Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Acceleration of thiol additive-free native chemical ligation by intramolecular S-S-acyl transfer

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1. Materials and Instruments

Boc-amino acids were purchased from Biosolve (Valkenswaard, Netherland) and NeoMPS (Strasbourg, France) and the MBHA resin was acquired from Novabiochem (Darmstadt, Germany). Coupling Agents were purchased from Roth (Karlsruhe, Germany), Chemcube (Bochum, Germany) and Novabiochem (Darmstadt, Germany). Water was purified by using a Milli-Q Ultra Pure Water Purification system from membraPure. Acetonitrile was obtained from VWR Chemicals (Darmstadt, Germany) and has HPLC grade. Dimethylformamide for peptide synthesis was acquired from Biosolve (Valkenswaard, Netherland).

Manual solid phase peptide synthesis was performed by using 2 mL polyethylene syringe reactors (from MultSynTech) equipped with a fritted disc. Automated linear solid phase Fmoc-synthesis was performed by using an Intavis ResPep parallel synthesizer equipped with micro scale columns.

Analytical HPLC was performed by using an EliteLaChrom instrument of Merck-Hitachi. Detection was achieved with a diode-array-detector scanning from 210 to 400 nm. For the peptides a RP-C18A Polaris column (5 µm particle, 250x4.6 mm, 100 Å pore size) was used. Column oven temperature was set to 55°C. For elution following solvents were used at a 0.8 ml/min flow rate:

A: 98.9 % H_2O, 1 % acetonitrile, 0.1 % TFA

B: 98.9 % acetonitrile, 1 % H_2O, 0.1 % TFA.

Unless indicated otherwise a linear gradient from 03% B to 60% B in 30 min was used.Crude peptides obtained by solid phase synthesis were purified by semipreparative HPLC using an Agilent 1100 series instrument with a RP-C18A Polaris column (5 µm particle, 250x10 mm, 220 Å pore size) from Varian with a flow rate of 6 mL/min. Detection of the signals was achieved with a UV/VIS-detector at 210 nm. For elution solvents were used as mentioned above for analytical HPLC. Analytical UPLC was performed by using an Acquity UPLC® system from Waters. Detection was achieved with a UV/Vis-detector at 210 nm. An X-Bridge C18 BEH 130 column (1.7 µm particle, 50x2.1 mm, 130 Å pore size) from Waters was used. Column oven temperature was set to 50°C. For elution solvents were used at a 0.5 ml/min flow rate as mentioned above for analytical HPLC. Unless indicated otherwise a linear gradient from 03% B to 60% B in 2 min was used. Mass analysis was performed with an ESI-MS instrument from Agilent 1100 series with A (98.9 % H_2O, 1 % acetonitrile, 0.1 % formic

acid) and B (98.9 % acetonitrile, 1 % H_2O, 0.1 % formic acid) with isocratic flow (50 % B) of 0.3 mL/min in 2 min. Analytical UPLC-MS was performed by using an Acquity UPLC® H-Class system from Waters. Detection was achieved with a PDA-detector at 210 nm and 280 nm and a QDa mass spectrometer. An X-Bridge C18 BEH 130 column (1.7 µm particle, 50x2.1 mm, 130 Å pore size) from Waters was used. Column oven temperature was set to 50°C. For elution solvents were used at a 0.5 mL/min flow rate as mentioned above for semipreparative HPLC. Unless indicated otherwise a linear gradient from 03% B to 60% B in 4 min was used.

Concentrations of peptide solutions were determined by using the absorption at 278 nm measured with ND-1000 spectrophotometer from Peqlab Biotechnology GmbH. The extinction coefficients of the peptides were calculated as the sum of the extinction coefficients of the tyrosine and tryptophane residues:

$$\varepsilon_{278}(\text{Tyr}) = 1400\ \text{L/(mol} \cdot \text{cm)}$$
$$\varepsilon_{278}(\text{Trp}) = 5600\ \text{L/(mol} \cdot \text{cm)}$$

2. Solid Phase Peptide Synthesis

2.1 Manual solid phase peptide synthesis using Boc/Cbz-strategy

Coupling: 4 eq. amino acid (final concentration 0.4 M), 3.9 eq. HCTU, 4 eq. HOBt and 8 eq. NMM were dissolved in DMF and transferred to the resin. After 30 min, the resin was washed five times with DMF. After coupling of the 12th residue the procedure was repeated once. The MPA-linkage was introduced as Trt-protected mercaptopropionic acid under the same conditions like an amino acid.

Capping: A mixture of 5 % Ac_2O and 6 % 2,6-lutidine in DMF was added to the resin for 3 min. The resin was washed five times with DMF and with CH_2Cl_2 before Boc cleavage.

Boc cleavage: 5 % *m*-cresol in TFA was added for 5 min. The resin was washed ten times with CH_2Cl_2 and five times with DMF prior to the next coupling.

Trt-cleavage: A mixture of TFA/TIS (95:5) was added to the resin for 5 min. This was repeated once. The resin was washed ten times with CH_2Cl_2 and five times with DMF prior to the next coupling.

Resin cleavage: After synthesis the crude peptide was cleaved from the MBHA resin (16:3:1, TFA:TFMSA:*m*-cresol, 2 h), extracted with TFA, precipitated in cold Et_2O and dissolved in H_2O/acetonitrile.

2.2 Fmoc-based automated solid phase synthesis

Fmoc-cleavage: The resin was treated twice for 3 min with 20% piperidine in DMF and washed with DMF.

Coupling: 4 eq. of a 0.4 M solution of the monomer in a 0.4 M solution of Oxyma in DMF, 3.8 eq. of a 0.4 M solution of HCTU in DMF and 8 eq. of a 4 M solution of NMM in DMF were mixed in a preactivation vessel and transferred to the resin. After 30 min the procedure was repeated once. The resin was washed with DMF before capping.

Capping: A mixture of 5 % Ac_2O and 6 % 2,6-lutidine in DMF was added to the resin. After 3 min the resin was washed with DMF.

3. Model Peptide Thioesters

3.1 Synthesis of model thioesters using Boc/Cbz-strategy

H-RIGELX-MPA-CYG-NH_2 1a$_{CYG}$

The synthesis of X = Gly (**1aG**$_{CYG}$), Ala (**1aA**$_{CYG}$), Asn (**1aN**$_{CYG}$), Ser (**1aS**$_{CYG}$), Leu (**1a**$_{CYG}$), Val (**1aV**$_{CYG}$), Pro (**1aP**$_{CYG}$) was performed by using 15 µmol resin-bound Fmoc-Gly.

1aG$_{CYG}$: 5 µmol (35 %), ε_{278} = 1400 L/mol · cm, $C_{44}H_{71}N_{13}O_{13}S_2$, 1054.2 g/mol, t_R = 1.3 min (UPLC), ESI-MS (m/z): calcd. 1054.5 $[M+H]^+$, 527.7 $[M+2H]^{2+}$, found 1054.4 $[M+H]^+$, 527.9 $[M+2H]^{2+}$.

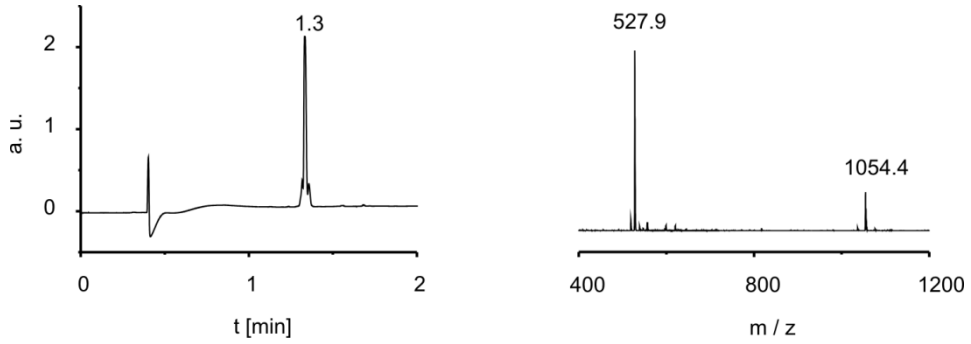


Figure S1: UPLC-trace (left) and ESI-MS (right) of purified **1aG**$_{CYG}$.

