provided an isolated, synthetic or recombinant μ-conotoxin peptide that is more potent than des Arg1-TIIIA at neuronal mammalian VSSCs.

25 In another aspect of the invention there is provided an isolated, synthetic or recombinant μ-conotoxin peptide that has a calculated log_{10}IC_{50} (M) value of ≤ -8.9, determined by displacement of ^{125}I TIIIA at neuronal mammalian VSSCs using the conditions described herein at example 5.
In another aspect of the invention there is provided an isolated, synthetic or recombinant \( \mu \)-conotoxin peptide that acts with a potency of from 1 pM to 10 \( \mu \)M at peripheral neuronal VSSCs as measured by displacement of \(^{125}\text{I}}\)-TIINA from sodium channels in membranes from dorsal root ganglia (DRG) (as described herein at example 5), the proportion of the sodium current blocked by the peptide in mammalian DRG (as described herein at example 11) or by inhibition of conduction of mylenated nerve bundles (also described herein at example 11). Those peptides with high potency are preferred.

In another aspect of the invention there is provided an isolated, synthetic or recombinant \( \mu \)-conotoxin peptide having an amino acid sequence as set out in Table 1 below, in which Z and pGlu represent pyroglutamate
Table 1 – General Peptide Sequences

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**Legend:**
- **PET/RIBE:** Protein engineered to include RIBE.
- **SiIB:** Site-specific integration bracket.

**Note:** The table represents a comparison of different constructs or sequences related to RIBE and SiIB with various combinations and modifications.
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|--------|---------|-------|-------|-------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| 10     | A15-TllIA | R     | H     | G     | C     | C     | K     | K     | G     | O     | K     | G     | C     | S     | S     | R     | A     | C     | R     | O     | -      | Q     | H     | C     | C     | -      |
| 11     | A15,A23-TllIA | R    | H     | G     | C     | C     | C     | K     | K     | G     | O     | K     | G     | C     | S     | S     | R     | A     | C     | R     | O     | -      | Q     | H     | C     | C     | A      |
| 12     | A2-TllIA   | R     | A     | G     | C     | C     | K     | K     | G     | O     | K     | G     | C     | S     | S     | R     | E     | C     | R     | O     | -      | Q     | H     | C     | C     | -      |
| 13     | A15,A23,A34-TllIA | R   | H     | G     | C     | C     | K     | K     | G     | O     | K     | G     | C     | S     | S     | R     | A     | C     | R     | O     | -      | Q     | H     | C     | C     | A      |
| 14     | Q2-TllIA   | R     | Q     | G     | C     | C     | C     | K     | G     | O     | K     | G     | C     | S     | S     | R     | E     | C     | R     | O     | -      | Q     | H     | C     | C     | A      |
| 15     | TllIA      | R     | H     | G     | C     | C     | K     | G     | O     | K     | G     | C     | S     | S     | R     | E     | C     | R     | O     | -      | A     | H     | C     | C     | -      |
| 16     | DesZ1, R11,R14,A15-SIIIB | -   | N     | -     | C     | C     | N     | G     | -     | -     | G     | C     | S     | S     | R     | W     | C     | R     | A     | H     | A     | R     | C     | C     | -      |
| 17     | DesZ1,R14, A15-SIIIB | -   | N     | -     | C     | C     | N     | G     | -     | -     | G     | C     | S     | S     | K     | W     | C     | R     | A     | H     | A     | R     | C     | C     | -      |
| 18     | A15,K23-TllIA | R   | H     | G     | C     | C     | K     | G     | O     | K     | G     | C     | S     | S     | R     | A     | C     | R     | O     | -      | Q     | H     | C     | C     | K      |
| 19     | DesZ1,R14, A15,A21-SIIIB | -   | N     | -     | C     | C     | N     | G     | -     | -     | G     | C     | S     | S     | K     | W     | C     | R     | A     | H     | A     | R     | C     | C     | A      |
| 20     | DesZ1,R14, A15,A21,A22-SIIIB | -   | N     | -     | C     | C     | N     | G     | -     | -     | G     | C     | S     | S     | K     | W     | C     | R     | A     | H     | A     | R     | C     | C     | A      |
| 45     | DesZ1,A15-SIIIB | -   | N     | -     | C     | C     | N     | G     | -     | -     | G     | C     | S     | S     | K     | W     | C     | K     | A     | H     | A     | R     | C     | C     | -      |</p>
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Particularly preferred are \( \mu \)-conotoxin peptides that selectively bind to mammalian neuronal VSSCs over skeletal muscle VSSCs. Preferably the peptides exhibit at least a 3-fold selectivity towards mammalian neuronal VSSCs over skeletal muscle VSSCs when measured by \(^{125}\text{I}-\text{TIIIA}\) displacement studies. Preferably the peptides exhibit selectivity for mammalian peripheral neuronal VSSCs over skeletal muscle VSSCs when measured by \(^{125}\text{I}-\text{TIIIA}\) displacement studies.

Thus, in another form of the present invention there is provided an isolated, synthetic or recombinant \( \mu \)-conotoxin peptide, which exhibits selectivity towards mammalian neuronal VSSCs over skeletal muscle VSSCs when measured by \(^{125}\text{I}-\text{TIIIA}\) displacement studies.

And in another aspect of the present invention is provided an isolated, synthetic or recombinant \( \mu \)-conotoxin peptide having a 3-fold or greater selectivity for mammalian neuronal VSSCs over skeletal muscle VSSCs when measured by \(^{125}\text{I}-\text{TIIIA}\) displacement studies.

Preferably the peptides have a 10-fold or greater, more preferably 100-fold or greater, and even more preferably a 1000-fold or greater selectivity towards mammalian neuronal VSSCs over skeletal muscle VSSCs when measured by \(^{125}\text{I}-\text{TIIIA}\) displacement studies.

In further aspect of the present invention there is provided an isolated, synthetic or recombinant \( \mu \)-conotoxin peptide having a 3-fold or greater selectivity for mammalian neuronal VSSCs over skeletal muscle VSSCs when measured by \(^{125}\text{I}-\text{TIIIA}\) displacement studies and which has the sequence:

\[
\text{Xaa1 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Lys Gly His Ala Arg Cys Cys}
\]

[SEQ ID NO 9]
where Xaa1 is pyroglutamate, or derivatives thereof, excluding the derivative of the sequence:

Xaa1 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Arg Asp His Ala Arg Cys Cys

[SEQ ID NO 109].

[SEQ. ID NO 9 and 109] are respectively conotoxins.

SIIIA and SIIIB are conotoxins from Conus striatus.

In another form of the present invention there is provided an isolated, synthetic or recombinant μ-conotoxin peptide, which exhibits selectivity towards mammalian peripheral neuronal VSSCs over skeletal muscle VSSCs.

Accordingly, in another aspect of the present invention is provided an isolated, synthetic or recombinant μ-conotoxin peptide having a 3-fold or greater selectivity for mammalian peripheral neuronal VSSCs over skeletal muscle VSSCs. Preferably these peptides have a 10-fold or greater, more preferably 100-fold or greater, and more preferably a 1000 fold or greater selectivity towards mammalian peripheral neuronal VSSCs over skeletal muscle VSSCs.

In another aspect of the present invention there is provided an isolated, synthetic or recombinant μ-conotoxin peptide having a 3-fold or greater selectivity for selectivity towards mammalian peripheral neuronal VSSCs over skeletal muscle VSSCs and which has the sequence:

Xaa1 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Lys Gly His Ala Arg Cys Cys

[SEQ ID NO 9]

or
Xaa1 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Arg Asp His Ala Arg Cys Cys

[SEQ ID NO 109]

or derivatives thereof, where Xaa1 is pyroglutamate.

In another aspect of the present invention there is provided a means for selectively inhibiting neuronal action potentials in the peripheral nervous system over skeletal muscle VSSCs by the use of a peptide having the sequence:

Xaa1 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Lys Gly His Ala Arg Cys Cys

[SEQ ID NO 9]

or

Xaa1 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Arg Asp His Ala Arg Cys Cys

[SEQ ID NO 109]

or derivatives thereof, where Xaa1 is pyroglutamate.

Preferably the peptides or their derivatives are used in the treatment of acute and chronic pain, by administering the peptide locally near or at the site of injury or tumour, or on sensory nerves using cuffs or deposits to produce regional nerve block. Preferably the compounds do not cross into the central nervous system following peripheral administration, such as via oral, i.v., subcutaneous, intramuscular, rectal, nasal, lung or perineural methods and are used in the treatment of cardiovascular and neurological diseases, cancer and conditions including chronic and gut pain.

In another aspect of the present invention is provided an isolated, synthetic or recombinant µ-conotoxin peptide selected from the following sequences of amino acids shown in Table 3 below:
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Preferably, in addition to being at least 3 fold more neuronally selective (peripheral and/or central) over skeletal muscle VSSCs when measured by $^{125}$I-TIIIA displacement studies, the peptides of this aspect of the invention are also highly potent ($\log_{10}IC_{50}$ (M) < -7.0, preferably < -8.0) at neuronal VSSCs, again when measured by $^{125}$I-TIIIA displacement studies (as described in example 5).

In a further form of the invention there is provided chimeric derivatives of TIIIA or derivatives thereof, and SIIIA or SIIIB or derivatives of SIIIA or SIIIB. Accordingly, there is provided an isolated, synthetic or recombinant chimeric derivative of TIIIA, SIIIA, SIIIB or derivatives thereof.

Preferred examples of such chimeras include an isolated, synthetic or recombinant chimera consisting of loop 2 and C-terminal tail region from TIIIA or derivative thereof, such as A15,A23,A24-TIIIA, and the N-terminal tail region and loops 1 and 3 from SIIIA or SIIIB, or derivatives of SIIIA or SIIIB, such as A15-SIIIB; and an isolated, synthetic or recombinant chimera consisting of loop 3, N-terminal and C-terminal regions of SIIIA or SIIIB, or derivatives of SIIIA or SIIIB, such as desZ1,A15,K21-SIIIB with loop 1 and loop 2 from TIIIA or derivatives of TIIIA, such as A15-TIIIA.

In another form of the invention there is provided an isolated, synthetic or recombinant $\mu$-conotoxin peptide which has selectivity between different subtypes of neuronal VSSCs, preferably mammalian neuronal VSSCs.

In a further aspect of the invention there is provided an isolated, synthetic or recombinant $\mu$-conotoxin peptide having selectivity for one or more of PN1 (Na$_v$1.7), PN3 (Na$_v$1.8), NaN (Na$_v$1.9), Type III (Na$_v$1.3), cardiac (Na$_v$1.5), Type VI (Na$_v$1.6), Type I (Na$_v$1.1) or Type II (Na$_v$1.2) VSSCs subtype over the muscle (Na$_v$ 1.4) VSSC subtype. Preferably the selectivity is 3-fold or greater, more preferably 10-fold or greater, more preferably 100 fold or greater, and even more preferably 1000-fold or greater. Preferably the peptide is selective for one or more peripheral
neuronal (ie. PN1 (Na_\text{v}1.7), PN3 (Na_\text{v}1.8), NaN (Na_\text{v}1.9), Type III (Na_\text{v}1.3), cardiac (Na_\text{v}1.5), Type VI (Na_\text{v}1.6)) VSSC subtypes over the muscle (Na_\text{v}1.4) VSSC subtype.

5 The peptides of the present invention are \(\mu\)-conotoxin peptides or derivatives thereof and thus have disulfide bonds between the first and fourth, second and fifth and third and sixth cysteine residues, respectively together with ability to block any VSSC subtype.

10 Preferably the derivatives of various forms of the invention are limited to the addition or deletion of no more than 3 amino acid residues and/or conservative amino acid substitution or side chain modification.

In another aspect of the invention there is provided an isolated, synthetic or recombinant \(\mu\)-conotoxin peptide having six cysteines and 3-disulfide bonds there between, and a substantially multi-turn topology with no \(\beta\)-sheet present in the active conformer.

In another aspect of the invention there is provided pharmaceutical compositions including a peptide as described above. The peptide may be in the form of a pharmaceutically acceptable derivative, and may be included with a pharmaceutically acceptable carrier.

In another aspect of the invention there is provided nucleic acid probes for use in finding active analogues of the above described peptides.

In another aspect of the invention there is provided the use of the above described peptides in an assay. Preferably the assay relates to finding compounds which are sodium channel inhibitors, including toxins contaminating foods.

30 In another aspect of the invention there is provided the use of the above described...
peptides for screening small molecule libraries to identify small molecules that are selective blocking agents at specific VSSC subtypes.

In another aspect of the invention there is provided the use of the above described peptides in assays where the blocking activity of the above described peptides at a particular VSSC subtype is compared to the blocking activity of a test sample at the same VSSC subtype.

In another aspect of the invention there is provided the use of the above described peptides in assays where the binding activity of the above described peptides at a particular VSSC subtype is compared to the binding activity of a test sample at the same VSSC subtype.

In another aspect of the invention there is provided the use of the above described peptides in assays where the ability of a test sample to displace the above described peptide at a particular VSSC subtype is determined.

In another aspect of the invention there is provided the use of the above described peptides as probes to analyse or characterise VSSCs and the nature and positions of residues comprising the μ-conotoxin binding sites on VSSCs.

In another aspect of the invention there is provided the use of the above described peptides in the prophylaxis or treatment of conditions such as pain including neuropathic, inflammatory and chronic pain, joint pain, gastrointestinal pain, visceral pain, stroke, epilepsy and cardiovascular conditions.

In another aspect of the invention there is provided the use of the above described peptides as neuromuscular blocking agents, local anaesthetic agents, analgesic agents and neuroprotective agents.
In another aspect of the invention there is provided the use of the above described peptides as blockers of TTX-sensitive sodium channels, in particular peripheral or central neuronal VSSCs (i.e., PN1 (Na\(_{v1.7}\)), PN3 (Na\(_{v1.8}\)), NaN (Na\(_{v1.9}\)), Type III (Na\(_{v1.3}\)), cardiac (Na\(_{v1.5}\)), type VI (Na\(_{v1.6}\)). Type I (Na\(_{v1.1}\)) or Type II (Na\(_{v1.2}\)).

In another aspect of the invention there is provided the use of the above described peptides as blockers of mammalian TTX-resistant sodium channels, or subtype specific sodium channels, such as the PN3/SNS/Na\(_{v1.8}\) channel.

In another aspect of the invention is nucleic acid sequences encoding the \(\mu\)-conotoxin peptides and encoding propeptides, and the propeptides.

**Detailed description of the invention**

The present invention is predicated on the surprising discovery of selected \(\mu\)-conotoxin peptides, and derivatives thereof which have increased potency than the previously known \(\mu\)-conotoxins. Also discovered were \(\mu\)-conotoxin peptides and derivatives thereof which showed surprising selectivity towards neuronal VSSC over muscle VSSC. Further, \(\mu\)-conotoxin peptides and derivatives thereof were discovered that were surprisingly selective for peripheral neuronal VSSC over skeletal muscle VSSC.

It should be understood that the terms conotoxin peptide or conotoxins are not limited to naturally occurring toxic peptides obtained from the genus *Conus* but rather simply indicates an initial source from which the peptides have been derived. Conotoxin peptides may be synthetically created, non-naturally occurring peptide derivatives. Conopeptides is an alternative term interchangeable with conotoxins and conotoxin peptides.

