The Synthesis of SO-3, a Conopeptide with High Analgesic Activity Derived from *Conus striatus*†

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The synthesis and characterization of the conopeptide, SO-3, originally derived from *Conus striatus* is reported. It contains 25 amino acid residues and three disulfide bridges and manifests 72% sequence identity with MVIIA, an N-type Ca^{2+} channel inhibitor of high analgesic activity. We evaluated SO-3 in several mouse models of pain. The results indicate that SO-3 is a potent, nonaddictive, analgesic agent.

Conotoxins are small peptide toxins that are encoded by individual genes and secreted by the venomous marine snails of the genus *Conus*. Most of them are 10–40 amino acids in length and contain 2–4 disulfide bridges. They act as paralytic toxins via effects on either voltage-gated ion channels, ligand-gated ion channels, or G-protein-linked receptors.[1–3] These properties have led to the use of certain conotoxins as tools for neurobiological research as well as for the treatment and diagnosis of neurological diseases. Of prominence in the therapeutic realm is MVIIA (ziconotide), an N-type calcium channel inhibitor from *Conus magus*. Intrathecally administered MVIIA displays approximately 1000-fold greater potency than morphine without any apparent development of tolerance.[4–6] Unfortunately, several untoward effects that accompany the administration of MVIIA, including dizziness, blurred vision, nystagmus, and sedation, may curtail its clinical applicability.[5] Ongoing efforts are focused on finding other analgesic conopeptides with lesser associated side effects.[7,8]

It has been known that the genes encoding the conotoxins and the post-translational modification of the gene products are highly changeable and environment-dependent among the different *Conus* species. The South China Sea is in a temperate zone and provides an optimal environment for *Conus* proliferation. To date, approximately 100 species of cone snails from this habitat have been identified. In a search for new conotoxins with analgesic activity or other therapeutic potential, more than 25 species of *Conus* inhabiting the South China Sea have been collected. Through gene screening, we have previously identified a peptide termed SO-3[9,10] that is highly homologous to MVIIA in DNA sequence. The results presented herein describe the synthesis and further characterization of SO-3. In addition, the analgesic effects of both SO-3 and MVIIA were examined in standard mouse and rat models of pain. The high biological activity of synthetic SO-3, in tandem with low adverse effects, suggests that it a promising new candidate in the analgesic drug arsenal.

Synthesis and Structure of SO-3. Using the designated folding conditions, a 20% overall yield of oxidized peptide was obtained in greater than 98% purity after two steps of HPLC purification (Figure 1). Using MALDI-TOF, the molecular mass (single isotope) of SO-3 was 2560.1 Da, in excellent agreement with the calculated molecular mass of 2561.1 Da. The three-dimensional solution structure of SO-3 was previously determined by ^{1}HNMR and shows that it contains a short antiparallel β-sheet involving residues 6–9, 19–21, and 24–25,[11] similar to MVIIA.[12] The disulfide bridge pattern was established as Cys^1–Cys^{16}, Cys^8–Cys^{20}, Cys^{15}–Cys^{25}, identical to the cystine framework found in MVIIA and other ω-conotoxins.[12–14] The CD spectrum of SO-3 was nearly identical to that of MVIIA (Figure 2).[15] This is expected insofar as SO-3 and MVIIA share 72% sequence identity and manifest identical disulfide bond motifs.

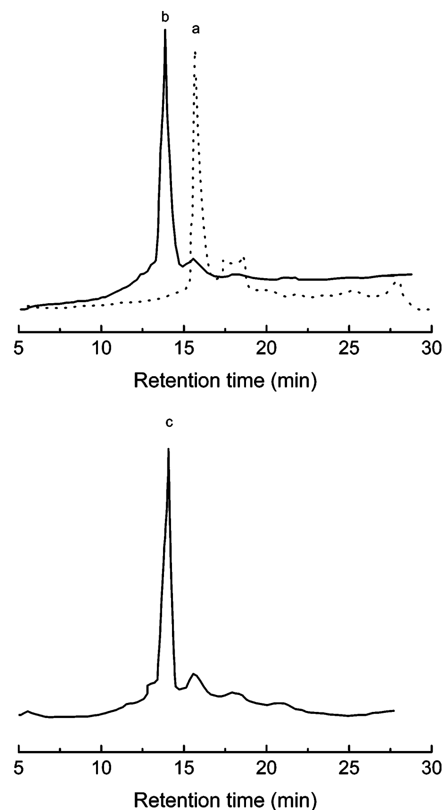

Figure 1. HPLC chromatograms of reduced, linear SO-3, and folded product: (a) reduced SO-3; (b and c) the oxidized, disulfide-bridged product formed in buffer containing GSH-GSSG and cysteine, respectively. Samples were applied to a Zorbax C_{18} column (4.6 × 250 mm) and eluted by implementing a 25 min linear gradient of 8–40% 0.1% TFA in acetonitrile at a flow rate of 1 mL/min.

Biological Activity. Goldfish Toxicity. The lethality of MVIIA after intramuscular injection into goldfish (*Carassius carassius*) was 60%, 80%, 100%, and 100% at doses of 0.5, 1.0, 3.0, and 8.5 μg/per goldfish, respectively. At the

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Table 1. Analgesic Effects of SO-3 and MVIIA in Mice Using the Hot-Plate Method[a]

dosage (μg/kg)[b]	reaction times (s) before administration[c]	reaction times (s) after administration[c]			
		0.5 h	1.5 h	2.5 h	4 h
normal saline	11.4 ± 2.3	11.8 ± 2.6	11.8 ± 2.7	11.2 ± 1.5	
SO-3 (0.5)	11.6 ± 5.2	23.4 ± 3.1	20.0 ± 6.2	15.8 ± 6.6	
MVIIA (0.5)	9.8 ± 1.9	20.0 ± 5.0	12.6 ± 6.5	11.4 ± 3.9	
SO-3 (1.0)	16.0 ± 5.2	43.0 ± 11.3	16.2 ± 5.0	15.0 ± 5.6	
MVIIA (1.0	12.6 ± 5.8	36.0 ± 2.6	13.8 ± 4.8	12.0 ± 3.3	
SO-3 (1.5)	13.6 ± 6.5	49.6 ± 12.5	39.6 ± 14.3	38.2 ± 19.9	29.4 ± 17.9
MVIIA (1.5)	10.8 ± 5.2	46.4 ± 11.8	26.0 ± 10.4	23.2 ± 6.9	19.6 ± 3.6
SO-3 (3.0)	13.0 ± 1.2	>60.0	59.8 ± 0.4	55.8 ± 8.4	51.8 ± 5.4
MVIIA (3.0)	10.2 ± 3.6	55.2 ± 6.9	43.0 ± 14.5	40.6 ± 17.3	37.0 ± 19.9

[a] $n = 5$, $p < 0.05$. [b] Intracerebral injection. [c] Values represent reaction times required to elicit jumping or licking of the hind paw after placement on a hot plate maintained at 55 °C.

Table 2. Analgesic Activities of SO-3 and MVIIA in Mice Determined by the Light Radiation Method[a]

dosage (μg/kg)[b]	reaction times (s) before administration[c]	reaction times (s) after administration[c]			
		1 h	2.5 h	3.3 h	4 h
normal saline		5.5 ± 1.4	6.2 ± 1.6	5.5 ± 0.6	5.7 ± 0.6
SO-3 (0.7)	4.6 ± 1.1	12.1 ± 2.4	10.0 ± 3.4	9.8 ± 3.2	8.1 ± 4.5
SO-3 (1.2)	5.6 ± 1.1	10.5 ± 3.6	8.5 ± 4.9	10.5 ± 3.3	7.66 ± 3.5
SO-3 (1.7)	4.4 ± 0.6	12.2 ± 3.6	12.9 ± 3.8	11.5 ± 4.2	10.4 ± 2.5
SO-3 (2.2)	7.2 ± 2.1	14.2 ± 3.2	12.4 ± 3.7	9.6 ± 2.7	7.3 ± 2.3
MVIIA (2.2)	7.9 ± 1.3	14.0 ± 2.9	12.0 ± 3.8	8.6 ± 4.7	7.7 ± 3.8
SO-3 (2.7)	6.1 ± 1.3	14.2 ± 3.0	11.9 ± 3.0	10.2 ± 6.6	8.8 ± 1.0
MVIIA (2.7)	7.7 ± 2.1	12.1 ± 3.9	11.9 ± 3.0	12.0 ± 3.5	9.0 ± 1.0
SO-3 (3.2)	5.8 ± 0.9	11.2 ± 4.1	11.8 ± 3.9	11.1 ± 4.1	11.6 ± 3.4
MVIIA (3.2)	5.7 ± 1.3	13.4 ± 3.9	12.9 ± 4.0	11.8 ± 3.2	11.9 ± 4.0

[a] $n = 10$, $p < 0.05$. The cutoff latency is 16 s. [b] Intracerebral injection. [c] Values represent reaction times required to observe a tail-flicking response following stimulus with light.

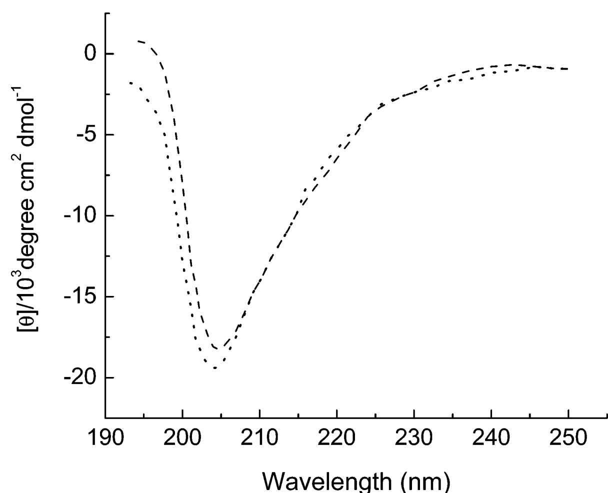

Figure 2. CD spectra of SO-3 and MVIIA. Measurements were made on a Jasco J-715 spectropolarimenter in H_2O at room temperature at a concentration of 0.08 mM SO-3 and 0.09 mM MVIIA in a 1 mm path length cell. Each curve represents the accumulation of eight individual scans for both SO-3 (dashed line) and MVIIA (dotted line).

same SO-3 dosages, all goldfish survived and were otherwise normal, with the exception of one that showed abnormal swimming behavior.

Analgesic Effects in Mice. The dose−response effects of SO-3 and MVIIA using the hot plate and light radiation pain models are presented in Tables 1 and 2. The EC_{50} value for SO-3 derived from the data in Table 1 is 0.75 ± 0.30 μg/kg. The EC_{50} value of MVIIA was not calculable from the limited data. However, for both the hot-plate and irradiation assays, SO-3 demonstrated analgesic effects that were comparable to or slightly better than those observed for identical doses of MVIIA. In the chemical stimulus test, the number of observed twists decreased from 19.9 ± 5.8 to 9.6 ± 4.7 after intracerebral administration of SO-3 at a dose of 0.7 μg/kg and indicate a 50% increase in analgesic efficacy. When higher doses of SO-3 and MVIIA (>3.2 μg/kg) were delivered, some tremor was noted. Overall, high doses of SO-3 resulted in slightly lower adverse effects (tremor) compared with identical dosages of MVIIA.

Analgesic Effects in Rats. The results of the tail-flicking reaction method, following inmersion of the subject's tail into hot water, indicate that the analgesic potency of SO-3 is also slightly higher than that of MVIIA at the two administered dosages (Table 3). In the mechanical stimulus tolerance model, the threshold for tail withdrawal increased by an average of 100 and 150 g after SO-3 was administered at doses of 1.2 and 2.0 μg/kg, respectively. Importantly, using the mechanical stimulus model, no drug tolerance was noted after a 15-day daily intrethecal bolus infusion regimen of 2 μg/kg of SO-3.

The above results show that the analgesic potency of SO-3 is more than 3000-fold and 50-fold greater than that of morphine administrated by systemic and intrathecal injection, respectively.[16] In all pain models implemented, SO-3 was shown to be comparable to MVIIA. In goldfish, no lethality was associated with SO-3 doses as high as 8.5 μg/per goldfish, despite 100% lethality being observed with MVIIA at the same dose. In mice, the LD_{50} of SO-3 was 13.5 mg/kg by intracerebral administration ($n = 8$), which is 18 000 times higher than the EC_{50}, suggesting that SO-3 has a high safety index. In summary, SO-3 displays significant therapeutic potential as a nociceptive agent, with analgesic potency similar to that of MVIIA as well as minimal side effects, low toxicity, and no apparent tolerance to analgesia.

Experimental Section

N-Fmoc-amino acids, DCC, HOBt, HBTU, TFA, and Rink resin were purchased from Advanced Chem Tech (Louisville, KY), glutathione (GSH, GSSG), DTT, and cysteine were obtained from Gibco (Carlsbad, CA), and Sephadex G-25 was purchased from Pharmacia (Peapack, NJ). Authentic MVIIA

Table 3. Analgesic Activities of SO-3 and MVIIA in Rats on Tail-Flicking Reaction Elicited by Tail Immersion in Hot (55 °C) Water[a]

dosage (μg/kg)[b]	reaction times (s) before administration[c]	reaction times (s) after administration[c]			
		1 h	2 h	3 h	4 h
normal saline	2.2 ± 0.5	2.0 ± 0.6	2.0 ± 0.6	2.0 ± 0.3	2.2 ± 0.6
SO-3 (1.2)	2.0 ± 0	3.3 ± 0.3	3.6 ± 0.4	3.5 ± 0.4	3.5 ± 0.4
MVIIA (1.2)	2.0 ± 0.1	2.8 ± 0.4	3.4 ± 0.6	2.9 ± 1.1	2.8 ± 0.6
SO-3 (2.0)	2.3 ± 0.4	3.2 ± 0.4	4.0 ± 0.8	4.5 ± 0.5	3.2 ± 0.4
MVIIA (2.0)	2.0 ± 0.2	2.5 ± 0.4	3.0 ± 0.7	3.1 ± 0.6	2.9 ± 0.4

[a] $n = 7$, $p < 0.05$. [b] Intrathecal injection. [c] Values represent reaction times required to observe a tail-flicking response following tail immersion in hot water.

was obtained from American Peptide Company (Sunnyvale, CA). All other reagents were analytically pure. Goldfish (25 ± 0.3 g) were obtained from Tong County Hatchery in Beijing. Kunming mice (20 ± 2 g) and Wistar rats (250 ± 20 g) were provided by the Academy of Military Medical Science (Beijing, China).

Peptide Synthesis. The protected peptides (0.1 mmol scale) corresponding to the sequence of SO-3 (CKAAGKPCS-RIAYNCCTGSCRSGKC-NH_2) and MVIIA (CKGKGAKCSR-LMY DCCTGSCRSGKC) were assembled by automated F-moc solid phase methodology on Rink resin using a Model 433A automated synthesizer (ABI, Foster City, CA). The peptide-resin was deprotected in a suspension comprised of 10 mL of TFA, 0.75 g of phenol, 0.25 mL of 1,2-ethanedithiol, 0.5 mL of thioanisole, and 0.5 mL of H_2O at room temperature for 2.5 h. The resin was separated from the peptide deprotection cocktail by filtration. The crude peptide was precipitated with dry, cold diethyl ether (150 mL) and purified by chromatography on Sephadex G-25 using 10% acetic acid as eluent. Fractions containing peptide were pooled and lyophilized. The resulting crude peptide was approximately 80% pure as determined by HPLC.

Peptide Folding. Because SO-3 and MVIIA contain six cysteine residues maintained in three disulfide bridges, folding under oxidative conditions produces several isomers. After screening of oxidation−reduction systems, buffers, salts, concentration of SO-3 or MVIIA, and temperature, two highly efficient folding conditions were selected: (a) 0.5 M NH_4Ac buffer (pH 7.9) containing 1 mM GSH, 0.1 mM GSSG, 1 mM EDTA, and 0.2 mg/mL SO-3 or MVIIA; (b) 0.5 M NH_4Ac buffer containing 1 mM cysteine, 1 mM EDTA, and 0.2 mg/mL SO-3 or MVIIA. Linear peptide SO-3 and MVIIA were folded for 48−72 and 24−48 h in condition a and b at 4 °C, respectively.

Peptide Purification and Characterization. Upon completion of the oxidation of SO-3 or MVIIA, the reaction mixtures were acidified (pH < 4.5) with acetic acid and filtered. The filtrate was directly loaded on a Zorbax 21.2 × 250 mm preparative C_{18} column using a preparative HPLC pump (Waters 2000 series, Milford, MA). The column was washed with buffer A (0.1% TFA in water), then eluted with a 40 min linear gradient of 10−40% buffer B (0.1% TFA in acetonitile) at a flow rate of 8 mL/min. The fractions that were 90% enriched in SO-3 and MVIIA were further purified by semi-preprative reversed-phase HPLC using a 9.4 × 250 mm Zorbax C_{18} column. The final product was obtained by conversion from the TFA salt to the acetate salt by application to a Sephadex G-25 column and elution with 20% acetic acid. The purity of the peptides was assessed by analytical reversed-phase HPLC using a Zorbax C_{18} column (4.6 × 250 mm) with a 25 min linear gradient of 8−40% buffer B (0.1% TFA in acetonitile) at a flow rate of 1 mL/min. The final products were ≥98% pure. Confirmation of the correct molecular mass was ascertained by mass spectrometry on a Voyager MALDI-TOF spectrometer. The disulfide bridge pattern was assigned by a method employing partial reduction of cysteine linkages followed by amino acid sequencing.[17] The HPLC chromatogram and CD spectrum of synthetic MVIIA were identical to those obtained with the authentic sample of MVIIA. Biological activity of the synthetic material was confirmed through toxicity and analgesic potency in comparison with authentic MVIIA.

CD Spectroscopy. CD spectra were measured between 190 and 250 nm on a Jasco J-715 spectropolarimeter. The peptides were dissolved in H_2O to final concentrations of 0.08 mM (SO-3) and 0.09 mM (MVIIA). A 1 mm path length quartz cell was employed. Each spectrum represents the accumulation of eight individual scans collected at 1.0 nm intervals at a bandwidth of 1.0 nm.

Determination of Toxicity and Analgesic Potency. The toxicity of SO-3 and MVIIA to red common goldfish (*Carassius carassius*) was determined using Adeyemo's method.[18] The peptides were dissolved in 0.9% NaCl solution and filtered through a 0.2 μm pore size syringe filter (Whatman, Maidstone, England). Intramuscular injections into 5−10 goldfish were performed with 4 μL aliquots of the aqueous peptide solutions. The analgesic effects of SO-3 and MVIIA on mice and rats were determined by standard hot-plate, light radiation, and acetic acid stimulus (on mice) and tail-flick latency and mechanical tail tests (on rats)[19−21] following either intracerebral or intrathecal bolus injections. In the hot-plate model, the hot plate was maintained at 55 ± 0.1 °C and the time required to elicit jumping or licking of hind paw was recorded. A cutoff latency of 60 s was implemented to prevent tissue damage. A light radiation apparatus (Institute of Materia Medica of Chinese Academy of Medical Science, Beijing) was applied to determine the time at which a tail-flicking response was observed (cutoff latency = 16 s). The light beam was focused on a small area on the lower back, approximately 1.5 cm before the base of the tail. The tail flick reaction was also determined by inserting the subject's tail into hot water (55 ± 0.2 °C, cutoff latency = 5 s). In the acid stimulus model, 0.4 mL of 1% acetic acid was intraperitoneally injected and, after 5 min, the number of twisting motions occurring within a 15 min interval was recorded. A Model XZC-A pressure apparatus (Academy of Medical Science of Sandong Province, China) was used to measure the threshold for rat tail withdrawal to painful mechanical stimulus. EC_{50} was obtained by the pharmacology-dose−response curve fitting (Origin Software, Northampton, MA) with the equation $y = A_1 + (A_2 - A_1)/(1 + 10\wedge(\log EC_{50} - x) \times \text{slope})$, where A_2 and A_1 are the maximum and minimum analgesic times in the presence of the highest dose of SO-3 and in the absence of SO-3, respectively.

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