1aA$_{CYG}$: 4 µmol (30 %), ε_{278} = 1400 L/mol · cm, $C_{45}H_{73}N_{13}O_{13}S_2$, 1068.3 g/mol, t_R = 1.4 min (UPLC), ESI-MS (m/z): calcd. 1068.5 $[M+H]^+$, 534.8 $[M+2H]^{2+}$, found 1068.5 $[M+H]^+$, 534.9 $[M+2H]^{2+}$.

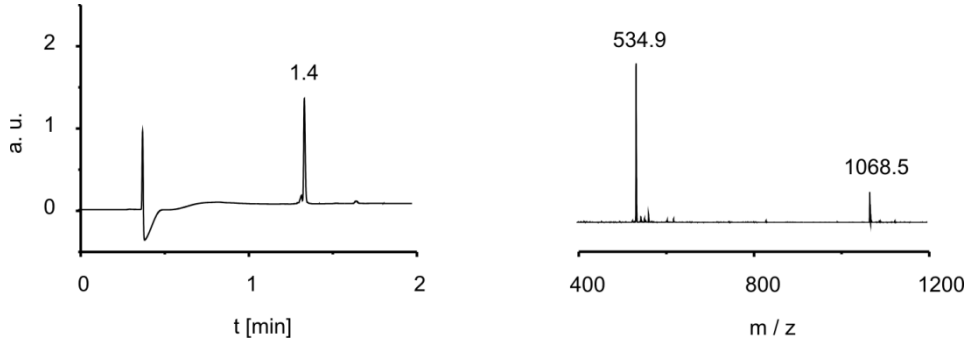


Figure S2: UPLC-trace (left) and ESI-MS (right) of purified **1aA**$_{CYG}$.

1aN$_{CYG}$: 2 µmol (15 %), ε_{278} = 1400 L/mol · cm, $C_{46}H_{74}N_{14}O_{14}S_2$, 1111.3 g/mol, t_R = 1.3 min (UPLC), ESI-MS (m/z): calcd. 1111.5 $[M+H]^+$, 556.3 $[M+2H]^{2+}$, found 1111.6 $[M+H]^+$, 556.4 $[M+2H]^{2+}$.

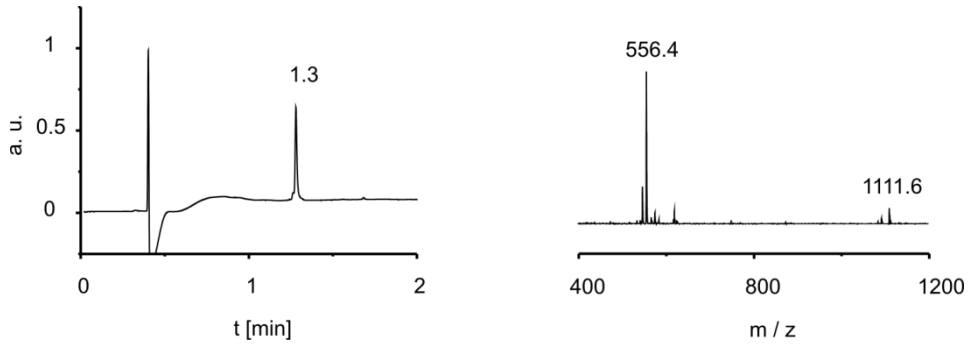


Figure S3: UPLC-trace (left) and ESI-MS (right) of purified **1aN**$_{CYG}$**.**

1aS$_{CYG}$: 3 µmol (20 %), ε_{278} = 1400 L/mol · cm, $C_{45}H_{73}N_{13}O_{14}S_2$, 1084.3 g/mol, t_R = 1.3 min (UPLC), ESI-MS (m/z): calcd. 1084.5 $[M+H]^+$, 542.7 $[M+2H]^{2+}$, found 1084.5 $[M+H]^+$, 542.9 $[M+2H]^{2+}$.

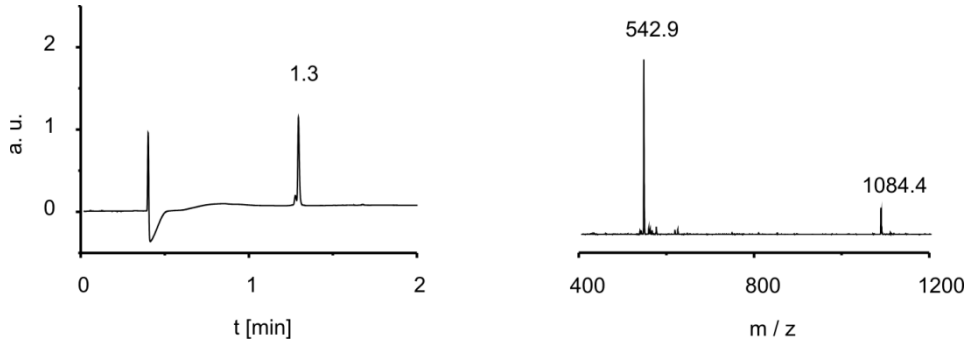


Figure S4: UPLC-trace (left) and ESI-MS (right) of purified **1aS**$_{CYG}$.

1a$_{CYG}$: 3 µmol (20 %), ε_{278} = 1400 L/mol · cm, $C_{48}H_{79}N_{13}O_{13}S_2$, 1110.4 g/mol, t_R = 1.6 min (UPLC), ESI-MS (m/z): calcd. 1110.5 $[M+H]^+$, 555.8 $[M+2H]^{2+}$, found 1110.6 $[M+H]^+$, 555.9 $[M+2H]^{2+}$.

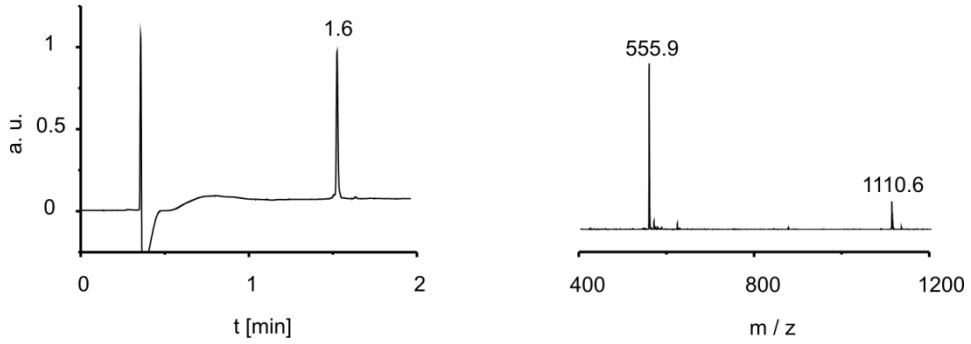


Figure S5: UPLC-trace (left) and ESI-MS (right) of purified **1a**$_{CYG}$.

1aV$_{CYG}$: 4 µmol (30 %), ε_{278} = 1400 L/mol · cm, $C_{47}H_{77}N_{13}O_{13}S_2$, 1096.3 g/mol, t_R = 1.5 min (UPLC), ESI-MS (m/z): calcd. 1096.5 $[M+H]^+$, 548.8 $[M+2H]^{2+}$, found 1096.5 $[M+H]^+$, 548.9 $[M+2H]^{2+}$.