The new derivatives of \(\mu\)-conotoxin peptides are based on the peptide TIIIA isolated from *Conus tulipa* and TIIIA and TIIIB, both isolated from *Conus striatus*. 
TIIIA  Arg His Gly Cys Cys Lys Gly Xaa2 Lys Gly Cys Ser Ser Arg Glu Cys
Arg Xaa2 Gln His Cys Cys
where Xaa2 is 4Hyp

[SEQ ID NO 8]

5  SIIIB  Xaa1 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Lys Gly His
    Ala Arg Cys Cys

[SEQ ID NO 9]

SIIIA  Xaa1 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Arg Asp
    His Ala Arg Cys Cys
where Xaa1 is pyroglutamate.

[SEQ ID NO 109]

These µ-conotoxin peptides have six cysteine residues and as such it is possible
to form disulfide bonds between pairs of cysteine residues. Preferably there are
disulfide bonds formed between the first and fourth, second and fifth and third and
sixth cysteine residues, respectively. It is also thought to have a substantially
multi-turn topology, with no evidence of β-sheet in the active conformer.

The presence of the disulfide bonds between pairs of cysteine residues acts to
constrain the peptide, and results in the formation of 'loops' of amino acid
residues. The first loop comprises the amino acid residues located between the
second and third cysteine residues. The second loop comprises the amino acid
residues located between the third and fourth cysteine residues. The third loop
comprises the amino acid residues located between the fourth and fifth cysteine
residues. The peptides and their derivatives may also have tails at either end, the
N-terminal tail comprises the amino acid residues attached to the first cysteine
residue, and the C-terminal tail comprises the amino acid residues attached to the
sixth cysteine residue. One or both tails may be omitted.

By way of example for TIIIA the loops and tails are as follows:-
N-ter tail Loop 1 Loop 2 Loop 3
Arg His Gly Cys Cys Lys Gly Xaa2 Lys Gly Cys Ser Ser Arg Glu Cys Arg Xaa2 Gln His
Cys Cys
C-ter tail

Likewise for SIIIB the loops and tails are as follows:-

N-ter tail Loop 1 Loop 2 Loop 3
Xaa1 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Lys Gly His Ala Arg Cys
Cys
C-ter tail

The new family of peptides has a general amino acid sequence of:

Xaa7 Xaa8 Xaa9 Cys Cys Xaa10 Xaa11 Xaa12 Xaa13 Xaa14 Cys Xaa13 Xaa14
Xaa15 Xaa16 Xaa17 Xaa18 Cys Xaa19 Xaa20 Xaa21 Xaa22 Xaa23 Cys Cys
Xaa24 Xaa25

[SEQ ID NO 110]

where Xaa7 to Xaa25 are independently any amino acid residue other than
cysteine or may be absent.

More particularly in the peptides of this family Xaa11 and Xaa14 are Gly and
Xaa15 and Xaa16 are Ser.

Specific examples of μ-conotoxin peptides according to the present invention are
provided in Table 1, as set out earlier. Unexpectedly, a number of the new
peptides of the invention have been found to both be highly potent inhibitors of
VSSCs and to preferentially bind at neuronal VSSCs in preference to muscle
VSSCs.
In a preferred embodiment of the invention Xaa9, Xaa12 and Xaa13 are absent and Xaa11 and Xaa14 are Gly and Xaa15 and Xaa16 are Ser, which provides peptides of the type that includes SIIIA and SIIIB and most derivatives thereof.

5 Preferably these peptides are limited to the addition or deletion of no more than 6 amino acid residues (including Xaa9, Xaa12 and Xaa13). Preferably the derivatives are also limited to conservative amino acid substitution or side chain modification.

10 It has been surprisingly found that SIIIB and a number of derivatives thereof (and also a few derivatives of TIIIA) exhibit significant binding selectivity towards neuronal mammalian VSSCs over skeletal muscle VSSCs. The peptides demonstrate selectivity to mammalian neuronal VSSCs over skeletal muscle VSSCs when measured by $^{125}\text{I}$-TIIIA displacement studies.

15 Particularly preferred are those peptides of the general SEQ. ID. NO. 110, or those of the more preferred embodiment where Xaa9, Xaa12 and Xaa13 are absent, which exhibit a 3-fold or greater selectivity towards mammalian neuronal VSSCs over skeletal muscle VSSCs when measured by $^{125}\text{I}$-TIIIA displacement studies.

20 Preferably the peptides have a 10-fold or greater, more preferably 100-fold or greater, and most preferably a 1000-fold or greater selectivity towards mammalian neuronal VSSCs over skeletal muscle VSSCs when measured by $^{125}\text{I}$-TIIIA displacement studies.

25 Even more surprising, despite the prior indications to the contrary for SIIIA, it was found that SIIIB, SIIIA and a number of derivatives thereof (and also a few derivatives of TIIIA) exhibit significant binding selectivity towards mammalian peripheral neuronal VSSCs over skeletal muscle VSSCs. Preferably the peptides demonstrate selectivity to mammalian peripheral neuronal VSSCs over skeletal muscle VSSCs when measured by $^{125}\text{I}$-TIIIA displacement studies or functional studies. Particularly preferred are those peptides of the general SEQ. ID. NO 110,
or those of the more preferred embodiment where Xaa9, Xaa12 and Xaa13 are absent, which exhibit a 3-fold or greater selectivity towards mammalian peripheral neuronal VSSCs over skeletal muscle VSSCs when measured by $^{125}$I-TIIIA displacement or functional studies. Preferably the peptides have a 10-fold or greater, more preferably 100-fold or greater, and most preferably a 1000-fold or greater selectivity towards mammalian peripheral neuronal VSSCs over skeletal muscle VSSCs when measured by $^{125}$I-TIIIA displacement studies.

A preferred form of these peptides includes peptides where Xaa9, Xaa12 and Xaa13 are absent and where Xaa7 is absent, pyroglutamate, Glu, Gln, Asp, Asn, Arg, Lys or His; Xaa8, Xaa9 and Xaa10 are independently selected from Arg, Lys, His, Glu, Gln, Asp and Asn; Xaa17 is Arg, Lys or His; Xaa18 is Trp, Ala, Tyr, His or Phe; Xaa19 is Arg, Lys, His; Xaa20 is Ala, Gly; Xaa21 is His, Lys or Arg; Xaa22 is Ala; Xaa23 is Arg or Lys; and Xaa24 and Xaa25 are independently Ala, Lys or absent.

Another preferred form of the invention includes those peptides which are more selective towards mammalian neuronal VSSCs than STX. Preferably the difference between log$_{10}$C$_50$ (M) for neuronal VSSCs and log$_{10}$C$_50$ (M) for skeletal VSSCs is < -0.450 when measured by $^{125}$I-TIIIA displacement studies.

Particularly preferred peptides of the invention include those peptides described in Table 3 - Selective peptide sequences, as set out earlier.

In another preferred embodiment of the invention Xaa23 is absent and Xaa11 and Xaa14 are Gly and Xaa15 and Xaa16 are Ser, which provides peptides of the type that include TIIIA and most derivatives thereof.

In this embodiment it is preferable for Xaa9 to be Gly, Xaa12 and 20 to be independently 4Hyp, Pro or be absent; and Xaa24 and Xaa25 are independently Ala, Lys or absent. Preferably Xaa7 is absent or Arg, His or Lys; Xaa8 is His, Lys,
Arg, Ala, Ser, Thr, Pro, Hyp, Gly, Gln, Asn, Asp or Glu; Xaa10 is His, Lys, Arg, Ala, Ser, Thr, Pro, Hyp, Gly, Gln, Asn, Asp or Glu; Xaa13 is His, Lys, Arg, Ala, Ser, Thr, Pro, Hyp, Gly, Gln, Asn, Asp, Glu or absent; Xaa17 is Arg, Lys or His; Xaa18 is Gln, Asn, Ala, Ser, Thr, Pro, Hyp or Gly; Xaa19 is Arg, Lys or His; Xaa21 is Ala, Ser, Thr, Pro, Hyp, Gly, Gln, Asn, Asp, or Glu; and Xaa22 is Arg, Lys or His.

Preferably the derivatives of IIIA are limited to the addition or deletion of no more than 3 amino acid residues of IIIA.

It is preferred that these peptides should have a greater potency on mammalian VSSCs than desR1-III A, or a potency \( < -8.9 \) based compared to the \( \log_{10} C_{50} \) (M) when measured by the displacement of \( ^{125}\text{I} \) IIIA binding from CNS or peripheral mammalian nerve membrane. Particularly preferred peptides of the invention include those peptides described in Table 2 – Potent Peptide Sequences, as set out earlier.

Another preferred form of the invention includes those peptides which are more selective towards mammalian peripheral neuronal VSSCs than IIIA. Unexpectedly, a number of the new peptides of the invention have been found to both be highly potent inhibitors of peripheral neuronal VSSCs and to preferentially bind at peripheral neuronal VSSCs in preference to muscle VSSCs.

The present invention is directed to the sequences as described above in Tables 1 to 3, and derivatives thereof. Preferably the derivatives are limited to conservative amino acid substitution or side chain modifications. Thus the \( \mu \)-conotoxin peptide of the invention may be naturally occurring peptides isolated from a cone snail such as IIIA, or derivatives or synthetic versions thereof.

The term "derivative" as used herein in connection with a naturally occurring conotoxin peptide, refers to a peptide which differs from the naturally occurring peptides by one or more amino acid deletions, additions, substitutions, or side-
chain modifications. All such derivatives according to the present invention have a binding affinity to neuronal voltage sensitive sodium channels.

Substitutions encompass amino acid alterations in which an amino acid is replaced with a different naturally-occurring or a non-conventional amino acid residue. Such substitutions may be classified as "conservative", in which case an amino acid residue contained in a polypeptide is replaced with another naturally-occurring amino acid of similar character either in relation to polarity, side chain functionality or size, for example Ser↔Thr↔Pro↔Hyp↔Gly↔Ala, Val↔Ile↔Leu, His↔Lys↔Arg, Asn↔Gln↔Asp↔Glu or Phe↔Trp↔Tyr. It is to be understood that some non-conventional amino acids may also be suitable replacements for the naturally occurring amino acids. For example Lys residues may be substituted by ornithine, homoarginine, nor-Lys, N-methyl-Lys, N, N-dimethyl-Lys and N, N, N-trimethyl-Lys. Lys residues can also be replaced with synthetic basic amino acids including, but not limited to, N-1-(2-pyrazoliny)-Arg, 2-(4-piperidiny)-Gly, 2-(4-piperidiny)-Ala, 2-[3-(2S)pyrrolininy]-Gly and 2-[3-(2S)pyrrolininy]-Ala. Tyr residues may be substituted with 4-methoxy tyrosine (MeY), meta-Tyr, ortho-Tyr, nor-Tyr, 125I-Tyr, mono-halo-Tyr, di-halo-Tyr, O-sulpho-Tyr, O-phospho-Tyr, and nitro-Tyr. Tyr residues may also be substituted with the 3-hydroxy or 2-hydroxy isomers (meta-Tyr or ortho-Tyr, respectively) and corresponding O-sulpho- and O-phospho derivatives. Tyr residues can also be replaced with synthetic hydroxyl containing amino acids including, but not limited to 4-hydroxymethyl-Phe, 4-hydroxyphenyl-Gly, 2,6-dimethyl-Tyr and 5-amino-Tyr. Aliphatic amino acids may be substituted by synthetic derivatives bearing non-natural aliphatic branched or linear side chains CnH2n+2 where n is a number from 1 up to and including 8. Examples of suitable conservative substitutions by non-conventional amino acids are given in WO02/064740, the entire contents of which is incorporated herein by reference. According to the present invention, substitutions are restricted to conservative substitutions.
Amino acid substitutions are typically of single residues, but may be of multiple residues, either clustered or dispersed.

Substitutions may also be "non-conservative", in which an amino acid residue which is present in a peptide is substituted with an amino acid residue having different properties, such as naturally-occurring amino acid from a different group (eg. substituting a charged or hydrophobic amino acid with alanine), or alternatively, in which a naturally-occurring amino acid residue is substituted with a non-conventional amino acid residue. It is thought that non-conservative substitutions to the amino acid residues of SIIIA, SIIIB and TIIIA may provide further analogues that are capable of blocking TTX-resistant sodium channels, or other subtype specific sodium channels.

Additions encompass the addition of one or more naturally occurring or non-conventional amino acid residues, although preferably not cysteine residues. Deletion encompasses the deletion of one or more amino acid residues, although preferably not cysteine residues.

As stated above the present invention includes peptides in which one or more of the amino acids other than Cys has undergone side chain modifications. Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with NaBH₄; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with NaBH₄; and N-acetylation.
The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

5 The carboxyl group may be modified by carbodiimide activation via O-acylisourea formation followed by subsequent derivatisation, for example, to a corresponding amide.

Acidic amino acids may be substituted with tetrazolyl derivatives of glycine and alanine, as described in WO02/0600923.

10 The tyrosine residue may be altered, for example by methoxylation at the 4-position. Tyrosine may also be altered by nitration with tetryl to form a 3-nitrotyrosine derivative. Examples of tyrosine derivatives are given in WO02/064740.

15 Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

20 Proline residue may be modified by, for example, hydroxylation in the 4-position.

Other derivatives contemplated by the present invention include a range of glycosylation variants. Altered glycosylation patterns may result from expression of recombinant molecules in different host cells. Ser, Thr and Hyp residues may be modified to contain an O-glycan, while Asn and Gln residues can be modified to form a N-glycan. In accordance with the present invention, the term "glycan" refers to an N-, S- or O-linked mono-, di-, tri-, poly- or oligosaccharide that can be attached to any hydroxy, amino or thiol group of natural of modified amino acids by synthetic or enzymatic methodologies known in the art. The monosaccharides making up the glycan can include D-xylose, D-altrose, D-glucose, D-mannose, D-
gulose, D-idose, D-galactose, D-talose, D-galactosamine, D-glucosamine, D-N-acetyl-glucosamine (GlcNAc), D-N-acetyl-galactosamine (GalNac), D-fucose or D-arabinose. These saccharides may be structurally modified ie., with one or more O-sulphate, O-phosphate, O-acetyl or acidic groups such as sialic acid, including combinations thereof. The glycan may also include similar polyhydroxyl groups, such as D-penicillamine 2,5 and halogenated derivatives thereof or polypropylene glycol derivatives. The glycosidic linkage is beta and 1-4 or 1-3, preferably 1-3. The linkage between the glycan and the amino acid may be alpha or beta, preferably alpha and is 1-.

A list of some amino acids having modified side chains and other unnatural amino acids is shown in Table 4.
<table>
<thead>
<tr>
<th>Non-conventional amino acid</th>
<th>Code</th>
<th>Non-conventional amino acid</th>
<th>Code</th>
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These types of modifications may be important to stabilise the peptide if administered to an individual or used as a diagnostic reagent.

Particularly preferred side chain modifications include the replacement of Tyr with MeY and/or replacement of Pro with Hyp.

These types of modifications, and others which involve more substantive side chain modifications, may be important to stabilise the peptide if administered to an individual or used as a diagnostic reagent, or to improve solubility or bioavailability, or to provide other pharmacologies. For example it is possible to extend or contract side chain length, or insert or remove functional groups to achieve these effects, eg by introduction of nitroxide donor groups.
The µ-conotoxins of the present invention are typically amidated at the C-terminal, however compounds with a free carboxyl terminus or other modifications at the C-terminal are considered to be within the scope of the present invention. Preferably the peptides are amidated or have a free carboxyl at the C-terminal.

