

Supporting Information for

Highly Efficient Solid Phase Synthesis of Large Polypeptides by Iterative Ligations of Bis(2-sulfanylethyl)amido (SEA) Peptide Segments

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1. General Methods

Reagents and solvents

2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium fluorophosphate (HBTU) and *N* α -Fmoc protected amino acids were obtained from Iris Biotech GmbH. Side-chain protecting groups used for the amino acids were Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(*t*Bu)-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Cys(S*t*Bu)-OH or Fmoc-Cys(Trt)-OH. Synthesis of *bis*(2-sulfanylethyl)aminotriyl polystyrene (SEA PS) resin **1** was carried out as described elsewhere.¹ Rink-PEG-PS resin (NovaSyn TGR) and PEGA¹⁹⁰⁰ resin were obtained from Novabiochem and Agilent Technologies, respectively. 4-Mercaptophenylacetic acid (MPAA), 3-mercaptopropionic acid (MPA), *tris*(2-carboxyethyl)phosphine hydrochloride (TCEP), *N,N,N',N'*-tetramethylazodicarboxamide (TMAD), triisopropylsilane (TIS), dimethyl sulfide (DMS), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), copper sulfate pentahydrate, aminoguanidine hydrochloride and sodium ascorbate were purchased from Sigma-Aldrich. All other reagents were purchased from Acros Organics or Merck and were of the purest grade available.

Peptide synthesis grade *N,N*-dimethylformamide (DMF), dichloromethane (CH₂Cl₂), diethylether (Et₂O), acetonitrile (CH₃CN), heptane, LC-MS-grade acetonitrile (CH₃CN, 0.1% TFA), LC-MS-grade water (H₂O, 0.1% TFA), *N,N*-diisopropylethylamine (DIEA), acetic anhydride (Ac₂O) were purchased from Biosolve and Fisher-Chemical. Trifluoroacetic acid (TFA) was obtained from Biosolve. Water was purified with a Milli-Q Ultra Pure Water Purification System.

Synthesis of 2-[2-(2-Azido-ethoxy)-ethylsulfonyl]-ethyl 4-nitrophenyl carbonate (N₃-ESOC-ONp) **3** and 2-(2-azidoethoxy)-ethanol were carried out as described in a previous paper.² *Tris*-(3-hydroxypropyltriazolylmethyl)amine (THPTA) and bicyclo[6.1.0]non-4-yn-9-ylmethyl (4-nitrophenyl) carbonate (BCN-ONp) were prepared as described elsewhere.^{3,4}

Analyses and purifications

Reactions were monitored by analytical LC-MS (Waters 2695 LC/ZQ 2000 quadripole) on a reverse phase column XBridge BEH300 C18 (3.5 μ m, 300 Å, 4.6 \times 150 mm) at 30 °C using a linear gradient of 0-100% of buffer B in buffer A over 30 min at a flow rate of 1 mL/min (buffer A = 0.1% TFA in H₂O; buffer B = 0.1% TFA in CH₃CN/H₂O: 4/1 by vol). The column eluate was monitored by UV at 215 nm and by evaporative light scattering (ELS, waters 2424). The peptide masses were measured by on-line LC-MS:

¹N. Ollivier, J. Dheur, R. Mhidia, A. Blanpain, O. Melnyk, *Org. Lett.* **2010**, *12*, 5238-5241.

²V. Aucagne, I. E. Valverde, P. Marceau, M. Galibert, N. Dendane, A. F. Delmas, *Angew. Chem. Int. Ed.* **2012**, *51*, 11320-11324.

³THPTA: T. R., Chan, R. Hilgraf, K. B. Sharpless, V. V. Fokin, *Org. Lett.* **2004**, *6*, 2853-2855.

⁴BCN-ONp: J. Dommerholt, S. Schmidt, R. Temming, L. J. A. Hendriks, F. P. J. T. Rutjes, J. C. M. van Hest, D. J. Lefeber, P. Friedl, F. L. van Delft, *Angew. Chem. Int. Ed. Engl.* **2010**, *49*, 9422-9425.

Ionization mode: ES+, m/z range 350–2040, capillary voltage 3 kV, cone voltage 30 V, extractor voltage 3 V, RF lens 0.2 V, source temperature 120 °C, desolvation temperature 350 °C. Calculated masses were based on average isotope composition. Samples were prepared using 10 µL aliquots of the reaction mixtures. The aliquots were quenched by adding 90 µL of 10% aqueous TFA, extracted with Et₂O to remove MPAA or MPA before analysis.

MALDI-TOF mass spectra were recorded with a Bruker Autoflex Speed using alpha cyano 4-hydroxycinnamic acid or sinapinic acid as matrix. The observed m/z corresponded to the monoisotopic ions, unless otherwise stated.

Preparative reverse phase HPLC of crude peptides were performed with an Autopurification prep HPLC–MS Waters system using a reverse phase column XBridge ODB prep C-18 (5 µm, 300 Å, 19 × 100 mm) and appropriate gradient of increasing concentration of buffer B in buffer A (flow rate of 25 mL/min). The fractions containing the purified target peptide were identified on-line using MS (ZQ 2000) quadrupole. Selected fractions were then combined and lyophilized.

2. Peptide sequences

-Assembly of peptide 9 (6a + 7a + 7b + 8a)

Segment **4a**: N₃-Esoc-ILKEPVQGA-SEA^{off}

Segment **7a**: C(S*t*Bu)HHLEPGG-SEA^{off}

Segment **7b**: C(S*t*Bu)HHLEPAG-SEA^{off}

Segment **8a**: CILKEPVHGA-NH₂

-Assembly of peptide 10 (6b + 7c + 7d + 8b), see Figure 5

Segment **4b**: N₃-Esoc -AAAAAKDYIRN-SEA^{off}

Segment **7c**: C(S*t*Bu)IIGKGRSYKGTVSITKSGIK-SEA^{off}

Segment **7d**: C(S*t*Bu)QPWSSMIPHEHSFLPSSYRGKDLQENY-SEA^{off}

Segment **8b**: CRNPRGEEGGPWCFTSNPEVRYEVCDIPQCSEV-NH₂

-Assembly of peptide 12 (6c + 7e + 7c + 7d + 8b), see Figure 6

Segment **4c**: N₃-Esoc -GQRKRRNTIHEAAAAAKDYIRN-SEA^{off}

Segment **7e**: C(S*t*Bu)LWFPFNSMSSGVKKEFGHEFDLYENKDYIRN-SEA^{off}

Segment **7c**: C(S*t*Bu)IIGKGRSYKGTVSITKSGIK-SEA^{off}

Segment **7d**: C(S*t*Bu)QPWSSMIPHEHSFLPSSYRGKDLQENY-SEA^{off}

Segment **8b**: CRNPRGEEGGPWCFTSNPEVRYEVCDIPQCSEV-NH₂

3. Peptide synthesis/purification/LC-MS characterization

3.1 Synthesis of SEA^{off} peptide segments

-Loading of the first amino acid on SEA-PS resin 1

SEA PS resin **1** (0.2 mmol, 0.175 mmol/g) was conditioned in CH₂Cl₂. Fmoc-Aa-OH (5 mmol) was dissolved in CH₂Cl₂ (2 mL) and added to the resin (when necessary few drops of DMF were added to favor dissolution of the amino acid). Bromo-tris-pyrrolidino phosphonium hexafluorophosphate (PyBrop, 466 mg, 5 mmol) was dissolved CH₂Cl₂ (1 mL) and added to the resin. DIEA (0.5 mL, 15 mmol) was then added to the resin, which was agitated during 2 h. The resin was then washed with CH₂Cl₂ (3 × 2 min) and treated with Ac₂O/DIEA/CH₂Cl₂: 10/5/85 by vol (10 mL, 2 × 10 min) to cap unreacted amino groups. Finally, the resin was washed with CH₂Cl₂ (5 × 2 min).

-Typical procedure for Fmoc-SPPS elongation using SEA-PS resin 1

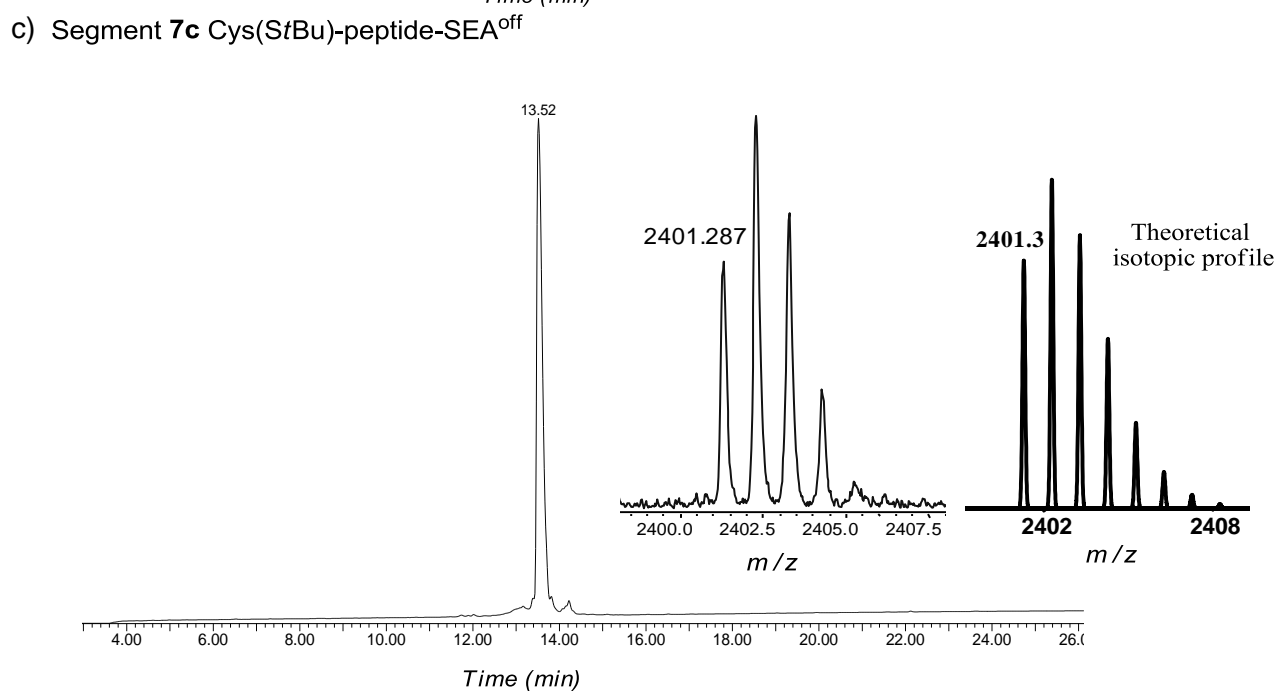
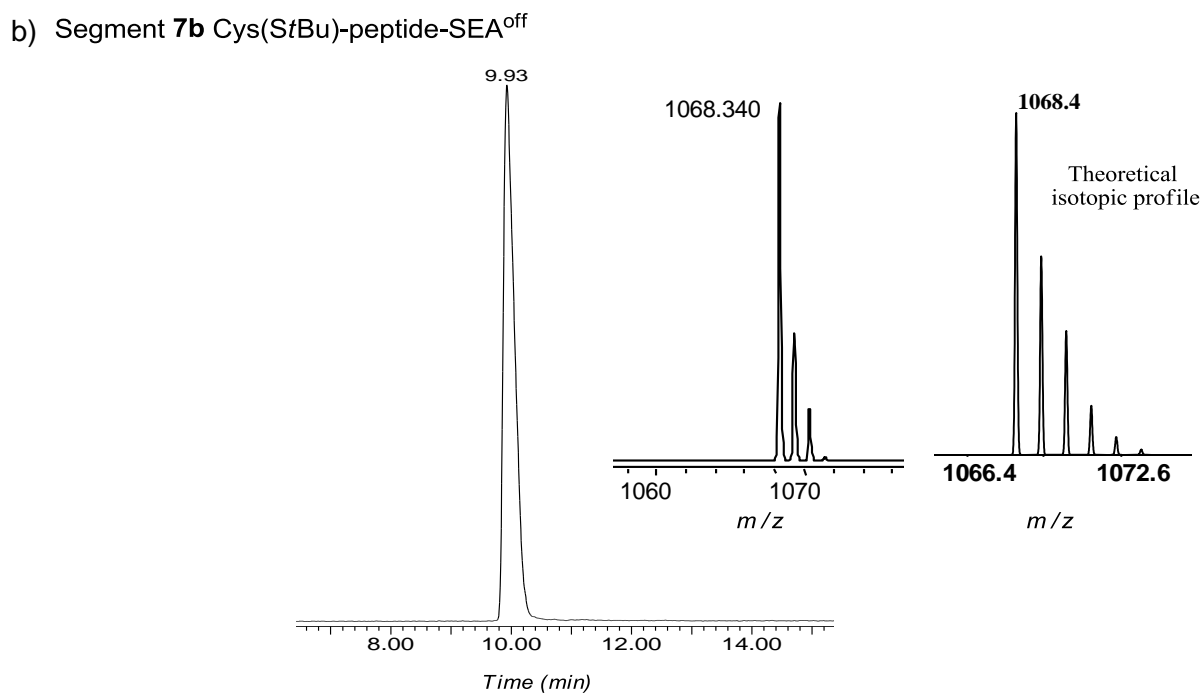
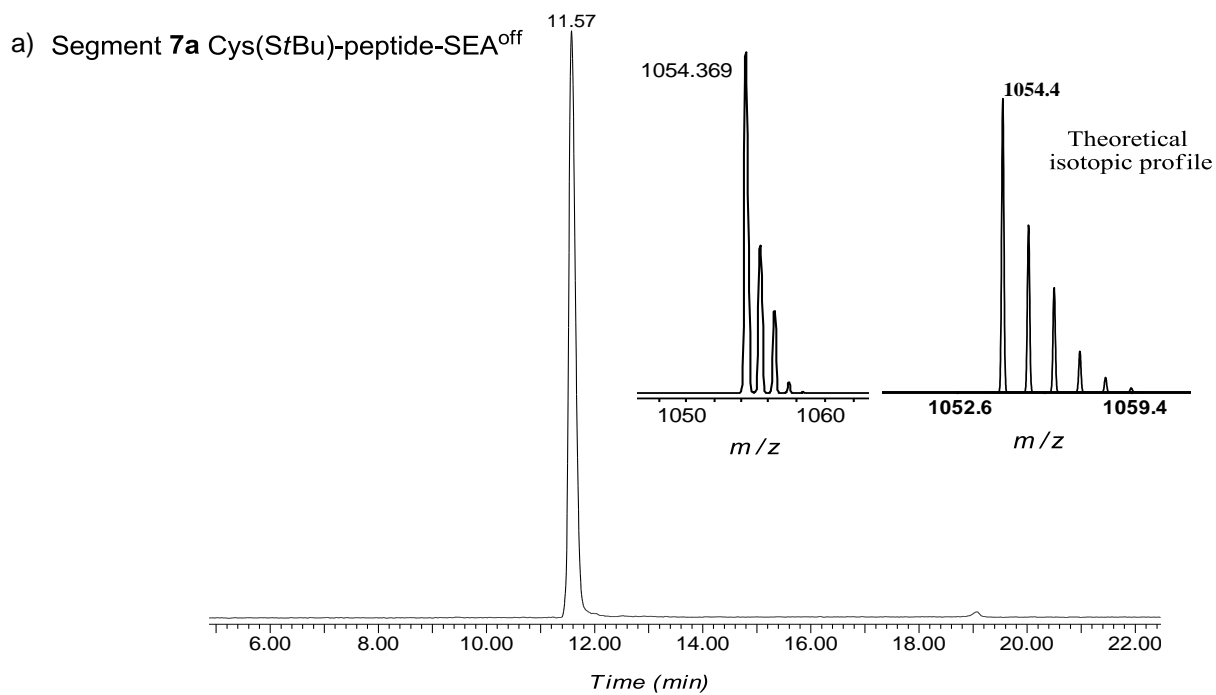
Peptide elongation was performed using standard Fmoc/*tert*-butyl chemistry on an automated peptide synthesizer (0.2 mmol scale). Couplings were performed using five fold molar excess of each Fmoc-L-amino acid, 4.5-fold molar excess of HBTU, and 10-fold molar excess of DIEA. A capping step was performed after each coupling with Ac₂O/DIEA in DMF. At the end of the synthesis, the resin was washed with CH₂Cl₂, diethylether (2 × 2 min) and dried in vacuo.

-Deprotection and cleavage of SEA^{on} peptide segments

Deprotection and cleavage were performed with TFA/TIS/DMS/H₂O: 90/2.5/2.5/2.5/2.5 by vol (20 mL) during 2 h. The peptide was precipitated in cold Et₂O/heptane: 1/1 by vol (150 mL), centrifugated, dissolved in deionized water, and lyophilized to give the crude SEA^{on} peptide segment.