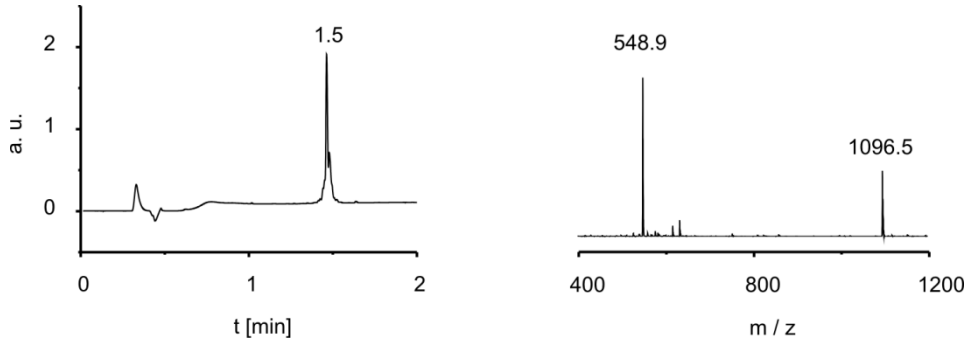


Figure S6: UPLC-trace (left) and ESI-MS (right) of purified **1aV**$_{CYG}$.

1aP$_{CYG}$: 2 µmol (15 %), ε_{278} = 1400 L/mol · cm, $C_{47}H_{75}N_{13}O_{13}S_2$, 1094.3 g/mol, t_R = 1.3 min (UPLC), ESI-MS (m/z): calcd. 1094.5 $[M+H]^+$, 547.8 $[M+2H]^{2+}$, found 1094.5 $[M+H]^+$, 547.8 $[M+2H]^{2+}$.

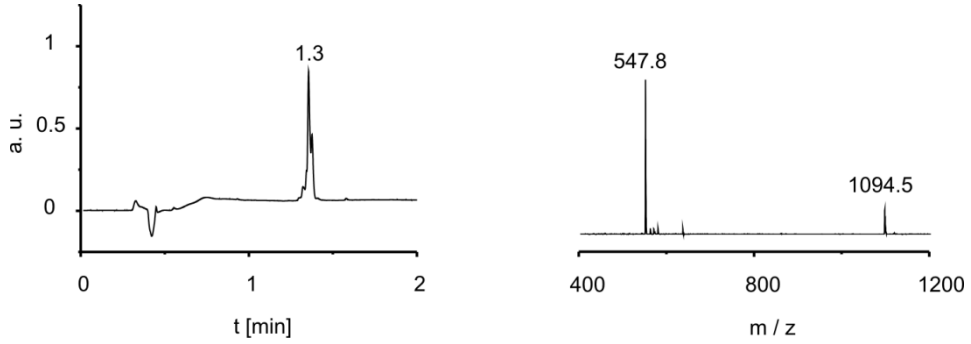


Figure S7: UPLC-trace (left) and ESI-MS (right) of purified **1aP**$_{CYG}$.

H-RIGELX-MPA-AYG-NH$_2$ 1a$_{AYG}$

The synthesis of X = Leu (**1a**$_{AYG}$) was performed by using 10 µmol resin-bound Fmoc-Gly.

1a$_{AYG}$: 1.5 µmol (15 %), ε_{278} = 1400 L/mol · cm, $C_{48}H_{79}N_{13}O_{13}S$, 1078.28 g/mol, t_R = 1.4 min (UPLC), ESI-MS (m/z): calcd. 1078.6 $[M+H]^+$, 539.8 $[M+2H]^{2+}$, found 1078.5 $[M+H]^+$, 539.9 $[M+2H]^{2+}$.

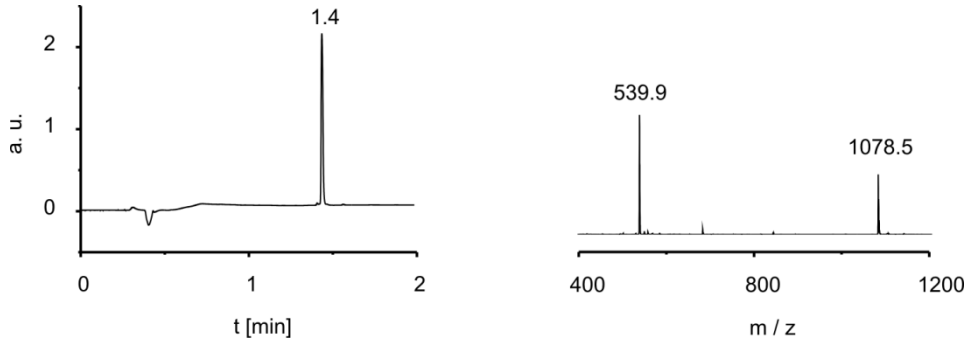


Figure S8: UPLC-trace (left) and ESI-MS (right) of purified **1a**$_{AYG}$.

The synthesis of X = Val (**1aV**$_{AYG}$) or X = Pro (**1aP**$_{AYG}$) was performed by using 7 µmol resin-bound Fmoc-Gly.

1aV$_{AYG}$: 1.8 µmol (26 %), ε_{278} = 1400 L/mol · cm, $C_{47}H_{77}N_{13}O_{13}S$, 1064.26 g/mol, t_R = 1.4 min (UPLC), ESI-MS (m/z): calcd. 1177.6 $[M+TFA]^+$, found 1176.5 $[M+TFA]^+$.

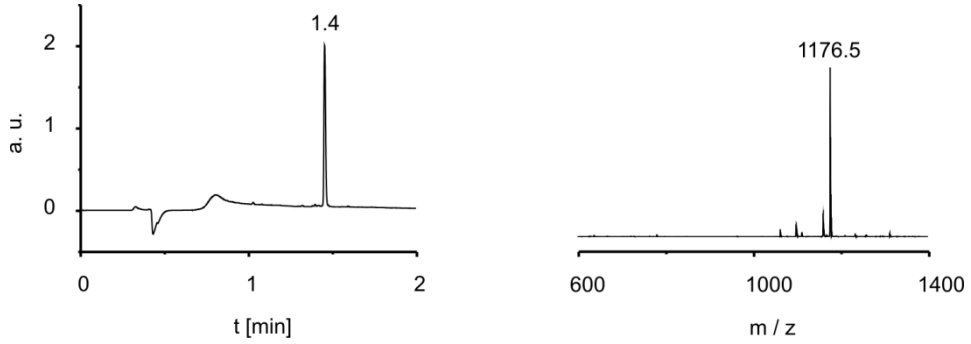


Figure S9: UPLC-trace (left) and ESI-MS (right) of purified **1aV**$_{AYG}$.

1aP$_{AYG}$: 2.2 µmol (2.3 mg, 31 %), ε_{278} = 1400 L/mol · cm, $C_{47}H_{75}N_{13}O_{13}S$, 1062.24 g/mol, t_R = 1.4 min (UPLC), ESI-MS (m/z): calcd. 1062.5 $[M+H]^+$, 531.8 $[M+2H]^{2+}$, found 1062.5 $[M+H]^+$, 531.9 $[M+2H]^{2+}$.