The peptides of the present invention may be in the form of a salt or pharmaceutically acceptable derivative thereof. The salts of the compound of formula I are preferably pharmaceutically acceptable, but it will be appreciated that non-pharmaceutically acceptable salts also fall within the scope of the present invention, since these are useful as intermediates in the preparation of pharmaceutically acceptable salts or may be useful in some applications, such as probes or assays.

The term "pharmaceutically acceptable derivative" includes pharmaceutically acceptable esters, prodrugs, solvates and hydrates, and pharmaceutically acceptable addition salts of the compounds or the derivatives. Pharmaceutically acceptable derivatives may include any pharmaceutically acceptable salt, hydrate or any other compound or prodrug which, upon administration to a subject, is capable of providing (directly or indirectly) a compound of the invention or an active metabolite or residue thereof.

The pharmaceutically acceptable salts include acid addition salts, base addition salts, salts of pharmaceutically acceptable esters and the salts of quaternary amines and pyridiniums. The acid addition salts are formed from a compound of the invention and a pharmaceutically acceptable inorganic or organic acid including but not limited to hydrochloric, hydrobromic, sulfuric, phosphoric, methanesulfonic, toluenesulfonic, benzenesulphonic, acetic, propionic, ascorbic, citric, malonic, fumaric, maleic, lactic, salicylic, sulfamic, or tartaric acids. The counter ion of quarternary amines and pyridiniums include chloride, bromide, iodide, sulfate, phosphate, methansulfonate, citrate, acetate, malonate, fumarate, sulfamate, and tartate. The base addition salts include but are not limited to salts
such as sodium, potassium, calcium, lithium, magnesium, ammonium and alkylammonium. Also, basic nitrogen-containing groups may be quaternised with such agents as lower alkyl halides, such as methyl, ethyl, propyl, and butyl chlorides, bromides and iodides; dialkyl sulfates like dimethyl and diethyl sulfate; and others. The salts may be made in a known manner, for example by treating the compound with an appropriate acid or base in the presence of a suitable solvent.

The compounds of the invention may be in crystalline form or as solvates (e.g. hydrates) and it is intended that both forms be within the scope of the present invention. The term "solvate" is a complex of variable stoichiometry formed by a solute (in this invention, a compound of the invention) and a solvent. Such solvents should not interfere with the biological activity of the solute. Methods of solvation are generally known within the art.

The term "pro-drug" is used in its broadest sense and encompasses those derivatives that are converted \textit{in vivo} to the compounds of the invention. Such derivatives would readily occur to those skilled in the art, and include, for example, compounds where a free hydroxy group is converted into an ester derivative or a ring nitrogen atom is converted to an N-oxide. Examples of ester derivatives include alkyl esters, phosphate esters and those formed from amino acids. They includes acetates, lactates and glutamates of the compounds of the invention. Any compound that is a prodrug of a compound of the invention is within the scope and spirit of the invention. Conventional procedures for the preparation of suitable prodrugs according to the invention are described in text books, such as "Design of Prodrugs" Ed. H. Bundgaard, Elsevier, 1985.

The term "pharmaceutically acceptable ester" includes biologically acceptable esters of compounds of the invention such as sulphonic, phosphonic and carboxylic acid derivatives.
It will be appreciated that the compounds of the invention may have at least one asymmetric centre, and therefore are capable of existing in more than one stereoisomeric form. The invention extends to each of these forms individually and to mixtures thereof, including racemates. Where possible the isomers may be separated conventionally by chromatographic methods or using a resolving agent. Alternatively the individual isomers may be prepared by asymmetric synthesis using chiral intermediates. Where the compound has at least one carbon-carbon double bond, it may occur in Z- and E- forms and all isomeric forms of the compounds being included in the present invention.

The peptides of the present invention retain the Cys residues and the characteristic disulphide-bonding pattern of \( \mu \)-conotoxin peptides. Derivatives may include additional Cys residues provided they are protected during formation of the disulphide bonds.

The earlier described peptides can be used in assays, such as an assay for finding other compounds that are sodium channel inhibitors.

The peptides could be labelled and used to establish binding assays to identify new molecules that act at or bind to the same site(s). For example, a labelled peptide ligand could have tritium included or may have radioactive iodine or similar attached through a Tyr or other appropriate residue. A Tyr scan through each peptide will establish a suitable location for incorporation of the Tyr. The inhibition of binding of such labelled peptides to tissue homogenates or expressed transporters by compounds or mixtures would permit identification of new peptides active at this site, including peptides present in serum and nerve and muscle tissue of mammals, including human tissues. The assay will also allow identification of non-peptide molecules that also act at the same site as \( \mu \)-conotoxin peptides, and that may have utility as orally active forms of these peptides. Labelled peptides will additionally permit autoradiographic studies to identify the location of the peptide binding across various tissues.
The µ-conotoxins of the present invention may be prepared using standard peptide synthetic methods followed by oxidative disulfide bond formation. For example, the linear peptides may be synthesised by solid phase methodology using BOC chemistry, as described by Schnoltzer et al (1992). Following deprotection and cleavage from the solid support the reduced peptides are purified using preparative chromatography. The purified reduced peptides are oxidised in buffered systems. The oxidised peptides are purified using preparative chromatography. Reduction/alkylation techniques can be used to determine the disulfide bond connectivities (e.g. example 3 hereafter) using well documented procedures (Shon KJ et al., (1997) Biochemistry, 36(31):9581-7; Bures et al., (1998) Biochemistry, 37(35):12172-7. The peptides can also be made using selective oxidative disulfide bond formation using the procedures outlined in Kent et al., (1998) Biopolymers 46(2):53-63.


The µ-conotoxins may also be prepared using recombinant DNA technology. A nucleotide sequence encoding the desired peptide sequence may be inserted into a suitable vector and protein expressed in an appropriate expression system. In some instances, further chemical modification of the expressed peptide may be appropriate, for example C-terminal amidation. Under some circumstances it may be desirable to undertake oxidative bond formation of the expressed peptide as a chemical step following peptide expression. This may be preceded by a reductive step to provide the unfolded peptide. Those skilled in the art may readily determine appropriate conditions for the reduction and oxidation of the peptide.
The invention further provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a μ-conotoxin peptide as described above.

It may also be possible to prepare antiidiotypic antibodies using techniques known to the art. These antiidiotypic antibodies and their use as therapeutic agents represent a further aspect of the present invention.

The nucleic acid molecules of the present invention may be DNA or RNA. When the nucleic acid molecule is in DNA form, it may be genomic DNA or cDNA. RNA forms of the nucleic acid molecules of the present invention are generally mRNA.

The nucleic acid molecules may be used in probes for use in finding active analogues of the above described peptides.

Although the nucleic acid molecules of the present invention are generally in isolated form, they may be integrated into or ligated to or otherwise fused or associated with other genetic molecules such as vector molecules and in particular expression vector molecules. Vectors and expression vectors are generally capable of replication and, if applicable, expression in either a prokaryotic cell or a eukaryotic cell, or both. Preferably, prokaryotic cells include *E. coli*, *Bacillus sp* and *Pseudomonas sp*. Preferred eukaryotic cells include yeast, fungal, mammalian and insect cells.

Accordingly, another aspect of the present invention contemplates a genetic construct comprising a vector portion and a gene capable of encoding a peptide according to the invention.

Preferably, the gene portion of the genetic construct is operably linked to a promoter on the vector such that said promoter is capable of directing expression of the gene portion in an appropriate cell.
The present invention extends to such genetic constructs and to prokaryotic or eukaryotic cells comprising same.

Chimeras of the μ-conotoxins of the present invention, with other conotoxins or additionally with other peptides or proteins, can be made to engineer the activity into other molecules, in some instances to produce a new molecule with extra functionality.

By way of an example, the amino acid residues of one or more of the loops may be incorporated into another peptide or compound, and may thereby provide sodium channel blocking activity to the new peptide or compound. Preferably the loop amino acids would be held in substantially the same conformation as they occur in the original peptide.

Chimeras of the μ-conotoxins of the present invention can be made to improve the potency, selectivity or stability of existing molecules. Non-limiting examples of chimeras include an isolated, synthetic or recombinant chimera consisting of: loop 2 and C-terminal tail region from TIIIA or derivative thereof, such as A15,A23,A24-TIIIA, and the N-terminal tail region and loops 1 and 3 from SIIIA or SIIIB, or derivatives of SIIIA or SIIIB, such as A15-SIIIB or consisting of loop 3, N-terminal and C-terminal regions of SIIIA or SIIIB, or derivatives of SIIIA or SIIIB, such as desZ1,A15,K21-SIIIB with loop 1 and loop 2 from TIIIA or derivatives of TIIIA, such as A15-TIIIA.

The combination of certain amino acid segments of TIIIA and SIIIA or SIIIB (and derivatives thereof) utilises the potency of some of the preferred TIIIA derivatives together with the neuronal selectivity of some of the preferred SIIIA and SIIIB derivatives.
Preferably the \( \mu \)-conotoxin peptides according to the invention have 15 to 30 amino acids, more preferably 17 to 27.

The peptides according to the invention may be part of a larger peptide. For example, the N-terminus "tail" region may be extended to any suitable length by introduction of additional amino acid residues. Similarly the C-terminus may also be extended by addition of a peptide "tail". In some cases the activity of the peptide can be improved by such modifications.

The peptides according to the present invention may be modified by biotinylation for use in biological assays, attachment of antibodies for targeting the site of action, attachment of sugars and lipids to improve permeability.

This method may be of use in a number of different applications. It may be used in the formation of assays or probes such as that earlier described, or in the treatment of certain medical conditions.

The invention also provides a method of treating diseases involving sodium channels, including diseases involving a range of TTX-sensitive and TTX-resistant sodium channel present in nerves, including \( \text{Na}_v1.1 \), \( \text{Na}_v1.2 \), \( \text{Na}_v1.3 \), \( \text{Na}_v1.5 \), \( \text{Na}_v1.6 \), \( \text{Na}_v1.7 \), \( \text{Na}_v1.8 \), \( \text{Na}_v1.9 \), and more preferably diseases involving \( \text{Na}_v1.3 \) \( \text{Na}_v1.7 \), \( \text{Na}_v1.6 \) and \( \text{Na}_v1.9 \). These diseases include those which involve pain, and epilepsy, stroke, cancer and cardiovascular diseases.

Accordingly, the invention also provides for the use of an isolated, synthetic or recombinant \( \mu \)-conotoxin peptide of the invention in the manufacture of a medicament for the prophylaxis or treatment of conditions such as pain including neuropathic, chronic, visceral and gastrointestinal pain, cancer, seizures, stroke, epilepsy and cardiovascular conditions.
Accordingly the invention provides a method for the treatment of pain, seizures, stroke, epilepsy, cancer or cardiovascular disease, which includes the step of administering to a mammal, an effective amount of an isolated, synthetic or recombinant \( \mu \)-conotoxin peptide of the invention.

5 Preferably the mammal is in need of such treatment, although the peptide may be administered in a prophylactic sense.

According to a further aspect of the present invention there is provided a composition which includes an isolated, synthetic or recombinant \( \mu \)-conotoxin peptide of the invention, such as a peptide sequence set out in any one of Tables 1 to 3 or such a sequence which has undergone one or more conservative amino acid substitutions, additions or deletions and a pharmaceutically acceptable carrier or diluent.

10 Preferably the composition is in the form of a pharmaceutically acceptable composition.

As will be readily appreciated by those skilled in the art, the route of administration and the nature of the pharmaceutically acceptable carrier will depend on the nature of the condition and the mammal to be treated. It is believed that the choice of a particular carrier or delivery system, and route of administration could be readily determined by a person skilled in the art. In the preparation of any formulation containing the peptide actives care should be taken to ensure that the activity of the peptide is not destroyed in the process and that the peptide is able to reach its site of action without being destroyed. In some circumstances it may be necessary to protect the peptide by means known in the art, such as, for example, micro encapsulation. Similarly the route of administration chosen should be such that the peptide reaches its site of action.
The pharmaceutical forms suitable for injectable use include sterile injectable solutions or dispersions, and sterile powders for the extemporaneous preparation of sterile injectable solutions. They should be stable under the conditions of manufacture and storage and may be preserved against oxidation and the contaminating action of microorganisms such as bacteria or fungi.

Those skilled in the art may readily determine appropriate formulations for the peptides or modified peptides of the present invention using conventional approaches. Identification of preferred pH ranges and suitable excipients, for example antioxidants, is routine in the art (see for example Cleland et al, 1993). Buffer systems are routinely used to provide pH values of a desired range and include carboxylic acid buffers for example acetate, citrate, lactate and succinate. A variety of antioxidants are available for such formulations including phenolic compounds such as BHT or vitamin E, reducing agents such as methionine or sulphite, and metal chelators such as EDTA.

The solvent or dispersion medium for the injectable solution or dispersion may contain any of the conventional solvent or carrier systems for peptide actives, and may contain, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about where necessary by the inclusion of various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include agents to adjust osmolality, for example, sugars or sodium chloride. Preferably, the formulation for injection will be isotonic with blood. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin. Pharmaceutical forms suitable for injectable use may be delivered by any
appropriate route including intravenous, intramuscular, intracerebral, intrathecal, epidural injection or infusion.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients such as these enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilised active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, preferred methods of preparation are vacuum drying or freeze-drying of a previously sterile-filtered solution of the active ingredient plus any additional desired ingredients.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations preferably contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in therapeutically useful compositions should be sufficient that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such a sucrose, lactose or saccharin may be added or a
flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier.

Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations, including those that allow specific delivery of the active peptide to specific regions of the gut.

The present invention also extends to any other forms suitable for administration, for example topical application such as creams, lotions and gels, or compositions suitable for inhalation or intranasal delivery, for example solutions or dry powders.

Parenteral dosage forms are preferred, including those suitable for intravenous, intrathecal, and intracerebral or epidural delivery.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except in so far as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.
It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired as herein disclosed in detail.

The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form. A unit dosage form can, for example, contain the principal active compound in amounts ranging from 0.25 µg to about 2000 mg. Expressed in proportions, the active compound is generally present in from about 0.25 µg to about 200 mg/ml of carrier. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

In performing the method according to the present invention the administration of the peptide may be performed in conjunction with other therapies useful in the treatment of the condition, disease or disorder. Accordingly the peptide may be administered substantially simultaneously or sequentially with other agents useful in the treatment of the conditions, diseases or disorders. Where the co-administration is simultaneous, the peptide may be formulated in a composition with one or more of the other agents. The co-administration of other agents can be performed via the same or different route to the route of administration for the peptide. Where the method is for the treatment or control of acute, chronic and/or neuropathic pain or migraine, the peptide may be administered substantially
simultaneously or sequentially with an analgesic agent selected from the group consisting of opioid analgesics, opioid receptor-like antagonists, GPCR antagonists of the MRG family, NMDA antagonists, substance P antagonists, COX 1 and COX 2 inhibitors, tricyclic antidepressants (TAC), selective serotonin reuptake inhibitors (SSRI), capsaicin receptor antagonists, anaesthetic agents, benzodiazepines, skeletal muscle relaxants, migraine therapeutic agents, anti-convulsants, anti-hypertensives, anti-arrhythmics, antihistamines, steroids, caffeine, N-type calcium channel antagonists, nicotinic receptor partial agonists and antagonists, vanilloid receptor antagonists and agonists, TNF-α antagonists and antibodies, inhibitors of tetrodotoxin-sensitive Na Channels, P-type Calcium channel inhibitors, endothelial antagonists and botulinum toxin. The peptide may also be administered simultaneously with two or more other agents, for example mixtures of SSRIs and noradrenaline reuptake inhibitors.

