Conotoxins are well known for their highly variable structures and functions. Here we report the identification of a novel conotoxin named mr1e from Conus marmoreus. mr1e is composed of 11 amino acid residues cross-linked by two disulfide bonds (CCHSSWCKHLC). The spacing of intercysteine loops in mr1e is exactly the same as that in α4/3 conotoxins. However, the native mr1e peptide co-eluted on reverse-phase HPLC with the regioselectively synthesized ribbon disulfide linkage isomer (C1-C4, C2-C3) but not the globular linkage isomer (C1-C3, C2-C4). Although this peptide has the same disulfide connectivity as the χ-conotoxins, their sequences do not share significant homology. Thus, mr1e could be defined as a novel conotoxin family. By intracranial injection into mice, mr1e showed an excitatory effect. The characterization of mr1e certainly enriches our understanding of conotoxins, and also opens an avenue for further structural and functional investigation.

Keywords conotoxin; Conus marmoreus; disulfide connectivity; mr1e

Conotoxins are a natural library of bioactive peptides with a population of approximately 50,000 of which only a small portion has been studied [12]. More research is
mr1e, a conotoxin from Conus marmoreus with a novel disulfide pattern

needed to explore the remainder.

In this study, we report the purification and characterization of a novel conotoxin, mr1e, from the venom of the mollusk-hunting snail Conus marmoreus. This peptide has a CC-C-C cysteine framework, like α4/3 conotoxins, but adopts a C1-C4, C2-C3 disulfide bond connectivity. Thus, mr1e might define a novel conotoxin family.

Materials and Methods

Materials

Sephadex G-15 was purchased from Amersham Biosciences (Uppsala, Sweden), Zorbax 300SB-C18 analytical HPLC columns (4.6 mm×250 mm, 9.2 mm×250 mm) were obtained from Agilent Technologies (Santa Clara, USA), and trifluoroacetic acid and acetonitrile were from Merck (Darmstadt, Germany). Reagents for amino-terminal sequencing, Fmoc-amino acids, and Fmoc-cys (Trityl)-resin were purchased from Applied Biosystems (Foster City, USA). Other reagents were of analytical grade.

Peptide purification

The venom apparatus of C. marmoreus was dissected out, cut into segments, and homogenized. The venom was extracted with 1.1% (V/V) acetic acid for 30 min at 0 °C. The homogenate was centrifuged at 10,000 g for 10 min at 4 °C, and the supernatant was collected. This procedure was repeated twice, and the supernatants were pooled, lyophilized, and stored at −20 °C. For conotoxin purification, the lyophilized crude venom was dissolved with 1.1% acetic acid and briefly centrifuged, the supernatant was loaded onto a Sephadex G-15 column (2.6 cm×100 cm) and eluted with 1.1% acetic acid at a flow rate of 0.5 ml/min. The eluted fractions were pooled and further fractionated on a Zorbax 300SB-C18 semipreparative column (9.2 mm×250 mm; Agilent 1100 reverse-phase HPLC). Further purification of peptide mr1e was carried out on a Zorbax 300SB-C18 analytical column (4.6 mm×250 mm). The purity of the prepared peptide was determined by mass spectrometry.

Peptide sequencing

The purified peptide mr1e was dissolved in 0.1 M Tris-HCl, 6 M guanidine-HCl (pH 8.5), and 0.01 M EDTA, and reduced with 100× overdose of diithiothreitol at 37 °C for 2 h. Then 4 μl of 4-vinylpyridine was added. After being mixed and flushed with N2, the mixture was incubated at room temperature for 3 h in darkness. The alkylated peptide was then purified by HPLC. The amino acid sequence of the pyridylethylated mr1e was determined by automated Edman degradation on an ABI 491A Procise protein sequencing system (Applied Biosystems).

Molecular mass determination

The linear peptide of mr1e was synthesized by standard Fmoc chemistry. The protected peptide was independently grown on a Wang resin, using the 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU)/N-hydroxybenzotriazole (HOBt) amino acid activation method. Solid phase peptide synthesis was carried out on a 433A peptide synthesizer (Applied Biosystems).

Two isomers of mr1e with different protecting groups on Cys pairs [Trt-C1, Acm-C2,4 (isomer A C1-C3, C2-C4) and Trt-C1,4, Acm-C2,3 (isomer B C1-C4, C2-C3)] were synthesized. During the cleavage of synthesized peptides from the resin, the residue side chains were simultaneously deprotected except Cys (Acm). The linear peptides were oxidized at room temperature with 50 mM NH4AC buffer (pH 8.0) overnight to form the disulfide bond between free Cys residues. The purified monocyclic peptides were then dissolved in 10% CH3CN, 4% trifluoroacetic acid. Then 0.5 mg/ml iodine was added to form the second disulfide bond between Cys residues protected by Acn. The final fully disulfide bonded peptides were then purified and co-eluted with the natural mr1e.