-Typical procedure for SEA^{off} segment purification

SEA^{on} peptide segment was oxidized into SEA^{off} peptide segment by using iodine in aqueous acetic acid just before the HPLC purification step. For this, SEA^{on} peptide segment (25 μmol) was dissolved in AcOH/H₂O: 1/4 by vol (final peptide concentration 0.5 mM). Iodine solution (200 mM in DMSO, 50 μmol, 250 μL) was added in one portion. After 30 s, dithiothreitol (DTT, 65 mM in water, 50 μmol, 775 μL) was added to quench the excess of iodine. DTT is unable to reduce disulfide bonds in the acidic conditions used for converting SEA^{on} into SEA^{off}. Thus, there is no risk to convert SEA^{off} group back to SEA^{on} or to remove *tert*-butylsulfenyl groups during this chemical step. Once DTT was added, the mixture was immediately purified by reversed-phase HPLC using a linear water-acetonitrile gradient to give SEA^{off} peptide segments. The analytical HPLC and MS analyses of the purified synthetic SEA^{off} peptide segments **7a**, **7b**, **7c**, **7d** and **7e** are shown Figure S1.



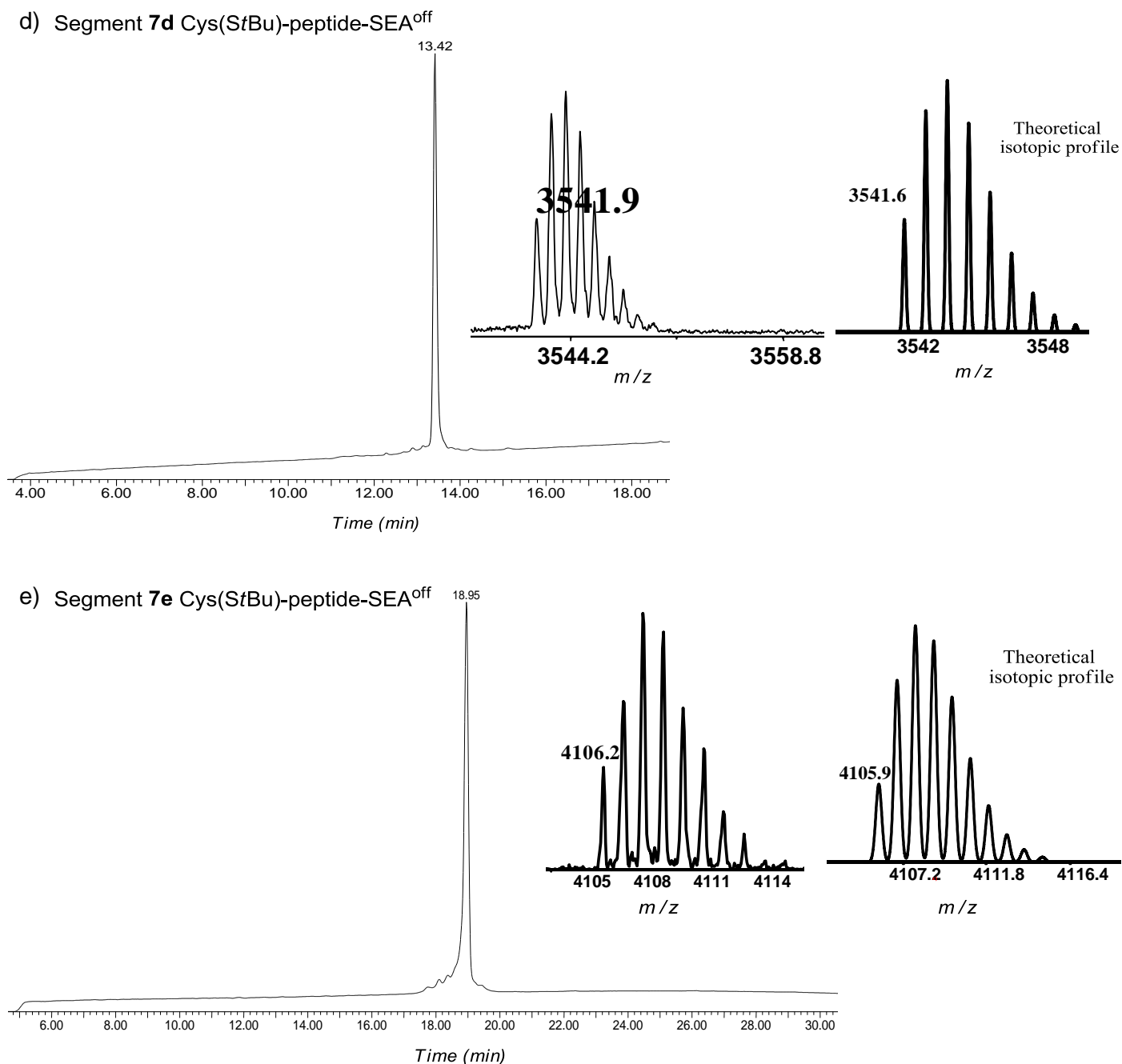


Figure S1. Analytical HPLC profiles ($\lambda=215$ nm) for purified synthetic peptide segments and Maldi-TOF data corresponding to each product.

a) Segment **7a**: 35 mg (25 % yield, 0.1 mmol scale), Maldi-TOF calc. for $[M+H]^+$: 1054.4, observed mass: 1054.4 (monoisotopic).

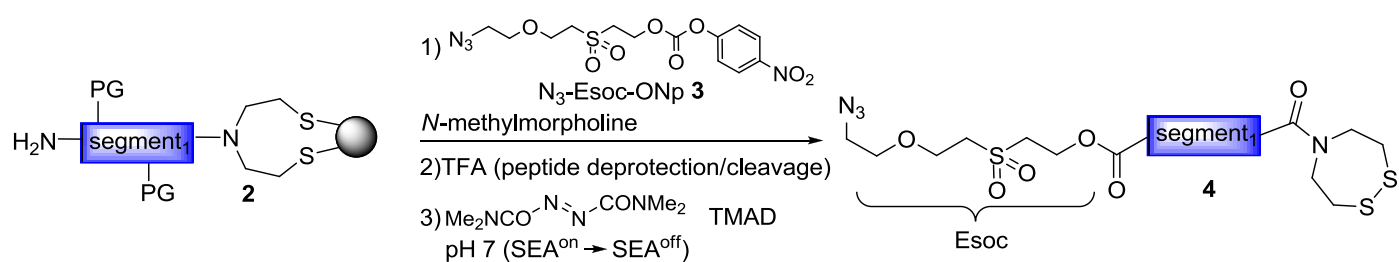
b) Segment **7b**: 40 mg (28 % yield, 0.1 mmol scale), Maldi-TOF calc. for $[M+H]^+$: 1068.4, observed mass: 1068.3 (monoisotopic).

c) Segment **7c**: 43 mg (28 % yield, 50 μ mol scale), Maldi-TOF calc. for $[M+H]^+$: 2401.3, observed mass: 2401.3 (monoisotopic).

d) Segment **7d**: 47 mg (23 % yield, 50 μ mol scale), Maldi-TOF calc. for $[M+H]^+$: 3541.6, observed mass: 3541.9 (monoisotopic).

e) Segment **7e**: 51 mg (21 % yield, 50 μ mol scale), Maldi-TOF calc. for $[M+H]^+$: 4105.9, observed mass: 4106.2 (monoisotopic).

3.2 Synthesis of N₃-Esoc-peptide-SEA^{off} **4** (Scheme 1)

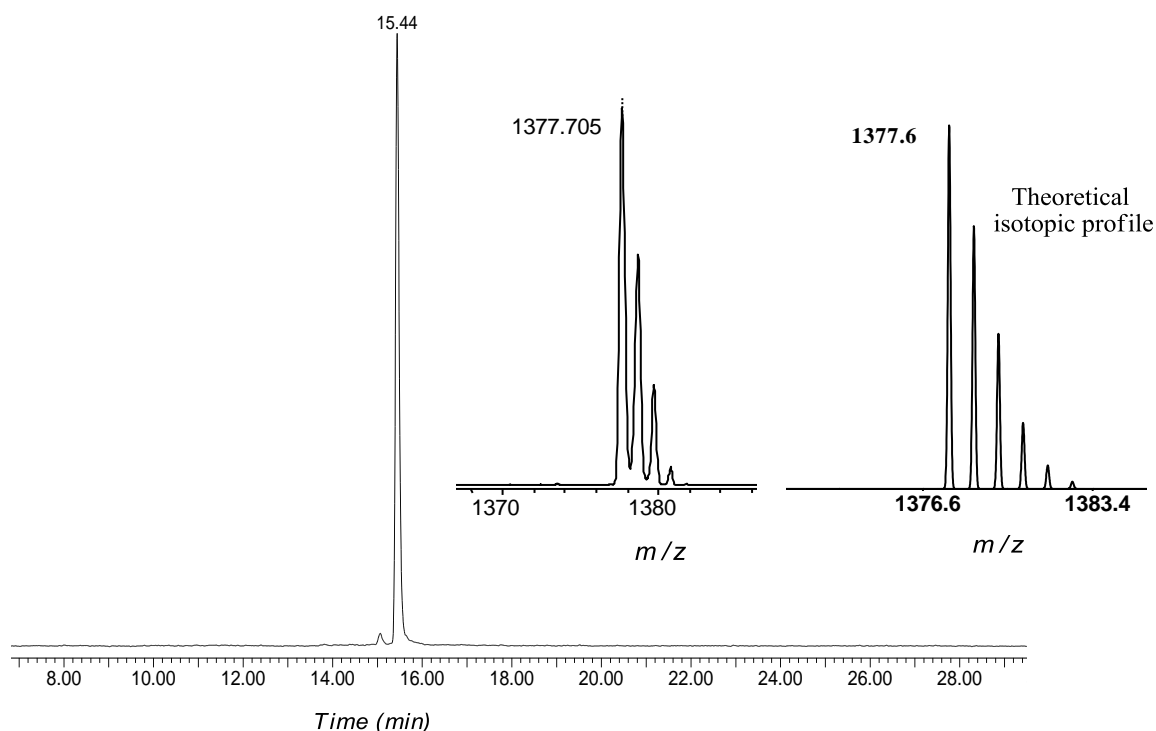


The peptide sequence was assembled on SEA PS resin **1** (0.2 mmol) using standard Fmoc/*tert*-butyl SPPS. 2-[2-(2-Azido-ethoxy)-ethylsulfonyl]-ethyl 4-nitrophenyl carbonate (N₃-ESOC-ONp) **3** (117 mg, 0.3 mmol, 1.5 eq) was then dissolved in minimum amount of DMF (2 mL) in the presence of *N*-methylmorpholine (44 μ L, 0.4 mmol, 2 eq) and added to the peptidyl resin **2** at room temperature. The bead suspension was agitated overnight. The peptide resin was then washed with DMF (2 \times 2 min), CH₂Cl₂ (2 \times 2 min) and Et₂O (2 \times 2 min) and dried *in vacuo*.

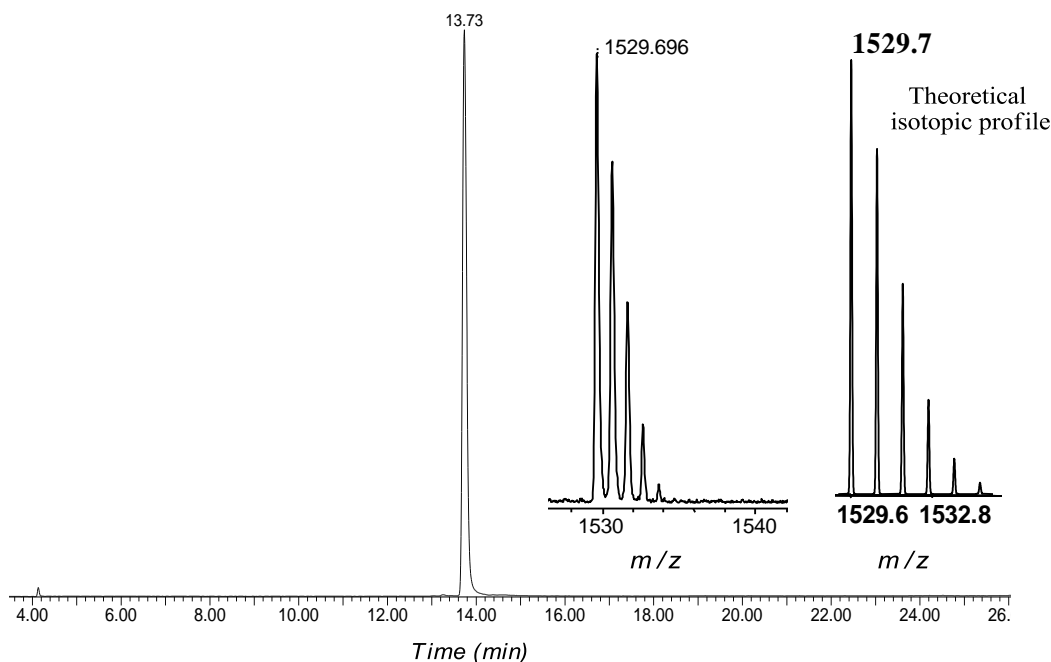
Deprotection and cleavage were performed with TFA/TIS/DMS/H₂O: 90/2.5/2.5/2.5 by volume (20 mL) during 2 h. The peptide was precipitated in cold Et₂O/heptane: 1/1 by volume (200 mL), dissolved in deionized water, and lyophilized to give the crude SEA^{on} peptide segments.

The crude segments N₃-Esoc-peptide-SEA^{on} (50 μ mol) were oxidized into SEA^{off} by using *N,N,N,N*-tetramethyl-azodicarboxamide (TMAD) (17 mg, 0.1 mmol, 2 eq) in 0.2 M pH 7.2 phosphate buffer (26 mL) at room temperature for 20 min (final peptide concentration 2 mM). The mixture was immediately purified by reversed-phase HPLC using a linear water-acetonitrile gradient containing 0.05 % TFA to give the peptide N₃-Esoc-peptide-SEA^{off} **4**. The analytical HPLC and MS analyses of the purified peptides N₃-Esoc-peptide-SEA^{off} **4a**, **4b** and **4c** are shown in Figure S2.

f) Segment **4a** N₃-Esoc-peptide-SEA^{off}



g) Segment **4b** N₃-Esoc-peptide-SEA^{off}



h) Segment **4c** N₃-Esoc-peptide-SEA^{off}

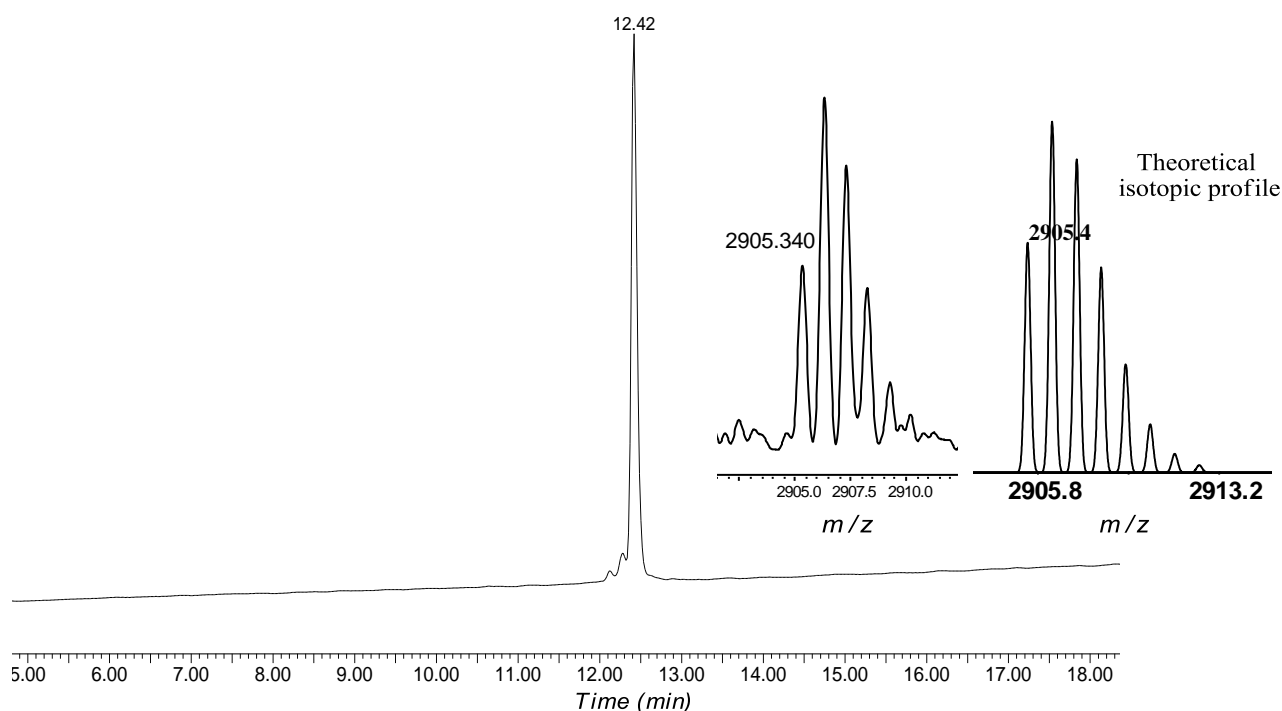


Figure S2. Analytical HPLC profiles ($\lambda=215$ nm) for purified synthetic peptide segments and MALDI-TOF data corresponding to each product.

f) Segment **4a**: N₃-Esoc-peptide-SEA^{off}: 39 mg (52 % yield, 50 μ mol scale), MALDI-TOF calc. for [M+H]⁺: 1377.6, observed mass: 1377.7 (monoisotopic).

g) Segment **4b**: N₃-Esoc-peptide-SEA^{off}: 38 mg (43 % yield, 50 μ mol scale), MALDI-TOF calc. for [M+H]⁺: 1529.7, observed mass: 1529.7 (monoisotopic).

h) Segment **4c**: N₃-Esoc-peptide-SEA^{off}: 61 mg (33 % yield, from 50 μ mol scale), MALDI-TOF calc. for [M+H]⁺: 2905.4, observed mass: 2905.3 (monoisotopic).