Where the analgesic agent is an opioid analgesic agent it is preferably selected from propoxyphene, meperidine, hydromorphone, hydrocodone, morphine, codeine and tramadol; their pharmaceutically active salts and their optical isomers.

Where the analgesic agent is an NMDA antagonist analgesic agent it is preferably selected from 2-piperdino-1-alkanol derivatives, dextromethorphan, eliprolil, and ifenprodil; their pharmaceutically active salts and their optical isomers.

Where the analgesic agent is a substance P antagonist analgesic agent it is preferably selected from 2-phenyl-piperidin-3-yl or 2-diphenylmethyl-1-azabicyclo[2.2.2]-octane-3-amine derivatives as described in U.S. Patent Application No. 2001/00336943 A1 (Coe et al.); their pharmaceutically active salts and their optical isomers.

Where the analgesic agent is a COX 2 inhibitor analgesic agent it is preferably selected from rofecoxib and celecoxib; their pharmaceutically active salts and their optical isomers.
Where the analgesic agent is an anaesthetic analgesic agent it is preferably selected from nitrous oxide, halothane, lidocaine, etidocaine, ropivacaine, chloroprocaine, sarapin and bupivacaine; their pharmaceutically active salts and their optical isomers.

Where the analgesic agent is a benzodiazepine analgesic agent it is preferably selected from diazepam, chlordiazepoxide, alprazolam, lorazepam, midazolam, L-365260; their pharmaceutically active salts and their optical isomers.

Where the analgesic agent is a skeletal muscle relaxant analgesic agent it is preferably selected from flexeril, carisoprodol, robaxisal, norgesic and dantrium; their pharmaceutically active salts and their optical isomers.

Where the analgesic agent is a migraine therapeutic agent it is preferably selected from elitriptan, sumatriptan, rizatriptan, zolmitriptan, and naratriptan; their pharmaceutically active salts and their optical isomers.

Where the analgesic agent is an anticonvulsant analgesic agent it is preferably selected from gabapentin, pregabalin, carbamazepine, and topiramate and valproic acid; their pharmaceutically active salts and their optical isomers.

Where the analgesic agent is a COX 1 inhibitor analgesic agent it is preferably selected from salicylic acid, acptomophen, diclofenac, piroxicam, indomethacin, ibuprofen, and naproxen; their pharmaceutically active salts and their optical isomers.

Where the analgesic agent is a tricyclic antidepressant analgesic agent it is preferably selected from amitriptyline, desipramine, perphenazine, protriptyline, and tranylcypromine their pharmaceutically active salts and their optical isomers.
Where the analgesic agent is a SSRI analgesic agent it is preferably selected from tramadol and milnacipran; their pharmaceutically active salts and their optical isomers.

Where the analgesic agent is a mixture of SSRI and Noradrenaline reuptake inhibitors, the latter is preferably selected from reboxetine and atomoxetine; their pharmaceutically active salts and their optical isomers.

The analgesic agent may also be selected from baclofen, clonidine, mexilitene, diphenyl-hydramine, hydroxysine, caffeine, prednisone, methylprednisone, decadron, paroxetine, sertraline, fluoxetine, Ziconotide.RTM. and levodopa; their pharmaceutically active salts and their optical isomers.

Where the analgesic agent is a TNF-α antagonist or antibody, the agent is preferably selected from etanercept, infliximab and thalidomide; their pharmaceutically active salts and their optical isomers.

Where the analgesic agent is an endothelial antagonist, the agent is preferably selected from bosentan and tesosentan; their pharmaceutically active salts and their optical isomers.

Where the analgesic agent is a vanilloid antagonist, the analgesic agent is preferably selected from ananamide, capsazepine, thiocarbamic acid derivatives (as described in WO02/16317 A1) and thiourea derivatives (as described in WO02/16318 A1); their pharmaceutically active salts and their optical isomers.

Where the analgesic agent is selected from nicotine receptor partial agonist it is preferably selected from:-

1,2,3,4,5,6-hexahydro-1,5-methano-pyrido[1,2-a][1,5]diazocin-8-one derivatives,

diazatetracyclo[9.3.1.0.sup.2,10.0.sup.4,8]pentadeca-2(10),3,8-triene derivatives,

10-aza-tricyclo[6.3.1.0.sup.2,7]dodeca-2(7),3,5-triene derivatives,
triazatetrcyclo[9.3.1.0.sup.2,10.0.sup.4,8] pentadeca-2(10),3,5,8-tetraene derivatives,
5,8,14-triazatetrcyclo[10.3.1.0.sup.2,11.0.sup.4,9] heptadeca-2(11),3,5,7,9-pentaene derivatives,
diazatetrcyclo[9.3.1.0.sup.2,10.0.sup.4,8] pentadeca-2(10),3,6,8-tetraene derivatives,
10-azatricyclo[6.3.1.0.sup.2,7] dodeca-2(7),3,5-triene derivatives,
5,7,14-triazatetrcyclo[10.3.1.0.sup.2,10.0.sup.4,8] hexadeca-2(10),3,5,8-tetraene derivatives,
5,8,15-triazatetrcyclo[11.3.1.0.sup.2,11.0.sup.4,9] heptadeca-2(11),3,5,7,9-pentaene derivatives,
5,14-diazatetrcyclo[10.3.1.0.sup.2,10.0.sup.4,8] hexadeca-2(10),3,5,8-tetraene derivatives, and
11-azatricyclo[7.3.1.0.sup.2,7] trideca-2(7),3,5-triene derivatives,
all of which are described in U.S. Patent Application No. 2001/00336943 A1 and
their pharmaceutically acceptable salts and their optical isomers.

Examples of conditions associated with acute, chronic and/or neuropathic pain
and inflammatory pain include soft tissue and peripheral damage, such as acute
trauma, osteoarthritis, rheumatoid arthritis, musculo-skeletal pain (particularly after
trauma), spinal pain, dental pain, myofascial pain syndromes, headache,
episiotomy pain, and burns; deep and visceral pain, such as heart pain, muscle
pain, eye pain, orofacial pain, for example, odontalgia, abdominal pain,
gynaecological pain, for example, dysmenorrhea, and labor pain; pain associated
with nerve and root damage, such as pain associated with peripheral nerve
disorders, for example, nerve entrapment and brachial plexus avulsions,
amputation, peripheral neuropathies, neuralgia, tic douloureux, atypical facial pain,
nerve root damage, pain and/or chronic nerve compression, and arachnoiditis;
pain associated with carcinoma, often referred to as cancer pain; pain associated
with AIDS, central nervous system pain, such as pain due to spinal cord or brain
stem damage; low back pain; sciatica; headache, including migraine, acute or
chronic tension headache, cluster headache, temporomandibular pain and maxillary sinus pain; ankylosing spondylitis, gout; post operative pain; phantom pains; diabetic neuropathy; shingles; and scar pain.

Examples of diseases or conditions of the urinary system include urinary and fecal incontinence. Examples of cardiovascular diseases or conditions include arrhythmias of various origins and coronary heart failure. Examples of mood disorders include depression, anxiety, cravings, an addictive disorder and withdrawal syndrome, an adjustment disorder, age-associated learning and mental disorders, anorexia nervosa, apathy, attention-deficit disorders due to general medical conditions, attention-deficit hyperactivity disorder, bipolar disorder, bulimia nervosa, chronic fatigue syndrome, chronic or acute stress, conduct disorder, cyclothymic disorder, depression, dysthymic disorder, fibromyalgia and other somatoform disorders, generalised anxiety disorder, incontinence, inhalation disorders, intoxication disorders, mania, obesity, obsessive-compulsive disorders and related spectrum disorders, oppositional defiant disorder, panic disorder, peripheral neuropathy, post-traumatic stress disorder, premenstrual dysphoric disorder, psychotic disorders, seasonal affective disorder, sleep disorders, social phobia, specific developmental disorders, selective serotonin reuptake inhibition (SSRI) "poop out" syndrome, and TIC disorders.

The invention will now be described with reference to the accompanying figures and examples, however it is to be understood that the particularity of the following description is not to supersede the generality of the preceding description of the invention.

Figure 1 is a model of the secondary structure of VSSCs, including the position of site 1 which is the μ-conotoxin binding site.

Figure 2 depicts the design of the Mu1A + ANCHOR PCR assay used for the production of mu class conopeptides.
Figure 3 depicts representative samples of DNA products produced by the PCR of Conus spp venom duct cDNA with the oligonucleotide primers Mu1A + ANCHOR. The 800bp (mu peptide) and 400bp ('linear' peptide) bands are indicated by arrows.

Figure 4 depicts nucleotide and amino acid sequences for the leader and mature peptide region of TIllIA.

Figure 5 depicts the 3D backbone structure and disulphide connectivities of GIllIB, TIllIA, PIllIA and SIllIB (left to right).

EXAMPLES

Example 1: Isolation of μ-conopeptides from Conus species by PCR based assay for mu conopeptides

μ-Conotoxins were isolated using molecular biology and assay-directed fractionation from several Conus species including Conus tulipa (TIllIA) and Conus striatus (SIllIA and SIllIB).

(a) Design of PCR primers

Redundant oligonucleotide primers suitable for PCR were designed from the amino acid sequences of GIllIA and manufactured using a contract supplier (Genset Pacific, Australia).

(b) Production of Coneshell venom duct cDNA

cDNA suitable for the production of either 5’RACE or 3’RACE PCR was manufactured from coneshell venom duct poly(A) mRNA using polymerase extension from a Not-1-dt(18)-ANCHOR primer. This single stranded cDNA was converted to double stranded (ds) cDNA using RNAase-H / DNase-1 method, blunt ended, and ligated with Marathon™ (Clontech) adaptors to both the 5’ and 3’ ends of the cDNA templates.
(c) PCR based assay for mu conopeptides

The PCR assay that is capable of producing conopeptides from the mu family is based on the PCR primers Mu1A plus ANCHOR (Figure 2). The Mu1A primer was based upon a comparison of nucleotide sequences from 6 different activity groups, and was considered specific for the mu group of peptides. PCR using the primers Mu1A plus ANCHOR normally produces two distinct DNA fragments on coneshell venom duct ds cDNA templates of ~800bp and ~400bp, respectively. Of these, the former produces the mu and mu-like genes and is present in virtually all Conus species (Figure 3).

(d) Sequencing and characterisation of mu conopeptide genes

The Mu1A + ANCHOR PCR products for all Conus species tested were electrophoresed in LMP agarose gels, the 800bp fragments were excised, purified, and cloned into ‘PCR direct’ TA-plasmid vectors (Invitrogen). The 800bp PCR DNA clone libraries were plated out, while recombinant clones were randomly selected and amplified in overnight cultures. Plasmid DNA was extracted from each culture and used as templates for DNA sequencing. Each clone was sequenced to the level required to produce unambiguous nucleotide data. The raw sequence data for each clone was formatted manually within the SeqNavigator software to ensure that no potential venom peptides of any type were overlooked. The nucleotide sequences were finally translated and catalogued.

This use of the Mu1A primer enabled the isolation of known μ-conotoxin, GIIIA in Conus marmoreus, C. tulipa and C. magus. spp as well as C. geographus, indicating that the method is successful. A peptide, TIIIA (SEQ. ID NO 8) which shares the same cysteine framework as GIIIA and PIIIA was also found in C. tulipa.

The nucleotide and amino acid sequence for the leader and mature peptide region of TIIIA was sequenced and is set out in figure 4. While TIIIA has the
same pattern of disulfide bridges as other μ-conotoxins, there are a number of differences in primary sequence.

Example 2. Preparation of TIIIA by fusion protein technology

It was possible to clone and functionally express a synthetic gene encoding for TIIIA. An A^{19}-TIIIA gene was constructed using two synthetic oligonucleotides with the optimal codon usage for *E. coli* expression. The protein is produced in the cytoplasm of *E. coli* thus minimising its degradation. The synthetic gene was flanked by KpnI and BamHI restriction sites to allow the insertion into pCP vector (Drevet P, et al (1997) *Protein Expr. Purif.* 10(3) 293-300). The resulting construct (pCP/TIIIA) encodes a fusion protein ZZ and TIIIA.

The final gene product ZZ-TIIIA, pCP/TIIIA was cultured in *E. coli*, BL21 (DE3) Lys S, and the hybrid was induced by IPTG (at OD 600 nm = 0.5) for 3 hours. The cells are harvested and lysed by 3 cycles of freeze and thawing. The lysate contained the soluble fusion protein. The disulfide bonds of ZZ-TIIIA are then formed in a mixture of reduced and oxidised glutathione. Purification and oxidation was then achieved in one step using IgG Sepharose affinity chromatography column. The produced and purified ZZ-TIIIA was cleaved with cyanogen bromide to release the conotoxin.

Example 3. Isolation of SIIIA and SIIIB from *C. striatus* using assay-directed fractionation.

SIIIA and SIIIB were isolated from the crude venom of *C. striatus* using assay-directed fractionation. RP-HPLC separated fractions that displaced ^125^I-TIIIA binding to mammalian VSSCs can be isolated to homogeneity by further HPLC and the active isolated peptides sequenced.

(a) Crude extract.
Active fractions were isolated from the crude venom of *C. striatus* using assay-directed fractionation to identify those RP-HPLC separated fractions that displaced ^125^I-TIII A binding to mammalian Na channels.

5 (b) Refractionation of active fractions
Fractions were rechromatographed by analytical RP-HPLC using a Zorbax 300SB-C18 (2.1 x 50mm 3.5μm) eluted at 0.2 ml/min with a linear gradient of 0-40% over 60min. Inhibition of ^125^I-TIII A binding identified active peaks.

10 (c) Reduction and alkylation
The purified peptides (~100 pmol) were fully reduced in the presence of TCEP and 50 mM ammonium acetate at pH 8 (37°C for 1 h) and then alkylated in the presence of maleimide (37°C, 1 h). The alkylated peptides were purified by RP-HPLC, applied to a Biobrene treated glass fibre filter, and analysed by Edman chemistry using an ABI model 470A protein sequencer. Alkylation of peptides with maleimide allowed their cysteine residues to be observed as PTH-cys-maleimide doublets (diastereomers).

(d) Pyroglutamate aminopeptidase digestion
Reduced and alkylated peptide dissolved in digestion buffer [sodium phosphate buffer, 100mmol/L; EDTA, 10mmol/L; dithiothreitol (DTT), 5mmol/L; glycerol, 5% v/v; pH 8] incubated at 4°C for 20 hrs then 25°C for 6 hrs followed by a further 20hrs at 4°C. The digested peptide were purified by RP-HPLC and sequenced.