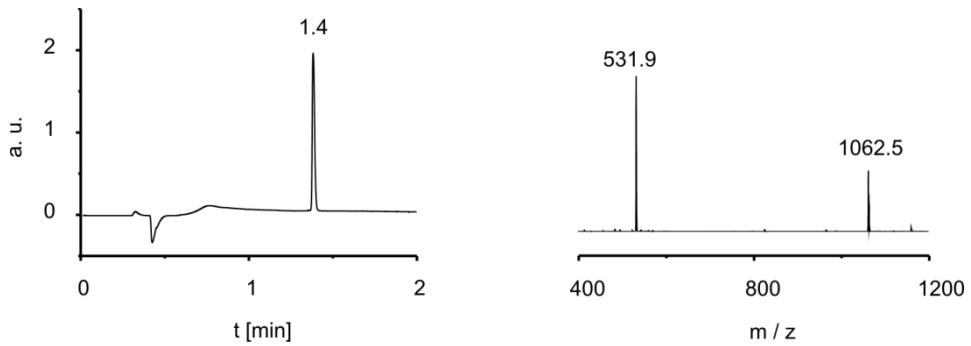


Figure S 10: UPLC-trace (left) and ESI-MS (right) of purified **1aP**$_{AYG}$.

H-RIGELX-MPA-CCCY-NH_2 1a$_{CCCY}$

The synthesis of X = Gly (**1aG**$_{CCCY}$), Ala (**1aA**$_{CCCY}$), Asn (**1aN**$_{CCCY}$) or Ser (**1aS**$_{CCCY}$) was performed by using 10 µmol resin-bound Fmoc-Gly.

1aG$_{CCCY}$: 900 nmol (15 %,), ε_{278} = 1400 L/mol · cm, $C_{48}H_{78}N_{14}O_{14}S_4$, 1203.48 g/mol, t_R = 1.5 min (UPLC), ESI-MS (m/z): calcd. 1201.5 $[2M+H]^+$, 601.2 $[2M+2H]^{2+}$, found 1201.4 $[2M+H]^+$, 601.3 $[2M+2H]^{2+}$.

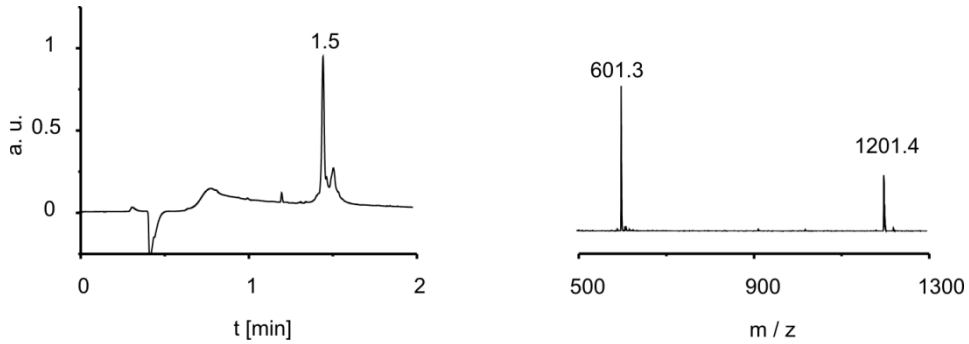


Figure S11: UPLC-trace (left) and ESI-MS (right) of purified **1aG**$_{CCCY}$.

1aA$_{CCCY}$: 1.0 µmol (17 %), ε_{278} = 1400 L/mol · cm, $C_{49}H_{80}N_{14}O_{14}S_4$, 1217.50 g/mol, t_R = 1.5 min (UPLC), ESI-MS (m/z): calcd. 1215.5 $[2M+H]^+$, 608.2 $[2M+2H]^{2+}$, found 1215.4 $[2M+H]^+$, 608.3 $[2M+2H]^{2+}$.

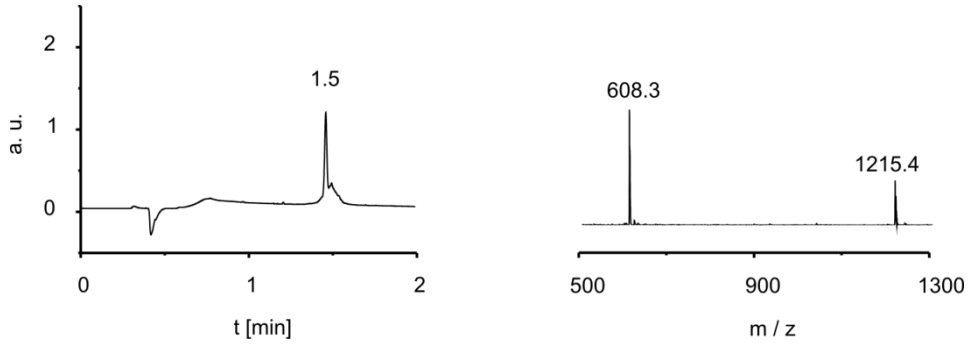


Figure S12: UPLC-trace (left) and ESI-MS (right) of purified **1aA**$_{CCCY}$.

1aN$_{CCCY}$: 550 nmol (10 %), ε_{278} = 1400 L/mol · cm, $C_{50}H_{81}N_{15}O_{15}S_4$, 1260.50 g/mol, t_R = 1.4 min (UPLC), ESI-MS (m/z): calcd. 1258.8 $[2M+H]^+$, 629.7 $[2M+2H]^{2+}$, found 1258.3 $[2M+H]^+$, 628.8 $[2M+2H]^{2+}$.

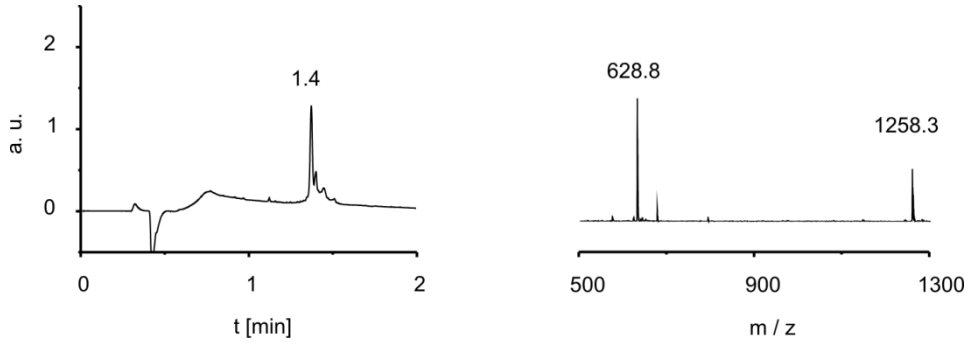


Figure S13: UPLC-trace (left) and ESI-MS (right) of purified **1aN**$_{CCCY}$.

1aS$_{CCCY}$: 660 nmol (9 %), ε_{278} = 1400 L/mol · cm, $C_{49}H_{80}N_{14}O_{15}S_4$, 1233.50 g/mol, t_R = 1.4 min (UPLC), ESI-MS (m/z): calcd. 1231.5 $[2M+H]^+$, 616.2 $[2M+2H]^{2+}$, found 1231.4 $[2M+H]^+$, 616.3 $[2M+2H]^{2+}$.

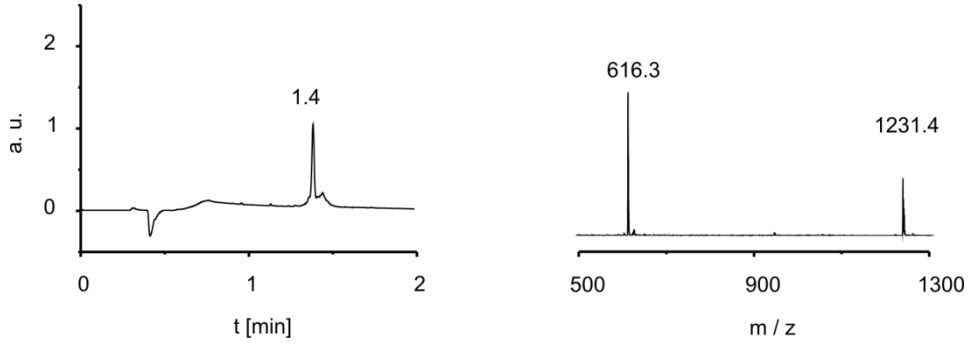


Figure S14: UPLC-trace (left) and ESI-MS (right) of purified **1aS**$_{CCCY}$.