3.3 Synthesis of peptide segment 8a and 8b

Peptide elongation was performed on Rink-PEG-PS resin (NovaSyn TGR, 0.25 mmol, 0.25 mmol/g) by using standard Fmoc/*tert*-butyl chemistry on an automated peptide synthesizer with HBTU/DIEA activation in DMF. A capping step was performed after each coupling with Ac₂O/DIEA in DMF. At the end of the synthesis, the Fmoc protecting group of the last amino acid was removed with 20% piperidine in DMF. Final deprotection and cleavage from the solid support were performed with TFA/H₂O/1,2-ethanedithiol/TIS: 94/2.5/2.5/1 by vol for 2 h (25 mL). The crude peptide was precipitated in cold Et₂O/heptane: 1/1 by vol (300 mL), centrifugated, solubilized in deionized water, and lyophilized. Purification was performed by reversed-phase HPLC using a linear water-acetonitrile gradient. The analytical HPLC and MS analyses of the purified peptides **8a** and **8b** are shown in Figure S3.

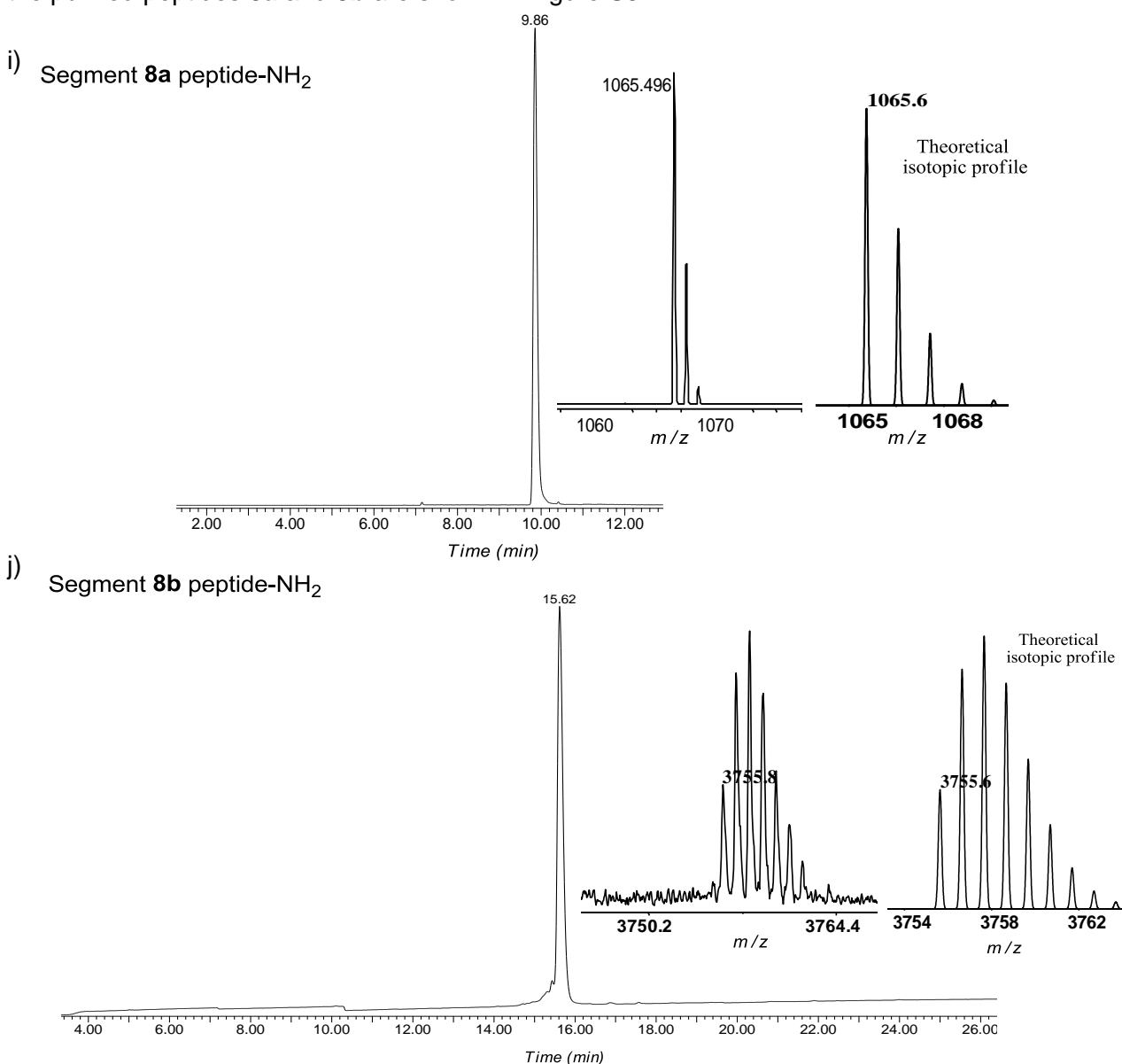
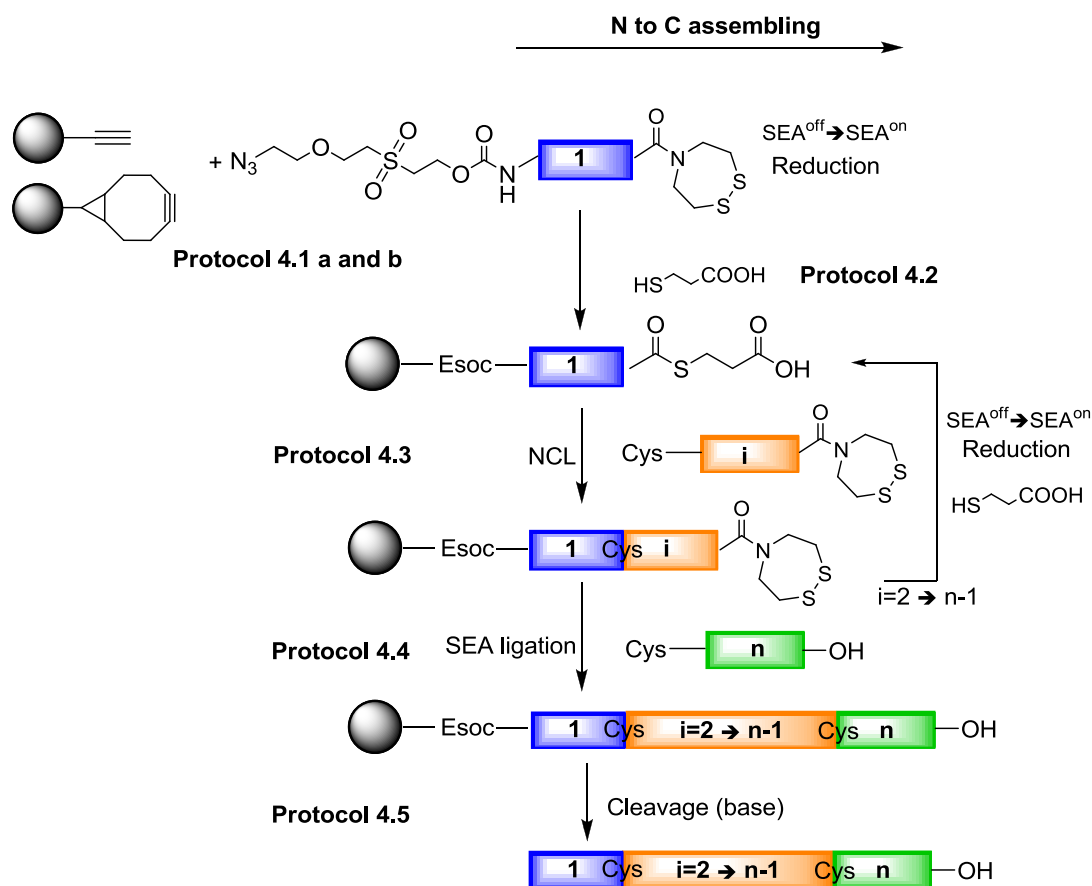


Figure S3. Analytical HPLC profiles ($\lambda=215$ nm) for purified synthetic peptide segments and Maldi-TOF data corresponding to each product.

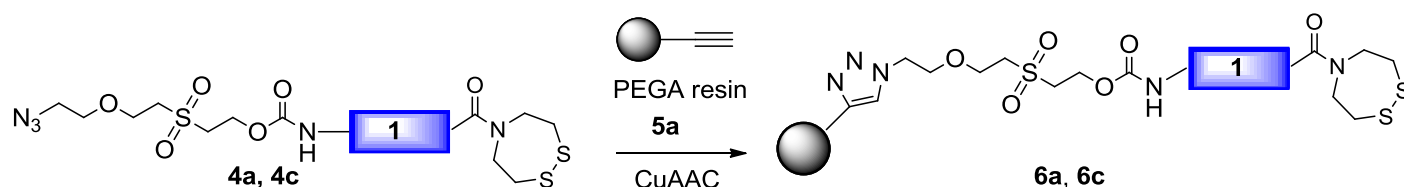
i) Segment **8a**: 77 mg (55% yield, 0.1 mmol scale), Maldi-TOF calc. for [M+H]⁺: 1065.6, observed mass: 1065.5 (monoisotopic).

j) Segment **8b**: 134 mg (13 % yield, 0.25 mmol scale), Maldi-TOF calc. for [M+H]⁺: 3755.6, observed mass: 3755.8 (monoisotopic).

4. General procedure for the solid phase protein total synthesis



4.1.a Coupling of the first N₃-Esoc-peptide-SEA^{off} segment to the solid support by CuAAC (Scheme 1)



- Preparation of alkyne resin **5a**

PEGA¹⁹⁰⁰ resin⁵ (0.2 mmol, 0.2 mmol/g) was conditioned in DMF. Pentynoic acid (64 mg, 0.65 mmol) was dissolved in DMF (2 mL) and added to the resin. 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium hexafluorophosphate 3-oxide (HATU, 228 mg, 0.6 mmol) was dissolved in DMF (1 mL) and added to the resin. DIEA (209 μ L, 1.2 mmol) was then added to the resin, which was then gently shaken during 2 h. The resin was then successively washed with DMF, CH₂Cl₂ and DMF.

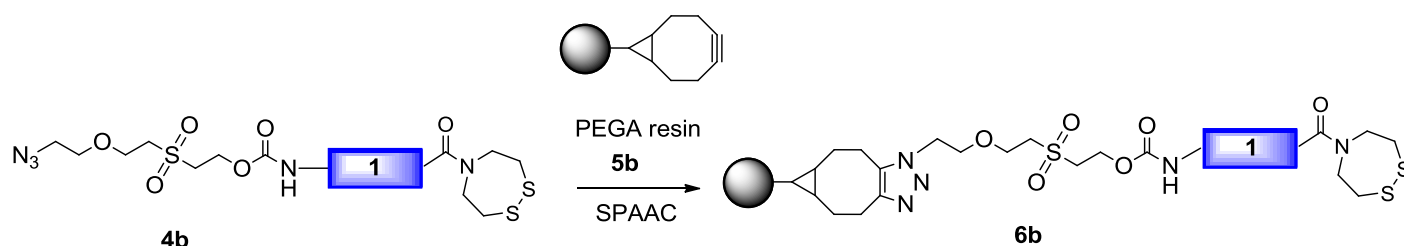
- CuAAC-mediated immobilisation

The CuAAC reactions were performed under a strict argon atmosphere, using solvents that had been freshly deoxygenated through four successive vacuum (15 mbar)/argon cycles.

⁵ M. Meldal, *Tetrahedron Lett.* **1992**, 33, 3077-3080

Alkyne resin **5a** (0.2 mmol/g, 6 μ mol) was washed with water then conditioned in a 9:1 mixture of 250 mM HEPES pH 7.5 buffer and MeOH, drained under argon, then the N₃-Esoc-tagged peptide **4a** or **4c** (3 μ mol) dissolved in 800 μ l of 9:1 HEPES/MeOH was added. A catalytic cocktail was prepared by dissolving successively aminoguanidine hydrochloride (0.33 mg, 3 μ mol), CuSO₄·5H₂O (3.7 mg, 15 μ mol), THPTA (32.6 mg, 75 μ mol) and sodium ascorbate (3 mg, 15 μ mol) in 200 μ l of 9:1 HEPES/MeOH, and was immediately added to the resin. The resin was stirred by bubbling argon for 2 h. Analysis of the supernatant confirmed a > 95 % consumption of the N₃-Esoc peptide as based on the integration of the HPLC peak. The resin was drained, then thoroughly washed with water, 6 M Guanidine.HCl / 200 mM EDTA, 0.1 % aqueous TFA solution, water, MeOH, DMF and finally water. HPLC analysis of the effluent confirmed that no starting peptide was non-covalently bound to the resin.

4.1.b Coupling of the first N₃-Esoc-peptide-SEA^{off} segment to the solid support by SPAAC (Scheme 1)



- Preparation of cyclooctyne resin **5b**

PEGA¹⁹⁰⁰ resin (0.2 mmol, 0.2 mmol/g) was conditioned in DMF. A 4:1 *exo/endo* mixture of bicyclo[6.1.0]non-4-yn-9-ylmethyl (4-nitrophenyl) carbonate (95 mg, 0.3 mmol) was dissolved in DMF (2 mL) and added to the resin, which was then gently shaken during 18 h. The resin was then thoroughly washed with DMF, CH₂Cl₂, 1% DIEA in DMF, and DMF.

- SPAAC-mediated immobilisation⁶

Cyclooctyne resin **5b** (0.2 mmol/g, 6 μ mol) was conditioned with water, then the N₃-Esoc-tagged peptide **4b** (3 μ mol) dissolved in 1 mL of a 0.1% TFA solution in 8:2 water/MeCN (pH~2) was added. The resin was gently shaken for 70 h. Analysis of the supernatant confirmed a > 97 % consumption of the N₃-Esoc peptide **4b** as based on the integration of the HPLC peak. Excess cyclooctyne was then capped by reaction with a solution of 2-(2-azidoethoxy)-ethanol (16 mg, 0.12 mmol) in 1 mL water for 2 h. The resin was washed with water, 6 M Guanidine.HCl, 0.1 % aqueous TFA solution, water, MeOH, DMF and finally water. HPLC analysis of the washing solutions confirmed that no starting peptide **4b** was non-covalently bound to the resin.

⁶ A. E. Wammes, M. J. Fischer, N. J. de Mol, M. B. van Eldijk, F. P. Rutjes, J. C. van Hest, F. L. van Delft, *Lab. Chip.* **2013**, *13*, 1863-1867.

4.2 Elongation cycle: Activation step (Figure 2)

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) (57 mg, 0.2 mmol) was dissolved in 6 M guanidine-HCl, 0.2 M pH 4.2 sodium acetate buffer (1 mL). 3-Mercaptopropionic acid (MPA, 50 μ L, final concentration 5 % by vol) was added. NaOH (5 M) was then used to adjust the pH to 4.1.

The SEA^{off} peptidyl resin (0.5 μ mol) was treated with the above solution (V = 100 μ L) and agitated at 37°C for 24 h. The solid support was then washed (3 \times 150 μ L) with a 6 M guanidine-HCl, 0.2 M pH 4.2 sodium acetate buffer.

4.3 Elongation cycle: Native Chemical Ligation (Figure 2)

4-Mercaptophenylacetic acid (MPAA, 57 mg, 0.2 mmol) was dissolved in 6 M guanidine-HCl, 0.2 M pH 7.2 sodium phosphate buffer (1 mL). NaOH (5 M) was then used to adjust the pH to 7.2.

The peptide segment (0.8 μ mol, 1.5 eq) to be ligated was dissolved in the above solution (V = 150 μ L, 3-5 mM final peptide concentration) and added to the growing peptidyl resin. The reaction mixture was gently shaken at 37°C for 24 h and then washed thoroughly (V = 5 \times 150 μ L) with 6 M guanidine-HCl, 0.2 M pH 7.2 sodium phosphate buffer to remove MPAA and the unreacted SEA^{off} peptide segment used in slight excess.