(a) Synthesis and oxidation
Analogues of the naturally occurring μ-conotoxins were manually synthesised, deprotected, and cleaved from resin in accordance with the methods previously described for other conotoxins (Nielsen et al., (1999) J. Mol. Biol. 289, 1405–
1421). The pure, reduced peptides (0.02-0.05 mM) were oxidised in either aqueous 0.33 M NH₄OAc, 0.5 M guanidine.HCl or in 2M (NH₄)₂SO₄ and 0.1M NH₄OAc (pH 7.5-8.0, adjusted with 0.1 M NH₄OH). The peptide solutions were then stirred for 2-4 days at 4°C in the presence of reduced and oxidised glutathione (molar ratio 1:100:10) to achieve oxidation. Oxidised peptides were purified by preparative RP-HPLC.

(b) Analysis
Peptides were quantified initially by triplicate amino acid analysis and then by RP-HPLC (HP 1100) using an external reference standard for each peptide. Mass spectra were acquired on a PE-Sciex API III triple quadrupole electrospray mass spectrometer (MS) in positive ion mode (m/z 500-2000, at 0.1-0.2-Da steps, declustering potentials of 10-90 V, and dwell times of 0.4-1.0 s). Data were deconvoluted (MacSpec 3.2, Sciex, Canada) to obtain the molecular weight from the multiply charged species. MS was used to confirm purity and to monitor peptide oxidation.

(c) Reduction and alkylation
The purified peptides (∼ 100 pmol) were fully reduced in the presence of TCEP and 50 mM ammonium acetate at pH 8 (37°C for 1 h) and then alkylated in the presence of maleimide (37°C, 1 h). The alkylated peptides were purified by RP-HPLC, applied to a Biobrene treated glass fibre filter, and analysed by Edman chemistry using an ABI model 470A protein sequencer. Alkylation of peptides with maleimide allowed their cysteine residues to be observed as PTH-cys-maleimide doublets (diastereomers)

Example 5. Radioligand binding assays - TIIIA

The ¹²⁵I-TIIIA radioligand binding assay was developed to quantify activity at muscle and neuronal sodium channels.
(a) Methods

Radioligand binding assays were performed on rat brain membrane (to test for TTX-S type II/Nap 1.2) and rat skeletal membrane membrane (to test for TTX-S µ1/Nap 1.4) incubated in the following (mM): 20 HEPES (pH 7.2), 75 
NaCl, 0.2 EDTA, 0.2 EGTA, 2 leupeptin, 0.5 units aprotinin, and 0.1% bovine 
serum albumin, as described previously for ω-conotoxins (Nielsen et al., 1999). 
For testing other peripheral neuronal subtypes including Na+1.7, Na+1.3 and 
Na+ 1.6) mammalian DRG is prepared in a similar manner as described for rat 
brain membrane.

125I-TIIIA was prepared using IODOGEN. 10 µl of 1 mg/ml TIIIA was added to 
50 µl Na phosphate buffer, pH 9.2 in a tube coated with IODOGEN, 0.5 mCi of 
Na125I was added and the mixture vortexed and incubated for 10 min. The 
iodinated compound was stored at 4°C, and used within 3 weeks. 125I-TIIIA 
eluted 1.5 min after TIIIA on a Zorbax SB 300 C18 column (gradient 0-30% B/ 
60 min) as a doublet peak on RP-HPLC due to mono-iodination on either 
histidine. Cold iodinated 127I-TIIIA was also prepared and quantified by HPLC. 
127I-TIIIA and 125I-TIIIA coeluted and MS confirmed it as the monoiodinated 

For displacement studies, 125I-TIIIA (7 pM diluted with assay buffer) were 
incubated with increasing concentrations of cold peptide. Saturation binding 

experiments were performed with freshly prepared 125I-TIIIA. Ligand binding 
was incubated for 1 hr at room temperature and filtered through Wallac GF/B 
filters treated with 0.6% polyethyleneimine using a Tomtec cell harvester and 
with washed with buffer (20mM HEPES, 125mM NaCl, pH 7.2). Data were counted 
on a scintillation counter and nonlinear regressions were fitted to each 
experiment with Prism software (GraphPad, San Diego, CA).

A comparison of the 125I-TIIIA assay (using the procedure described above)
with the standard $^3$H-STX assay using the procedure described in Nielsen et al., 2002 J. Biol. Chem. 277, 27247-27255 was made to assess the effectiveness of the new assay (see below).

5  (b) Results

TIIIA was found to significantly inhibit $^3$H-STX binding. Further studies indicated that $^{125}$I-TIIIA was found to label the same VSSCs as $^3$H-STX with little non-specific binding (10% or less). It was found to inhibit rat muscle and nerve, mammalian peripheral nerve, TTX sensitive sodium channels, human brain TTX sensitive sodium channels and fish brain sodium channels, making it a useful alternative binding assay to $^3$H-STX. Peptides tested against $^{125}$I-TIIIA displaced the ligand with higher potency than against the $^3$H-STX assay giving rise to an assay with higher sensitivity. The assay can be performed under different conditions to improve the sensitivity. The assay may also be useful in the detection of TTX, STX and other small molecules that bind to site 1 VSSCs, including those present in foods that pose a threat to human health.

20 Potency (-log1C$_{50}$ (M)) and extent of inhibition (%) of $[^3]$H-STX and $^{125}$I-TIIIA binding to VSSCs by $\mu$-conotoxins and TTX.

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<th>Rat brain</th>
<th>Rat muscle</th>
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<td>$^3$H-STX</td>
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<td>Rat muscle</td>
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Example 6. Displacement of $^{125}$I-TIIIA - Potency and Selectivity of derivatives

(a) Experimental procedures

The displacement study procedure for ligand binding experiments was conducted in accordance with that described in Example 5 above.

(b) Experimental data

A summary of potencies and selectivity of several of the peptides of the invention and comparative compounds is given below in Table 6 (high potency) and Table 7 (high selectivity). The potency and selectivity are determined by displacement of $^{125}$I-TIIIA from rat brain and skeletal muscle VSSCs and values are presented as log$_{10}$C$_{50}$ (M). To help interpret results, a 100-fold selectivity for neuronal over muscle VSSC will correspond to a value of −2 in table 6.

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<tr>
<td>4</td>
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<td>-6.340</td>
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(c) Analysis
As can be seen from the data in Table 6, SIIIA, SIIIB and TIIIA and several of their analogues are highly potent inhibitors of rat neuronal VSSCs (which correspond to both type II central neuronal and Na_{1.2} peripheral neuronal VSSCs). Analysis of
the affinity of TIIIA, SIIIB and a number of derivatives, including Ala and Gln analogues of the charged residues in TIIIA, has produced a number of \( \mu \)-conotoxin derivatives, such as A15-TIIIA, A2-TIIIA and A15,A23-TIIIA that are particularly potent at neuronal VSSCs. In general, TIIIA analogues are more potent inhibitors of neuronal VSSCs than SIIIB analogues. The former, analogues of and chimeras of, preferred analogues are also potent at skeletal muscle VSSCs. Therefore these peptides are valuable pharmacological inhibitors of a range of VSSCs, including the highly specific inhibition of a range of TTX-sensitive and TTX-resistant sodium channels present in nerves, including \( \text{Na}_v 1.1, \text{Na}_v 1.2, \text{Na}_v 1.3, \text{Na}_v 1.5, \text{Na}_v 1.6, \text{Na}_v 1.7, \text{Na}_v 1.8, \text{Na}_v 1.9 \), and preferably \( \text{Na}_v 1.2, \text{Na}_v 1.3, \text{Na}_v 1.6, \text{Na}_v 1.7, \text{Na}_v 1.8 \) and \( \text{Na}_v 1.8 \) (see Goldin, A.L. et al (2000) *Neuron* **28**, 365-368.).

Amongst the analogues tested, A15-TIIIA was found to be the most potent peptide inhibitor at rat brain sodium channels, irrespective of whether radiolabelled TIIIA or the STX was used in the assay (these assays require different conditions). In fish brain, STX and TIIIA were full inhibitors of \( ^{125}\text{I} \)-TIIIA binding, while TTX only displaced ~60% of peptide binding, indicating that TIIIA bound to neuronal TTX-resistant Na channels in vertebrates at the evolutionary level of Pisces and higher levels, including mammals.

The peptides were tested on skeletal muscle sodium channels to measure selectivity. Of the naturally occurring \( \mu \)-conotoxins, TIIIA is the most potent inhibitor of neuronal VSSCs, however, it is also potent at the skeletal muscle VSSCs, so displays only modest selectivity. SIIIB and SIIIA are naturally occurring \( \mu \)-conotoxins that display selectivity for neuronal over skeletal muscle VSSCs (>10-fold and 2.2-fold, respectively). The selectivity is significantly enhanced by making a number of substitutions ie, >1000-fold for desZ1,R14,A15,D21-SIIIB, >100-fold for A18-SIIIA, respectively. In general, the SIIIA and SIIIB analogues tested are more selective for neuronal VSSCs compared to the TIIIA series.

Analogue that are both potent and selective for neuronal VSSCs (ie desZ1,R14,A15,K21-SIIIB has a selectivity index of −2.82, and a potency of −8.58)
are useful tools for probing neuronal VSSCs.

Example 8. \(^1\)H NMR and Modelling Studies

(a) Methods

All NMR experiments were recorded on a Bruker ARX 500 spectrometer equipped with a z-gradient unit or on a Bruker DMX 750 spectrometer equipped with an x,y,z-gradient unit. Peptide concentrations were ~2 mM. \(\mu\)-Conotoxins were examined in 95% H2O, 5% D2O (pH 3.0 and 5.5; 275–298 K). \(^1\)H NMR experiments recorded were NOESY (Jeener, J. et al (1979) J. Chem. Phys. 71, 4546–4553; Kumar, A. et al (1980) Biochem. Biophys. Res. Commun. 95, 1–6) with mixing times of 150, 200, and 400 ms, TOCSY (Bax, A., and Davis, D. G. (1985) J. Magn. Reson. 65, 355–360) with a mixing time of 80 ms, DQF-COSY (Rance, M., et al (1983) Biochem. Biophys. Res. Commun. 177, 479–485), and E-COSY in 100% D2O (Greisinger, C. et al (1987) J. Magn. Reson. 75, 474–492). All spectra were run over 6024 Hz (500MHz) or 8192 Hz (750 MHz) with 4 K data points, 400–512 FIDs, 16–64 scans, and a recycle delay of 1 s. The solvent was suppressed using the WATERGATE sequence (Piotto, M. et al (1992) J. Biolmol. NMR 2, 661). The spectra were processed using UXNMR as described previously (Nielsen, K. J., et al (1999) J. Mol. Biol. 289, 1405–1421) and using Aurelia; subtraction of background was used to minimise T1 noise. Chemical shift values were referenced internally to DSS at 0.00 ppm. Secondary H\(\alpha\) shifts were measured using random coil shift values of Wishart et al. (Wishart, D. S., et al (1995) J. Biomol. NMR 5, 67–81). \(^3\)J\(_{\text{NH-H}}\) coupling constants were measured as previously described (Nielsen, K. J. et al (1999)).

**Distance Restraints and Structure Calculations**

Peak volumes in NOESY spectra were classified as strong, medium, weak, and very weak, corresponding to upper bounds on interproton distance of 2.7, 3.5, 5.0, and 6.0 Å, respectively. Lower distance bounds were set to 1.8 Å. Appropriate
pseudoatom corrections were made (Wüthrich, K., et al (1983) J. Mol. Biol. 169, 949–961), and distances of 0.5 and 2.0 Å were added to the upper limits of restraints involving methyl and phenyl protons, respectively. $^3J_{\text{NH-H}_2}$ coupling constants were used to determine $\phi$ dihedral angle restraints (Pardi, A. et al (1984) J. Mol. Biol. 180, 741–751), and in cases where $^3J_{\text{NH-H}_2}$ was 6–8 Hz and it was clear that a positive dihedral angle was not present, $\phi$ was restrained to -100 ±70°.

$^3J_{\text{NH-H}_2}$ coupling constants, together with relevant NOESY peak strengths, were used to determine $\chi_1$ dihedral angle restraints (Wagner, G. et al (1987) J. Mol. Biol. 196, 611–639). Where there was no diastereospecific assignment for a prochiral pair of protons, the largest upper bound for the two restraints was used. Where stereospecific assignments were established, these distances were specified explicitly.


(b) Structure results

The solution structures $\mu$-conotoxins are notoriously difficult to determine as they exhibit broad lines indicative of conformational exchange (GIIIA and GIIIB), or at
least two distinct conformations due to cis/trans isomerisation of one of the hydroxyprolines in loop 1. As a result, the constraints derived from the poor quality NMR data do not result in well resolved or reliable 3D structures. In contrast, the $^1$H NMR spectra of TIIIa, SIIIA, SIIIB and their analogues show no evidence of conformational exchange. For example, the peakwidths are narrow, the peak dispersion is high and the NOEs are plentiful. This is surprising given the relatively large number of glycine residues in loop 1 of TIIIa, SIIIA and SIIIB compared to GIIIA and PIIB.

High-resolution 3D NMR structures of the SIIIB, SIIIA (not shown) and TIIIa $\mu$-conotoxins were determined and compared to the published structure of GIIIB (Hill, J. M. et al (1996) Biochemistry 35, 8824–8835), and GIIIA (not shown) and PIIB (Nielsen et al., (2002) J. Biol. Chem 277) and are depicted in Figure 5. This reveals a global similarity in structure, but with a number of significant differences. A comparison of secondary H$\alpha$ shifts indicate the peptides have a similar conformation to GIIIA and GIIIB in the N- and C-terminal regions, however, the central structure is significantly different. GIIIB is described as comprising a short stretch of distorted $\alpha$-helix, and an antiparallel $\beta$-sheet joined by a $\alpha$-hairpin. However, like the major conformation of PIIB there is no $\beta$-sheet region in SIIIA, SIIB or TIIIa, nor is there any evidence of multiple conformations. Instead, the structures of these peptides are composed largely of several well-defined turns.

The measurements of H$\alpha$ chemical shifts, H$\beta$ shifts and $^3$J$_{\text{NH-HH}}$ coupling constants revealed that all analogues of TIIIa, SIIIA and SIIIB had the same overall fold, with an unusually high retention of backbone and side chain structural integrity across the set. This indicates that this class of peptides has a well-maintained scaffold useful for pharmacophore development and could provide useful templates from which to probe the molecular architecture of the outer vestibule of the Na channel, and from which to design and develop new molecules that selectively inhibit a
range of TTX-sensitive and TTX-resistant sodium channels present in nerves, including Na_v 1.1, Na_v 1.2, Na_v 1.3, Na_v 1.5, Na_v 1.6, Na_v 1.7, Na_v 1.8, Na_v 1.9.

Examples 9 to 11 below are prophetic examples describing testing methods which have not as yet been used with the peptides of the invention.

Prophetic Example 9. Antinociceptive efficacy of μ-conotoxins in rats with neuropathic pain secondary to a chronic constriction injury of the sciatic nerve

(i) Materials

(a) Animals

Adult male Sprague-Dawley rats were purchased from the Animal Resources Centre (ARC), Perth, Australia, and the Herston Medical Research Centre, The University of Queensland. Rats were housed in a temperature controlled environment (21 ± 2°C) with a 12h/12h light/dark cycle. Food and water were available ad libitum.