Bioassay

The biological activity of mr1e was studied by intracranial injection into mice. The toxin mr1e was dissolved in normal saline solution at different concentrations. Male Kunming mice (4 weeks old, 25–30 g body weight) were injected intracranially with 20 μl mr1e solution (n=4 for each dose). The control animals were injected with the same volume of normal saline solution (n=4). The symptoms of injected mice were observed for 2 h.

Results

mr1e purification and sequencing

The crude venom extract from C. marmoreus was first size-fractionated on a Sephadex G-15 column. The major
mr1e, a conotoxin from Conus marmoreus with a novel disulfide pattern

The peak indicated by an arrow in Fig. 1(B) was confirmed to contain a peptide with 11 amino acid residues after being reduced and pyridylethylated, CCHSSWCKHLC. The determined molecular mass of this toxin was 1301.7 Da, consistent with the calculated mass (1301.8 Da) assuming that two disulfide bonds are formed. This toxin shares the same cysteine framework with \( \alpha_4/3 \) conotoxins, with four and three residues in two cysteine loops, respectively. However, the disulfide bond connectivity of this toxin is not like that of \( \alpha \)-conotoxins (C\(_1\)-C\(_3\), C\(_2\)-C\(_4\)) but similar to the disulfide bond connectivity of \( \chi \)-conotoxins (C\(_1\)-C\(_4\), C\(_2\)-C\(_3\)). Therefore, this conotoxin was designated mr1e. According to the classical nomenclature of Conus peptides [1], the two small letters “mr” represent the Conus species from which the conotoxin was identified, number 1 indicates the disulfide framework, and the letter “e” indicates the order of discovery. Before the identification of this peptide, four conotoxins with a CC-C-C framework had been purified from C. marmoreus, namely \( \chi \)-MrlA, MrlB, MrlC and MrlD.

**Assignment of disulfide pattern of mr1e**

Although mr1e has a typical \( \alpha \)-conotoxin cysteine framework, its sequence does not share the conserved Pro residue at the third position in the first intercysteine loop of \( \alpha \)-conotoxins. To clarify the disulfide bond connectivity of mr1e, two isomers of mr1e with different disulfide pairing were chemically synthesized and selectively oxidized. We did not synthesize the isomer with C\(_1\)-C\(_2\), C\(_3\)-C\(_4\) pairing, because vicinal disulfide bonds are extremely rare. Successful peptide synthesis and the formation of each disulfide bond were confirmed by mass spectrometry. To our surprise, the native mr1e co-eluted with the isomer with ribbon pairing (C\(_1\)-C\(_4\), C\(_2\)-C\(_3\)) but not the isomer with globular pairing (C\(_1\)-C\(_3\), C\(_2\)-C\(_4\)) (Fig. 2), which clearly indicates that mr1e has the same topological fold as \( \chi \)-conotoxins.

**Bioassay**

Intracranial injection into mice showed that mr1e has an excitatory effect. When 25 \( \mu \)g mr1e were injected, the mice looked agitated which lasted for about 30 min without obvious behavior symptom. But injection of 100 \( \mu \)g mr1e immediately elicited stiffening of the body which lasted for about 10 min. Until 30 min after injection, the mice could not move due to the rigid paralysis of the rear legs. All the injected mice recovered after 30 min (data not shown).

Fig. 1 Purification of conotoxin mr1e from Conus marmoreus by reverse-phase HPLC  (A) The major peak of the crude venom extraction was fractionated on a semipreparative C18 column by a three-step gradient [0%–20% buffer B (0.1% trifluoroacetic acid in acetonitril) in 0–5 min, 20%–35% buffer B in 5–35 min, 35%–90% buffer B in 35–45 min] with a flow rate of 2 ml/min. The peak indicated by the arrow is mr1e. (B) mr1e was further purified on an analytical C18 column using the following gradient: 0–5 min, 0%–20% buffer B; 5–35 min, 20%–35% buffer B; 35–45 min, 35%–100% buffer B. The flow rate was 0.8 ml/min.
**Discussion**

In this paper, we report the purification and characterization of a novel conotoxin, mr1e, from the venom of *C. marmoreus*. This toxin might present a new family of conotoxins because it shares the same cysteine framework as α4/3 conotoxins but adopts distinct disulfide connectivity.

mr1e is composed of only 11 residues, four of which are cysteines in a CC-C-C pattern. The spacing of the intercysteine loops is exactly the same as α4/3 conotoxins, but mr1e does not show any sequence homology with other α4/3 conotoxins. In particular, mr1e does not have Pro at the third position in the first intercysteine loop, which is highly conserved in all the published α-conotoxins. This Pro residue proved to be critical for the globular disulfide pairing of α-conotoxins (C1-C3, C2-C4). Mutation of this Pro into either bulky charged side chain residue Lys or small side chain residue Ala switched the original globular conformation into ribbon conformation (C1-C4, C2-C3) [13]. Thus, the clarification of the disulfide pairing of mr1e became the primary interest.