After each ligation, an aliquot of resin was cleaved with NaOH (V = 100 μ L, 0.01M) for 2 min. TFA (10% in water, V = 10 μ L, final pH 1-2) was added to quench the reaction before the LC-MS.

4.4 Termination step: Introduction of the last Cys peptide segment using SEA Ligation (Figure 3)

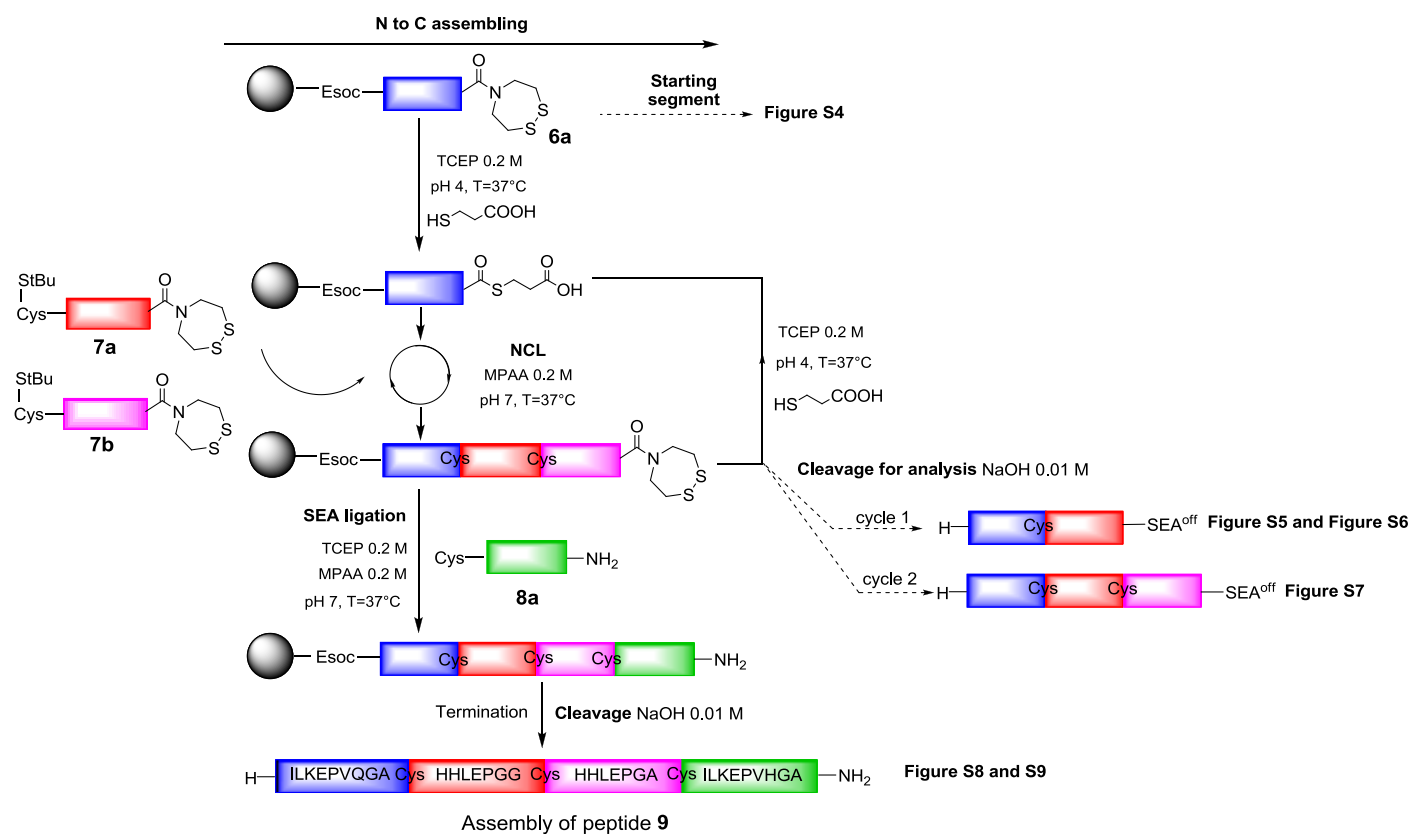
TCEP-HCl (57 mg, 0.2 mmol) and MPAA (33 mg, 0.2 mmol) were dissolved in 6 M guanidine-HCl, 0.2 M pH 7.2 sodium phosphate buffer (1 mL). NaOH (5 M) was then added to adjust the pH to 7.1.

The Cys peptide (0.8 μ mol, 1.5 eq) to be ligated was dissolved in the above solution (V = 150 μ L, 3-5 mM final peptide concentration) and added to the polymer support. The reaction mixture was gently shaken at 37°C for 24 h and then washed thoroughly with 6 M guanidine-HCl, 0.2 M pH 7.2 sodium phosphate buffer (V = 5 \times 150 μ L).

4.5 Cleavage step

The peptidyl resin was treated with 6 M guanidine, NaOH (0.01 M) pH 12 (V = 400 μ L) for 2 min. The solid support was washed with 6 M guanidine-HCl, 0.2 M pH 4.1 sodium acetate (V = 200 μ L) and 10% aqueous TFA (V = 60 μ L, final pH 1-2). The combined filtrates were purified by reversed-phase HPLC using a linear water-acetonitrile gradient containing 0.05 % TFA to give the purified peptides **9-12**.

5. Assembly of four peptide segments: synthesis of peptide 9



a) Starting segment

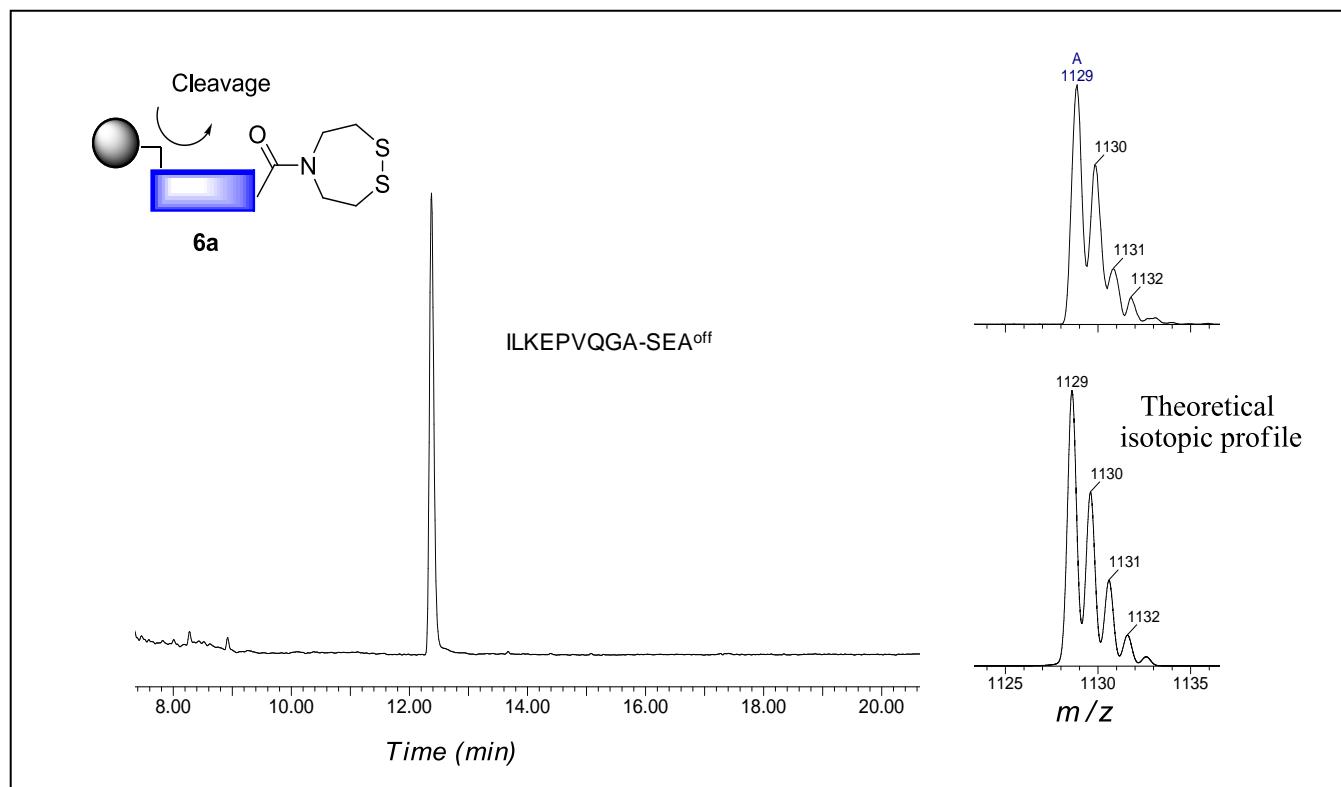


Figure S4. Analytical LC-MS profile (ELS detection) corresponding to the cleavage of peptidyl resin **6a**. ESI analysis calc. for $[M+H]^+$: 1129.4, observed: 1129 (monoisotopic composition).

a) Elongation cycle 1

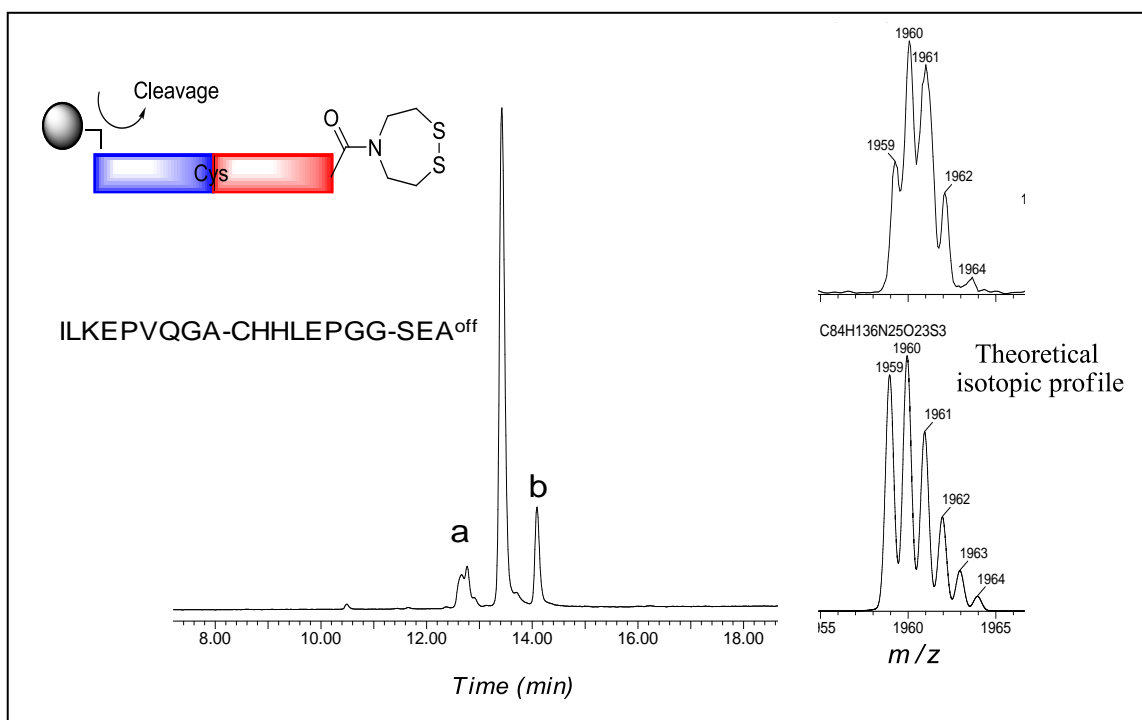


Figure S5. Analytical LC-MS profile (ELS detection) corresponding to the cleavage of the peptidyl resin after the first elongation cycle. Peak a is the target peptide, ESI analysis calc. for $[M+H]^+$: 1960.3, observed : 1960 (monoisotopic composition). Peak "a" is due to peptide **7a** whereas peak "b" corresponds to MPAA. Therefore, additional washing steps were carried out to remove **7a** and MPAA before the second elongation cycle. Note that target polypeptide showed the insertion of only one copy of internal segments **7a** in the peptide chain.

b) Beads supernatant of elongation cycle 1 (stability of SEA^{off} during NCL)

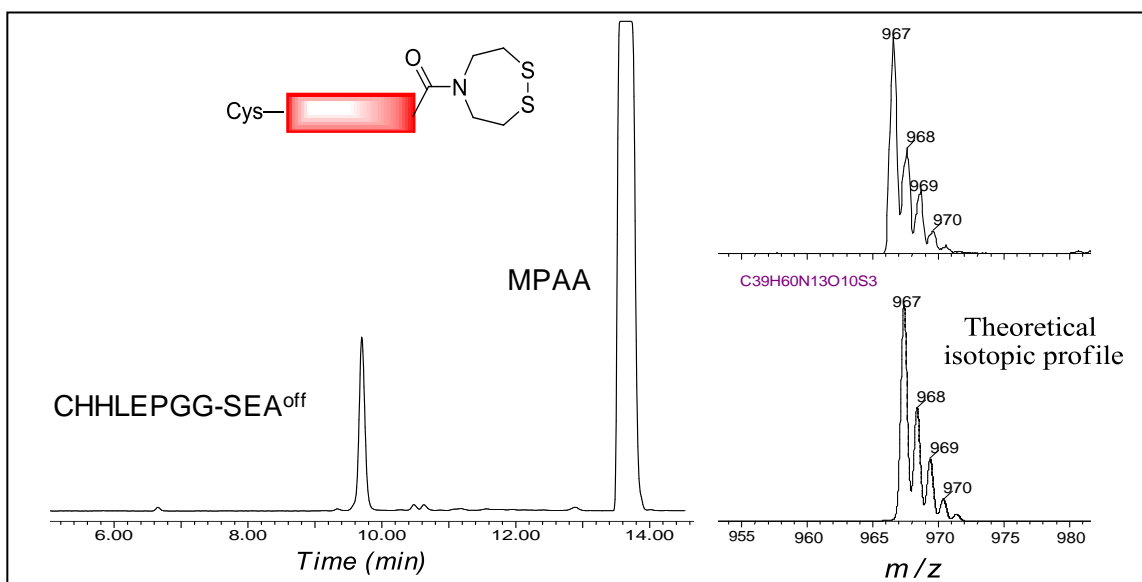


Figure S6. Analytical LC-MS profile (ELS detection) corresponding to the supernatant of the first elongation cycle. This analysis shows the removal of *tert*-butylsulfenyl group and importantly that SEA^{off} group remains in off state throughout the NCL reaction in the presence of MPAA. No oligomerization or cyclization side-products were detected. Peptide Cys-HHLEPPGG-SEA^{off}, ESI analysis calc. for $[M+H]^+$: 966.4, observed : 967 (monoisotopic composition).

c) Elongation cycle 2

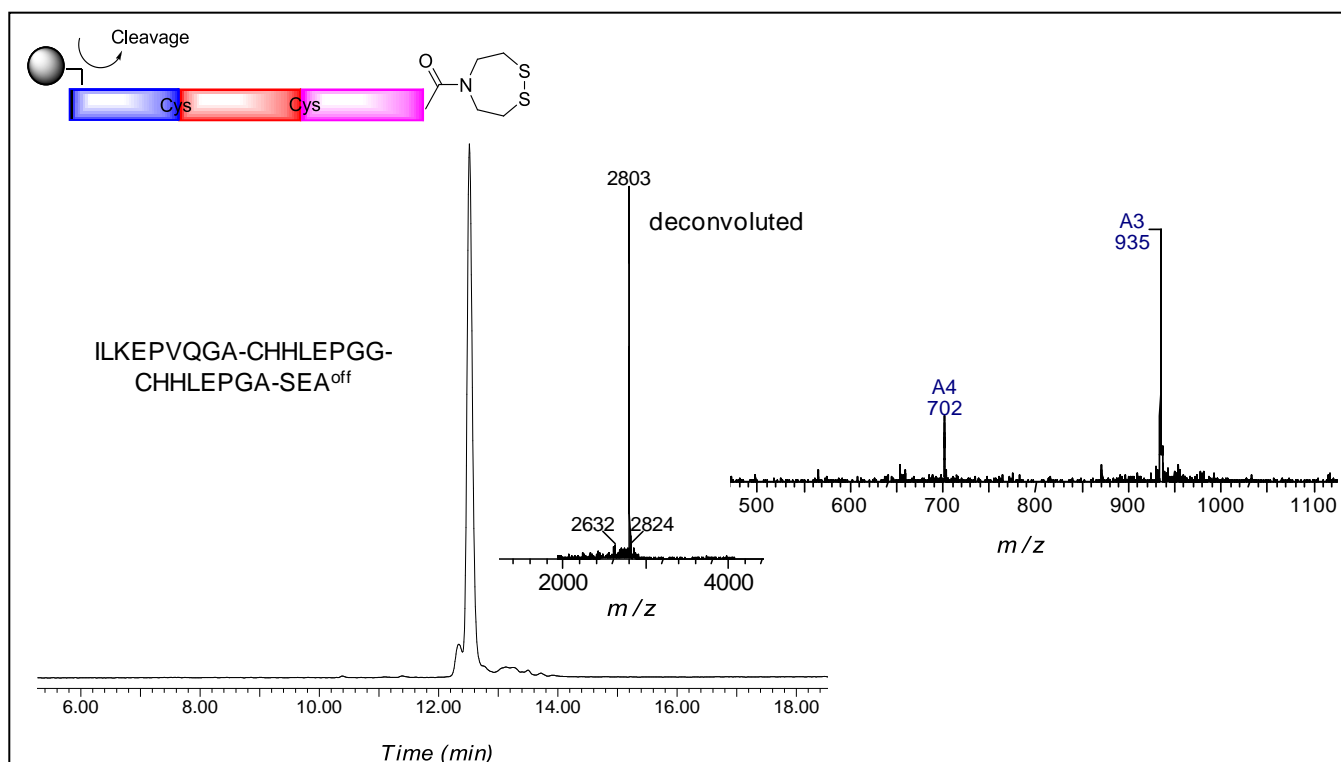


Figure S7. Analytical LC-MS profile (ELS detection) corresponding to the cleavage of the peptidyl resin after the second elongation cycle. Target peptide, ESI analysis calc. for M: 2804.3, found deconvoluted: 2803 (average isotopic composition). Note that target polypeptide showed the insertion of only one copy of internal segments **7b** in the peptide chain.