(b) Reagents and materials

Isoflurane (Forthane) was obtained from Abbott Australasia Pty Ltd (Sydney, Australia). Sodium benzylpenicillin vials (600 mg) were purchased from CSL Ltd (Melbourne, Australia). Normal saline ampoules were obtained from Delta West Pty Ltd (Perth, Australia) and heparinised saline (50 IU/5 ml) was purchased from Astra Pharmaceuticals Pty Ltd (Sydney, Australia). Single lumen polyethylene tubing (I.D. 0.2 mm, O.D. 0.6 mm) was purchased from Auburn Plastics and Engineering Pty Ltd (Sydney, Australia). Sterile siliconised silk sutures (Dysilk™) were obtained from Dyneck Pty Ltd (Adelaide, South Australia) and Michel clips were purchased from Medical and Surgical Requisites Pty Ltd (Brisbane, Australia).

(ii) Method

(a) Chronic Constriction Injury (CCI) of the Sciatic Nerve

Rats were anaesthetised with ketamine (80 mg/kg) and xylazine (8 mg/kg), administered by intraperitoneal injection, and a chronic constriction injury (CCI) of
the sciatic nerve was produced according to the method of Bennett GJ & Xie YK. (1988) Pain; 33(1):87-107. Briefly, the left common sciatic nerve was exposed at mid-thigh level by blunt dissection through the biceps femoris. Proximal to the trifurcation, ~ 10 mm of nerve was freed of adhering tissue and four loose ligatures (3.0 silk) were tied around the sciatic nerve (~ 1 mm apart). The incision was closed in layers. After surgery, rats received benzylpenicillin (60 mg s.c.) to prevent infection and were kept warm during surgical recovery. Rats were housed singly for 14 days prior to opioid or vehicle administration. Rats were inspected daily from the time of CCI-surgery with regard to posture of the affected hindpaw, exploring behaviour, body weight and water intake, and any signs of autotomy.

(b) Intrathecal Catheter Insertion

Ten to eleven days post CCI-surgery or in untreated controls, rats were deeply anaesthetised with a mixture of ketamine (80 mg kg\(^{-1}\)) and xylazine (8 mg/kg) administered as a single intraperitoneal (i.p.) injection. Prior to surgery, the back and neck regions of the rat were shaved and the skin cleansed with betadine surgical scrub. The rat was then placed in a prone position and the L6 lumbar vertebra was located by palpation of the tuber sacrales of the os ilium (Hebel & Stromberg 1976). A 6 cm incision was made in the midline of the back, 3 cm caudal and 3 cm cephalad to L6. A subcutaneous pocket (for the intrathecal catheter) was formed by blunt dissection with scissors on both sides of the incision. The fascia covering the superficial muscles of the back were cut in a 5 mm V-shaped incision that encompassed L5. Additional 5 mm caudal incisions were made parallel to L6. The fascia was then retracted and the lumbar muscles surrounding the base of L5 and L6 were removed, as was the m. interspinalis between the spinous processes of L5-L6.

Following removal of the L6 spinous processes with rongeurs, the soft tissue beneath the L5 iliac arch was removed, exposing the dura mater. The dural membrane was pierced with a 23G needle, releasing clear CSF. A polyethylene catheter (O.D. 0.6 mm, I.D. 0.2 mm; 20 cm in length) pre-filled with saline, was
carefully advanced a distance of 1 cm into the intrathecal space and a small volume of saline (20 mL) was administered through the catheter. If leakage of saline around the catheter was observed, the rat was excluded from further experimentation. After successful completion of the 'leak test', the intrathecal (i.t.) catheter was fixed with dental cement onto the surrounding muscle ~ 2 cm from L5, exteriorised through a subcutaneous (s.c.) tunnel to a small incision at the base of the neck and sutured in position. After suturing of the lumbar muscles and skin, rats received benzylpenicillin (50000 IU i.p.) and enrofloxacin (5 mg·kg\(^{-1}\) s.c.) to prevent infection and were kept warm during recovery from anaesthesia.

Following completion of the surgery, rats were housed singly for a recovery period of 3-4 days prior to i.t. drug administration. On the day following surgery, the local anaesthetic, lignocaine (2%, 20 mL) was administered via the i.t. catheter. If complete paralysis of both hind legs was not observed, rats were excluded from further experimentation.

\(\mu\)-conotoxin peptides can also be delivered at higher doses intravenously in CCI model rats to produce antinociception and locally to nerve bundles to produce sensory nerve block.

(c) Drugs Administered

Peptides were prepared in 5 mM sodium acetate buffer at pH 5.5 at delivered to rats in a single bolus dose of 0.2–30 nmoles. Stock solutions of the peptides were quantified relative to an amino acid analysed stock solution by reversed phase HPLC with u.v. detection. Morphine HCl powder was purchased from the Royal Brisbane Hospital Central Pharmacy (Herston, Queensland) and dissolved in normal saline to prepare a stock concentration of 10 \(\mu\)g/10 \(\mu\)l (morphine base). Each rat received 3.5–50 nmol (10-15 \(\mu\)l) of morphine. All dilutions were made with normal saline. All i.t. injections were followed by a saline flush (20 \(\mu\)L) to ensure complete peptide delivery into the intrathecal space.
(d) Storage of Stock Solutions
Aliquots (10 μL) of stock solutions were stored at −20°C prior to use for animal experimentation. Immediately prior to experimentation, aliquots of the relevant compound were thawed at room temperature and then diluted to the required concentration with sterile saline to achieve the desired final concentration for subsequent i.t. Unused portions were discarded to waste to ensure that compounds only underwent one freeze-thaw cycle.

(e) Intrathecal Drug Dosing
10 On day 14 post-CCI surgery, individual groups of drug-naïve-CCI rats received an i.t. bolus injection of μ-conotoxin, morphine or saline in a volume of 10−15 μL. Antinociception was assessed using von Frey filaments until responses returned to baseline.

(f) Assessment of antinociception: CCI rats using Von Frey filaments
Tactile allodynia, the distinguishing feature of neuropathic pain, was quantified using Von Frey filaments which were used to apply a non-noxious mechanical stimulus (light pressure) to the hindpaw. Rats were transferred to wire mesh testing cages (20 cm x 20 cm x 20 cm) and allowed to acclimatise for 10 min. Von Frey filaments were used to determine the lowest mechanical threshold required for a brisk paw withdrawal reflex. Briefly, starting with the Von Frey filament that produced the lowest force, the filament was applied to the plantar surface of the hindpaw until the filament buckled slightly. Absence of a response after 5 s prompted use of the next filament of increasing weight. Filaments used produced a buckling weight of 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 g and these were calibrated regularly. A score of 20 g was given to animals that did not respond to any of the Von Frey filaments. Paw withdrawal thresholds (g) were converted to area under the curve (AUC).
(g) Verification of correct i.t. catheter placement
At the completion of each experiment, malachite green dye (30 μL) was injected via the i.t. catheter whilst rats were lightly anaesthetised with O₂:CO₂ (50%:50%). Thirty seconds later, rats were decapitated and the spinal column was exposed surgically. Data from rats where there was evidence of subcutaneous dye leakage at the site where the catheter entered the back muscles above L6 or failure of the dye to distribute at least 3-4 cm along the spinal cord, were excluded from the analysis.

(h) Data Analysis
The area under the degree of antinociception versus time curve (AUC values) for each peptide was calculated from time = 0 to 3 h. A dose-response curve for each peptide was constructed by plotting AUC values versus the i.t. peptide dose (expressed in nmol per rat).

Prophetic Example 10. Phrenic nerve-diaphragm studies
The selectivity and potency of μ-conotoxins at peripheral neuronal VSSCs was measured using well known techniques (Lewis RJ. et al. (1993) Toxicon. 31 1039-1050).

The left hemidiaphragm with intact phrenic nerve was excised from mice or rats within 30 sec of being killed. Each hemidiaphragm is trimmed to 8-10 mm width and placed in Ringer of the following composition (mM): NaCl 135.0; KCl, 5.0; CaCl₂, 2.0; MgCl₂, 1.0; KH₂PO₄, 1.0; NaHCO₃, 15.0; and glucose, 11.0. Ringer was bubbled with carbogen (95 % O₂/5% CO₂) throughout the experiments, with preparations maintained under 1.0 g tension in 5-ml organ baths at 37° (pH 7.4). The μ-conotoxin is added to the ringer and equilibrated. For each experiment, increasing concentrations (ranging from 10⁻¹² to 10⁻⁶M) of μ-conotoxin are used. Contractile responses to electrical stimulation, either indirectly (via the phrenic nerve) or directly (via the muscle), are recorded via isometric force transducers.
Preparations are stimulated at 0.1 Hz (0.2 msec, 150% of threshold) or directly through the muscle (0.1 Hz with 2.0 msec pulses at a nominal 35 V).

Prophetic Example 11. Electrophysiological Experiments

Electrophysiological experiments were conducted to further investigate the effect of μ-conotoxins on TTX-S and TTX-R sodium channels in native tissue containing peripheral neuronal, central neuronal and skeletal VSSCs.

(a) Tissue Preparation

(i) Dissociation of Nodose and DRG Neurons - Sensory neurons from rat nodose ganglia and dorsal root ganglia (DRG) are isolated using previously described methods (Jeglitsch, G. et al., (1998) J. Pharmacol. Exp. Ther. 284, 516-525; Nicholson, G.M. et al., (1998) Pflugers Arch. 436, 117-126). Young rats (10-21 days) were killed by cervical dislocation and the nodose and DRG carefully removed. The ganglia are placed in physiological saline solution containing collagenase (~1.0 mg/ml type 2 Worthington Biochemical Corp., NJ) and incubated for 1 h at 37°C in 95% air and 5% CO2 for 24-48 hr. Neurons from the nodose ganglia that are clear and round are selected for experiments. Small diameter cells (~20 mm) from the DRG are used as these have been previously been reported to predominantly express TTX resistant Na+ currents (Elliott, A.A. and Elliott, J.R. (1993) J. Physiol. 463, 39-56).

(ii) Hippocampal CA1 Neurons

Dissociation of Hippocampal CA1 Neurons - Young rats (14-21 days) are anaesthetised under CO2 and decapitated with an animal guillotine. The brain is removed and transferred to ice cold artificial cerebrospinal fluid (ACSF containing 124 mM NaCl, 26 mM NaH2CO3, 3 mM KCl, 1.3 mM MgSO4, 2.5 mM NaH2PO4 and 20 mM glucose). The brain was mounted in a vibratome and bathed in ice cold ACSF equilibrated with 95 % O2 and 5% CO2 while the 500 mm thick slices were prepared. Brain slices are incubated for 30 min with 200 U/ml papain (Worthington Biochem.), 1.1 mM cysteine (Sigma), 0.2 mM EDTA and 13.4 mM
mercaptoethanol, at 35°C. Following incubation the CA1 region is located, removed and gently triturated using a fire polished Pasteur pipette. Neurons of 10–15 mm are used, with cells that are flat, swollen or grainy in appearance avoided.


(b) Electrophysiological Recordings

Whole cell Na+ currents are recorded using the patch clamp technique. Patch pipettes (GC150F, Harvard Apparatus Ltd., Edenbridge, Kent, UK) were prepared that have resistances of between 1-2 MΩ (nodose and DRG neurons) and 6-10 MΩ (CA1 neurons) when filled with pipette solution. Whole cell Na+ currents from nodose and DRG neurons were made using a List EPC 7 amplifier (List Medical), voltage steps were generated by a PC (Dell Pentium) running pClamp (Axon Instruments Inc, Union City, CA). Whole cell Na+ currents from CA1 neurons were made using an Axopatch 1D amplifier (Axon Instruments Inc), with voltage steps generated using a PC (Osborne 486-SX) running custom software (Ju, Y., Saint, D. A. and Gage, P. W. (1992) Br. J. Pharmacol. 107, 311-316; Hammarström, A.K. and Gage P.W. (1998) J. Physiol. 510, 735-741; Ju, Y.K. et al (1996) J. Physiol. 497, 337-347). For mylenated nerve bundles, field electrodes are used to measure compound action potentials as described in the literature (Weber et al., 2002; Gold et al., 2003).

(c) Solution and Toxins

To record Na+ currents from DRG and nodose neurons, patch pipettes are filled with the following solution (mM): CsF 135, NaCl 10, N-hydroxyethylpiperazine-N-ethanesulphonic acid (HEPES) 5, with pH adjusted 7.2 with CsOH. The bath solution contained (mM): NaCl 50, KCl 3, tetraethylammonium chloride (TEA) 90, CdCl2 0.1, glucose 7.7, HEPES 10, with pH adjusted to 7.4 with TEA-OH. To record Na+ currents from CA1 neurons, the patch pipette solution contained the
following solution (mM); CsF 125, NaF 5, KC1 10, TES 10, with pH adjusted to 7.4 with KOH. The bath solution contained (mM); NaCl 135, KC1 5, MgCl2 3, CaCl2 1, CoCl2 5, CsCl 5, TES 10, with pH adjusted to 7.4 with NaOH.

To record Na+ currents from mylenated nerve bundles, patch pipette and bath solution conditions are those described in Gold et al., 2003.

(s) Data Analysis
For CA1 Neurons, three distinct Na+ currents are measured; a transient TTX sensitive Na+ current (TTX-S $I_{NaT}$), a transient TTX resistant Na+ current (TTX-R $I_{NaT}$), and a persistent TTX sensitive Na+ current (TTX-S $I_{NaP}$). The amplitude of evoked TTX-S $I_{NaT}$ is measured at its peak after subtraction of the current evoked in the presence of TTX (0.5-1 μM). The amplitude the TTX-R $I_{NaT}$ is measured at least 2 min following the addition of 0.5-1 μM TTX. The amplitude of TTX-S $I_{NaP}$ is measured at the end of a 400 ms voltage step after subtraction of the current evoked in the presence of TTX (0.5-1 μM). All values are expressed as means ± SEM with “n” indicating the number of cells in a given series of experiments. Comparisons of two means are made using Student’s two-tailed unpaired t test. The more potent μ-conotoxins identified in the 125I-TIIIA binding assay had a potency of 1 μM or greater in the assays described above.

Example 12
The activity of μ-conotoxins was determined at sodium channels expressed in Xenopus oocytes as described below.

Oocyte injection and recording
Oocytes (stage V–VI) were surgically removed from mature Xenopus laevis frogs anaesthetized by immersion in 0.1% 3-aminobenzoic acid ethyl ester (MS-222).
The follicular cell layer was removed by incubating oocytes in Ca\(^{2+}\)-free solution containing (mM): 96 NaCl, 2 KCl, 1 MgCl\(_2\), 5 HEPES (pH 7.4), plus 2 mg/ml collagenase (Sigma Type 1) for 2 hr at room temperature. Oocytes were rinsed several times, sorted and maintained at 18 °C in ND96 storage solution, which contained (mM): 96 NaCl, 2 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 5 HEPES, 5 pyruvate, plus 50 μg/ml gentamycin (pH 7.4).

cRNA was synthesized in vitro from linearised template cDNA using an Ambion mMessage mMachine kit. cDNA (1–50 ng) encoding Nav1.2, 1.3, 1.6 and 1.7 Na\(^{+}\) channels were injected with each subunit cRNA using a precision injector (Drummond). Injected cells were maintained in ND96 solution at 18 °C for 2–14 days prior to experiments.

