Supporting Information

Synthesis of cysteine-rich peptides by native chemical ligation without use of exogenous thiols

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Contents

1. General Information……. p3
a. Materials
b. HPLC
c. Mass spectrometry
d. Amino acid sequence analysis
e. Automated solid-phase synthesis (Boc-strategy)
f. Ligation time-courses

2. Experimental Section……..…..…. p4-14
a. Synthesis of peptide segments
b. Ligation time-courses by the presence of TCEP in a concentration-dependent manner: **1** + **2**
c. Ligation time-courses: **3** + **4**
d. One-pot synthesis of ProTx-I (3SS)
e. Disulfide structure determination of ProTx-I
f. Synthesis of [Ile^{14}]-ProTx-I (6SH)
g. Synthesis of orexin A (4SH)
h. Synthesis of kurtoxin (8SH)
i. Ligation time-courses: NCL of model thioesters **5**-**8**

1. General Information……..

a. Materials

All reagents and solvents were obtained from the Peptide Institute, Inc. (Osaka, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Nacalai Tesque, Inc. (Kyoto, Japan), Watanabe Chemical Industries, Ltd. (Hiroshima, Japan) and Sigma-Aldrich Co. LLC. (St. Louis, MO).

b. HPLC

Preparative HPLC was carried out on a Shimadzu liquid chromatograph Model LC-8A (Kyoto, Japan) with a DAISO-PAK SP-120-5-ODS-BIO (30 x 250 mm) and the following solvent systems: 0.1% TFA in H_2O (A) and 0.1% TFA in CH_3CN (B) at a flow rate of 20 mL min^{-1} (room temperature) with detection at 220 nm. Analytical HPLC was performed on a Shimadzu liquid chromatograph Model LC-10AT (Kyoto, Japan) with a DAISO-PAK SP-120-5-ODS-BIO (4.6 x 150 mm) and the following solvent systems: 0.1% TFA in H_2O (A) and 0.1% TFA in CH_3CN (B) at a flow rate of 1 mL min^{-1} (40 °C) with detection at 220 nm.

c. Mass spectrometry

ESI MS: mass spectra of all peptide segments were observed with an Agilent G1956B LC/MSD detector using an Agilent 1100 series HPLC system; observed masses were derived from the experimental m/z values for all observed protonation states of a molecular species, using the program ChemStation®.

d. Amino acid sequence analysis

Amino acid sequence analysis was performed with a gas-phase sequencer (Shimadzu PPSQ-21A).

e. Automated solid-phase synthesis (Boc-strategy)

Automated peptide synthesis was performed on an ABI433A (Forester, CA, USA) peptide synthesizer. The peptide chain was elongated using *in situ* neutralization protocols of coupling with Boc-amino acid/HCTU/6-Cl-HOBt/DIEA (4/4/4/6 equiv) in NMP (single coupling, 30 min). The acetyl capping was performed using acetic anhydride/NMP in the presence of DIEA after each coupling step. The following side-chain-protected amino acids were employed: Arg(Tos), Asn(Xan), Asp(OcHex), Cys(MeBzl), Glu(OcHex), His(Bom), Lys(ClZ), Ser(Bzl), Thr(Bzl), Trp(For), Trp(Hoc), Tyr(BrZ).

f. Ligation time-courses

Ligation time-courses were plotted for the reaction of *C*-segment peptides (1.0 equiv. 2.0 mM) with *N*-segment thioesters (1.3 equiv. 2.6 mM) in the presence of thiol additives: MPAA (100 mM), thiophenol (100 mM), or thiol-additve free.

Aliquots of 3 μL were taken from the reaction mixture at various time intervals and quenched with 45 μL of 3% TFA in water and neutral TCEP solution 5μL (0.5 M, Sigma-Aldrich), and analyzed by analytical HPLC at 220 nm. The extent of ligation was quantified by integration of the ligated products as a fraction of the sum of the unreacted *C*-segment peptides and the ligated products by HPLC.

2. Experimental Section……
a. Synthesis of peptide segments

[Cys(Acm)$^{2, 9}$]-ProTx-I (1-14)-SCH_2CH_2CO-Leu-NH_2
H-EC(Acm)RYWLGGC(Acm)SAGQT-SCH_2CH_2CO-L-NH_2 (1)
The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Thr(Bzl)-SCH_2CH_2CO-Leu-MBHA resin (0.53 g, 0.25 mmol) according to the general automated SPPS procedure. The peptide resin was treated by HF/*p*-cresol (v/v, 90/10) at -2 °C to -5 ºC for 1 h to give a crude product, which was purified by preparative HPLC to yield 0.12 g (25%). Analytical HPLC: Rt, 11.7 min (20-40% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1873.1, found 1872.0.