d) Termination step

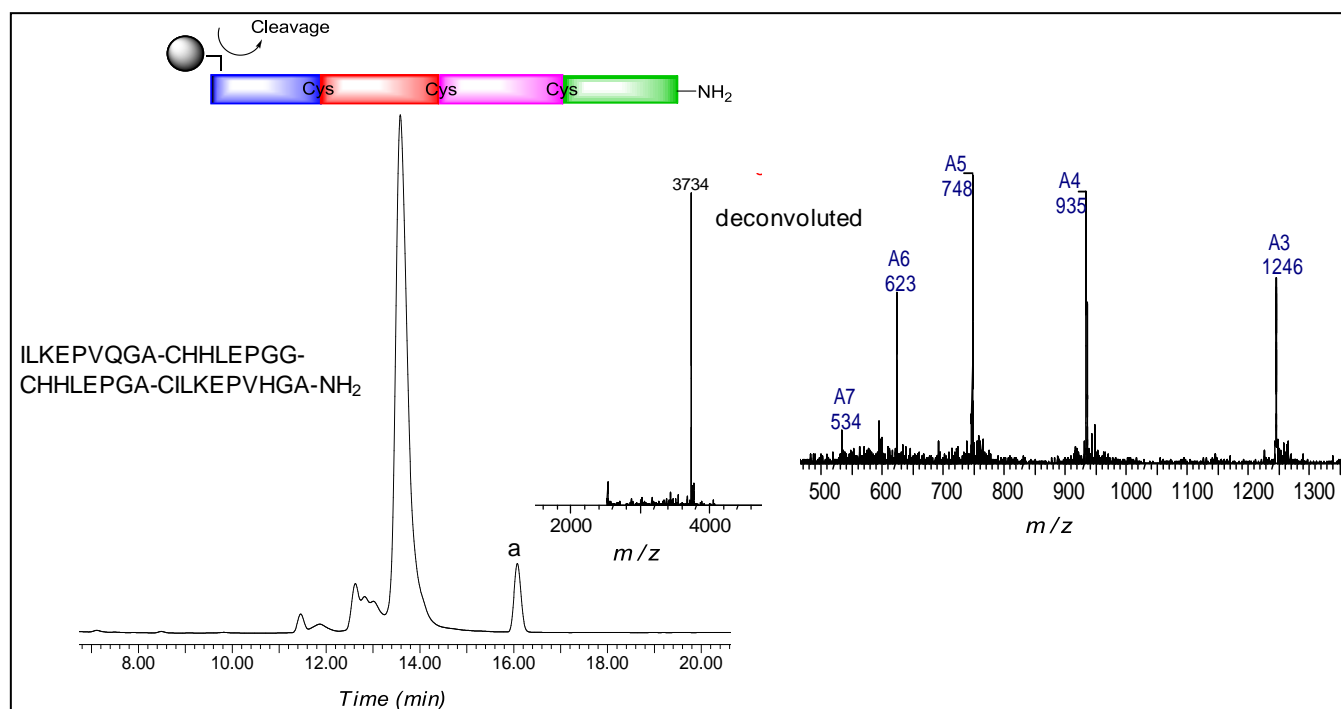


Figure S8. Analytical LC-MS profile (ELS detection) for the crude peptide **9**. Main peak corresponds to peptide **9**. ESI analysis calc. for M: 3734.3, found deconvoluted: 3734 (average isotopic composition). Peak a corresponds to MPAA.

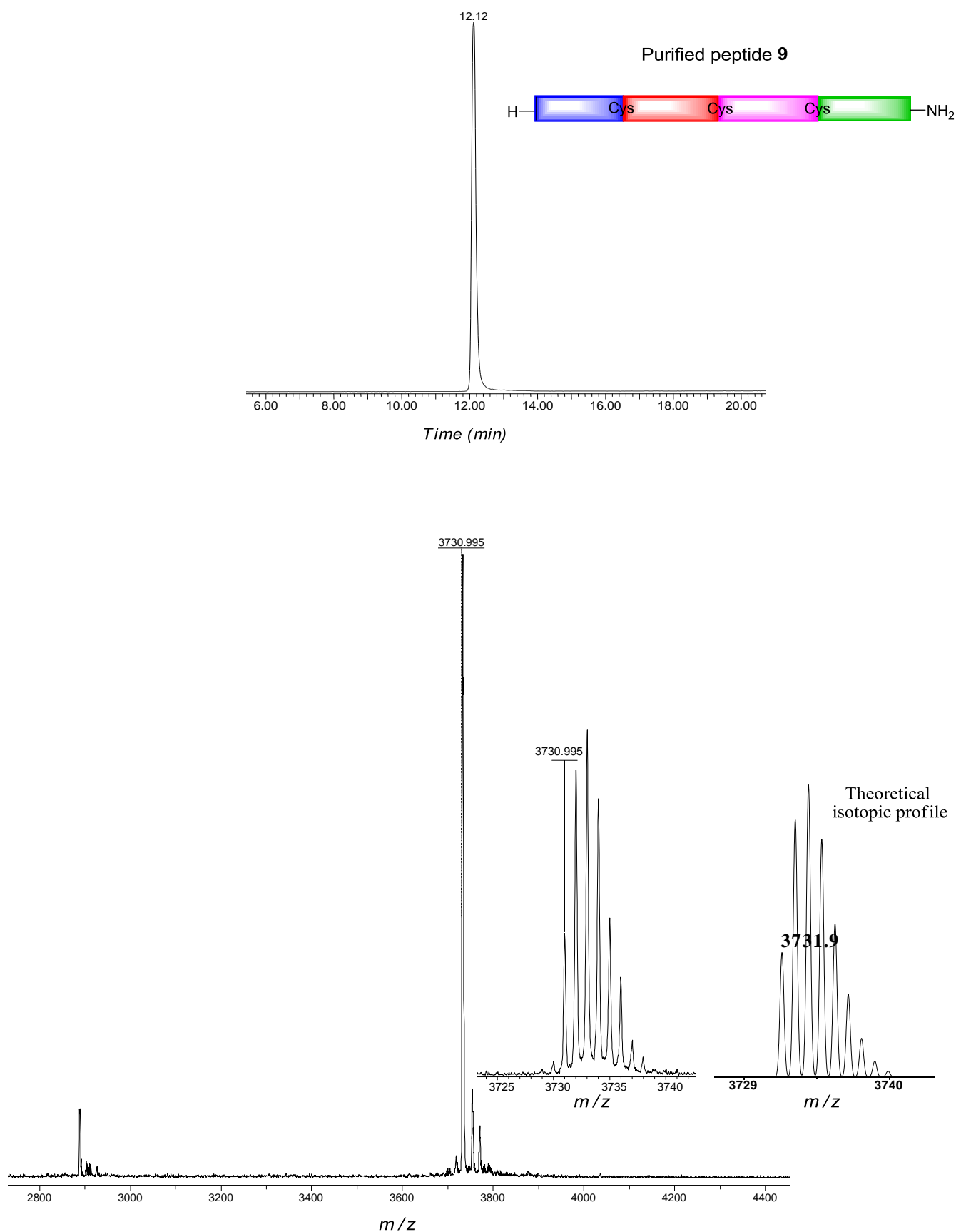
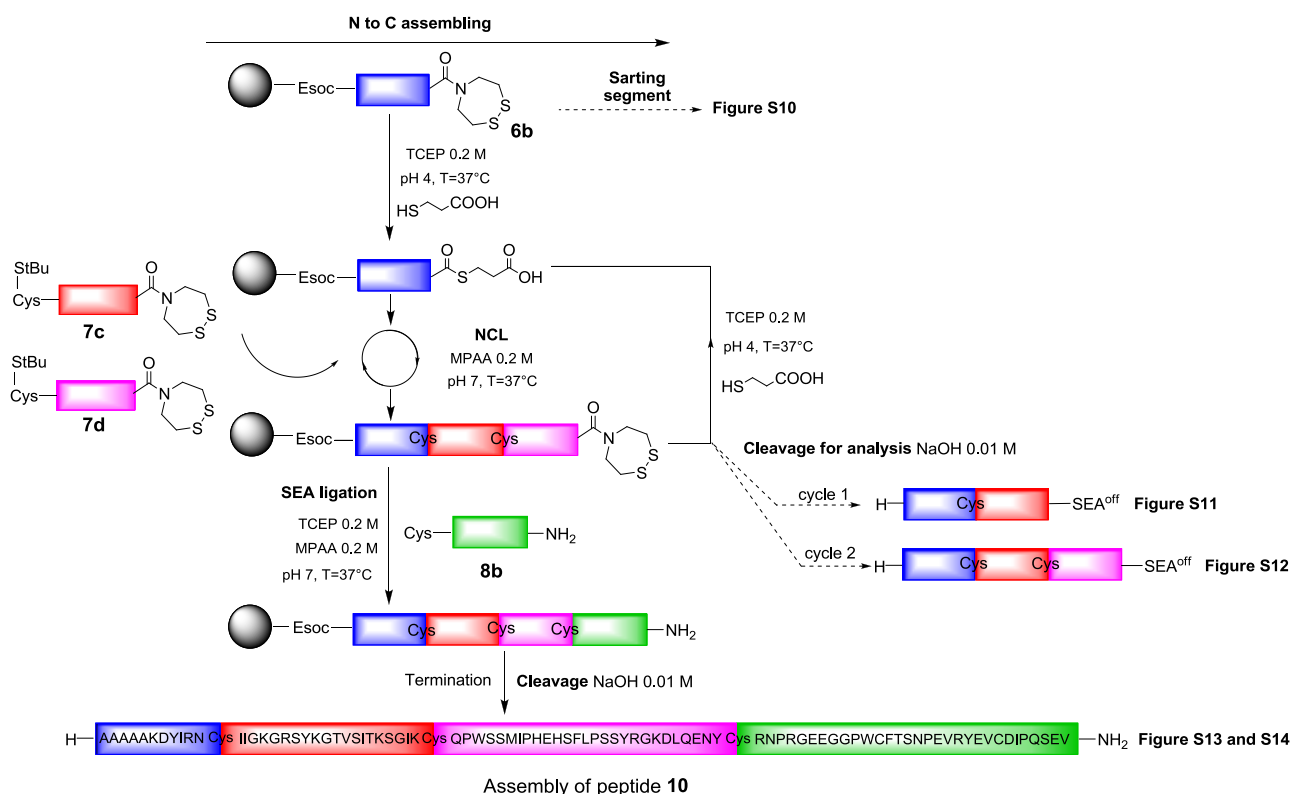


Figure S9. Analytical HPLC profile (detection at 215 nm) and MALDI-TOF analysis for purified peptide 9. 4.3 mg (21% yield, 4.5 μ mol scale). MALDI-TOF calc. for $[M+H]^+$: 3731.9, observed: 3730.9 Da (monoisotopic composition).

6. Assembly of four peptide segments: synthesis of peptide 10



a) Starting segment

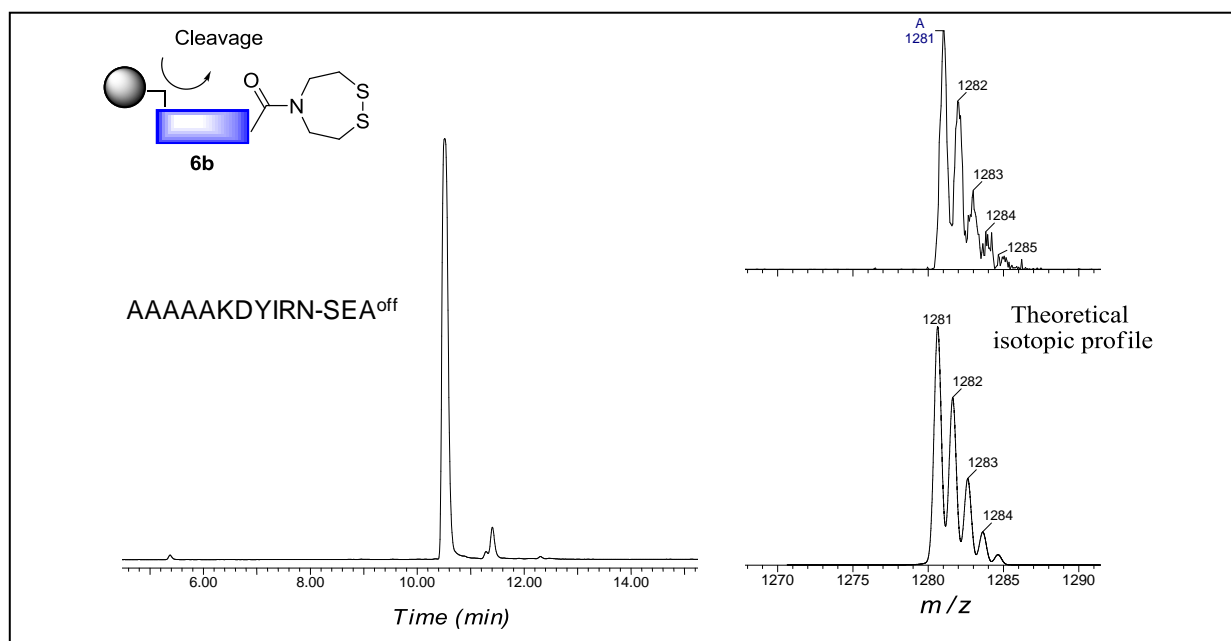


Figure S10. Analytical LC-MS profile (ELS detection) corresponding to the cleavage of peptidyl resin **6b**. ESI analysis calc. for $[M+H]^+$: 1281.51, observed: 1281 (monoisotopic composition).

b) Cycle 1

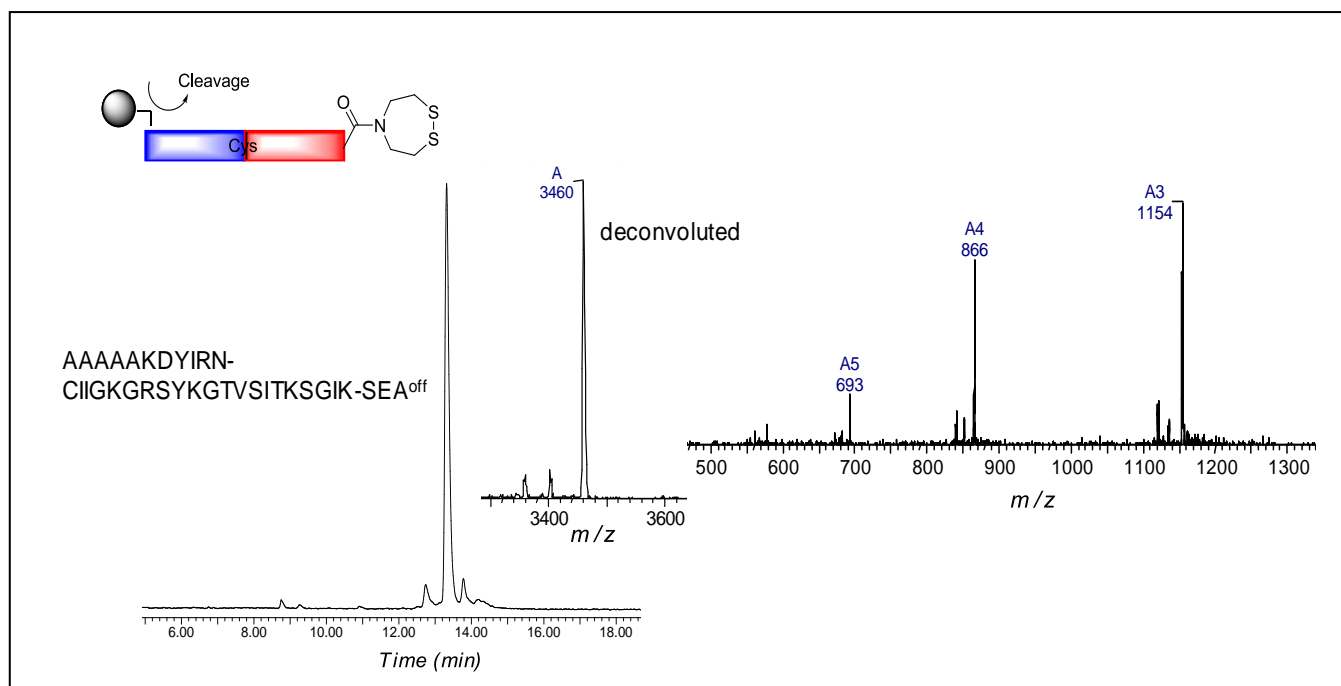


Figure S11. Analytical LC-MS profile (ELS detection) corresponding to the cleavage of the peptidyl resin after the first elongation cycle. Target peptide, ESI analysis calc. for M: 3459.1, found deconvoluted: 3460 (average isotopic composition).