Electrophysiological recordings were made in a solution containing (mM): 81 NaCl, 5 KCl, 5 HEPES, 3 CaCl\(_2\), 2 MgCl\(_2\) adjusted to pH 7.5 with NaOH. Depolarization-activated Na\(^{+}\) currents were recorded using a two-electrode voltage clamp with virtual ground circuit (Geneclamp 500B amplifier; Axon Instruments Inc., Union City, CA). Voltage and current electrodes were filled with 3M KCl and had resistances that ranged from 0.2–1.0 MΩ in the recording solution. Depolarization-activated Na\(^{+}\) currents were evoked from a holding potential of −70 mV by test voltages generated using pCLAMP 8.0 software and a Digidata 1200 series interface (Axon Instruments Inc). Membrane currents were filtered at 1–2 kHz and sampled at 10 kHz. Capacitative and leak currents were subtracted online using a −P/6 pulse protocol. Oocytes were continuously perfused with recording solution at a flow rate of 2 ml/min at room temperature (≈ 22 °C). Cells showing < 5 % change in current amplitude over a 10 min incubation period were used in these studies to
avoid problems associated with current run-down. All recordings were performed in a small volume (200 µl) Perspex bath. µ-Conotoxins were added directly into the bath to a final concentration of 1 or 3 µM. Recordings were made for at least 5 min in the presence of each toxin until the current amplitude stabilized before washout.

For each of the µ-conotoxins studied, the rate of onset of toxin block and rate of the measurable recovery from block during toxin washout were obtained from single exponential fits to the data using Prism software (GraphPad). The Kd for each toxin can be calculated from this kinetic data.

**Results**

As expected, µ-conotoxins active in displacing ¹²⁵I-TIIIA from rat brain were also active in inhibiting sodium current through Naᵥ1.2 expressed in oocytes. Surprisingly, several µ-conotoxins had activity to inhibit sodium current through peripheral sodium channels, including Naᵥ 1.1, 1.3, 1.6 and 1.7. Specifically, Q20-TIIIA (Seq. ID no 111) at 1 µM inhibited 40% of sodium current through Naᵥ1.7, and desZ1-SIIIB (Seq ID. No. 41) at 3 µM inhibited 25% current through Naᵥ1.7. Thus the µ-conotoxins of the present invention are able to inhibit peripheral neuronal forms of the voltage sensitive sodium channel.

It will be appreciated by a person skilled in the art that the numerous variations and/or modifications may be made to the invention as shown the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.
Throughout this specification and any claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference to any prior art in this specification is not, and should not be taken as an acknowledgment or any form or suggestion that that prior art forms part of the common general knowledge in Australia.
CLAIMS

1. An isolated, synthetic or recombinant \( \mu \)-conotoxin peptide comprising following sequence

\[
\text{Arg His Gly Cys Cys Lys Gly Xaa2 Lys Gly Cys Ser Ser Arg Glu Cys Arg Xaa2 Gln}
\text{His Cys Cys}\quad \text{[SEQ ID NO 8]}
\]

where Xaa2 is 4Hyp;
and derivatives thereof but excluding the des Arg1 derivative and derivatives of the sequence

\[
\text{His Gly Cys Cys Lys Gly Xaa5 Xaa3 Gly Cys Ser Ser Arg Xaa3 Cys Arg Xaa5 Gln}
\text{His Cys Cys}\quad \text{[SEQ ID NO 6]}
\]

where Xaa3 is Glu or \( \gamma \)-carboxy Glu, Xaa4 is Gln or pyro-Glu, Xaa5 is Pro or hydroxy-Pro and Xaa6 is Trp or bromo-Trp.

2. An isolated, synthetic or recombinant \( \mu \)-conotoxin peptide according to claim 1 further excluding des Arg1 derivatives.

3. An isolated, synthetic or recombinant \( \mu \)-conotoxin peptide according to claim 1 or 2 wherein the derivatives are limited to the addition or deletion of no more than 3 amino acid residues and/or to conservative amino acid substitution or side chain modification.

4. An isolated, synthetic or recombinant \( \mu \)-conotoxin peptide according to any one of claims 1 to 3 which is more potent than des Arg1-TIIIA [Seq. ID. No. 42] at neuronal mammalian voltage sensitive sodium channels ("VSSCs").

5. An isolated, synthetic or recombinant \( \mu \)-conotoxin peptide according to any one of claims 1 to 3 with a potency of \( \leq -8.9 \), as determined from a calculated
log_{10}C_{50} (M) value by displacement of $^{125}$I TIIIA at neuronal (rat brain) VSSCs using the displacement study method described herein in example 5.

6. An isolated, synthetic or recombinant $\mu$-conotoxin peptide according to claim 1 with a potency of from 1 pM to 10 $\mu$M at peripheral neuronal VSSCs as measured by
   a) displacement of $^{125}$I-TIIIA from sodium channels in membranes from dorsal root ganglia (DRG) by the method described herein in example 5; or
   b) the proportion of the sodium current blocked by the peptide in mammalian DRG by the method as described herein in example 11; or
   c) inhibition of conduction of mylenated nerve bundles as described herein in example 11.

7. An isolated, synthetic or recombinant $\mu$-conotoxin peptide according to any one of claims 1 to 6 with selectivity for mammalian neuronal VSSCs over skeletal muscle VSSCs when measured by $^{125}$I-TIIIA displacement studies.

8. An isolated, synthetic or recombinant $\mu$-conotoxin peptide according to claim 7 with a 3-fold or greater selectivity for mammalian neuronal VSSCs over skeletal muscle VSSCs when measured by $^{125}$I-TIIIA displacement studies.

9. An isolated, synthetic or recombinant $\mu$-conotoxin peptide having a 3-fold or greater selectivity for mammalian neuronal VSSCs over skeletal muscle VSSCs when measured by $^{125}$I-TIIIA displacement studies and which has the sequence:

$$\text{Xaa1 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Lys Gly His Ala Arg Cys Cys}$$

[SEQ ID NO 9]

and derivatives thereof, excluding the derivative of the sequence:
Xaa1 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Arg Asp His Ala Arg Cys Cys

[SEQ ID NO 109]

where Xaa1 is pyroglutamate.

10. An isolated, synthetic or recombinant μ-conotoxin peptide according to claim 9 having for a 10-fold or greater selectivity for mammalian neuronal VSSCs over skeletal muscle VSSCs when measured by $^{125}$I-TIIIA displacement studies.

11. An isolated, synthetic or recombinant μ-conotoxin peptide according to claim 10 having for a 100-fold or greater selectivity for mammalian neuronal VSSCs over skeletal muscle VSSCs when measured by $^{125}$I-TIIIA displacement studies.

12. An isolated, synthetic or recombinant μ-conotoxin peptide according to claim 11 having for a 1000-fold or greater selectivity for mammalian neuronal VSSCs over skeletal muscle VSSCs when measured by $^{125}$I-TIIIA displacement studies.

13. An isolated, synthetic or recombinant μ-conotoxin peptide according to any one of claims 9 to 12 wherein the derivatives are limited to the addition or deletion of no more than 3 amino acid residues and/or to conservative amino acid substitution or side chain modification.

14. An isolated, synthetic or recombinant μ-conotoxin peptide according to any one of claims 1 to 13 having selectivity for mammalian peripheral neuronal VSSCs over skeletal muscle VSSCs when measured by $^{125}$I-TIIIA displacement studies.

15. An isolated, synthetic or recombinant μ-conotoxin peptide having a 3-fold or greater selectivity for selectivity towards mammalian peripheral neuronal VSSCs over skeletal muscle VSSCs and which has the sequence:
Xaa1 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Lys Gly His Ala Arg Cys Cys

[SEQ ID NO 9]

or

5

Xaa1 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Arg Asp His Ala Arg Cys Cys

[SEQ ID NO 109]

where Xaa1 is pyroglutamate,

and derivatives thereof.

10

16. An isolated, synthetic or recombinant μ-conotoxin peptide according to claims 15 wherein the derivatives are limited to the addition or deletion of no more than 3 amino acid residues and/or to conservative amino acid substitution or side chain modification.

15

17. An isolated, synthetic or recombinant μ-conotoxin peptide comprising the following sequence:

Xaa7 Xaa8 Xaa9 Cys Cys Xaa10 Xaa11 Xaa12 Xaa13 Xaa14 Cys Xaa13 Xaa14

Xaa15 Xaa16 Xaa17 Xaa18 Cys Xaa19 Xaa20 Xaa21 Xaa22 Xaa23 Cys Cys

Xaa24 Xaa25

[SEQ ID NO 110]

where Xaa7 to Xaa25 are independently any amino acid residue other than cysteine or may be absent,

excluding peptides having the sequence set out in [SEQ ID NO 1 to 7 and 109].

25

18. An isolated, synthetic or recombinant μ-conotoxin peptide according to claim 17 wherein Xaa11 and Xaa14 are Gly and Xaa15 and Xaa16 are Ser.

30

19. An isolated, synthetic or recombinant μ-conotoxin peptide according to claim 17 or 18 wherein no more than 6 residues are absent.
20. An isolated, synthetic or recombinant \( \mu \)-conotoxin peptide according claim 19 wherein Xaa9, Xaa12 and Xaa13 are absent and Xaa11 and Xaa14 are Gly and Xaa15 and Xaa16 are Ser.

5

21. An isolated, synthetic or recombinant \( \mu \)-conotoxin peptide according to claim 17 or 18 wherein
   Xaa9, Xaa12 and Xaa13 are absent;
   Xaa7 is absent, pyroglutamate, Glu, Gln, Asp, Asn, Arg, Lys or His;
10   Xaa8, Xaa9 and Xaa10 are independently selected from Arg, Lys, His, Glu, Gln, Asp and Asn;
   Xaa17 is Arg, Lys or His;
   Xaa18 is Trp, Ala, Tyr, His or Phe;
   Xaa19 is Arg, Lys, His; Xaa20 is Ala, Gly;
15   Xaa21 is His, Lys or Arg;
   Xaa22 is Ala;
   Xaa23 is Arg or Lys; and
   Xaa24 and Xaa25 are independently Ala, Lys or absent.

20

22. An isolated, synthetic or recombinant \( \mu \)-conotoxin peptide according claim 17 or 18 wherein Xaa23 is absent.

23. An isolated, synthetic or recombinant \( \mu \)-conotoxin peptide according claim 22 wherein Xaa9 is Gly;

25   Xaa12 and 20 are independently 4Hyp, Pro or be absent; and
   Xaa24 and Xaa25 are independently Ala, Lys or absent.

24. An isolated, synthetic or recombinant \( \mu \)-conotoxin peptide according claim 23 wherein Xaa7 is absent or Arg, His or Lys;

30   Xaa8 is His, Lys, Arg, Ala, Ser, Thr, Pro, Hyp, Gly, Gln, Asn, Asp or Glu;
   Xaa10 is His, Lys, Arg, Ala, Ser, Thr, Pro, Hyp, Gly, Gln, Asn, Asp or Glu;
Xaa13 is His, Lys, Arg, Ala, Ser, Thr, Pro, Hyp, Gly, Gln, Asn, Asp, Glu or absent;
Xaa17 is Arg, Lys or His;
Xaa18 is Gln, Asn, Ala, Ser, Thr, Pro, Hyp or Gly;
Xaa19 is Arg, Lys or His;
Xaa21 is Ala, Ser, Thr, Pro, Hyp, Gly, Gln, Asn, Asp, or Glu; and
Xaa22 is Arg, Lys or His.

25. An isolated, synthetic or recombinant μ-conotoxin peptide according to any
one of claims 17 to 24 which exhibits a 3-fold or greater selectivity towards
mammalian neuronal (peripheral and/or central) VSSCs over skeletal muscle
VSSCs when measured by $^{125}\text{I}$-TIIIA displacement studies.

26. An isolated, synthetic or recombinant μ-conotoxin peptide according to any
one of claims 17 to 25 which is more selective towards mammalian neuronal
VSSCs than STX.

27. An isolated, synthetic or recombinant μ-conotoxin peptide according to
claim 26 wherein the difference between $\log_{10}C_{50}$ (M) for neuronal VSSCs and
$\log_{10}C_{50}$ (M) for skeletal VSSCs is < -0.450 when measured by $^{125}\text{I}$-TIIIA
displacement studies.

28. An isolated, synthetic or recombinant μ-conotoxin peptide according to any
one of claims 17 to 27 with a potency of ≤ -7.0, as determined from a calculated
$\log_{10}C_{50}$ (M) value by displacement of $^{125}\text{I}$ TIIIA at neuronal (rat brain) VSSCs
using the displacement study method described herein in example 5.

29. An isolated, synthetic or recombinant μ-conotoxin peptide according to
claim 28 with a potency of ≤ -8.0, as determined from a calculated $\log_{10}C_{50}$ (M)
value by displacement of $^{125}\text{I}$ TIIIA at neuronal (rat brain) VSSCs using the
displacement study method described herein in example 5.
30. An isolated, synthetic or recombinant μ-conotoxin peptide as set out in Table 1 and derivatives thereof.

31. An isolated, synthetic or recombinant μ-conotoxin peptide as set out in Table 1.

32. An isolated, synthetic or recombinant μ-conotoxin peptide as set out in Table 1 which selectively binds to mammalian neuronal VSSCs over skeletal muscle VSSCs.

33. An isolated, synthetic or recombinant μ-conotoxin peptide as set out in Table 1 which selectively binds to mammalian peripheral neuronal VSSCs over skeletal muscle VSSCs when measured by $^{125}$I-TIIIA displacement studies.

34. An isolated, synthetic or recombinant μ-conotoxin peptide as set out in Table 1 with a potency of ≤ -7.0, as determined from a calculated log$_{10}$IC$_{50}$ (M) value by displacement of $^{125}$I TIIIA at neuronal (rat brain) VSSCs using the displacement study method described herein in example 5.

35. An isolated, synthetic or recombinant μ-conotoxin peptide as set out in Table 1 with a potency of ≤ -8.0, as determined from a calculated log$_{10}$IC$_{50}$ (M) value by displacement of $^{125}$I TIIIA at neuronal (rat brain) VSSCs using the displacement study method described herein in example 5.

36. An isolated, synthetic or recombinant μ-conotoxin peptide as set out in Table 1 with a potency of ≤ -8.9, as determined from a calculated log$_{10}$IC$_{50}$ (M) value by displacement of $^{125}$I TIIIA at neuronal (rat brain) VSSCs using the displacement study method described herein in example 5.
37. An isolated, synthetic or recombinant μ-conotoxin peptide as set out in Table 2.

38. An isolated, synthetic or recombinant μ-conotoxin peptide as set out in Table 3.

39. A composition comprising an isolated, synthetic or recombinant μ-conotoxin peptide according to any one of claims 1 to 38 and a pharmaceutically acceptable carrier or diluent.

40. The composition according to claim 39 wherein the peptide is set out in Table 1.

41. The composition according to claim 39 or 40 comprising one or more active agents in addition to the peptide.

42. Use of an isolated, synthetic or recombinant conotoxin μ-conotoxin according to any one of claims 1 to 38, or a salt, ester, amide, solvate, prodrug or cyclised derivative thereof, in the manufacture of a medicament for the treatment or prophylaxis of disease mediated by sodium channels or in the control or treatment of pain.

43. The use according to claim 42 in the manufacture of a medicament for the treatment or prophylaxis of epilepsy, stroke, cancer and cardiovascular disease or in the treatment of pain.