Indeed, mr1e has a ribbon disulfide connectivity (C1-C4, C2-C3), like previously reported χ-conotoxins (Table 1). χ-Conotoxins constitute a unique family of conotoxins and act on a special target noradrenaline transporter [11]. Interestingly, all the χ-conotoxins identified so far, as well as mr1e, are from the same species, *C. marmoreus*. Sequence comparison clearly showed that mr1e has distinct features compared with χ-conotoxins. First, the spacing of the second intercysteine loop is different. There are three residues in this loop of mr1e, but only two residues exist in the corresponding loop of χ-conotoxins. Second, the sequence of the first intercysteine loop is also significantly different between mr1e and other χ-conotoxins. Based on the Ala-scanning study of χ-MrIA,
the residues in this loop are all of great importance for the activity or structure of this toxin [14]. It would thus be plausible to speculate that mr1e might have a different biological function.

The bioactivity of mr1e was preliminarily explored by intracranial injection into mice, a method popularly used for conotoxin studies [20,21,22,23]. The descriptive behavior symptoms induced by different conotoxins are often taken as qualitative and suggestive information for further study [21,23]. The injection bioassay showed that mr1e could induce an excitatory effect on mice. The symptoms produced by mr1e are similar to those induced by mr1e could induce an excitatory effect on mice. The symptoms produced by different conotoxins are of interest for conotoxin studies [20,21,22,23]. The descriptive behavior symptoms induced by different conotoxins are of interest for conotoxin studies [20,21,22,23].

Table 1 Sequence comparison of conotoxin mr1e, from Conus marmoreus, with other two-disulfide-bond conotoxins

<table>
<thead>
<tr>
<th>Conotoxin</th>
<th>Sequence</th>
<th>Family</th>
<th>Disulfide connectivity</th>
<th>Target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mr1e</td>
<td>CCHSSWCKHLC</td>
<td>Unknown</td>
<td>C---C---C</td>
<td>Unknown</td>
<td>This work</td>
</tr>
<tr>
<td>MrIA (CMrVIB/mr10a)</td>
<td>NGVCCGYKLCOC</td>
<td>γ/λ</td>
<td>C---C---C</td>
<td>Noradrenaline transporter</td>
<td>[14,16,17]</td>
</tr>
<tr>
<td>MrIB</td>
<td>VGVCCGYKLCOC</td>
<td>γ/λ</td>
<td>C---C---C</td>
<td>Noradrenaline transporter</td>
<td>[14,16,17]</td>
</tr>
<tr>
<td>CMrVIA (MrIC)</td>
<td>VCCGYKLCOC</td>
<td>γ/λ</td>
<td>C---C---C</td>
<td>Noradrenaline transporter</td>
<td>[14,16,17]</td>
</tr>
<tr>
<td>CMrX (MrID)</td>
<td>GICCGVSFCYOC</td>
<td>γ/λ</td>
<td>C---C---C</td>
<td>Noradrenaline transporter</td>
<td>[14,16,17]</td>
</tr>
<tr>
<td>ImI</td>
<td>GCCSDPRCAWRC*</td>
<td>α</td>
<td>C---C---C</td>
<td>Acetylcholine receptor</td>
<td>[18]</td>
</tr>
<tr>
<td>PnIA</td>
<td>GCCSLPPCAANNPDYC*</td>
<td>α</td>
<td>C---C---C</td>
<td>Acetylcholine receptor</td>
<td>[19]</td>
</tr>
<tr>
<td>TIA</td>
<td>FNWRCCILPACRRNHKFC*</td>
<td>ρ</td>
<td>C---C---C</td>
<td>Adrenoreceptor</td>
<td>[11]</td>
</tr>
<tr>
<td>p5a</td>
<td>GCCPKQMRCCCTL*</td>
<td>τ</td>
<td>C---C---C</td>
<td>Unknown</td>
<td>[10]</td>
</tr>
</tbody>
</table>

The conserved Pro residue in the first intercysteine loop is shadowed. *C-terminal amidation; O, hydroxyproline.

Conotoxins are well known for their extremely diversified structures and functions, easily seen by comparing the two-disulfide-bond conotoxins described in Table 1. These toxins possess typically 10–19 residues including four cysteines. However, they show highly divergent cysteine frameworks, disulfide pairing, non-cysteine sequences, and biological functions. Furthermore, the same cysteine framework could form different disulfide connectivities, such as the contrast between mr1e and 4/3 conotoxin. Toxins with the same disulfide linkage might have different targets and functions. For instance, p-TIA has the same topological fold as 4/7 conotoxin, but it targets adrenoreceptors instead of acetylcholine receptors. The mechanism of the diversity of conotoxins is still unclear. The identification of mr1e certainly enriches our understanding of conotoxins, and also opens the way for further structural and functional investigation.

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