[Cys(Acm) $^{16, 21, 28}$]-ProTx-I (15-35)
H-CC(Acm)KHLVC(Acm)SRRHGWC(Acm)VWDGTFS-OH (2)
The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Ser(Bzl)-PAM resin (0.41 g, 0.25 mmol) according to the general automated SPPS procedure. The peptide resin was treated by HF/*p*-cresol (v/v, 90/10) in the presence of $MeONH_2$.HCl (10 equiv.) at -2 °C to -5 ºC for 1 h to give a crude product, which was purified by preparative HPLC to yield 0.14 g (21%). Analytical HPLC: Rt, 13.5 min (20-40% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 2694.1, found 2693.6.

ProTx-I (1-14)-SCH_2CH_2CO-Leu-NH_2
H-ECRYWLGGCSAGQT- SCH_2CH_2CO-L-NH_2 (3)
The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Thr(Bzl)-SCH_2CH_2CO-Leu-MBHA resin (0.53 g, 0.25 mmol) according to the general automated SPPS procedure. The peptide resin was treated by HF/*p*-cresol (v/v, 90/10) at -2 °C to -5 ºC for 1 h to give a crude product, which was purified by preparative HPLC to yield 0.11 g (26%). Analytical HPLC: Rt, 12.8 min (20-40% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1731.0, found 1730.4.

ProTx-I (15-35)
H-CCKHLVCSRRHGWCVWDGTFS-OH (4)
The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Ser(Bzl)-PAM resin (0.41 g, 0.25 mmol) according to the general automated SPPS procedure. The peptide resin was treated by HF/*p*-cresol /1,4-butanedithiol (v/v, 80/5/15) in the presence of $MeONH_2 \cdot HCl$ (10 equiv.) at -2 °C to -5 ºC for 1 h to give a crude product, which was purified by preparative HPLC to yield 88 mg (14%). Analytical HPLC: Rt, 14.2 min (20-40% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 2480.9, found 2480.4.

[Ile ^{14}]-ProTx-I (1-14)-SCH_2CH_2CO-Leu-NH_2
H-ECRYWLGGCSAGQI -SCH_2CH_2CO-L-NH_2
The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Ile-SCH_2CH_2CO-Leu-MBHA resin (0.50 g, 0.25 mmol) according to the general automated SPPS procedure. The peptide resin was treated by HF/*p*-cresol (v/v, 90/10) at -2 °C to -5 ºC for 1 h to give a crude product, which was purified by preparative HPLC to yield

0.16 g (38%). Analytical HPLC: Rt, 18.1 min (20-40% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1743.0, found 1742.5.

Orexin A (1-11)-SCH_2CH_2CO-Leu-NH_2
XPLPDCCRQKT-*SCH_2CH_2CO-**L**-NH_2 (X= pyroglutamic acid)*
The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Thr(Bzl)-SCH_2CH_2CO-Leu-MBHA resin (0.53 g, 0.25 mmol) according to the general automated SPPS procedure. The peptide resin was treated by HF/*p*-cresol (v/v, 90/10) at -2 °C to -5 ºC for 1 h to give a crude product, which was purified by preparative HPLC to yield 0.14 g (38%). Analytical HPLC: Rt, 7.1 min (10-60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1471.8, found 1471.3.

Orexin A (12-33)
*H-**CSCRLYELLHGAGNHAAGILTL**-NH_2*
The peptide was assembled using an ABI 433A peptide synthesizer on a MBHA resin (0.42 g, 0.25 mmol) according to the general automated SPPS procedure. The peptide resin was treated by HF/*p*-cresol (v/v, 90/10) in the presence of $MeONH_2 \cdot HCl$ (10 equiv) at -2 °C to -5 ºC for 1 h to give a crude product, which was purified by preparative HPLC to yield 56 mg (10%). Analytical HPLC: Rt, 19.1 min (10-60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 2312.7, found 2312.1.

Kurtoxin (1-23)-SCH_2CH_2CO-Leu-NH_2
*H-**KIDGYPVDYWNCKRICWYNNKYCNDL**-SCH_2CH_2CO-**L**-NH_2*
The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Leu-SCH_2CH_2CO-Leu-MBHA resin (0.55 g, 0.35 mmol) according to the general automated SPPS procedure. The peptide resin was treated by HF/*p*-cresol /1,4-butanedithiol (v/v, 80/5/15) at -2 °C to -5 ºC for 1 h to give a crude product, which was purified by preparative HPLC to yield 0.29 g (24%). Analytical HPLC: Rt, 19.6 min (20-40% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 3488.0, found 3487.4.

Kurtoxin (24-63)
*H-**CKGLKADSGYCWGWTLSCYCQGLPDNARIKRSGRCRA**-OH*
The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Ala-Pam resin (0.36 g, 0.25 mmol) according to the general automated SPPS procedure. The peptide resin was treated by HF/*p*-cresol (v/v, 80/20) at -2 °C to -5 ºC for 1 h to give a crude product, which was purified by preparative HPLC to yield 62 mg (6.0%). Analytical HPLC: Rt, 17.3 min (20-40% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 4124.7, found 4124.3.

*H-**LYRAGCGGGT**-SCH_2CH_2CO-**L**-NH_2*
The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Thr(Bzl)-SCH_2CH_2CO-Leu-MBHA resin (0.42 g, 0.20 mmol) according to the general automated SPPS procedure. The peptide resin was treated by HF/*p*-cresol (v/v, 85/15) at -2 °C to -5 ºC for 1 h to give a crude product which was purified by preparative HPLC to yield 76 mg (31%). Analytical HPLC: Rt, 9.7 min (10-60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1154.4, found 1154.5.

*Model thiolactone (**5**)*

H-*LYRAGCGGGT*-SCH_2CH_2CO-*L*-NH_2 (20 mg, 17 μmol) was dissolved in 33 mL of ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 30 mM TCEP, 30 mM sodium ascorbate, pH 7.8), and the solution was incubated at room temperature. The mixture was allowed to react under stirring for 3 h and the reaction mixture was directly subjected to preparative HPLC to yield 12 mg (74%). Analytical HPLC: Rt, 10.4 min (1-60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 936.1, found 935.6.

*H-**LYRAGC**(Acm)**GGGT**-SCH_2CH_2CO-**L**-NH_2 (7)*

The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Thr(Bzl)-SCH_2CH_2CO-Leu-MBHA resin (0.32 g, 0.15 mmol) according to the general automated SPPS procedure. The peptide resin was treated by HF/*p*-cresol (v/v, 85/15) at -2 °C to -5 ºC for 1 h to give a crude product which was purified by preparative HPLC to yield 85 mg (46%). Analytical HPLC: Rt, 13.0 min (1-60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1225.4, found 1225.5.

*Model thioester of Ac-Cys-NH_2 (**6**)*

7 (20 mg, 16 μmol) and Ac-Cys-NH_2 (0.10 g, 0.62 mmol) were dissolved in 3.0 mL of ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 50 mM sodium ascorbate, pH 7.8), and the solution was incubated at room temperature. The mixture was allowed to react under stirring for 2 h and the reaction mixture was directly subjected to preparative HPLC to yield 14 mg (75%). Analytical HPLC: Rt, 10.5 min (1-60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 1169.3, found 1169.0.

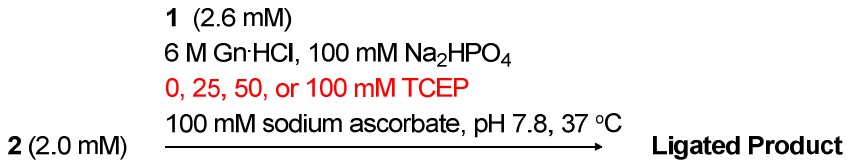
*Model thioester of MPAA (**8**)*

7 (30 mg, 25 μmol) was dissolved in 6.0 mL of ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 50 mM MPAA, 25 mM TCEP, pH 7.8), and the solution was incubated at room temperature. The mixture was allowed to react under stirring for 24 h and the reaction mixture was directly subjected to preparative HPLC to yield 11 mg (37%). Analytical HPLC: Rt, 13.2 min (1-60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 1175.3, found 1174.6.

*H-**CSPGYS**-NH_2*

The peptide was assembled using an ABI 433A peptide synthesizer on a MBHA resin (0.42 g, 0.25 mmol) according to the general automated SPPS procedure. The peptide resin was treated by HF/*p*-cresol (v/v, 85/15) at -2 °C to -5 ºC for 1 h to give a crude product which was purified by preparative HPLC to yield 0.11 g (72%). Analytical HPLC: Rt, 8.6 min (1-60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 611.7, found 612.3.

b) Ligation time-courses in the presence of TCEP in a concentration-dependent manner: 1 + 2

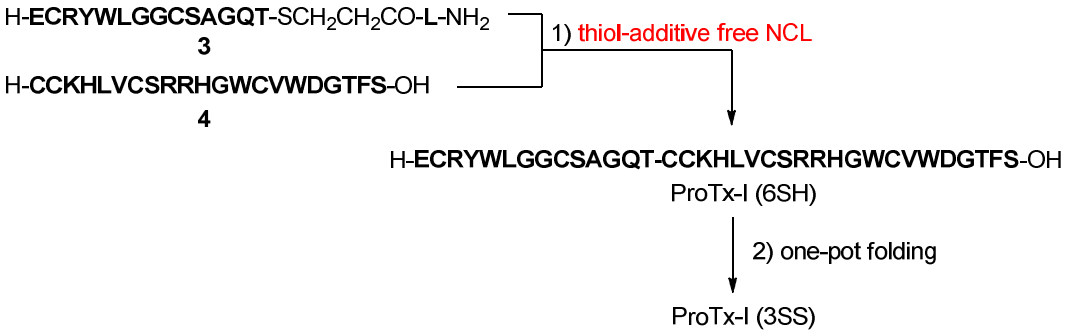


The *C*-segment peptide **2** (1.0 equiv. 2.0 mM) and the *N*-segment thioester **1** (1.3 equiv. 2.6 mM) were dissolved in 6 M Gn·HCl, 100 mM Na_2HPO_4, 0, 25, 50, or 100 mM TCEP, 100 mM sodium ascorbate buffer (pH 7.8) at 37 °C. Reaction aliquots were acidified with TFA to quench the reaction and the progress of ligation was analyzed by analytical HPLC.

c) Ligation time-courses: 3 + 4

The *C*-segment peptide **4** (1.0 equiv. 2.0 mM) and the *N*-segment thioester **3** (1.3 equiv. 2.6 mM) were dissolved in 6 M Gn·HCl, 100 mM Na_2HPO_4, 50 mM TCEP, 100 mM sodium ascorbate buffer (pH 7.8) at 37 °C in the presence of thiol additives: MPAA (100 mM), PhSH (100 mM), or thiol-additve free. Reaction aliquots were acidified with TFA to quench the reaction and the progress of ligation was analyzed by analytical HPLC.

d) One-pot Synthesis of ProTx-I (3SS)



The *N*-segment thioester **3** (5.0 mg, 2.9 μmol) and the *C*-segment peptide **4** (5.5 mg, 2.2 μmol) were dissolved in 1.1 mL of ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 100 mM TCEP, 100 mM sodium ascorbate, pH 7.8), and the solution was incubated at 37 °C. The mixture was allowed to react under stirring for 20 h and was diluted with 1 M Gn·HCl/1 M NH_4OAc buffer (pH 7.8, 0.55 mL) containing reduced form glutathione (68 mg, 0.22 mmol), oxidized form glutathione (54 mg, 89 μmol). After storage at 4 °C for 24 h, the reaction mixture was directly subjected to preparative HPLC to yield 5.0 mg (56%). Analytical HPLC: Rt, 18.7 min (20-40% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 3987.5, found 3987.0.

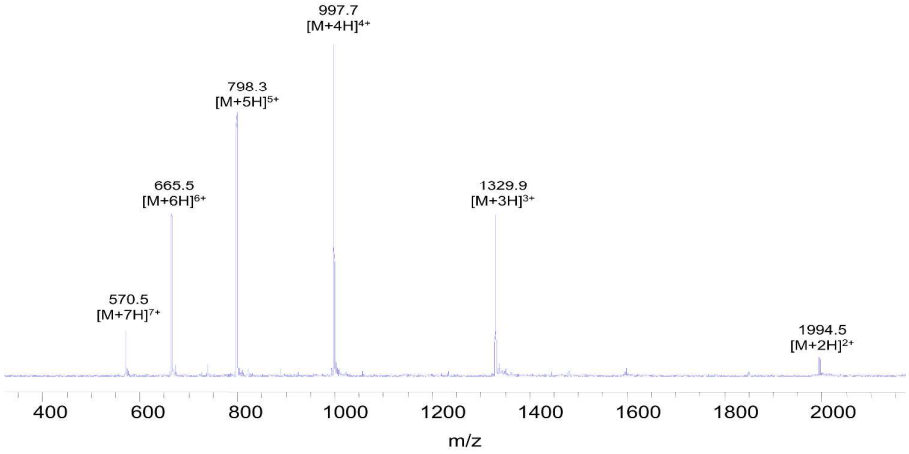


Figure S1. ESI MS profiles of ProTx-I (3SS).

e) Disulfide structure determination of ProTx-I

The synthetic peptide was digested with thermolysin to obtain its cystine segments as shown in Table S1. The assignment of cystine segment consisting of one disulfide linkage could readily reveal one of three disulfide linkages in the ProTx-I molecule to be linked between Cys^{9}-Cys^{21}. The cystine segment containing two adjacent Cys residues with two disulfide linkages, for which two possible disulfide modes could be considered, was subjected to Edman degradation to assign its disulfide structure with the guidance of the cycles detecting diPTH cystine. However, Edman degradation could not discriminate between two possible disulfide modes because diPTH cystine could be detected at the same cycles in both structures (Table S2). To avoid this obstacle, the acetyl group was introduced to the *N*-terminus and the ε-amino group at Lys^{17} of the parent molecule prior to enzymatic digestions. This provided a cystine segment having three peptide chains linked by two disulfide linkages, in which the peptide chain originating from the *N*-terminus was tagged with the acetyl group. This procedure was successfully applied to discriminating two possible structures by Edman degradation. DiPTH cystine was detected at the cycles indicated at the upper strand in Table S2, implying that the cystine segment should have the upper type of structure. From these results, the synthetic ProTx-I was confirmed to have the native disulfide pairing, *i.e.* Cys^{2}-Cys^{16}, Cys^{9}-Cys^{21} and Cys^{15}-Cys^{28}.

Table S1. Cystine segments obtained by enzymatic digestion of synthetic ProTx-I and Ac-ProTx-I

Assignment	Sequence	ESI MS : MW	Cycle(s) detecting $(PTH\text{-}Cys)_2$
(6-10)/(20-21)	LGGCS/VC	653.8 (653.8)[a]	4
(1-3)/(11-18)/(27-28)	ECR/AGQTCCKH/WC	1557.0 (1556.8)	5,6
Ac-(1-3)/(11-18)/(27-28)	**Ac**-ECR/AGQTCCK(**Ac**)H/WC	1640.9 (1640.8)	5

[a]( ): The values in parentheses are theoretical values.

Table S2. Two possible disulfide structures of cystine segment containing two adjacent Cys residues and ratios of $(PTH\text{-}Cys)_2$ that should appear in each cycle during Edman degradation

Peptide	Cystine segments with 2 SS* and its expected cycle(s) to detect $(PTH\text{-}Cys)_2$	cycle 4	5	6		cycle 4	5	6
ProTx-I	ECR AGQTCCKH WC	0	1	1	**Ac**-ECR AGQTCCK(Ac)H WC	0	1	0
	ECR AGQTCCKH WC	0	1	1	**Ac**-ECR AGQTCCK(Ac)H WC	0	0	1

* Two possible disulfide modes can be considered.

Acetylation of ProTx-I

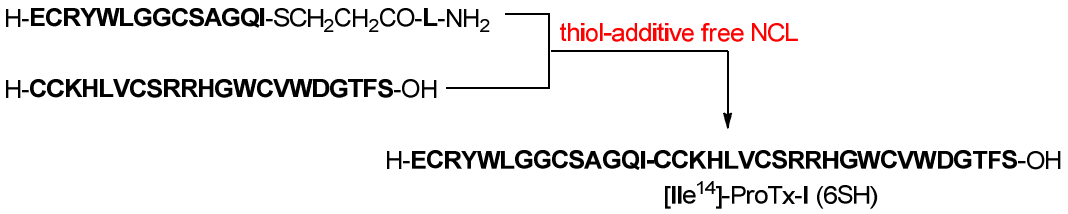
The folded peptide (0.2 mg, 0.05 μmol) was dissolved in 0.2 mL of 0.1 M sodium phosphate buffer (pH 8.3). To the solution AcOSu (0.02 mg, 0.12 μmol) was added, and the mixture was allowed to react for 10 min at room temperature. After the pH was adjusted to < 2 by the addition of TFA, the mixture was directly applied to HPLC (YMC-Pak ODS column 4.6 x 150 mm) with a gradient elution of 10-60% CH_3CN in 0.1% TFA to obtain the acetylated peptide.

Ac-ProTx-I, MW measured by ESI MS: 4071.2 (theoretical value: 4071.6).

Enzymatic digestion of ProTx-I

Each of ProTx-I and Ac-ProTx-I (0.2 mg) was digested by thermolysin (10 μL, conc.: 1 mg/mL H_2O) in 0.1 M NH_4OAc (0.3 mL, pH 6.5) containing 1 mM $CaCl_2$ at 37 ºC for 2 h. The respective segments obtained were isolated by RP-HPLC on a YMC Pak ODS column (4.6 x 150 mm) using a linear gradient (1-30% CH_3CN in 0.1% TFA for 30 min) and characterized by sequence analysis and mass spectroscopy. The data were shown in Table S1.

f) Synthesis of [Ile^{14}]-ProTx-I (6SH)



The *N*-segment thioester (11 mg, 6.4 μmol) and the *C*-segment thioester (12 mg, 4.9 μmol) were dissolved in 2.5 mL of ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 100 mM TCEP, 100 mM sodium ascorbate, pH 7.8), and the solution was incubated at 37 °C. The mixture was allowed to react under stirring for 32 h and then treated with

DTT (45 mg, 0.30 mmol). After 30 min the pH was adjusted to < 2 by the addition of 1 M HCl aq. The reaction mixture was directly subjected to preparative HPLC to yield 8.8 mg (45%). Analytical HPLC: Rt, 19.4 min (20-40% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 4005.6, found 4005.0.

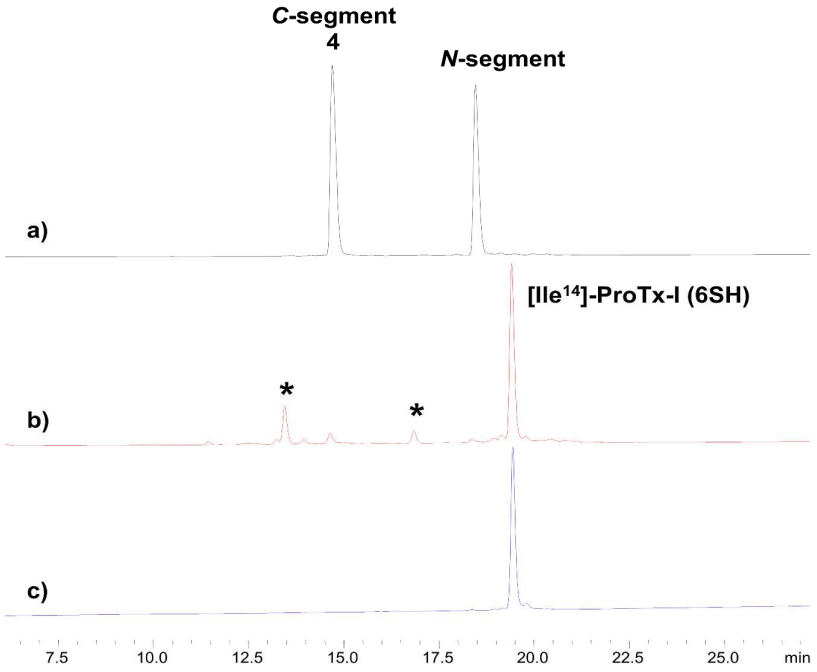


Figure S2. HPLC profiles of the reaction for the synthesis of [Ile^{14}]-ProTx-I (6SH). a) NCL (t = 0 h), b) NCL (t = 32 h), c) purified product. HPLC conditions: column, DAISO-PAK SP-120-5-ODS-BIO (4.6 x 150 mm); elution, 20-40% CH_3CN in 0.1% TFA (25 min) at 40 °C; flow rate, 1.0 mL/min; detection, 220 nm.
* thiolactone of the *N*-segment.

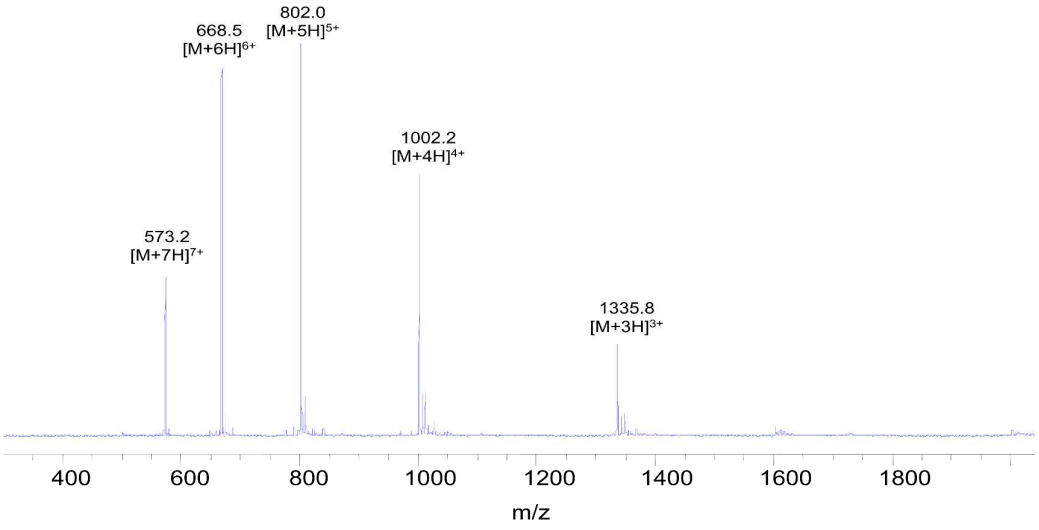
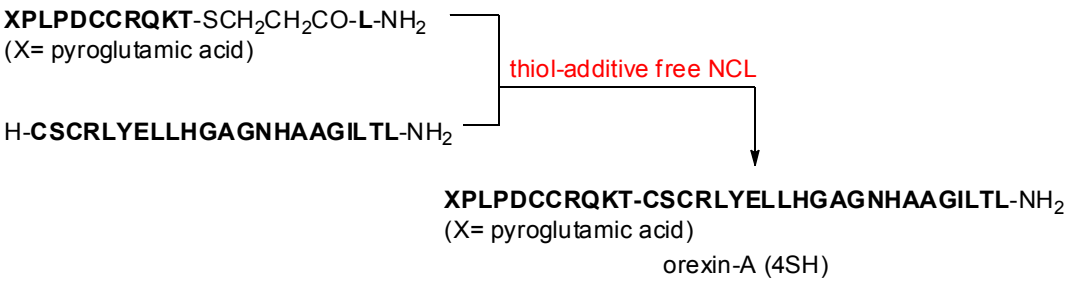


Figure S3. ESI MS profiles of [Ile^{14}]-ProTx-I (6SH).

g) Synthesis of orexin A (4SH)



The *N*-segment thioester (8.3 mg, 5.6 μmol) and the *C*-segment thioester (10 mg, 4.3 μmol) were dissolved in 2.2 mL of ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 100 mM TCEP, 100 mM sodium ascorbate, pH 7.8), and the solution was incubated at 37 °C. The mixture was allowed to react under stirring for 24 h and then treated with DTT (40 mg, 0.26 mmol). After 30 min the pH was adjusted to < 2 by the addition of 1 M HCl aq. The reaction mixture was directly subjected to preparative HPLC to yield 9.4 mg (61%). Analytical HPLC: Rt, 20.9 min (10-60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 3566.1, found 3564.6.

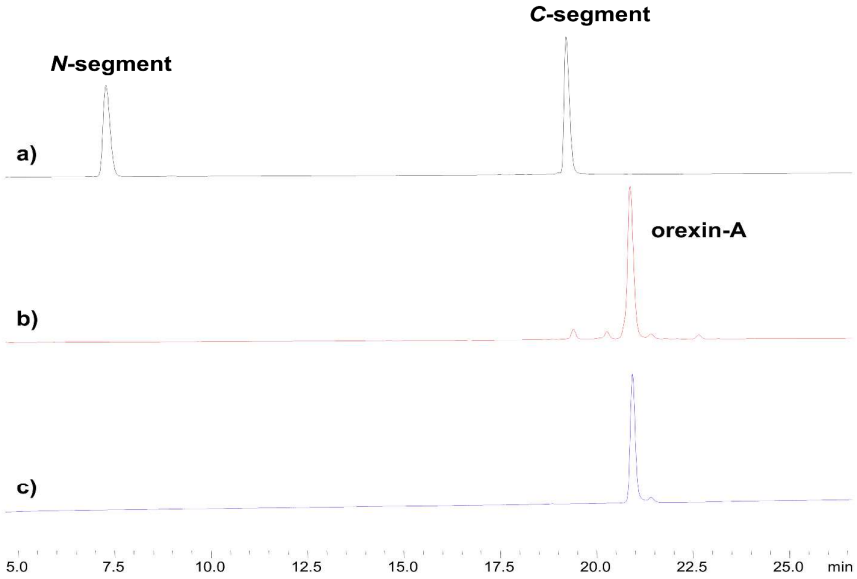


Figure S4. HPLC profiles of the reaction for the synthesis of orexin-A (4SH)
a) NCL (t = 0 h), b) NCL (t = 24 h), c) purified product. HPLC conditions: column, DAISO-PAK SP-120-5-ODS-BIO (4.6 x 150 mm); elution, 10-60% CH_3CN in 0.1% TFA (25 min) at 40 °C; flow rate, 1.0 mL/min; detection, 220 nm.

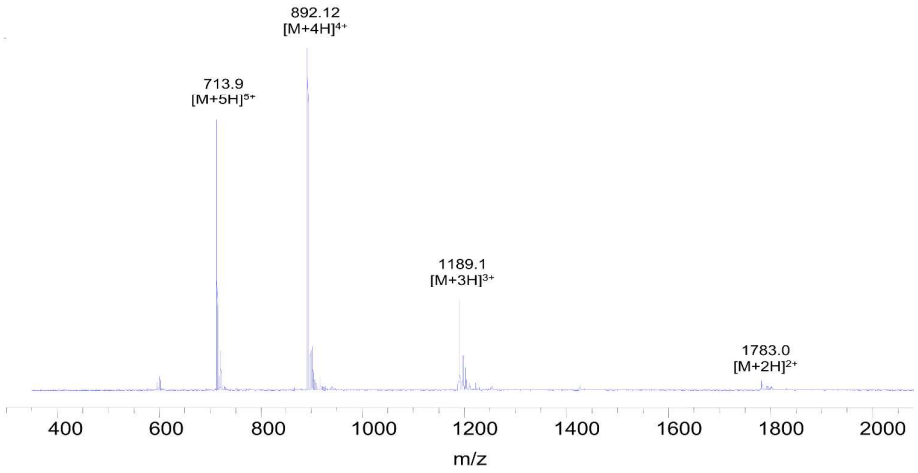
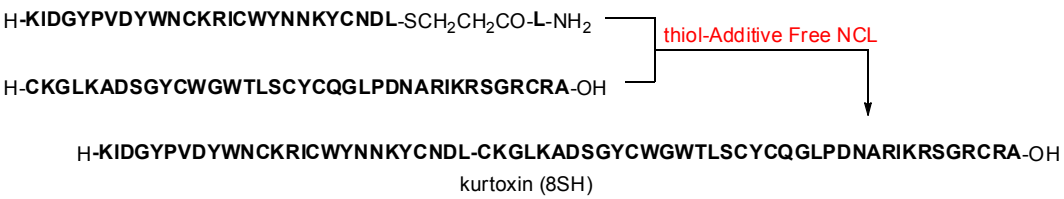


Figure S5. ESI MS profiles of orexin A (4SH).

h) Synthesis of kurtoxin (8SH)



The *N*-segment thioester (11 mg, 3.2 μmol) and the *C*-segment thioester (10 mg, 2.4 μmol) were dissolved in 1.2 mL of ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 100 mM TCEP, 100 mM sodium ascorbate, pH 7.8), and the solution was incubated at 37 °C. The mixture was allowed to react under stirring for 18 h and then treated with DTT (22 mg, 0.15 mmol). After 30 min the pH was adjusted to < 2 by the addition of 1 M HCl aq. The reaction mixture was directly subjected to preparative HPLC to yield 9.9 mg (55%). Analytical HPLC: Rt, 22.4 min (20-40% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 7394.4, found 7393.7

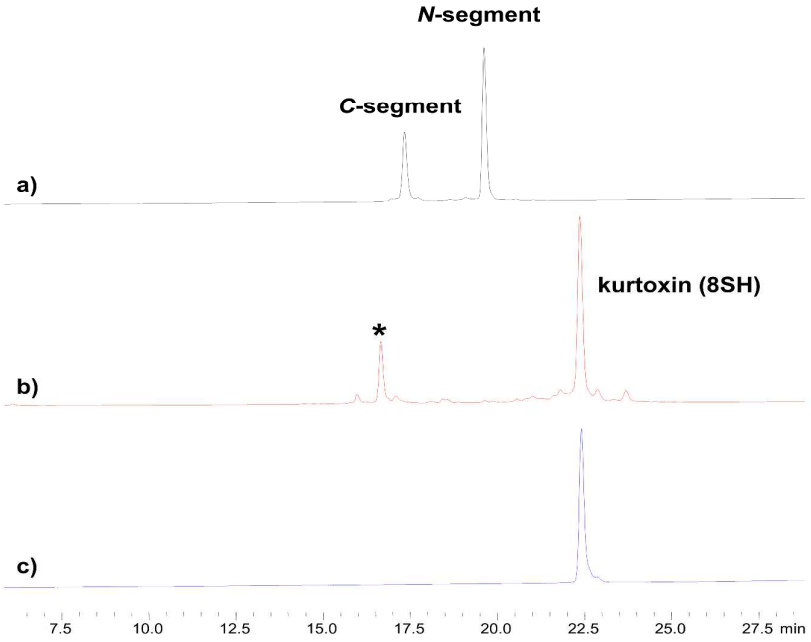


Figure S6. HPLC profiles of the reaction for the synthesis of kurtoxin (8SH)
a) NCL (t = 0 h), b) NCL (t = 18 h), c) purified product. HPLC conditions: column, DAISO-PAK SP-120-5-ODS-BIO (4.6 x 150 mm); elution, 20-40% CH_3CN in 0.1% TFA (25 min) at 40 °C; flow rate, 1.0 mL/min; detection, 220 nm.
* thiolactone and hydrolysate of the *N*-segment.

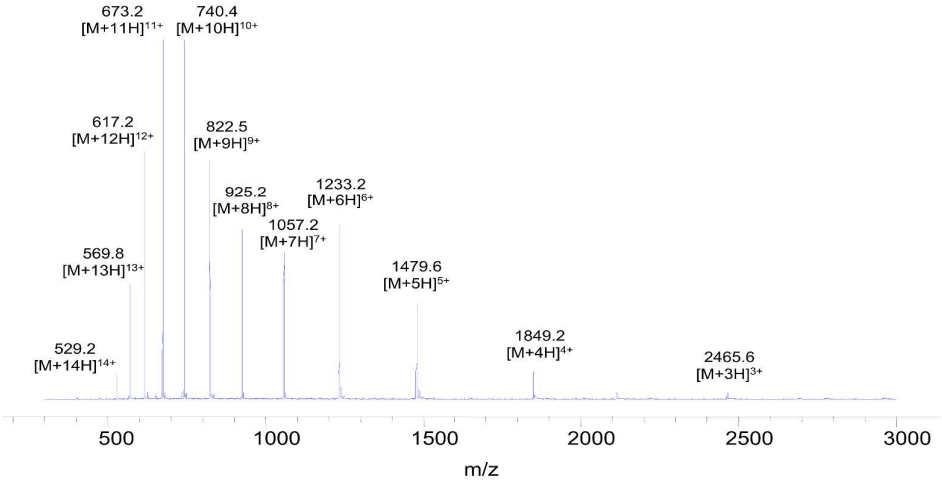


Figure S7. ESI MS profiles of kurtoxin (8SH).

i) Ligation time-courses: NCL of model thioesters 5-8.
The *C*-segment peptide (1.0 equiv. 2.0 mM) and the *N*-segment thioester **5-8** (1.5 equiv. 3.0 mM) were dissolved in 6 M Gn·HCl, 100 mM Na_2HPO_4, 25 mM TCEP buffer (pH

7.5) at room temperature. Reaction aliquots were acidified with TFA to quench the reaction and the progress of ligation was analyzed by analytical HPLC.