1aL$_{CCCY}$: 900 nmol (15 %), ε_{278} = 1400 L/mol · cm, $C_{52}H_{86}N_{14}O_{14}S_4$, 1259.58 g/mol, t_R = 1.6 min (UPLC), ESI-MS (m/z): calcd. 1257.5 $[2M+H]^+$, 629.3 $[2M+2H]^{2+}$, found 1257.4 $[2M+H]^+$, 629.3 $[2M+2H]^{2+}$.

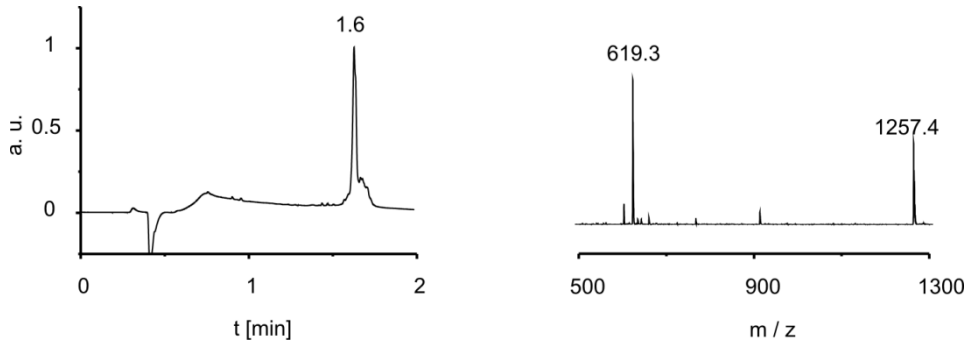


Figure S15: UPLC-trace (left) and ESI-MS (right) of purified **1aL**$_{CCCY}$.

H-YIGELX-MPA-G-NH$_2$ 1b$_G$

The synthesis of X = Gly (**1bG$_G$**), Ala (**1bA$_G$**), Asn (**1bN$_G$**), Ser (**1bS$_G$**) or Leu (**1b$_G$**) was performed by using 10 µmol resin-bound Fmoc-Gly.

1bG$_G$: 3.2 µmol (32 %), ε_{278} = 1400 L/mol · cm, $C_{35}H_{54}N_8O_{11}S$, 794.92 g/mol, t_R = 1.8 min (UPLC, 4 min), ESI-MS (m/z): calcd. 795.4 $[2M+H]^+$, 398.2 $[2M+2H]^{2+}$, found 794.4 $[M+H]^+$, 397.8 $[M+2H]^{2+}$.

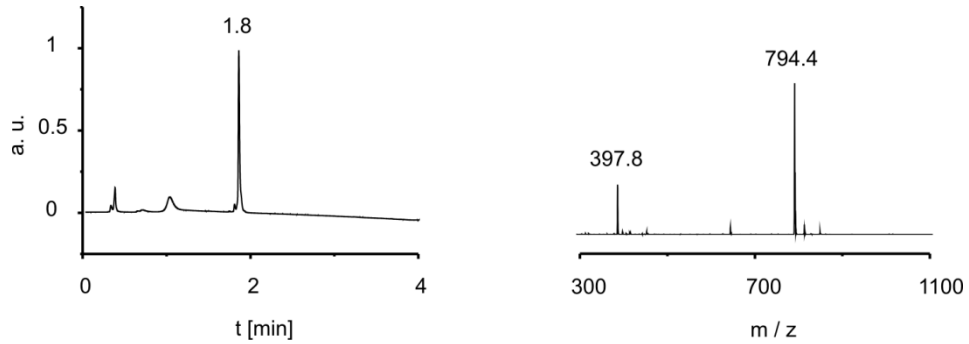


Figure S16: UPLC-trace (left) and ESI-MS (right) of purified **1bG$_G$**.

1bA$_G$: 3.2 µmol (32 %), ε_{278} = 1400 L/mol · cm, $C_{36}H_{56}N_8O_{11}S$, 808.94 g/mol, t_R = 1.9 min (UPLC, 4 min), ESI-MS (m/z): calcd. 809.4 $[M+H]^+$, 405.2 $[M+2H]^{2+}$, found. 808.4 $[M+H]^+$, 404.8 $[M+2H]^{2+}$.

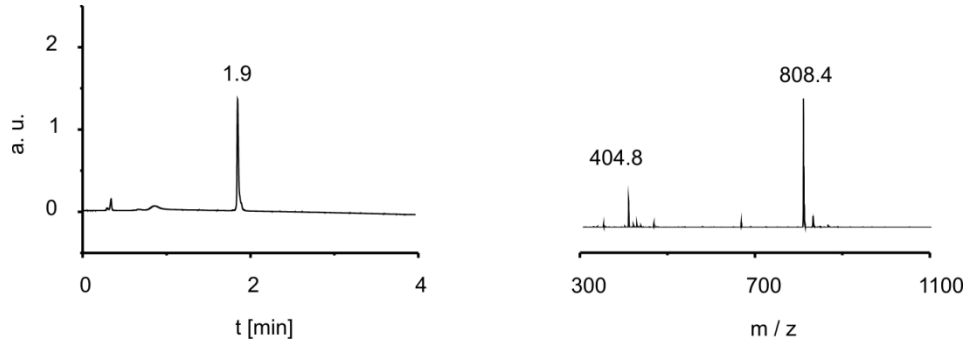


Figure S17: UPLC-trace (left) and ESI-MS (right) of purified **1bA$_G$**.

1bN$_G$: 1.9 µmol (20 %), ε_{278} = 1400 L/mol · cm, $C_{37}H_{57}N_9O_{12}S$, 851.97 g/mol, t_R = 1.7 min (UPLC, 4 min), ESI-MS (m/z): calcd. 852.4 $[M+H]^+$, 426.7 $[M+2H]^{2+}$, found 851.4 $[M+H]^+$, 426.4 $[M+2H]^{2+}$.

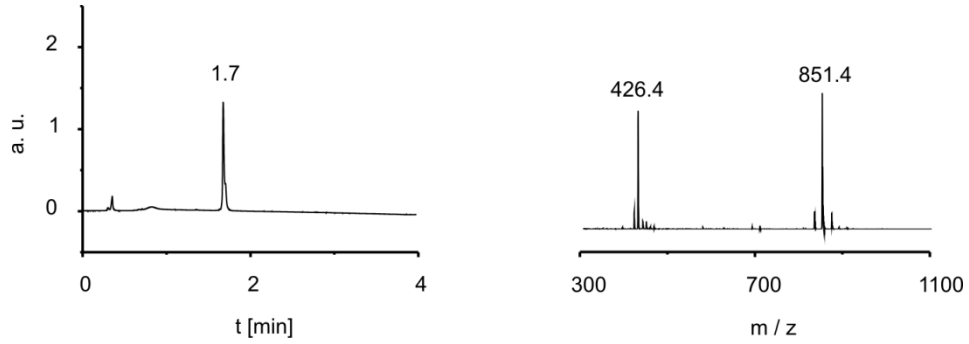


Figure S18: UPLC-trace (left) and ESI-MS (right) of purified **1bN**$_G$.

1bS$_G$: 2.4 µmol (24 %), ε_{278} = 1400 L/mol · cm, $C_{36}H_{56}N_8O_{12}S$, 824.94 g/mol, t_R = 1.7 min (UPLC, 4 min), ESI-MS (m/z): calcd. 825.4 $[M+H]^+$, 413.2 $[M+2H]^{2+}$, found 824.4 $[M+H]^+$, 412.8 $[M+2H]^{2+}$.

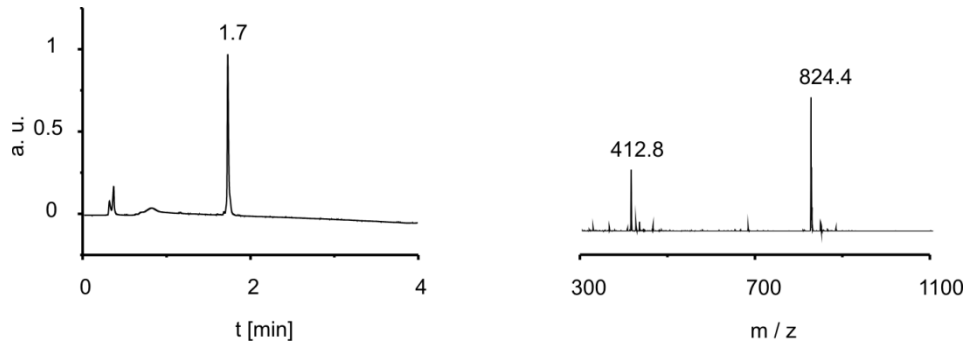


Figure S19: UPLC-trace (left) and ESI-MS (right) of purified **1bS**$_G$.

1b$_G$: 2.2 µmol (22 %), ε_{278} = 1400 L/mol · cm, $C_{39}H_{62}N_8O_{11}S$, 851.02 g/mol, t_R = 2.3 min (UPLC, 4 min), ESI-MS (m/z): calcd. 851.4 $[M+H]^+$, 426.2 $[M+2H]^{2+}$, found 850.4 $[M+H]^+$, 425.8 $[M+2H]^{2+}$.

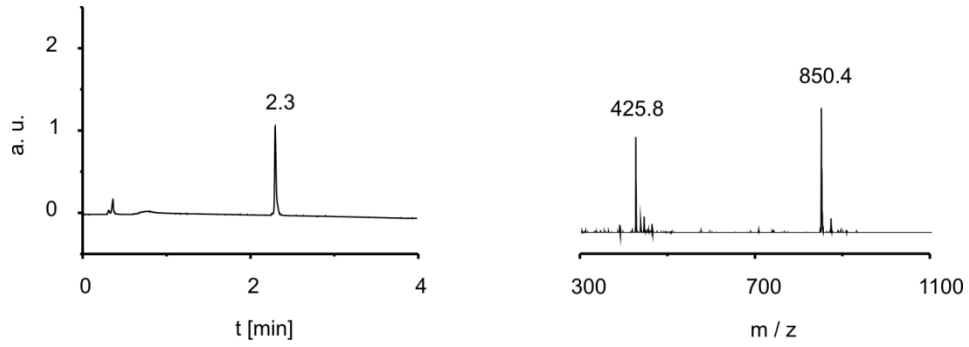
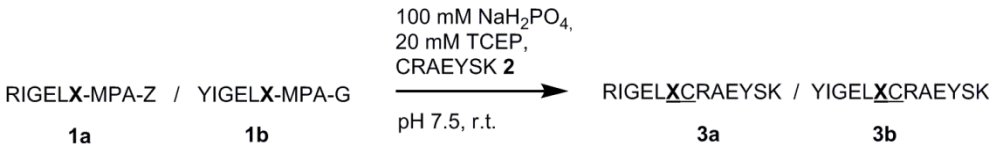


Figure S20: UPLC-trace (left) and ESI-MS (right) of purified **1bN**$_G$.

3.2 Ligations of the model thioesters



Scheme S1: Ligation reactions between peptide **1a**/**1b** and peptide **2**.

The lyophilized model peptides **1a**/**1b** (1 eq.) and **2** (1.5 eq.) were dissolved in degassed ligation buffer (100 mM NaH_2PO_4, 20 mM TCEP, pH 7.5, 2 mM thioester concenctration) and the reaction was gently agitated at room temperatur. Aliquots were taken from the reaction mixture at various time intervals and quenched with 0.1% TFA in water and analyzed by analytical HPLC. The yield was calculated based on the peak areas of the thioester **1a**/**1b** versus the desired ligation products **3a/3b** at 280 nm.

RIGELGCRAEYSK **3aG**: $C_{62}H_{105}N_{21}O_{19}S$, 1480.69 g/mol, t_R = 18.6 min (analyt. HPLC), ESI-MS (m/z): calcd. 1480.8 $[M+H]^+$, found 1480.6 $[M+H]^+$.

YIGELGCRAEYSK **3bG**: $C_{65}H_{102}N_{18}O_{20}S$, 1487.68 g/mol, t_R = 20.2 min (analyt. HPLC), ESI-MS (m/z): calcd. 2972.4 $[2M+H]^+$, found 2972.6 $[2M+H]^+$.

RIGELACRAEYSK **3aA**: $C_{63}H_{107}N_{21}O_{19}S$, 1494.72 g/mol, t_R = 18.9 min (analyt. HPLC), ESI-MS (m/z): calcd. 1494.8 $[M+H]^+$, found 1494.6 $[M+H]^+$.

YIGELACRAEYSK$_2$ **3bA**: $C_{66}H_{104}N_{18}O_{20}S$, 1501.71 g/mol, t_R = 20. min (analyt. HPLC), ESI-MS (m/z): calcd. 3000.5 $[2M+H]^+$, found 3000.9 $[2M+H]^+$.

RIGELNCRAEYSK **3aN**: $C_{64}H_{108}N_{22}O_{20}S$, 1537.72 g/mol, t_R = 18.8 min (analyt. HPLC), ESI-MS (m/z): calcd. 1537.8 $[M+H]^+$, found 1538.0 $[M+H]^+$.

YIGELNCRAEYSK **3bN**: $C_{64}7_{105}N_{19}O_{19}S$, 1544.73 g/mol, t_R = 19.2 min (analyt. HPLC), ESI-MS (m/z): calcd. 1543.7 $[2M+2H]^{2+}$, found 1543.9 $[2M+2H]^{2+}$.

RIGELSCRAEYSK **3aS**: $C_{63}H_{107}N_{21}O_{20}S$, 1510.72 g/mol, t_R = 21.2 min (analyt. HPLC), ESI-MS (m/z): calcd. 1510.8 $[M+H]^+$, found 1510.5 $[M+H]^+$.

YIGELSCRAEYSK **3bS**: $C_{66}H_{104}N_{18}O_{21}S$, 1517.71 g/mol, t_R = 19.5 min (analyt. HPLC), ESI-MS (m/z): calcd. 1516.7 $[2M+2H]^{2+}$, found 1517.0 $[2M+2H]^{2+}$.

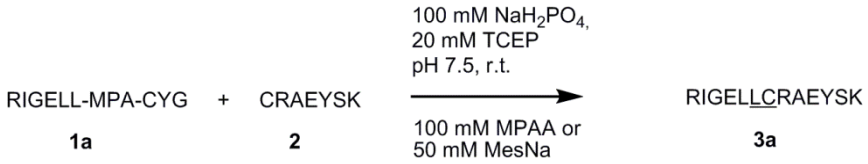
RIGELLCRAEYSK **3a**: $C_{66}H_{113}N_{21}O_{19}S$, 1536.80 g/mol, t_R = 18.7 min (analyt. HPLC), ESI-MS (m/z): calcd. 1536.8 $[M+H]^+$, found 1536.6 $[M+H]^+$.

YIGELLCRAEYSK **3b**: $C_{69}H_{110}N_{18}O_{20}S$, 1543.79 g/mol, t_R = 22.5 min (analyt. HPLC), ESI-MS (m/z): calcd. 1543.8 $[M+H]^+$, found 1543.6 $[M+H]^+$.

RIGELVCRAEYSK **3aV**: $C_{65}H_{111}N_{21}O_{19}S$, 1522.77 g/mol, t_R = 20.1 min (analyt. HPLC), ESI-MS (m/z): calcd. 1522.8 $[M+H]^+$, found 1522.9 $[M+H]^+$.

RIGELPCRAEYSK **3aP**: $C_{65}H_{109}N_{21}O_{19}S$, 1520.76 g/mol, t_R = 19.4 min (analyt. HPLC), ESI-MS (m/z): calcd. 1520.8 $[M+H]^+$, found 1520.9 $[M+H]^+$.

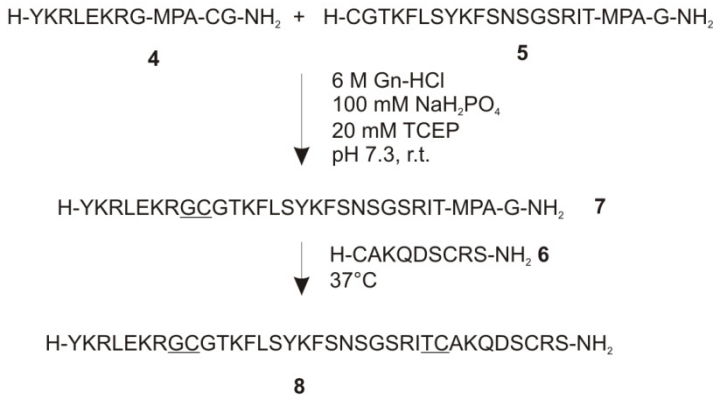
3.3 Ligations of the model thioester 1a with thiol additives



Scheme S2: Ligation reactions between thioester **1a** and peptide **2** using thiol additives.

The ligation buffer (100 mM NaH_2PO_4, 20 mM TCEP, pH 7.5) was equipped either with MPAA (final concentration: 100 mM) or MesNa (final concentration: 50 mM) and the final pH of the solution was measured, if required readjusted to 7.5 and degassed. The lyophilized model peptides **1a** (1 eq.) and **2** (1.5 eq.) were then dissolved in the ligation buffer (2 mM thioester concenctration) and the reaction mixture was gently agitated at room temperatur. Aliquots were taken from the reaction at various time intervals and quenched with 0.1% TFA in water and analyzed by UPLC-MS. The yield was calculated based on the peak areas of the thioester **1a** versus the desired ligation products **3a** at 280 nm.

4. Sequential Native Chemical Ligation



Scheme S3: Sequential ligation of peptide segments **4**, **5** and **6** to yield **8**, a 34mer out of secretory human type II phospholipase A ($sPLA_2$).

H-YKRLEKRG-MPA-CG-NH_2 4

The synthesis was performed via Boc/Cbz-strategy by using 10 µmol resin-bound Fmoc-Gly. 1.8 µmol (18 %), ε_{278} = 1400 L/mol · cm, $C_{54}H_{93}N_{19}O_{14}S_2$, 1296.56 g/mol, t_R = 1.5 min (UPLC-MS), ESI-MS (m/z): calcd. 648.8 $[M+2H]^{2+}$, 432.9 $[M+3H]^{3+}$, found 649.0 $[M+2H]^{2+}$, 433.2 $[M+3H]^{3+}$.

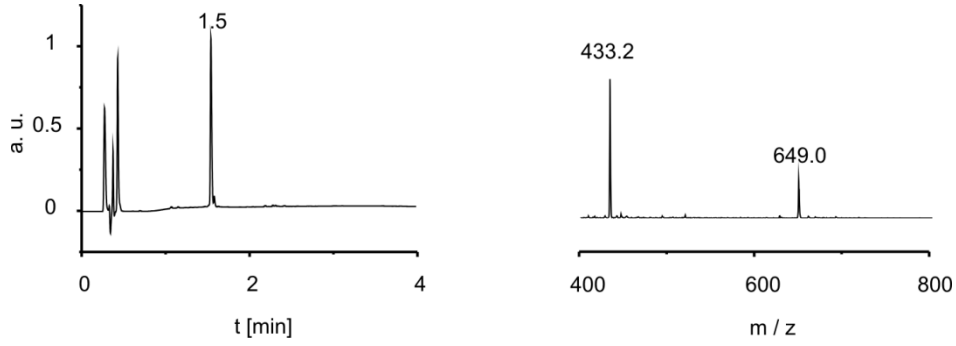


Figure S21: UPLC-trace (left) and ESI-MS (right) of purified **4**.

H-CGTKFLSYKFSNSGSRIT-MPA-G-NH_2 5

The synthesis was performed via Boc/Cbz-strategy by using 20 µmol resin-bound Fmoc-Gly. 2.3 µmol (12 %), ε_{278} = 1400 L/mol · cm, $C_{93}H_{146}N_{26}O_{28}S_2$, 2140.44 g/mol, t_R = 2.3 min (UPLC-MS), ESI-MS (m/z): calcd. 1070.5 $[M+H]^{+}$, 714.0 $[M+2H]^{2+}$, found 1071.1 $[M+H]^{+}$, 714.6 $[M+2H]^{2+}$,

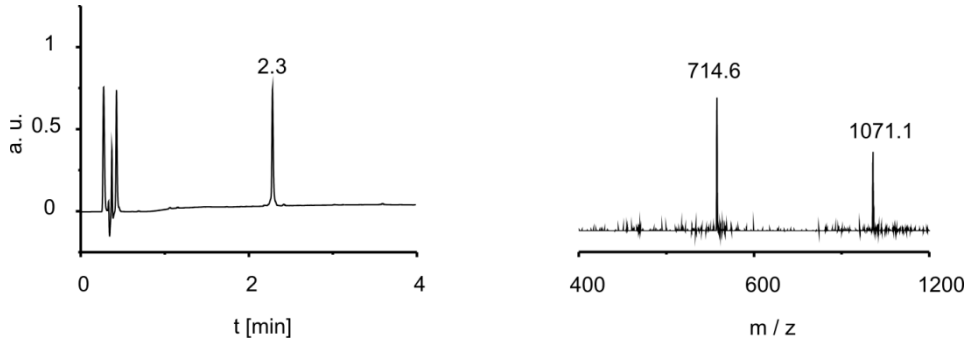


Figure S22: UPLC-trace (left) and ESI-MS (right) of purified **5**.

H-CAKQDSCRS-NH_2 6

The synthesis was performed via automated Fmoc-synthesis in a 10 µmol scale. 3.7 µmol (30 %), $C_{36}H_{65}N_{15}O_{14}S_2$, 996.12 g/mol, t_R =0.4 min (UPLC-MS), ESI-MS (m/z): calcd. 996.4 $[M+H]^+$, 498.7 $[M+2H]^{2+}$, found 996.4 $[M+H]^+$, 499.0 $[M+2H]^{2+}$.