c) Cycle 2

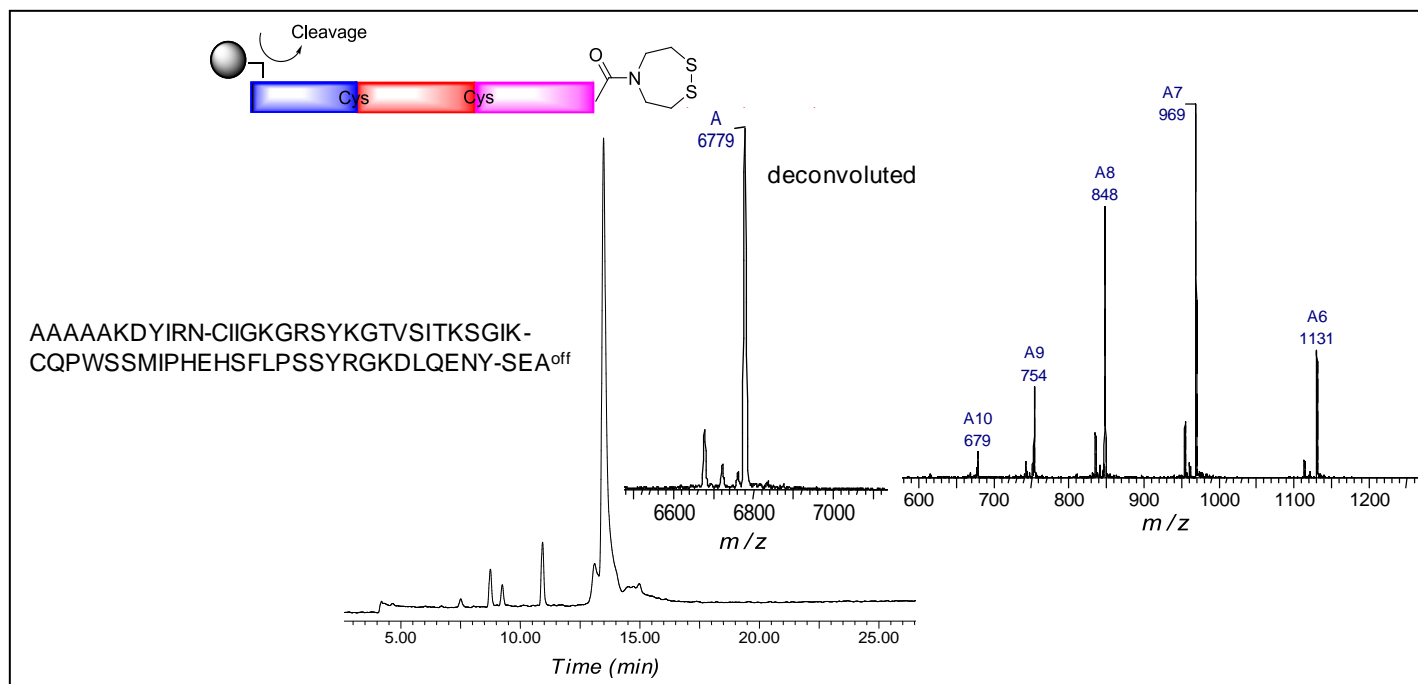


Figure S12. Analytical LC-MS profile (ELS detection) corresponding to the cleavage of the peptidyl resin after the second elongation cycle. Target peptide, ESI analysis calc. for M: 6779.7, found deconvoluted: 6779 (average isotopic composition).

d) Termination

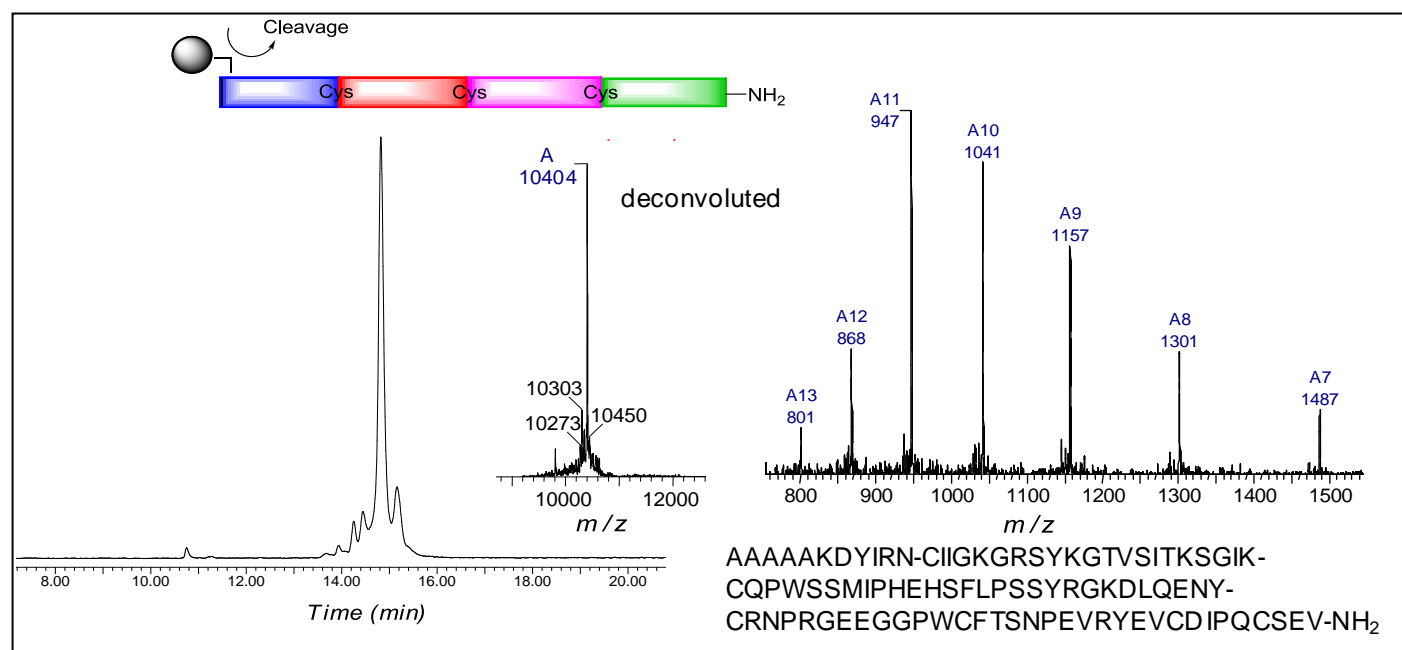
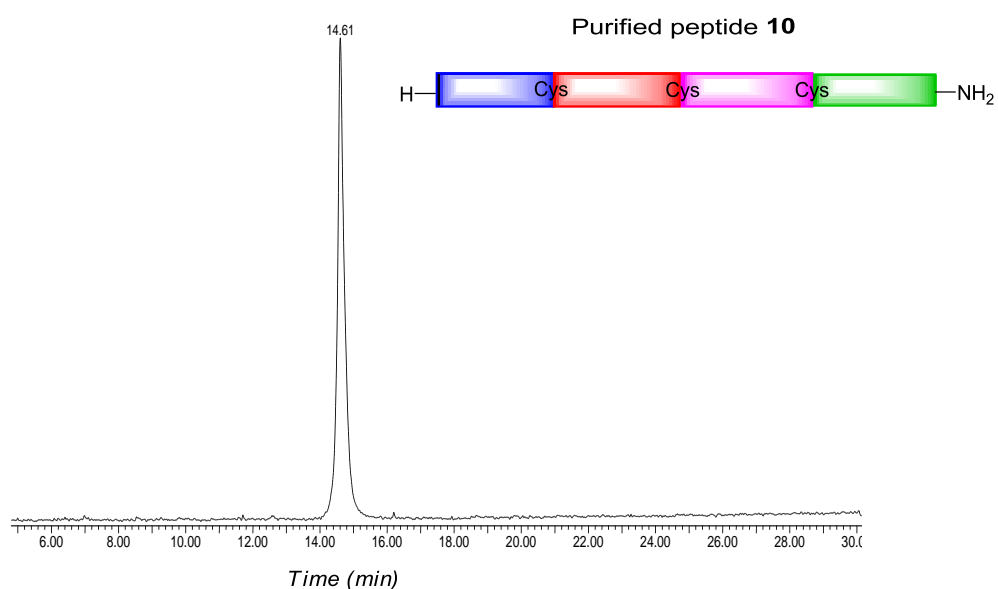


Figure S13. Analytical LC-MS profile (ELS detection) for the crude peptide **10**. Main peak corresponds to peptide **10**. ESI analysis calc. for M: 10400.6, found deconvoluted: 10404 (average isotopic composition).



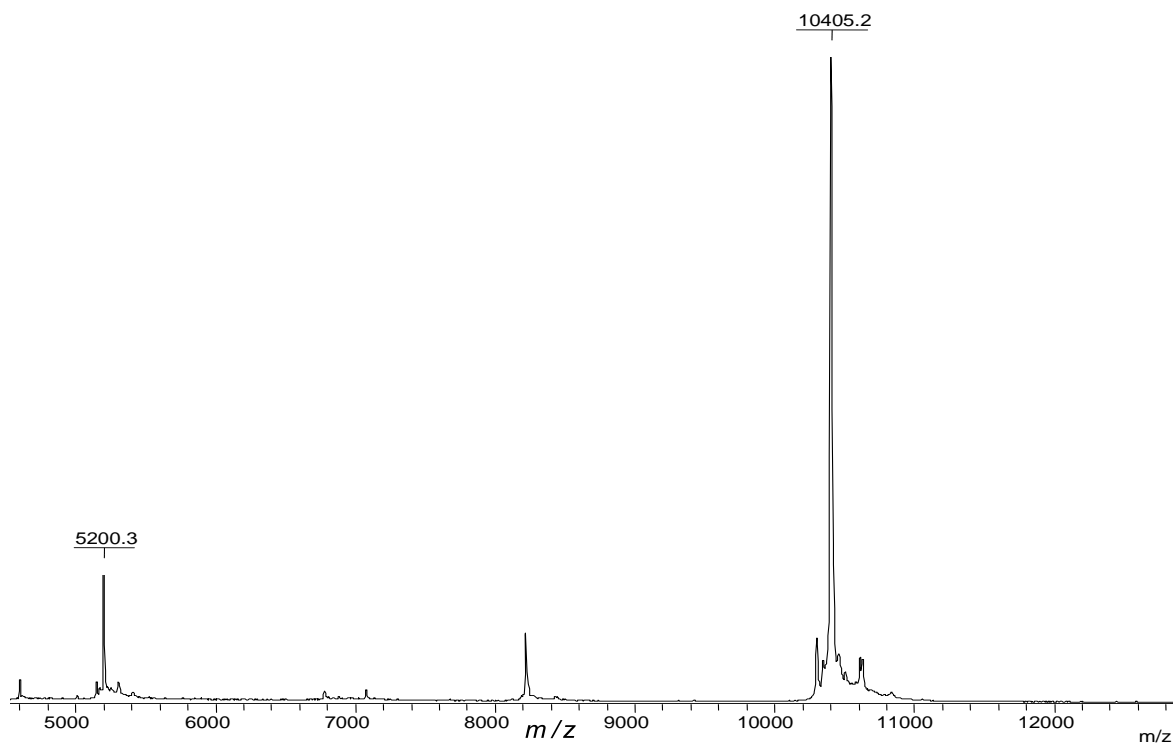
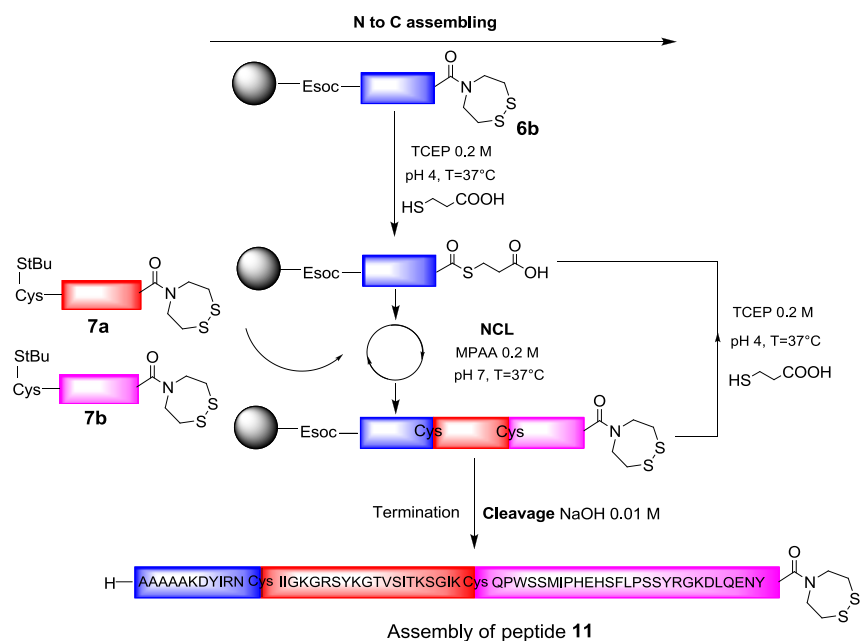


Figure S14. Analytical HPLC profile (detection at 215 nm) and MALDI-TOF analysis for purified peptide **10**. 1.1 mg (18 % yield, 0.5 μ mol peptide resin), MALDI-TOF calc. for $[M+H]^+$: 10401.6, observed mass: 10405.2 (average isotopic composition).

7. Synthesis of large SEA^{off} peptide segments: synthesis of peptide **11**



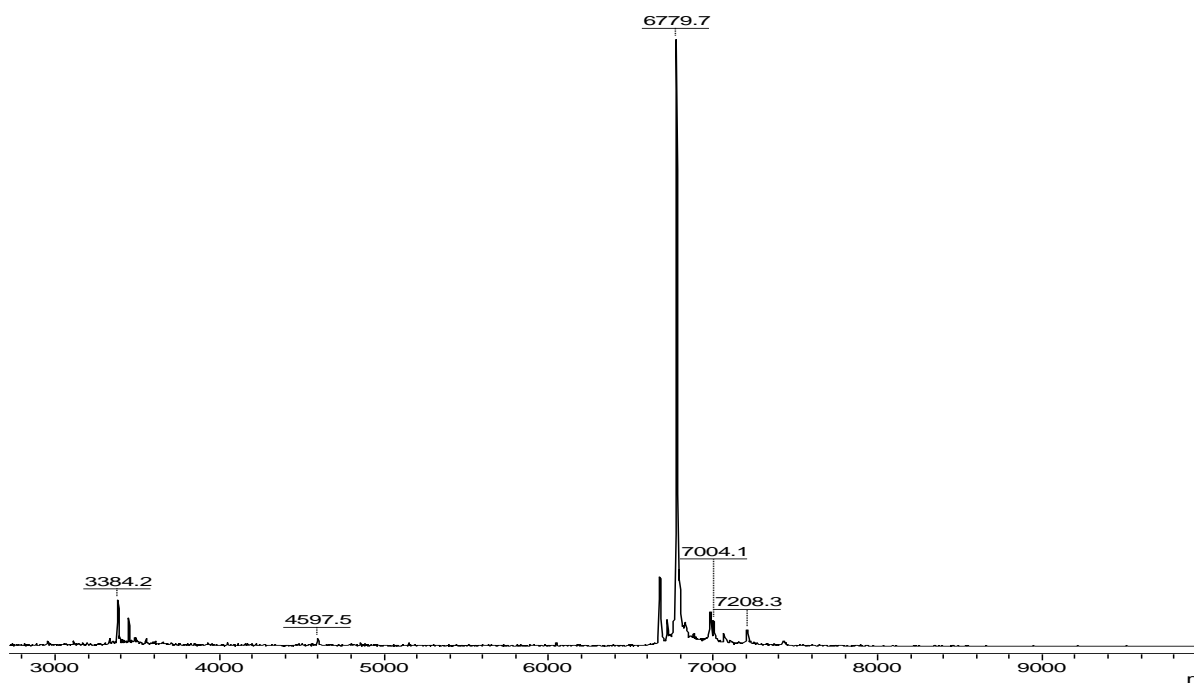
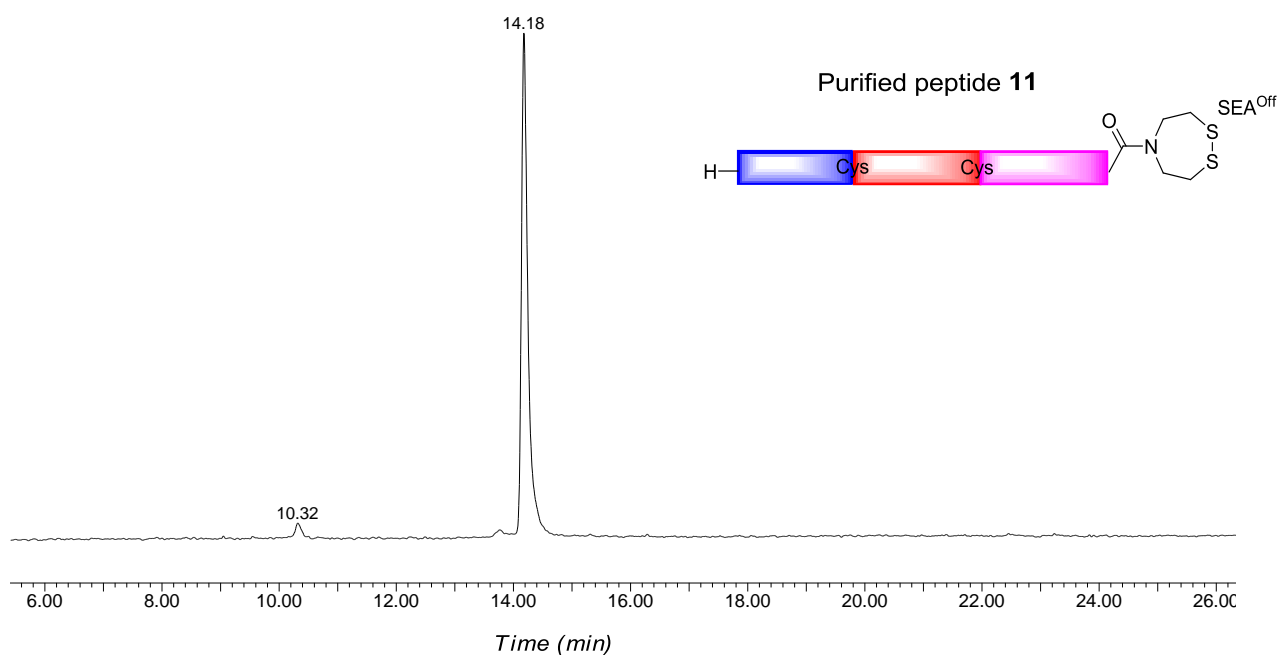
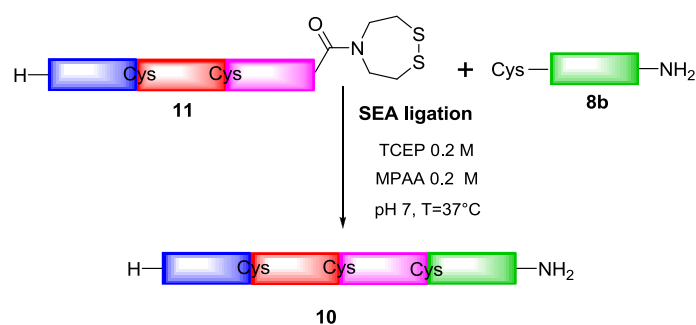


Figure S15. Analytical HPLC profile (detection at 215 nm) and Maldi-TOF analysis for purified peptide **11**. 0,92 mg (22 % yield, 0.5 μ mol peptide resin), Maldi-TOF calc. for $[M+H]^+$: 6779.7, observed mass: 6779.7 (average isotopic composition).

8. Functionality of large SEA^{off} peptide segments: assembly of peptide **10** in solution



TCEP-HCl (28 mg, 0.1 mmol) and MPAA (16 mg, 0.1 mmol) was dissolved in 6 M guanidine-HCl, 0.2 M pH 7.2 sodium phosphate buffer (1 mL). NaOH (5 M) was then added to adjust the pH to 7.1.

Peptide **11** (0.8 mg, 0.1 μ mol) and peptide segment **8b** (0.4 mg, 0.1 μ mol, 1 eq) were dissolved in the above solution ($V= 33 \mu$ L, 3 mM final peptide concentration). The reaction mixture was shaken at 37°C for 48 h and the ligation was monitored by LC-MS (Figure S16). After completion of the reaction, the mixture was diluted with water-TFA 0.1% (1 mL), extracted with Et₂O (3 \times 500 μ L) and purified by reversed-phase HPLC using a linear water-acetonitrile gradient containing 0.05 % TFA to give the purified ligation product **10** (0.22 mg, 19 % yield). The analytical HPLC chromatograms of purified peptide **10** produced using solid phase approach or by ligation of peptides **11** and **8b** in solution are shown in Figure S17.

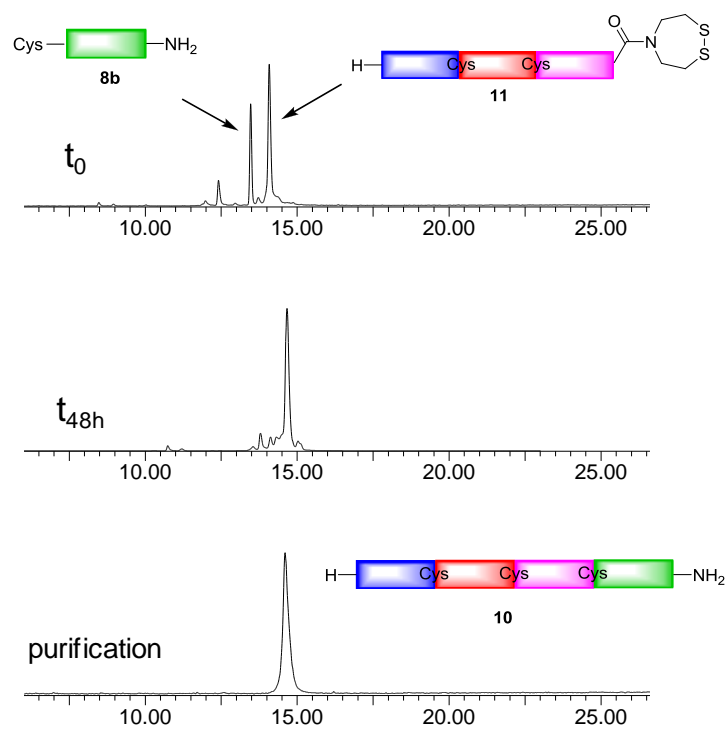


Figure S16. Monitoring of the ligation of SEA^{off} peptide **11** with peptide **8b** by HPLC (ELSD detection).

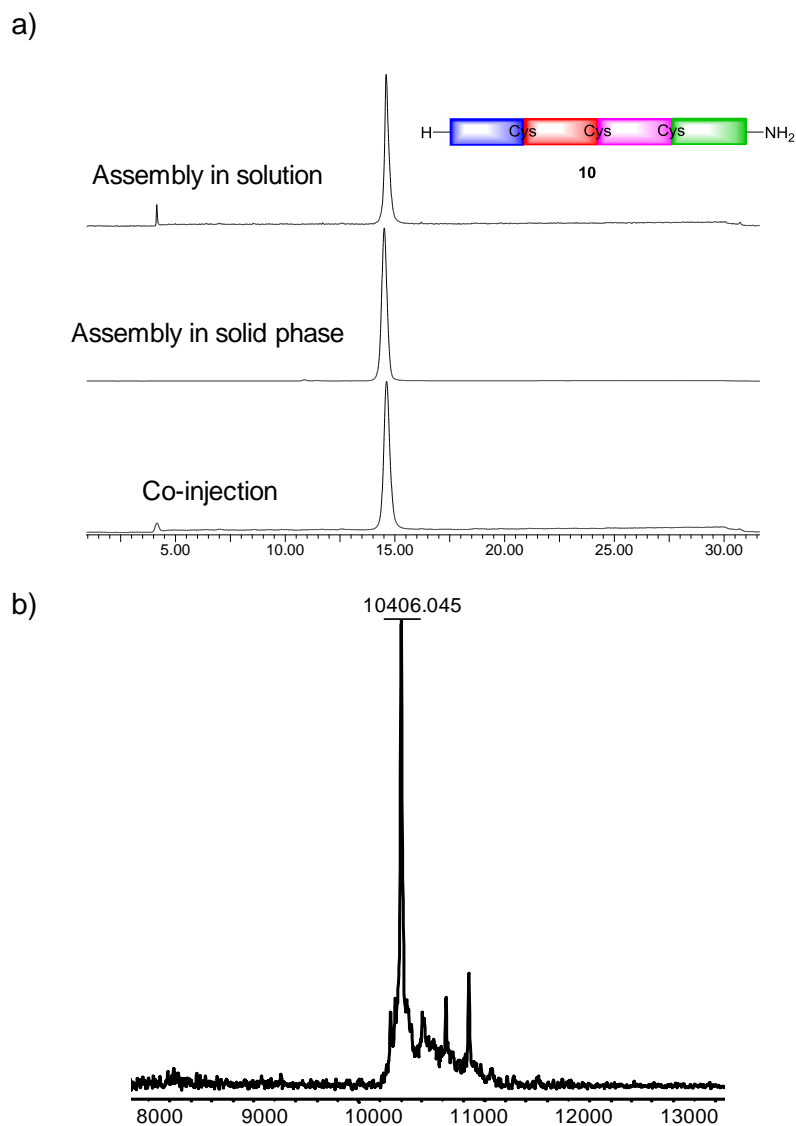
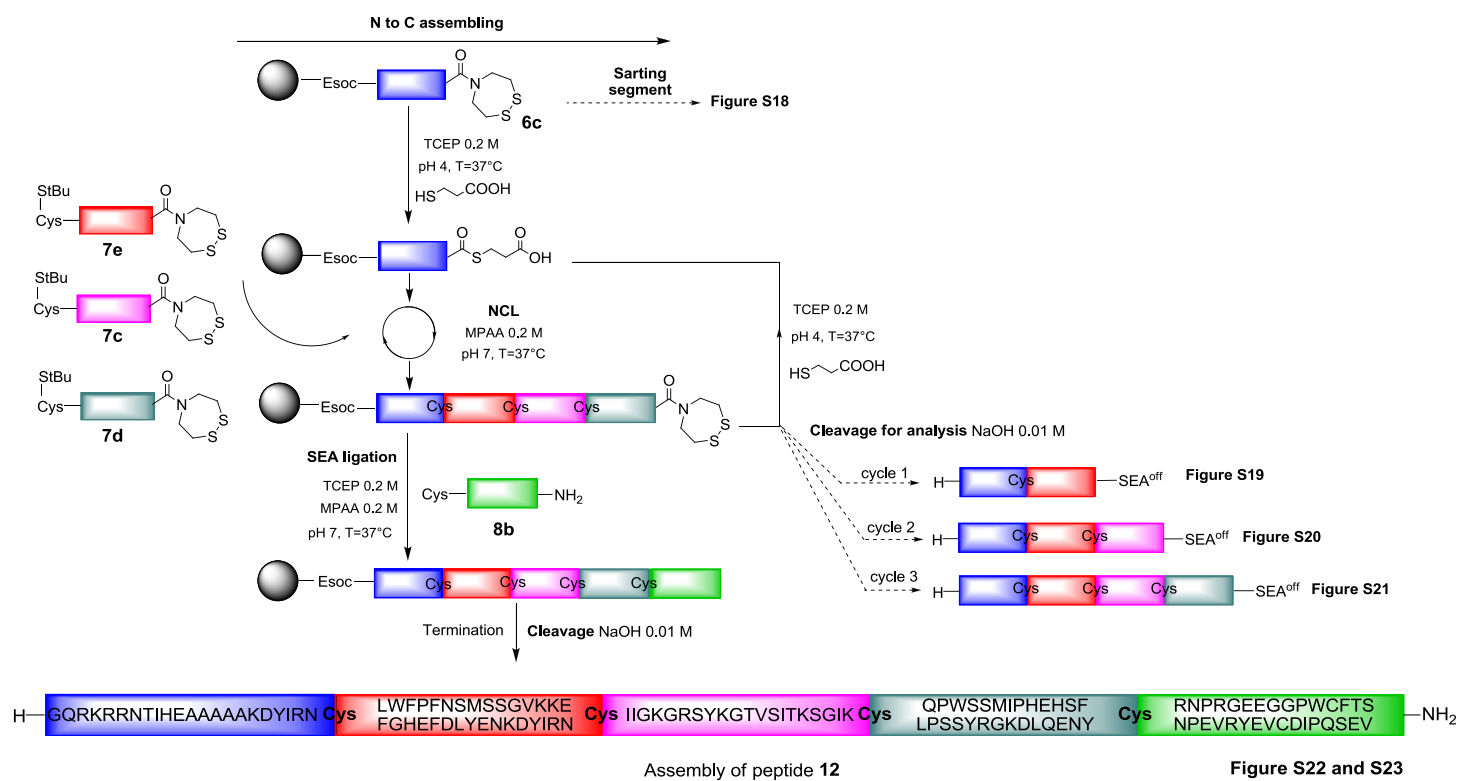


Figure S17. (a) HPLC analysis (ELS detection) of the purified peptide **10** obtained using the solution approach, the solid phase approach or both compounds were co-injected. (b) Maldi-TOF spectrum of peptide **10** synthesized using the solution approach.

9. Assembly of five peptide segments: synthesis of peptide 12



a) Starting segment

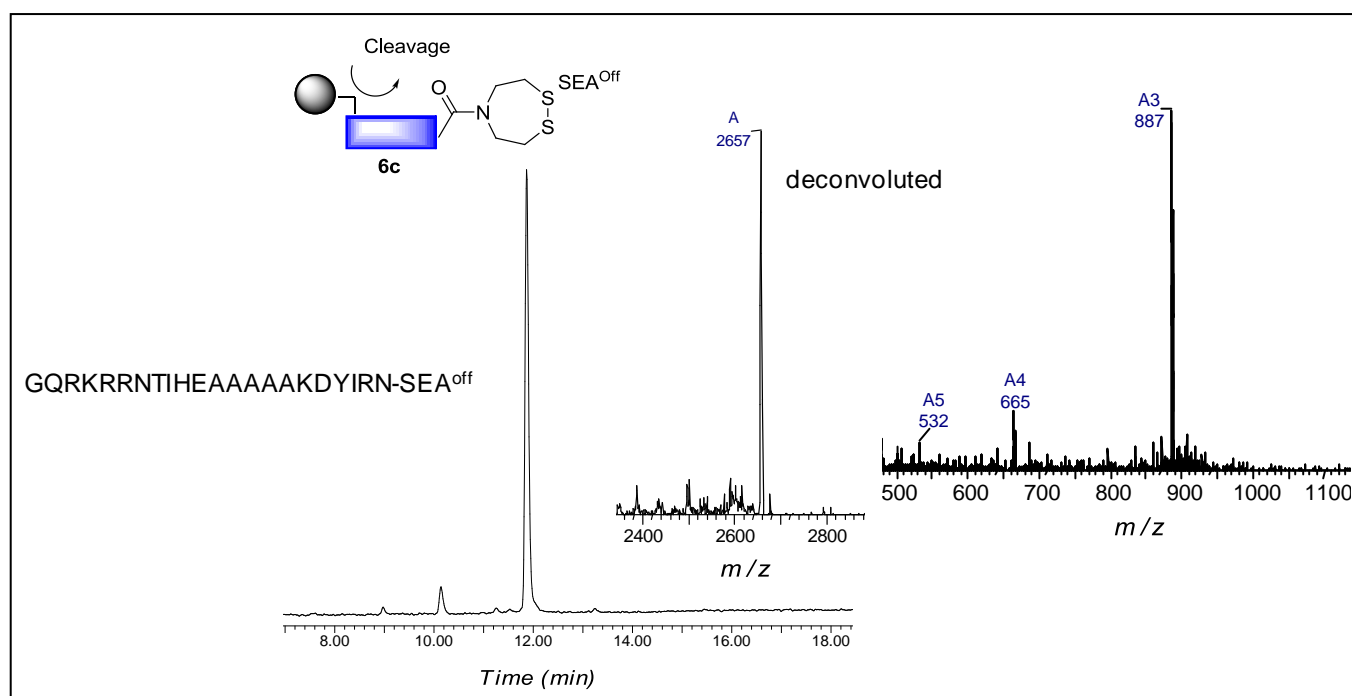


Figure S18. Analytical LC-MS profile (ELS detection) corresponding to the cleavage of peptidyl resin **6c**. ESI analysis calc. for $[M+H]^+$: 2657.1, observed: 2657 (average isotopic composition).

b) Elongation cycle 1

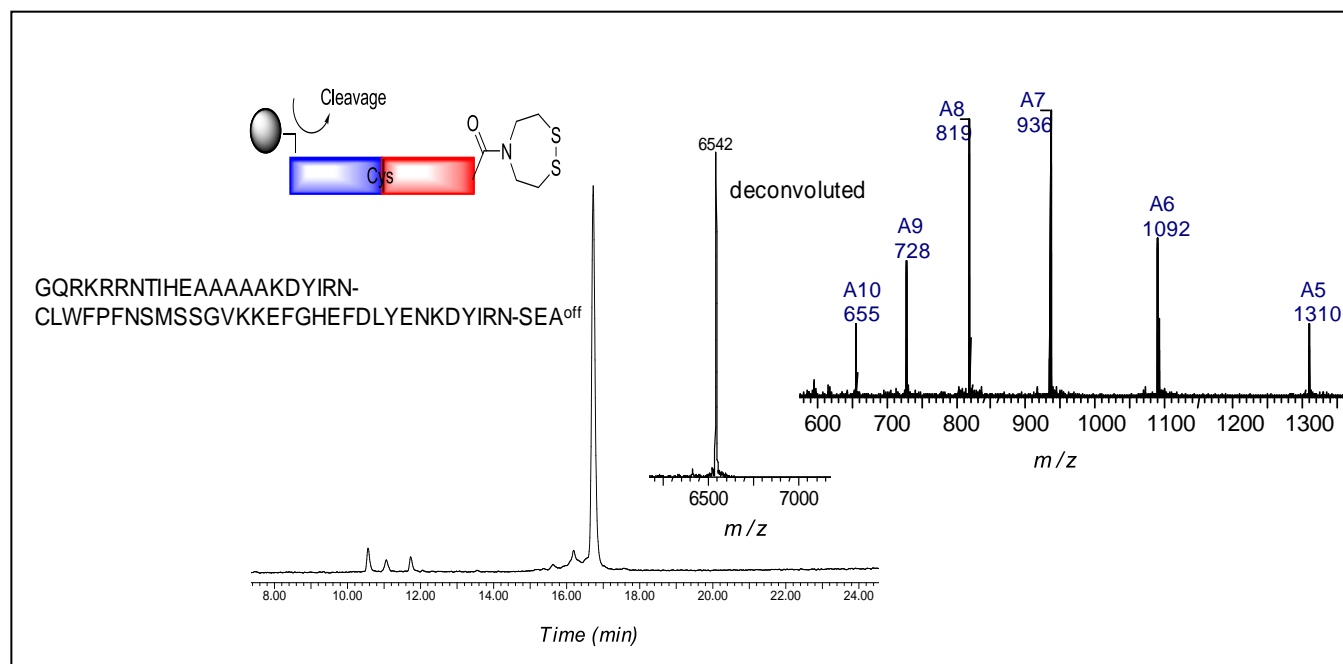


Figure S19. Analytical LC-MS profile (ELS detection) corresponding to the cleavage of the peptidyl resin after the first elongation cycle. Target peptide, ESI analysis calc. for M: 6541.6, found deconvoluted: 6542 (average isotopic composition).

c) Elongation cycle 2

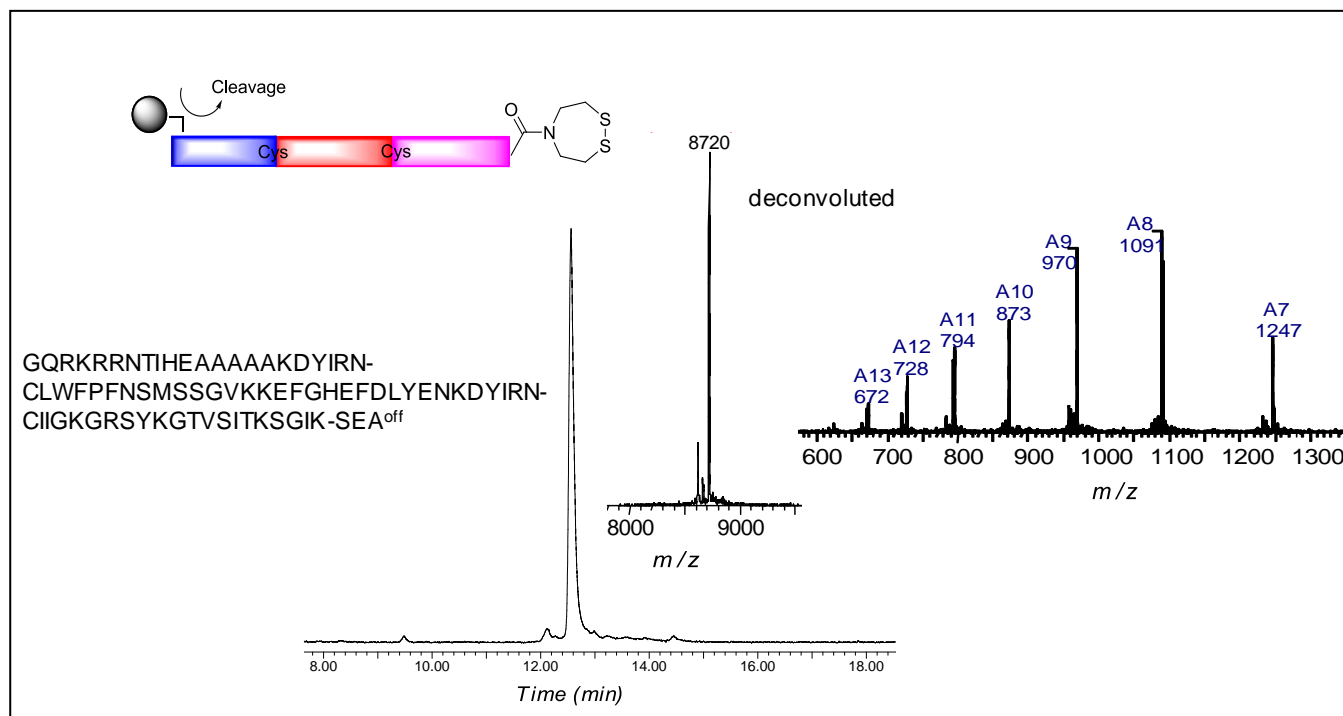


Figure S20. Analytical LC-MS profile (ELS detection) corresponding to the cleavage of the peptidyl resin after the second elongation cycle. Target peptide, ESI analysis calc. for M: 8719.9, found deconvoluted: 8720 (average isotopic composition).

d) Elongation cycle 3

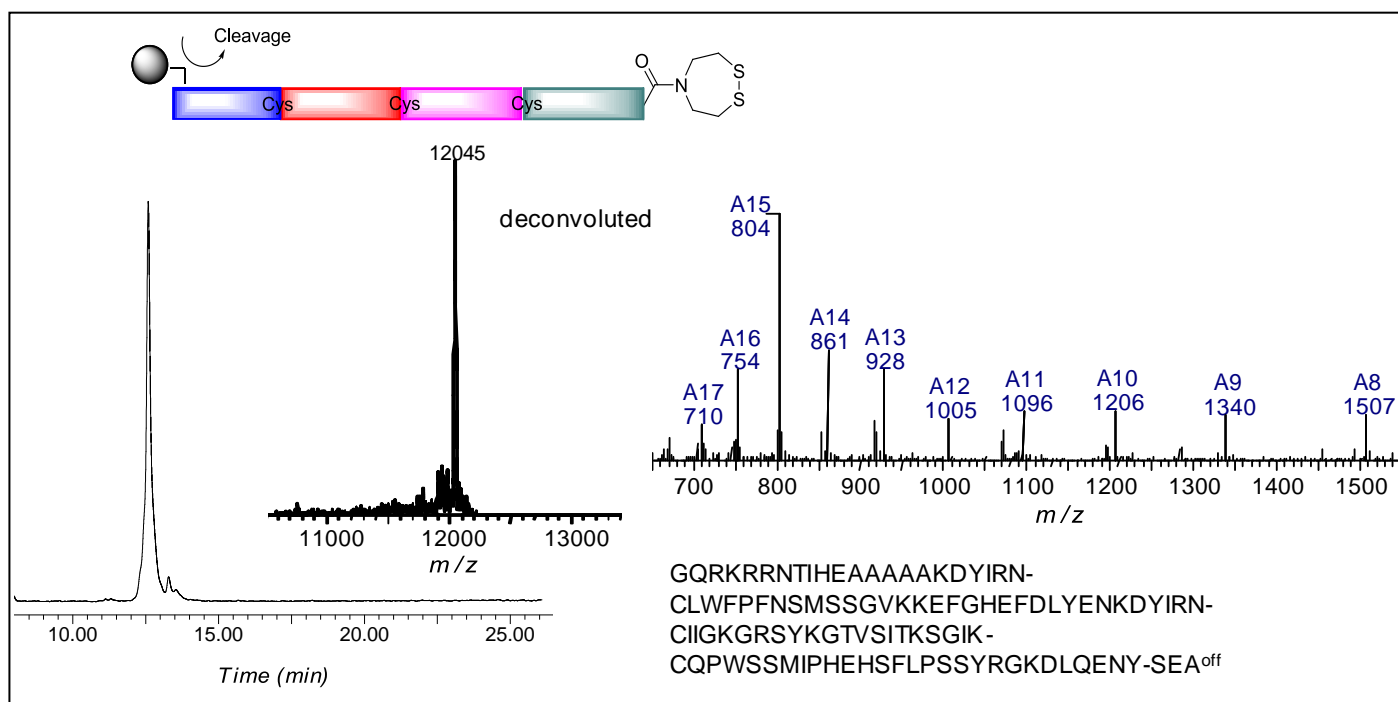


Figure S21. Analytical LC-MS profile (ELS detection) corresponding to the cleavage of the peptidyl resin after the third elongation cycle. ESI analysis calc. for M: 12040.6, found deconvoluted: 12045 (average isotopic composition).

d) Termination step

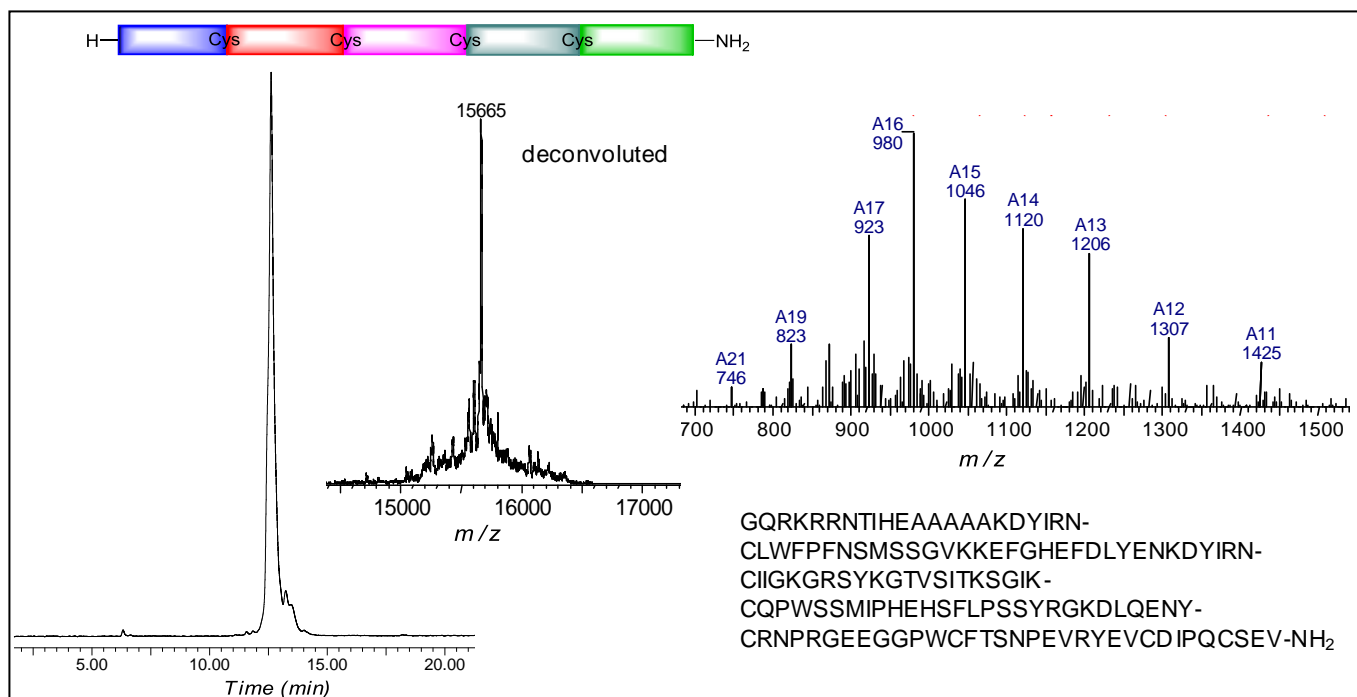


Figure S22. Analytical LC-MS profile (ELS detection) for the crude peptide **12**. ESI analysis calc. for M: 15661.5, found deconvoluted: 15665 (average isotopic composition).

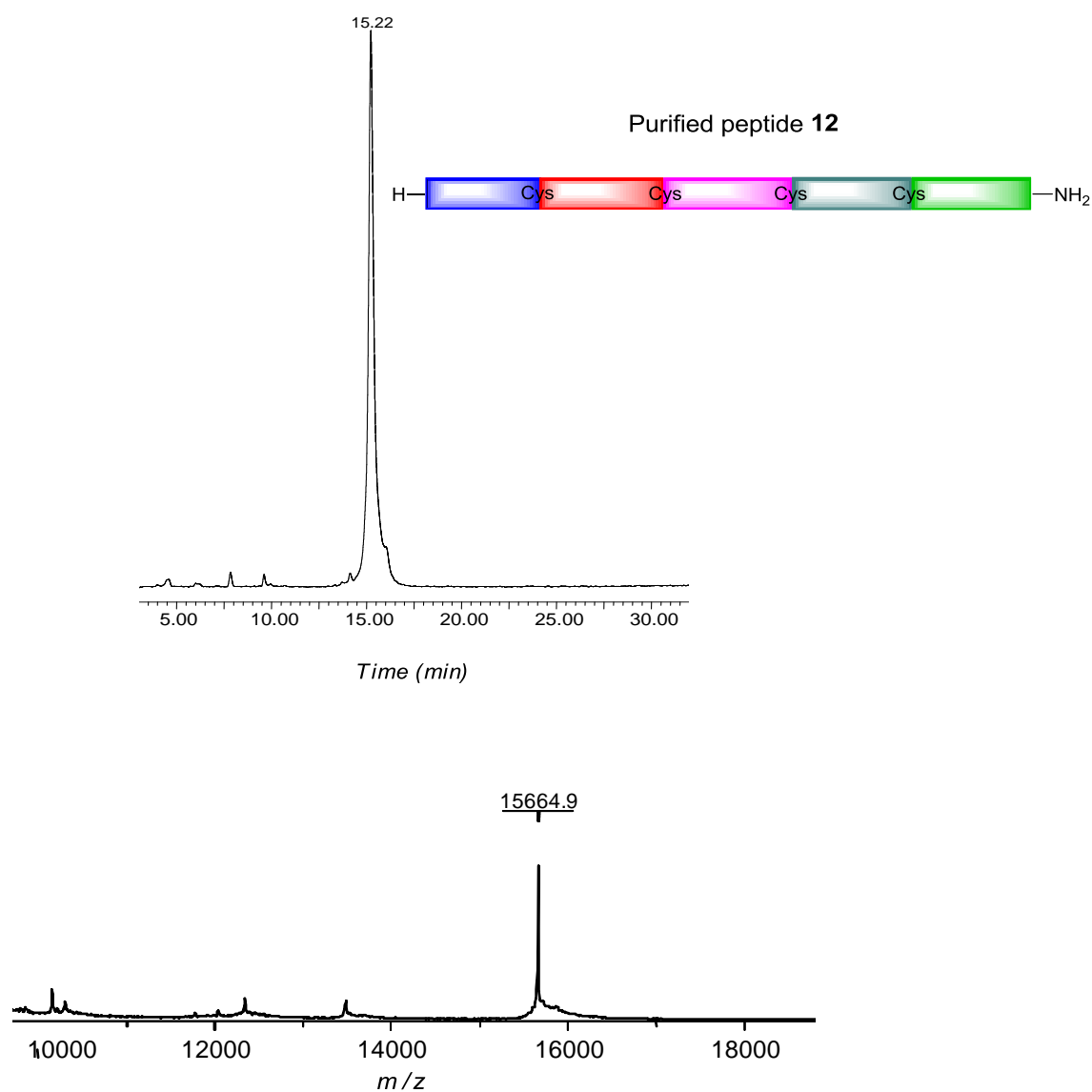


Figure S23. Analytical HPLC profile (detection at 215 nm) and MALDI-TOF analysis for purified peptide **12**. 0.6 mg (6.5 % yield, 0.5 μ mol scaled), MALDI-TOF calc. for $[M+H]^+$: 15662.5, observed mass: 15664.9 (average isotopic composition).