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Supporting Information

N-Chlorosuccinimide, an Efficient Peptide Disulfide Bond Forming Reagent in Aqueous Solution

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1. General Procedures

Fmoc-amino acid derivatives, Fmoc-Rink Amide AM resin and 2-CTC resin were obtained from IRIS Biotech (Marktredwitz, Germany). Fmoc-Cys(*S*-Tmp)-OH was prepared according to our previously published protocol.[1] Diisopropylcarbodiimide (DIC) and TFA were obtained from Aldrich (Milwaukee, USA). Oxyma was obtained from Luxembourg Industries Ltd. (Tel Aviv, Isreal). DMF, CH_2Cl_2, Et_2O, acetonitrile, DMSO and piperidine (HLPC grade) were obtained from SDS (Peypin, France). All reagents and solvents were used as received.

Room temperature (rt) refers to ambient temperature. Solid-phase syntheses were carried out manually in polypropylene syringed containing a polyethylene frit. Solvents and soluble reagents were removed by suction. Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 2 × 5 min). Washings between deprotection and coupling were performed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Following the final coupling or deprotection the resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and dried under a stream of air. Yields for peptides refer to the area of the chromatographic product peak recorded at 220 nm.

High resolution mass spectrometry (HRMS) measurements were recorded on Thermo Scientific LTQ-FT Ultra spectrometer. Mass values are quoted within the error limits of ±5 ppm mass units. ESI refers to the electrospray ionization technique.

Analytical high pressure liquid chromatography (HPLC) was carried out on a Waters instrument comprising a separation module (Waters 2695), automatic injector, photodiode array detector (Waters 2998) and system controller (Empower login), with an SunFire C18 analytical reversed-phase HPLC column (2.1 mm × 100 mm, 5 µm) or Xbridge BEH130 C18 reversed-phase analytical column (4.6 mm × 100 mm, 3.5 µm). UV measurements were recorded at 254 and 220 nm, and linear gradients of acetonitrile (0.036% TFA) into water (0.045% TFA) over 8 min were used at a flow rate of 1.0 mL·min^{-1} and a run time of 11 min.

LCMS was carried out on a Waters Micromass ZQ spectrometer using a SunFire C18 analytical reversed-phase HPLC column (2.1 mm × 100 mm, 5 µm). Linear gradients of acetonitrile (0.07% formic acid) into water (0.1% formic acid) over 8 min were used at a flow rate of 1.0 mL·min^{-1} and a run time of 11 min.

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2. General Methods Peptide Synthesis

General Method 1: Peptide Synthesis

Fmoc-Rink-Amide AM resin (0.45 mmol/gram, 1 equiv.) was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) followed by washing with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). The protected Fmoc-amino acids (3 equiv.) were incorporated using DIC (3 equiv.) and Oxyma (3 equiv.) in DMF, as a coupling system, with 5 min preactivation for 1 h at rt. Washes between couplings and deprotections were performed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Completion of the coupling was monitored by the Kaiser test.[2] Following elongation a microcleavage was performed, 5 mg of dry resin was treated with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was evaporated with a stream of argon, precipitated with Et_2O, centrifuged and the pellet was redissolved in H_2O/CH_3CN (1:1) for analysis by HPLC and LCMS.

General Method 2: Deprotection of *S*-Tmp protecting groups

The resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Deprotection was achieved by treatment with 0.1 M *N*-methylmorpholine in 5% dithiothreitol (DTT)/DMF (3 × 5 min) and subsequently washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min).

General Method 3: Disulfide Formation

The fully deprotected peptide was dissolved in H_2O or H_2O/acetonitrile. A solution of NCS (1.0-2.2 equiv.) in H_2O or H_2O/acetonitrile was added and the mixture was shaken for 15 min at room temperature. Subsequently, the mixture was frozen in N_2 (l) and lyophilized.

General Method 4: Microcleavage

Dry resin (5 mg) was treated with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was evaporated with a stream of argon, precipitated with Et_2O, centrifuged and the pellet was redissolved in H_2O/CH_3CN (1:1) for analysis by HPLC and LCMS.

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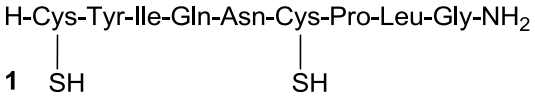
3. Peptide Synthesis

H-oxytocin(2 x SH)-NH_2 (1)



Peptide **1** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (1.11 g, 0.5 mmol, 0.45 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc, and washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). The Cys protecting group *S*-Tmp was removed according to General Method 2. A microcleavage was performed according to General Method 4 and subsequent HPLC analysis found that peptide **1** was obtained in 97**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 4.0 min). **HRMS** observed $[M+H]^+$ 1009.4594, required $[M+H]^+$ 1009.4594.

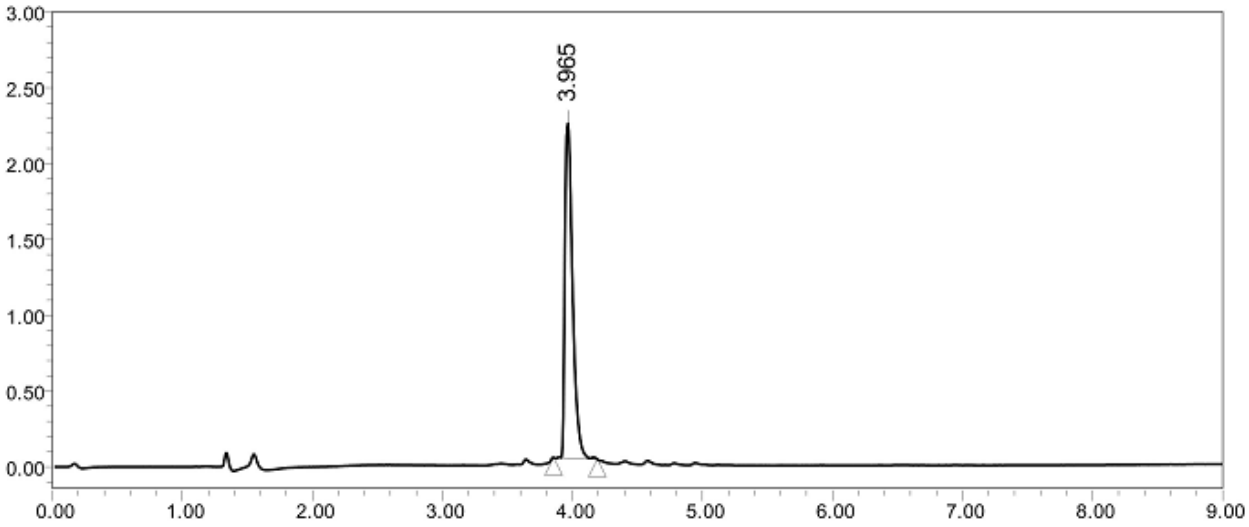
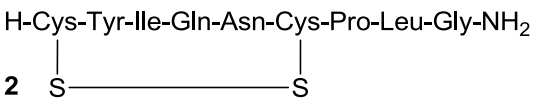


Figure S-1: HPLC chromatogram of peptide **1**

H-oxytocin-NH_2 (2)



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Peptide **2** was prepared by removing *S*-Tmp from the resin containing peptide **1** (5 mg, 2.5 µmol) according to General Method 2, followed by cleavage according to General Method 4. The crude peptide was oxidized according to General Method 3 and subsequently analyzed by HPLC and LCMS. For the best example, HPLC analysis found that peptide **2-5** was obtained in 95**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 3.9 min). **HRMS** observed $[M+H]^+$ 1007.4437, required 1007.4443.

Oxytocin	H_2O/CH_3CN	NCS equiv.	Purity (%)
2-1	1:0	1.5	88
2-2	1:1	1.5	94
2-3	1:3	1.5	94
2-4	1:1	1.1	94
2-5	1:1	1.5	95
2-6	1:1	2.0	94

Table 1: Results of oxytocin NCS oxidation under different conditions

H-oxytocin-NH_2 (2-1)

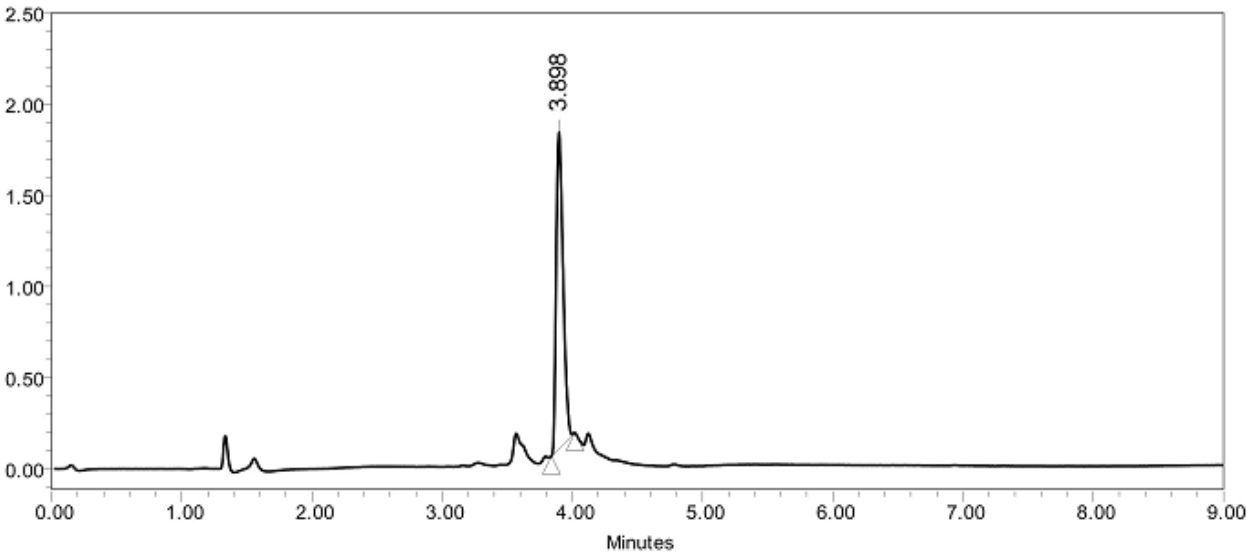

Figure S-2: HPLC chromatogram of peptide **2-1**

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H-oxytocin-NH_2 (2-2)

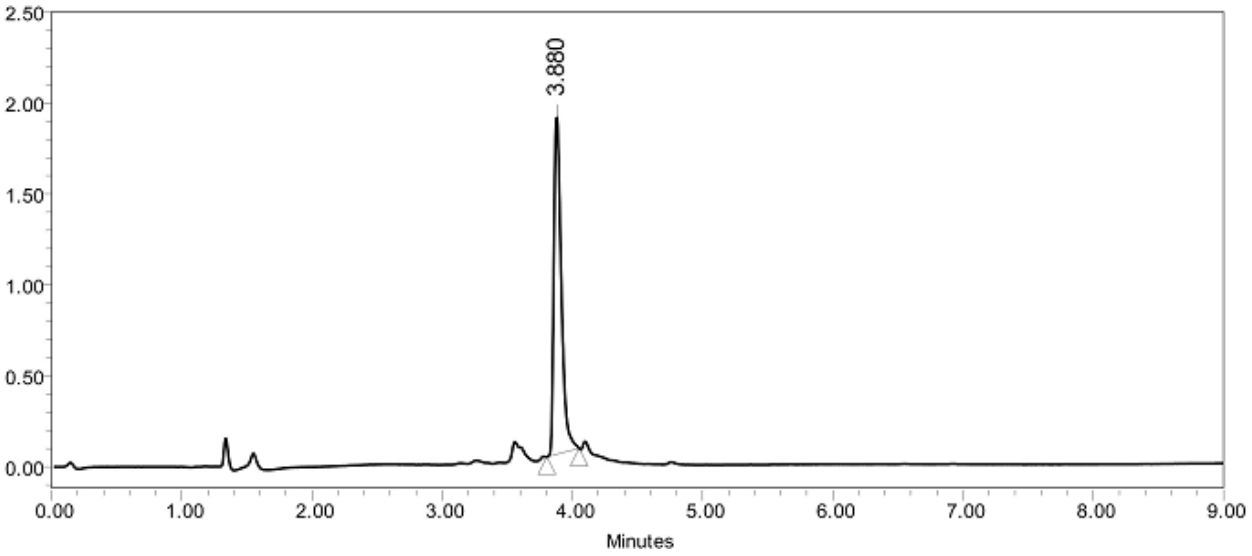

Figure S-3: HPLC chromatogram of peptide **2-2**

H-oxytocin-NH_2 (2-3)

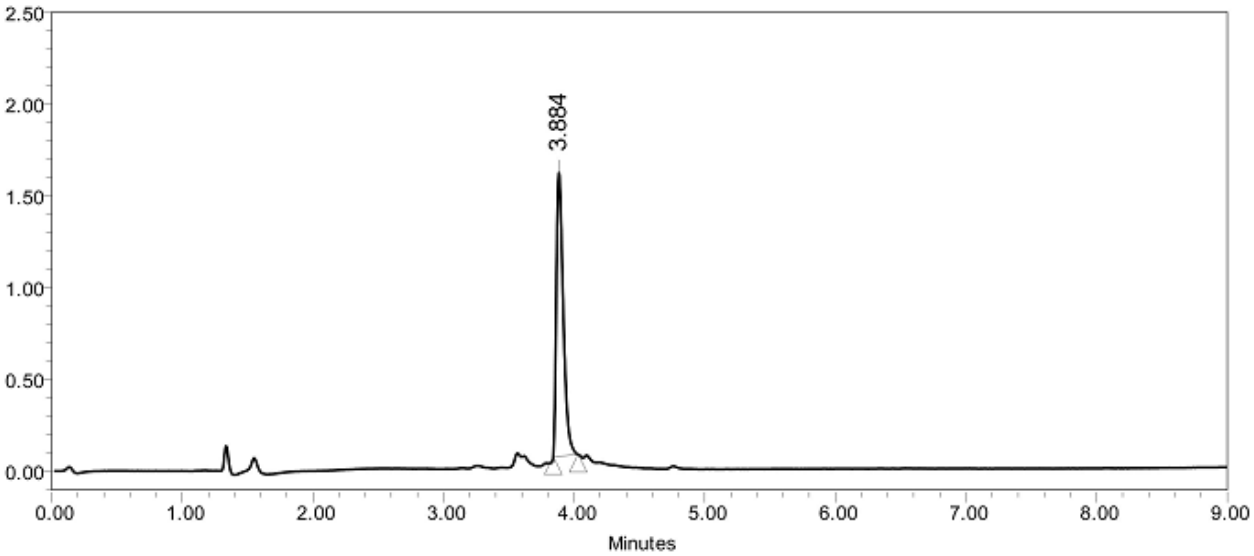

Figure S-4: HPLC chromatogram of peptide **2-3**

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H-oxytocin-NH_2 (2-4)

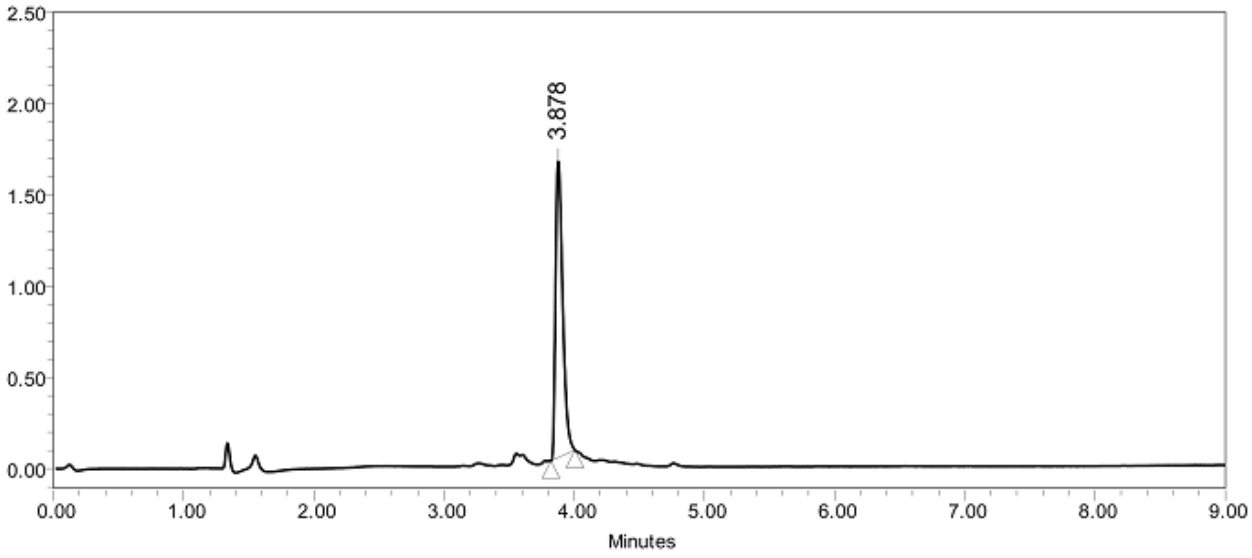

Figure S-5: HPLC chromatogram of peptide **2-4**

H-oxytocin-NH_2 (2-5)

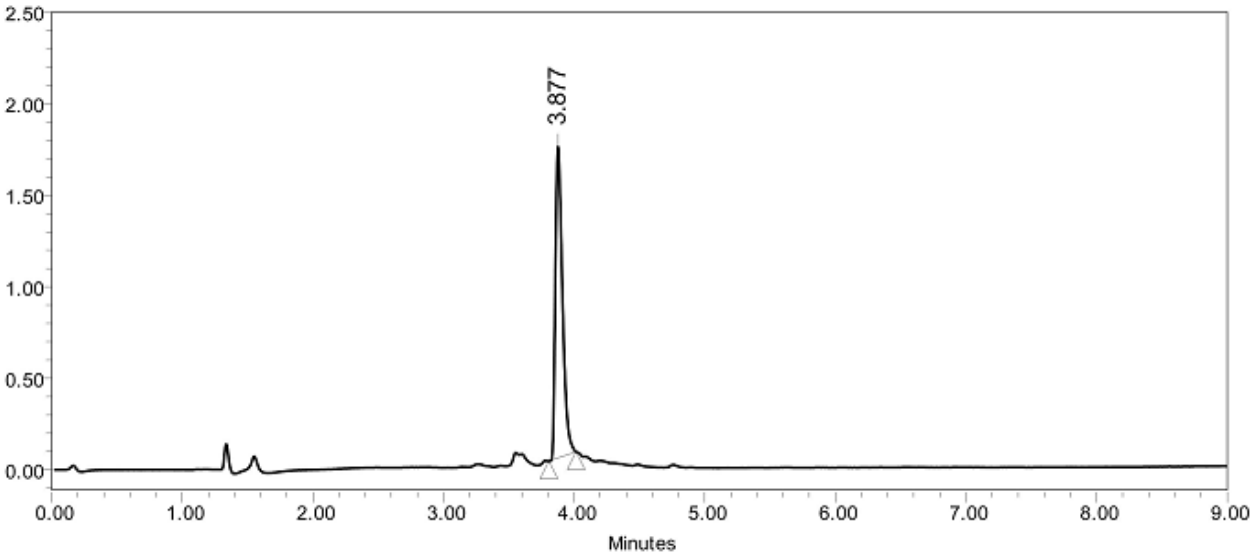

Figure S-6: HPLC chromatogram of peptide **2-5**

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H-oxytocin-NH_2 (2-6)

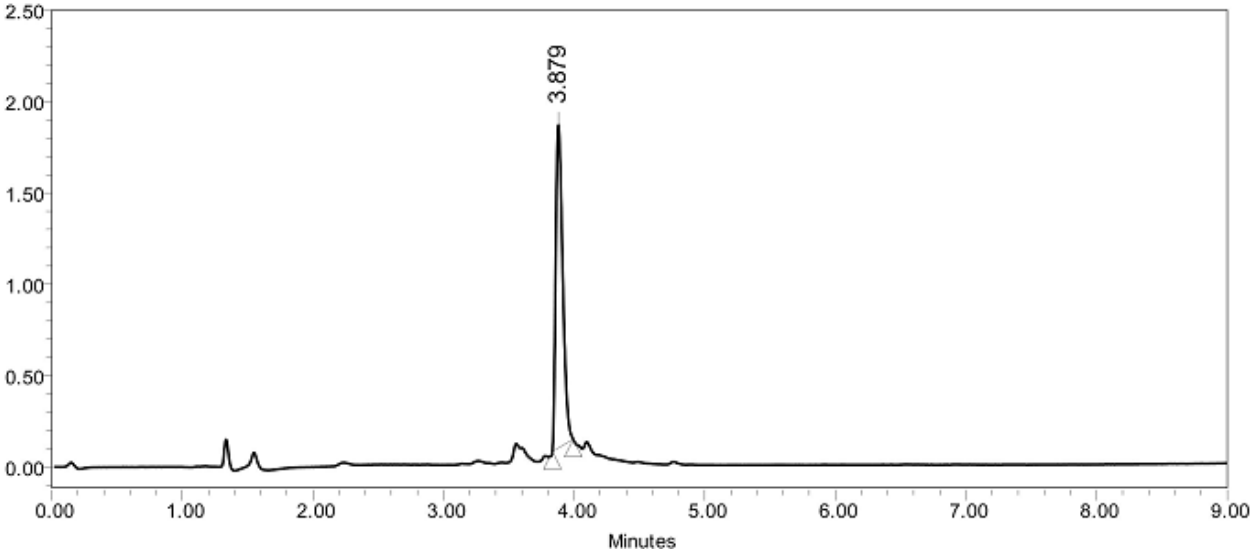

Figure S-7: HPLC chromatogram of peptide **2-6**

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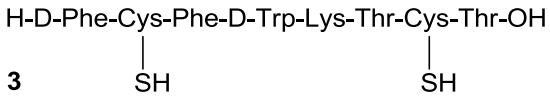
H-Octreotate(2 x SH)-OH (3)



The 2-CTC resin (1g, 1.6 mmol, 1.6 mmol/g) was washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Fmoc-Thr(tBu)-OH (238.2 mg, 0.6 mmol) and DIPEA (0.865 ml, 5 mmol) in CH_2Cl_2 were added to the resin and the resin was shaken for 1 h at rt. The resin was capped by the addition of methanol (0.8 mL) and shaken for 10 min. The resin was washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min) and dried over air. The Fmoc loading was determined by treating a small portion of resin (5 mg) with piperidine-DMF (1:4) (1 mL) for 20 min and dilited to 25 mL in a volumetric flask. The solution was quantified by UV (290 nm) to give a loading of 0.48 mmol/g. The resin (156.3 mg, 0.075 mmol) containing Fmoc-Thr(tBu) was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min) followed by peptide elongation according to General Method 1. Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc, and washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). A microcleavage was performed according to General Method 4 and subsequent HPLC analysis found that peptide **3** was obtained in 94**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 4.7 min). **HRMS** observed $[M+H]^+$ 1035.4427, required $[M+H]^+$ 1035.4427.

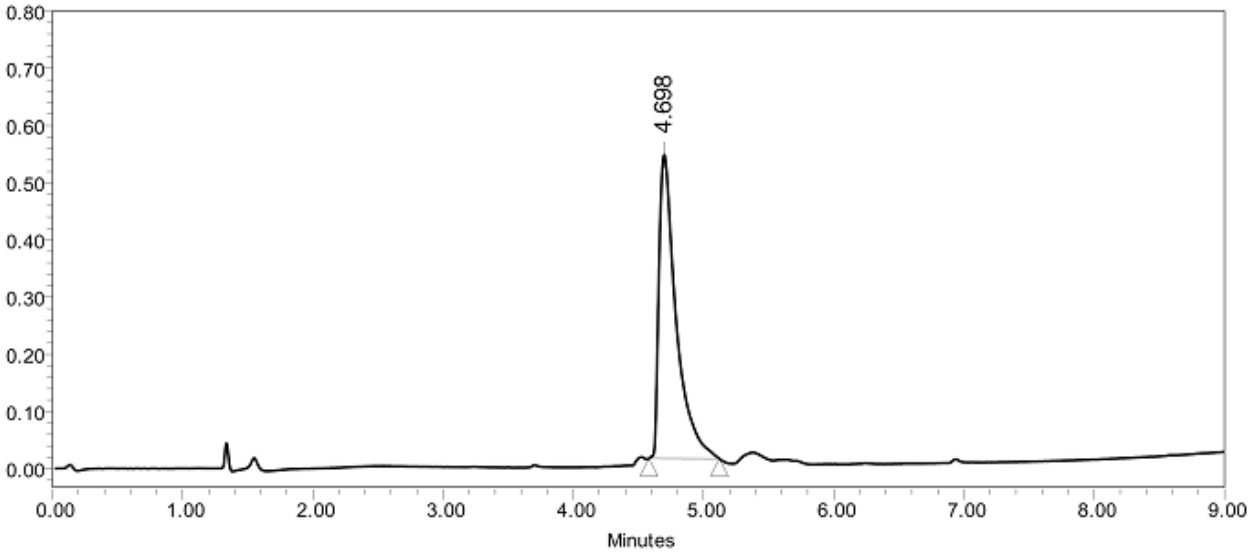


Figure S-8: HPLC chromatogram of peptide **3**

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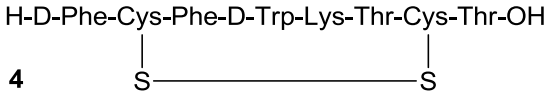
H-Octreotate-OH (4)



Peptide **4** was prepared by cleaving 5 mg of resin (**3**) according to General Method 4. The crude peptide was dissolved in H_2O/acetonitrile (1:1) and oxidized according to General Method 3 with NCS (2.0 equiv.) in H_2O/acetonitrile, lyophilized and subsequently analyzed. HPLC analysis found that peptide **4** was obtained in 84**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 4.4 min). **HRMS** observed [M] 1032.4198, required 1032.4197.

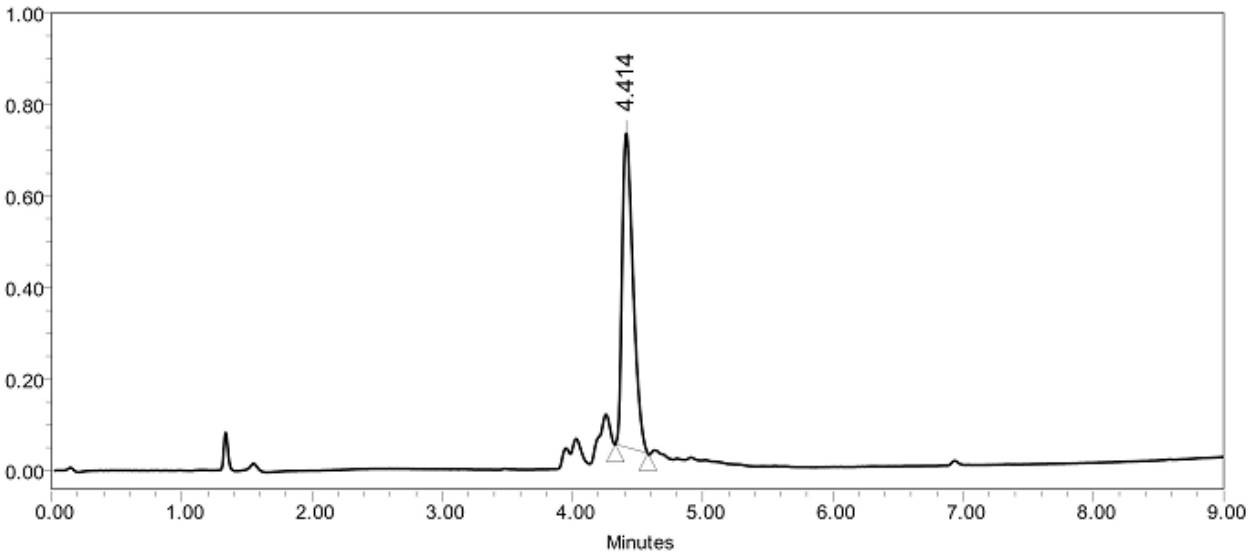


Figure S-9: HPLC chromatogram of peptide **4**

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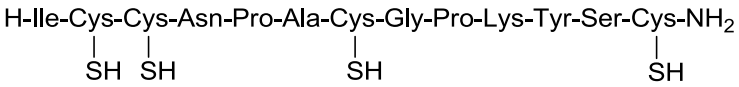
H-SI-Conotoxin(4 x SH)-NH_2 (5)



Linear SI conotoxin (**3**) was prepared according to General Method 1 using Fmoc-Rink-Amide AM resin (166.7 mg, 0.075 mmol, 0.45 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc, and washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). A microcleavage was performed according to General Method 4 and subsequent HPLC analysis found that peptide **5** was obtained in 85**%** purity (linear gradient from 0% to 30% acetonitrile over 8 min, t_R : 7.2 min). **HRMS** observed $[M+H]^+$ 1357.5492, required $[M+H]^+$ 1357.5520.

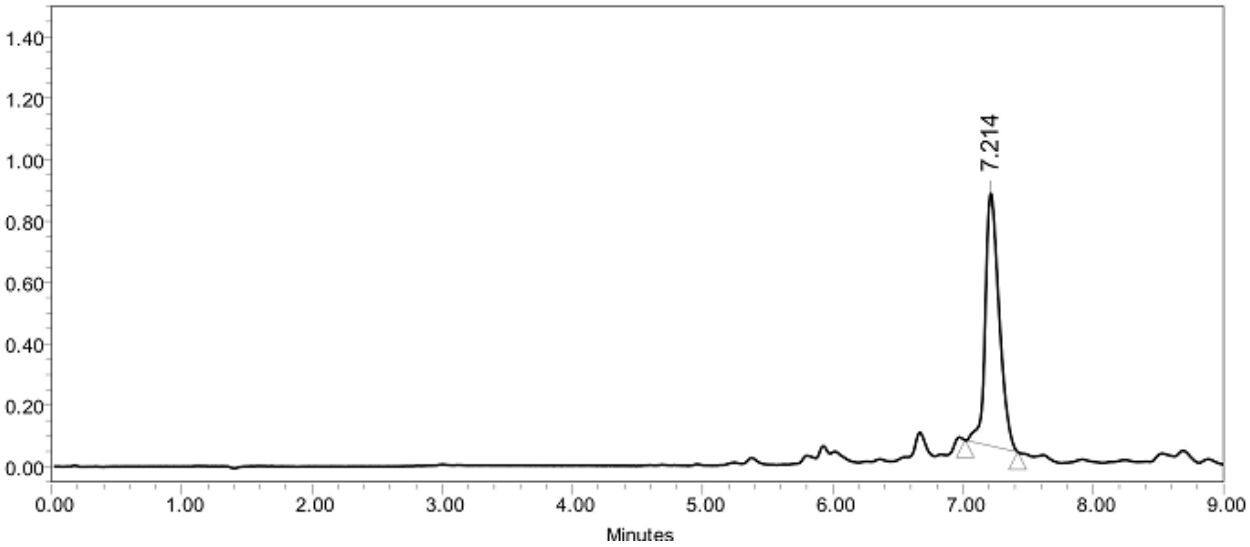


Figure S-10: HPLC chromatogram of peptide **5**

H-SI-Conotoxin-NH_2 (6)

Peptide **6** was prepared by cleaving 5 mg of resin (**5**) according to General Method 4. The crude peptide was dissolved in water and oxidized according to General Method 3 with NCS (2.2 equiv.) in H_2O/acetonitrile and subsequently analyzed. HPLC and LCMS analysis found that peptide **6** was obtained in two different disulfide connectivities in a ratio of 1:4 (linear gradient from 0% to 30% acetonitrile over 8 min, t_R : 7.2 and 7.5 min). **HRMS** observed $[M+H]^+$ 1353.5191, required $[M+H]^+$ 1353.5210.

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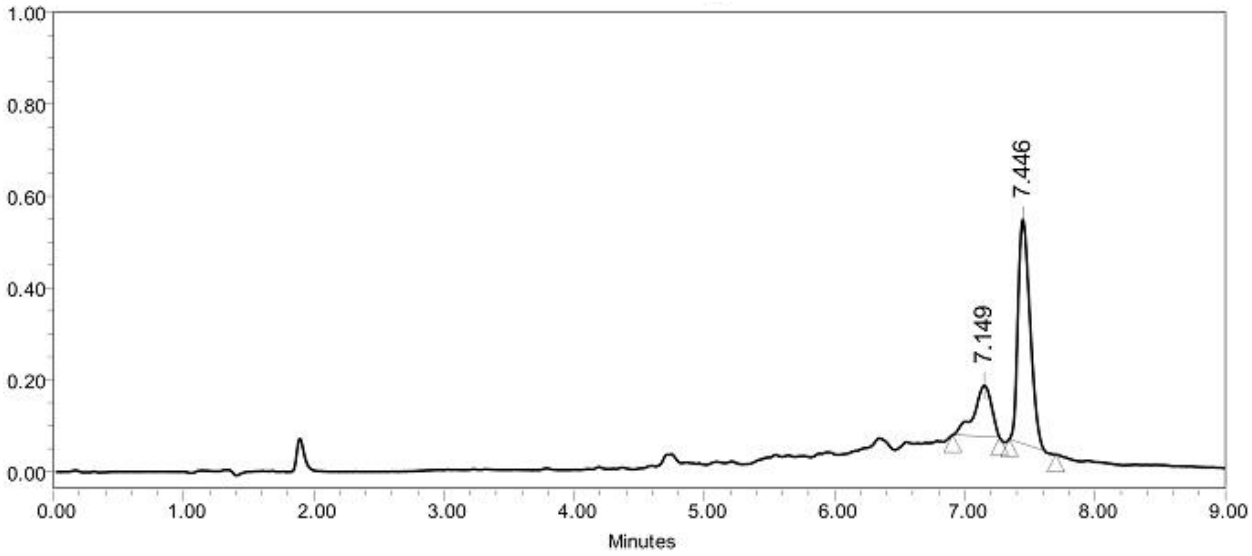

Figure S-11: HPLC chromatogram of peptide **6**

4. References

1. Postma, T. M.; Giraud, M.; Albericio, F., *Org. Lett.* **2012,** *14*, 5468-5471.
2. Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I., *Analytical Biochemistry* **1970,** *34*, 595-598.