44. A method for the treatment or prophylaxis cardiovascular and neurological diseases, cancer and the treatment or control of pain which includes the step of administering to a mammal an effective amount an isolated, synthetic or recombinant conotoxin μ-conotoxin according to any one of claims 1 to 38, or a salt, ester, amide, solvate, prodrug or cyclised derivative thereof.
45. A method of selectively inhibiting neuronal action potentials in the peripheral nervous system over skeletal muscle VSSCs by the use of a peptide having the sequence:

\[
\text{Xaa1 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Lys Gly His Ala Arg Cys Cys} \quad [\text{SEQ ID NO 9}]
\]

or

\[
\text{Xaa1 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Arg Asp His Ala Arg Cys Cys} \quad [\text{SEQ ID NO 109}]
\]

where Xaa1 is pyroglutamate,

and derivatives thereof, the derivatives limited to the addition or deletion of no more than 3 amino acid residues and/or to conservative amino acid substitution or side chain modification thereof.

46. Use of an isolated, synthetic or recombinant \( \mu \)-conotoxin peptide according to any one of claims 1 to 38 in the treatment or prophylaxis of stroke, epilepsy or cardiovascular conditions, or pain including neuropathic pain, chronic pain, gastrointestinal pain, inflammatory, joint and visceral pain.

47. Use of an isolated, synthetic or recombinant \( \mu \)-conotoxin peptide according to any one of claims 1 to 38 as neuromuscular blocking agents, local anaesthetic agents, analgesic agents and neuroprotective agents.

48. Use of an isolated, synthetic or recombinant conotoxin \( \mu \)-conotoxin according to any one of claims 1 to 38 as nucleic acid probes or in assays.
49. Use of an isolated, synthetic or recombinant conotoxin \( \mu \)-conotoxin according to any one of claims 1 to 38 to screen molecule libraries to identify molecules which are selective blocking agents at specific VSSC subtypes.
AMENDED CLAIMS
[received by the International Bureau on 04 October 2004 (04.10.04);
original claims 1-2, 17 amended; remaining claims unchanged (2 pages)]

1. An isolated, synthetic or recombinant \( \mu \)-conotoxin peptide comprising following sequence

\[
\text{Arg His Gly Cys Cys Lys Gly Xaa2 Lys Gly Cys Ser Ser Arg Glu Cys Arg Xaa2 Gln}
\]

\[
\text{His Cys Cys }
\]

where Xaa2 is 4Hyp;

and derivatives thereof but excluding the des Arg1 derivative and derivatives of the

\[
\text{sequence}
\]

\[
\text{His Gly Cys Cys Lys Gly Xaa5 Xaa3 Gly Cys Ser Ser Arg Xaa3 Cys Arg Xaa5 Gln}
\]

\[
\text{His Cys Cys }
\]

where Xaa3 is Glu or \( \gamma \)-carboxy Glu, Xaa5 is Pro or hydroxy-Pro.

2. An isolated, synthetic or recombinant \( \mu \)-conotoxin peptide according to claim 1 further excluding any des Arg1 derivatives.

3. An isolated, synthetic or recombinant \( \mu \)-conotoxin peptide according to claim 1 or 2 wherein the derivatives are limited to the addition or deletion of no more than 3 amino acid residues and/or to conservative amino acid substitution or side chain modification.

4. An isolated, synthetic or recombinant \( \mu \)-conotoxin peptide according to any one of claims 1 to 3 which is more potent than des Arg1-TIIIA [Seq. ID. No. 42] at neuronal mammalian voltage sensitive sodium channels ("VSSCs").

5. An isolated, synthetic or recombinant \( \mu \)-conotoxin peptide according to any one of claims 1 to 3 with a potency of \( \leq -8.9 \), as determined from a calculated
Xaa1 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Lys Gly His Ala Arg Cys Cys

[SEQ ID NO 9]

or

Xaa1 Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Arg Asp His Ala Arg Cys Cys

[SEQ ID NO 109]

where Xaa1 is pyroglutamate,
and derivatives thereof.

16. An isolated, synthetic or recombinant \(\mu\)-conotoxin peptide according to claims 15 wherein the derivatives are limited to the addition or deletion of no more than 3 amino acid residues and/or to conservative amino acid substitution or side chain modification.

17. An isolated, synthetic or recombinant \(\mu\)-conotoxin peptide comprising the following sequence:

Xaa7 Xaa8 Xaa9 Cys Cys Xaa10 Xaa11 Xaa12 Xaa13 Xaa14 Cys Xaa15 Xaa16
Xaa17 Xaa18 Cys Xaa19 Xaa20 Xaa21 Xaa22 Xaa23 Cys Cys Xaa24 Xaa25

[SEQ ID NO 110]

where Xaa7 to Xaa25 are independently any amino acid residue other than cysteine or may be absent,

excluding peptides having the sequence set out in [SEQ ID NO 1 to 7 and 109].

18. An isolated, synthetic or recombinant \(\mu\)-conotoxin peptide according to claim 17 wherein Xaa11 and Xaa14 are Gly and Xaa15 and Xaa16 are Ser.

19. An isolated, synthetic or recombinant \(\mu\)-conotoxin peptide according to claim 17 or 18 wherein no more than 6 residues are absent.
Design of the *Mu1A + ANCHOR* PCR assay used for the production of *mu* class conopeptides.

**Primer Sequences**

*Mu1A* 5' - AAGAGGGATCCATAGCATGTTCTGTAATCAAACGG - 3'
*ANCHOR* 5' - AACTGGAAGAATTCCGCGCCGCAGGAAT - 3'

_Figure 2_
Representative samples of DNA products produced by the PCR of *Conus* spp venom duct cDNA with the oligonucleotide primers *MuI A* + *ANCHOR*. The 800bp (mu peptide) and 400bp ('linear' peptide) bands are indicated by arrows.

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*Figure 3*
Nucleotide and amino acid sequence for the leader and mature peptide region of TIIIA

```
ATG ATG TCT AAA CTG GGA GTC TTG TTG ACC ATC TGT CTG CTT
M M S K L G V L L T I C L L
Met Met Ser Lys Leu Gly Val Leu Leu Thr Ile Cys Leu Leu

CTG TTT CCC CTT ACT GCT CTG CCG ATG GAT GGA GAT GAA CCT
L F P L T A L P M D G D E P
Leu Phe Pro Leu Thr Ala Leu Pro Met Asp Gly Asp Glu Pro

GCA ATC CGA CCT GCA ATG CGT ATG CAG GAC AAC ATT TCA CTC
A I R P A M R M Q D N I S S
Ala Ile Arg Pro Ala Met Arg Met Gln Asp Asn Ile Ser Ser

GAG CAG CAT CCC TTG TTT GAG GAG AGA CAC GGA TGT TGC AAG
E Q H P L F E E R H G C C K
Glu Glu His Pro Leu Phe Glu Glu Arg His Gly Cys Cys Lys

GGG CCG AAG GGA TGC TCC TCC AGA GAA TGC AGA CCC CAA CAT
G P K G C S S R E C R P Q H
Gly Pro Lys Gly Cys Ser Ser Arg Glu Cys Arg Pro Gln His

TGT TGC GGT CGA CGA TAA
C C G R R stop
Cys Cys Gly Arg Arg
```

Figure 4
SEQUENCE LISTING

110> The University of Queensland

120> NOVEL mu-CONOTOXIN PEPTIDES

130> 12442470/JGC

150> AU 2003902131
151> 2003-05-05

160> 110

170> PatentIn version 3.0

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213> Conus purpureascens

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1 5 10 15
Lys Xaa His Arg Cys Cys
20

Arg Asp Cys Cys Thr Xaa Xaa Lys Lys Cys Lys Asp Arg Glu Cys Lys
1 5 10 15

Xaa Glu Arg Cys Cys Ala
20

X at positions 6, 7 and 17 is hydroxy proline
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1   5  10  15
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20

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Ala Arg Cys Cys
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   1   5  10  15
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| 1  | 5  | 10 | 15 |

Arg Cys Cys

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Arg Cys Cys Ala Ala

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Arg Cys Cys Lys
Artificial

28

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Arg Cys Cys Ala Asp
20

29

19

PRT

29

Asn Cys Cys Asn Gly Gly Cys Ser Ser Arg Ala Cys Arg Ala His Ala
1 5 10 15

Arg Cys Cys

30

19

PRT

30

Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Ala Asp His Ala
1 5 10 15

Arg Cys Cys

31

19

PRT

Artificial
Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Arg Ala His Ala
1      5     10     15
Arg Cys Cys

Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Arg Lys His Ala
1      5     10     15
Arg Cys Cys

Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Arg Ala His Ala
1      5     10     15
Arg Cys Cys

Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Arg Ala His Ala
1      5     10     15
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Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Arg Ala His Ala
1      5     10     15
Arg Cys Cys Ala Lys

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| Asn | Cys | Cys | Asn | Gly | Gly | Cys | Ser | Ser | Lys | Ala | Cys | Arg | Ala | His | Ala |
| 1   | 5   | 5   | 10  | 15  |     |     |     |     |     |     |     |     |     |     |     |
| Arg | Cys | Cys |     |     |     |     |     |     |     |     |     |     |     |     |     |
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|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
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| 1   | 5   | 5   | 10  | 15  |     |     |     |     |     |     |     |     |     |     |     |
| Arg | Cys | Cys |     |     |     |     |     |     |     |     |     |     |     |     |     |
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|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Asn | Cys | Cys | Asn | Gly | Gly | Cys | Ser | Ser | Lys | Trp | Cys | Arg | Ala | Tyr | Ala |
| 1   | 5   | 5   | 10  | 15  |     |     |     |     |     |     |     |     |     |     |     |
| Arg | Cys | Cys |     |     |     |     |     |     |     |     |     |     |     |     |     |
|<210> | 37 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|<211> | 21 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
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|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Asn | Cys | Cys | Asn | Gly | Gly | Cys | Ser | Ser | Lys | Trp | Cys | Arg | Ala | His | Ala |
| 1   | 5   | 5   | 10  | 15  |     |     |     |     |     |     |     |     |     |     |     |
| Arg | Cys | Cys |     |     |     |     |     |     |     |     |     |     |     |     |     |
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1      5      10      15
Arg Cys Cys

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1      5      10      15
Arg Cys Cys

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Arg Cys Cys
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1 5 10 15
Arg Cys Cys

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1 5 10 15
Xaa Gln His Cys Cys Ala
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Asn Cys Cys Asn Gly Gly Cys Ser Ser Arg Trp Cys Lys Gly His Ala
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Arg Cys Cys

<400> 43

Asn Cys Cys Asn Gly Gly Cys Ser Ser Arg Trp Cys Lys Ala His Ala
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Arg Cys Cys Ala
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1 5 10 15

Arg Cys Cys Ala
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1 5 10 15

Arg Cys Cys Ala Ala
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1 5 10 15
Arg Cys Cys Ala Ala
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Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Lys Gly His Ala
1  5  10  15
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1  5  10  15

Arg Cys Cys Lys
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1  5  10  15

Ala Cys Cys

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1  5  10  15

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Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Lys Ala His Ala
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Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Lys Lys His Ala
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Artificial

Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Lys Ala His Ala
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Arg Cys Cys Ala Lys
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Arg Cys Cys Ala Lys
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His Ala Arg Cys Cys

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Cys Cys
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1      5   10       15
Gln His Cys Cys
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(1) .. (22)
X at position 6 is Hyp

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1   5   10   15
His Ala Arg Cys Cys Ala
20
81
19
PRT
Artificial

misc_feature
(1) .. (19)
X at position 14 is Hyp

81
Asn Cys Cys Asn Gly Gly Cys Ser Ser Arg Ala Cys Arg Xaa Gln His
1   5   10   15
Cys Cys Ala

82
21
PRT
Artificial

misc_feature
(1)\dots(21)
X at positions 6 and 16 is Hyp

Asn Cys Cys Lys Gly Xaa Lys Gly Cys Ser Ser Lys Trp Cys Arg Xaa
1 5 10 15
Gln His Cys Cys Ala
20

misc_feature
(1)\dots(22)
X at position 6 is Hyp

Asn Cys Cys Lys Gly Xaa Lys Gly Cys Ser Ser Arg Ala Cys Lys Gly
1 5 10 15
His Ala Arg Cys Cys Ala
20
84
19
PRT
Artificial

misc_feature
(1)..<(19)
X at position 14 is Hyp

Asn Cys Cys Asn Gly Gly Cys Ser Ser Lys Trp Cys Arg Xaa Gln His
1 5 10 15
Cys Cys Ala

85
21
PRT
Artificial

Asn Cys Cys Asn Gly Gly Cys Ser Ser Arg Ala Cys Lys Gly His Ala
1 5 10 15
Arg Cys Cys Ala Ala
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86
23
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 1    5    10    15

His Ala Arg Cys Cys Ala Ala
 20

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Cys Cys Ala Ala
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X at position 1 is pyroglutamate
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A. **CLASSIFICATION OF SUBJECT MATTER**

Int. Cl.? : C07K 7/08, 14/435; A61K 38/10, 38/17; A61P 25/04

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)

See Below Under Electronic Data Base Consulted

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)

STN Subsequence Search based on the compounds of Table 1; STN Files Medline, WPIDS; Keyword: mu conotoxin

C. **DOCUMENTS CONSIDERED TO BE RELEVANT**

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<td>US 5670622 A (SHON et al) 23 September 1997 See especially Seq. ID 1, Col. 4</td>
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[X] Further documents are listed in the continuation of Box C [X] See patent family annex

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

“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

“E” earlier application or patent but published on or after the international filing date

“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

“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)

“V” 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

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

“&” document member of the same patent family

Date of the actual completion of the international search: 30 July 2004

Date of mailing of the international search report: 5 AUG 2004

Name and mailing address of the ISA/AU

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R.L. POOLEY
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Form PCT/ISA/210 (second sheet) (January 2004)
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<td>Mari et al., “Conopeptides: Unique pharmacological agents that challenge current peptide methodologies”, Chimica Oggi (Chemistry Today), June 2003, page 43-48 &lt;br&gt;See especially Table II</td>
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Box No. 1  Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material
      
      ☑ a sequence listing
      
      ☐ table(s) related to the sequence listing

   b. format of material
      
      ☑ in written format
      
      ☐ in computer readable form

   c. time of filing/furnishing
      
      ☑ contained in the international application as filed
      
      ☐ filed together with the international application in computer readable form
      
      ☐ furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:
# INTERNATIONAL SEARCH REPORT

**International application No.**

PCT/AU2004/000583

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### Box No. II  Observations where certain claims were found unsearchable (Continuation of Item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. □ Claims Nos.:
   - because they relate to subject matter not required to be searched by this Authority, namely:

2. **X** Claims Nos. 17 (in part), 19 (in part), 21-29 (in part), 42-44 (in part), 46-49 (in part)
   - because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
     - The sequences defined in these claims encompass a very large and indeterminate range of sequences such that a meaningful search could not be drafted. Accordingly, the search of these sequences was limited to the sequences listed in the tables.

3. □ Claims Nos.:
   - because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

---

### Box No. III Observations where unity of invention is lacking (Continuation of Item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. □ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. □ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. □ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. □ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

---

**Remark on Protest**

□ The additional search fees were accompanied by the applicant's protest.

□ No protest accompanied the payment of additional search fees.
This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX