Identification of neuropeptide Y-like conopeptides from the venom of *Conus betulinus*

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**Neuropeptide Y** (NPY) is a ubiquitous endocrine neuropeptide found in vertebrate and invertebrate. In our present work, two NPY-like exocrine conopeptides (designated as cono-NPYs) were first identified in the venom of cone snails. Both cono-NPYs showed sequence characteristics of invertebrate NPYs, suggesting that some exocrine venom peptides are probably evolved from the preexisting endocrine peptides during the evolution of cone snails.

**Keywords** conopeptide; neuropeptide Y; evolution

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**Introduction**

Conotoxins and conopeptides are a group of exocrine venom peptides produced by cone snails [1–5]. Conotoxins typically contain two or more disulfide bonds, whereas conopeptides contain only one or no disulfide bond. Cone snails utilize these exocrine venom peptides to capture their preys or defend themselves from their predators. These peptides target at a wide range of ion channels and neurotransmitter receptors, therefore, they can be used as probes or tools in neuroscience, and some are being developed as therapeutic drugs.

Neuropeptide Y (NPY) is a ubiquitous endocrine neurotransmitter found in the brain and autonomic nervous system of vertebrate and invertebrate. NPY is associated with a number of biological functions, such as feeding behavior, energy balance, blood pressure, circadian rhythms, sexual behavior, memory processing, cognition, anxiety, and epilepsy [6–8]. Till now, five NPY receptors have been identified in mammals. These receptors are all G-protein-coupled receptors (GPCRs) [9].

In the present work, we identified two NPY-like exocrine conopeptides from the venom of *Conus betulinus*. These two conopeptides showed NPY-specific sequence motif, suggesting that they are probably evolved from the preexisting endocrine NPYs during the evolution of cone snails.

**Materials and Methods**

**Materials**

Specimens of *C. betulinus* were collected from South China Sea near Sanya City, Hainan Province. Trypsin was purchased from Sigma-Aldrich (St Louis, USA). Pepmap C18 reverse-phase semi-analytical column was from Applied Biosystems (Carlsbad, USA).

**Peptide purification**

The venom ducts of *C. betulinus* were dissected and homogenized in 0.1% aqueous trifluoroacetic acid (TFA). After centrifugation (12,000 g, 15 min), the supernatant was applied to high-performance liquid chromatography (HPLC). The peptides were eluted from the C18 reverse-phase column by an acetonitrile gradient composed of solvent A (0.1% aqueous TFA) and solvent B (acetonitrile plus 0.1% TFA). The eluted fractions were manually collected, lyophilized, and analyzed by mass spectrometry.

**Peptide sequencing and trypsin digestion**

The peptide sequence of the purified cono-NPY was determined by automated Edman’s degradation method. In addition, cono-NPY was digested by trypsin, and the resultant peptide fragments were analyzed by mass spectrometry.

**Bioassay**

The purified cono-NPYs were dissolved in saline at the final concentration of 1.0 mg/ml, respectively. Then, different dosages were injected into the brain intraventricle of Kunming genus mice (body weight 14.5 ± 1.0 g). The control animals were injected with the same volume of saline.
Results and Discussion

Identification of cono-NPYs from the venom of *C. betulinus*

The crude venom extract from *C. betulinus* was subjected to HPLC as shown in Fig. 1(A). Each eluted peak was manually collected and subjected to molecular mass measurement and amino acid sequence analysis. As shown in Fig. 1(B), two highly homologous conopeptides were identified by direct peptide sequencing. Both of them contained 37 amino acids and differed at only two residues. The two peptides contained no cysteine residues, so they belonged to conopeptides.

Amino acid sequence alignment showed that both peptides were homologous to the known endocrine NPYs, especially to invertebrate NPYs (Fig. 2). Both of them contained an NPY-specific C-terminal motif (RXRY/F) [10,11]. Therefore, they were named cono-NPY1 and cono-NPY2, respectively. Since the C-terminals of all known NPYs are amidated, we deduced that the C-terminal of the cono-NPYs is also amidated. The measured molecular

![Figure 1](image1.png)  
**Figure 1** Purification and characterization of cono-NPYs  
(A) HPLC purification of cono-NPYs from the venom of *C. betulinus*. The crude venom extract was subjected to HPLC, and the peptides were eluted from the C18 reverse-phase column by an acetonitrile gradient. The peaks of cono-NPY1 and cono-NPY2 were labeled. (B) The measured amino acid sequences and molecular masses of cono-NPYs. The different residues between cono-NPY1 and cono-NPY2 are shaded. The theoretical molecular masses are shown in parentheses.

![Figure 2](image2.png)  
**Figure 2** Amino acid sequence alignment of cono-NPYs and other known NPYs  
The deduced vertebrate and invertebrate ancestral NPYs were shown at the first and last lines, respectively. The highly conserved positions are shaded.
masses of both cono-NPYs were in good agreement with their theoretical values [Fig. 1(B)].

To further confirm the deduced amino acid sequence, we subjected cono-NPY1 to trypsin digestion as shown in Fig. 3. The resultant peptide fragments were then analyzed by mass spectrometry as listed in Table 1. The measured molecular masses of the resultant peptide fragments were well consistent with their theoretical values, suggesting that the measured amino acid sequence of cono-NPY1 was correct.

Bioassay of cono-NPYs
The bioactivities of cono-NPYs were assayed by intraventricular injection of these peptides into mice brain [12,13]. At the dose of 20 μg/mouse, the mice showed signs of hyperactivity, such as jumping, rapid circling, and tail flicking. The symptoms appeared 20 s after injection and lasted for 40–60 s. Then, the mice calmed down and recovered. At low dosage (10 μg/mouse), the symptoms were less strong.

In this study, we identified two NPY-like conopeptides for the first time. Both of them had an NPY-specific sequence motif. Intraventricular injection of cono-NPY into mice brain produced biological effects; however, it remains to be clarified if their real receptors are NPY receptors or related GPCRs.

Possible evolutionary pathway of NPYs from invertebrate to vertebrate and from endocrine to exocrine
Larhammar [10] deduced an ancestral NPY sequence by comparing the sequences from different lineages of diverse species. Meanwhile, de Jong-Brink et al. [14] deduced a putative invertebrate prototype of NPY/NPF sequence as shown in Fig. 2. The comparison of these NPY-family peptides clarifies the evolution of NPYs from invertebrates to vertebrates and indicates that cono-NPYs identified in our study should also belong to the NPY family.

NPYs are endocrine regulatory peptides secreted by neurons. However, cono-NPYs were isolated from the exocrine venom of cone snails, suggesting that some exocrine venom peptides are probably evolved from the pre-existing endocrine peptides during the evolution of cone snails. A similar finding that the previously identified conopeptide conopressin-G is homologous to the endocrine vasopressin [15,16] also supports this possible evolutionary pathway.

The function of the cono-NPYs is completely unknown. Since they are present in the venom of cone snails, they should target at certain receptors of the cone snail’s preys and be helpful to prey capture. However, the physiological functions of the endocrinial NPYs seem unrelated to the possible functions of the exocrinal cono-NPYs. It is possible that cono-NPYs have evolved new functions during evolution from the endocrine peptide to the exocrine peptide. In the future work, we will try to identify the target of cono-NPYs by various methods.

![HPLC profile of the trypsin-digested cono-NPY1](image)

Figure 3 HPLC profile of the trypsin-digested cono-NPY1 The purified cono-NPY1 was digested by trypsin in 100 mM Tris–acetate buffer (pH 8.0) containing 1 mM EDTA at 37°C for 2 h. After incubation, the digestion mixture was acidified to pH 2.0 by TFA and loaded onto a C18 reverse-phase column. The peptide fragments were eluted from the column by an acetonitrile gradient. The eluted peaks were manually collected, lyophilized, and analyzed by mass spectrometry.

<table>
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<tr>
<th>Peak no. in Fig. 3</th>
<th>Measured molecular mass</th>
<th>Theoretical molecular mass</th>
<th>Corresponding peptide fragment</th>
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<tr>
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<td>1636.8</td>
<td>TVSDPPARPAVFHSR</td>
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<tr>
<td>6</td>
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<td>4244.9</td>
<td>TVSDPPARPAVFHSREELMNYVRELNYFAIVGRPRF-NH₂</td>
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</tbody>
</table>

The amino acid sequence of cono-NPY1 and the potential trypsin cleavage sites (underlined) were shown as follows: TVSDPPARPAVFHSREELMNYVRELNYFAIVGRPRF-NH₂.
Funding

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References