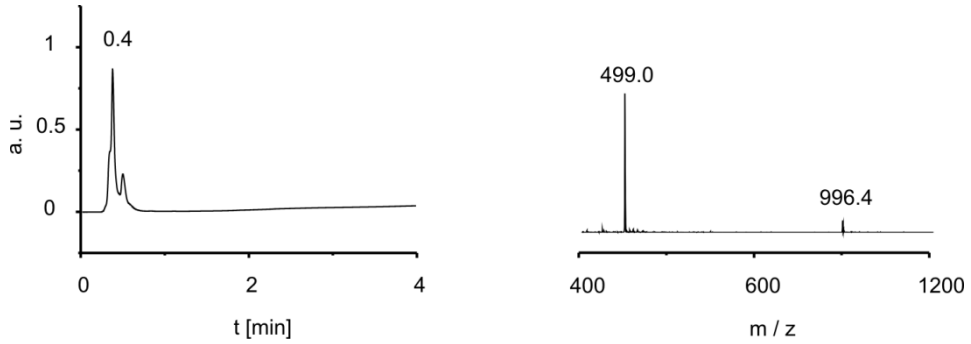


Figure S23: UPLC-trace (left) and ESI-MS (right) of purified **6**.

First ligation: The lyophilized peptides (40 nmol **4**, 60 nmol **5**) were dissolved in 12 µL degassed buffer (6 M GnHCl, 100 mM NaH_2PO_4, 20 mM TCEP, pH 7.0,) and gently agitated at room temperature. The ligation was complete after 4 h as indicated by UPLC-MS-analysis. The yield of the ligation product **7** was determined to 89 % (integration of peak area at 280 nm). The formation of cyclized **5** was observed as a side reaction.

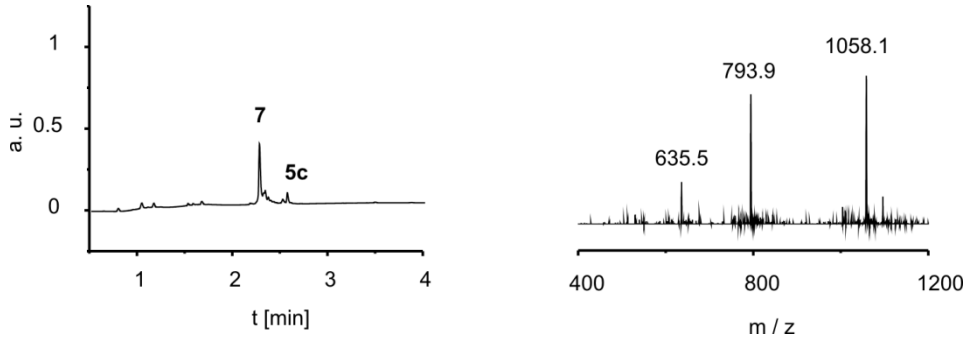


Figure S24: UPLC-MS trace (left) of the ligation between **4** and **5** after 4 h at 210 nm and ESI-MS (right) of **7** (ESI-MS (m/z): calcd. 1057.6 $[M+3H]^{3+}$, 793.4 $[M+4H]^{4+}$, 634.9 $[M+5H]^{5+}$, found 1058.1 $[M+3H]^{3+}$, 793.9 $[M+4H]^{4+}$, 635.5 $[M+5H]^{5+}$). **5c** = cyclized **5**.

Second ligation: After 4 h a solution of peptide **6** (90 nmol) 8 µL degassed buffer (6 M GnHCl, 100 mM NaH_2PO_4, 20 mM TCEP, pH 7.0) was added to the ligation mixture and the mixture was incubated at 37°C. The formation of the full-length ligation product was confirmed by UPLC-MS-analysis after 23 h (65 %, determined by integration of peak area at 280 nm). The amount of **5c** was less than 10 %.

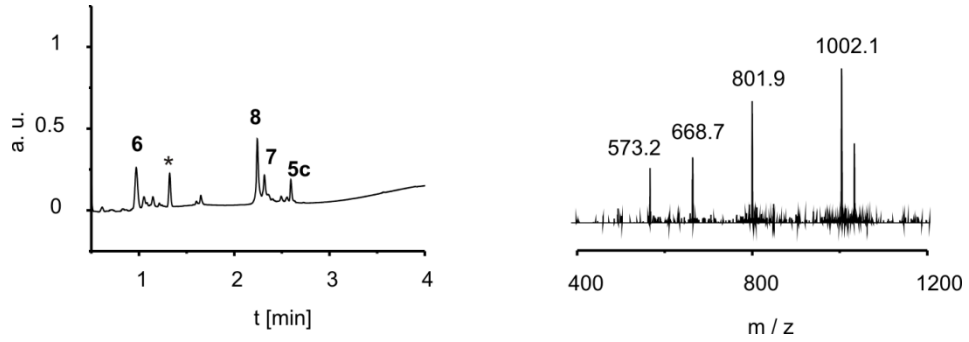
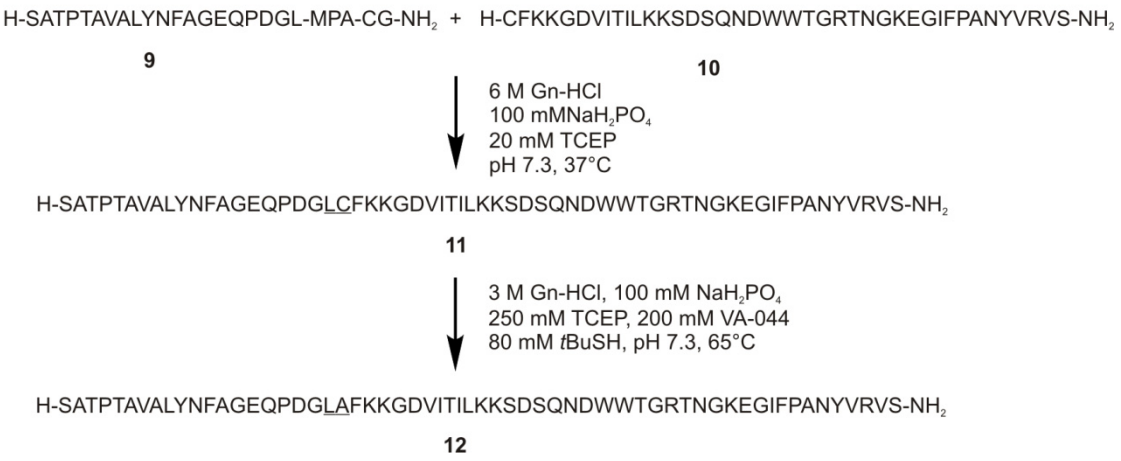


Figure S25: UPLC-MS trace (left) of the ligation reaction after 23 h at 210 nm and ESI-MS (right) of **8** (ESI-MS (m/z): calcd. 1001.8 $[M+4H]^{4+}$, 801.6 $[M+5H]^{5+}$, 668.2 $[M+6H]^{6+}$, 572.9 $[M+7H]^{7+}$, 501.4 $[M+8H]^{8+}$, found 1002.1 $[M+4H]^{4+}$, 801.9 $[M+5H]^{5+}$, 668.7 $[M+6H]^{6+}$, 573.2 $[M+7H]^{7+}$, 501.2 $[M+8H]^{8+}$. * = non-peptidic material

5. One-Pot Synthesis of YSC84 SH3 Domain



Scheme S4: Synthesis of YSC84 SH3 domain **12** by using a one-pot ligation desulfurization approach.

H-SATPTAVALYNFAGEQPGDL-MPA-CG-NH_2 9

The synthesis was performed by using 12 µmol resin-bound Fmoc-Gly. Removal of cyclohexyl protecting group (cHex) of aspartate side chain was not complete under these conditions. 2 µmol (15%), $C_{98}H_{149}N_{25}O_{33}S_2$, 2269.51 g/mol, ε_{278} = 1400 L/mol · cm, t_R = 1.9 min (UPLC-MS), ESI-MS (m/z): calcd. 1135.0 $[M+2H]^{2+}$, 757.0 $[M+3H]^{3+}$, found 1135.0 $[M+2H]^{2+}$, 757.4 $[M+3H]^{3+}$.

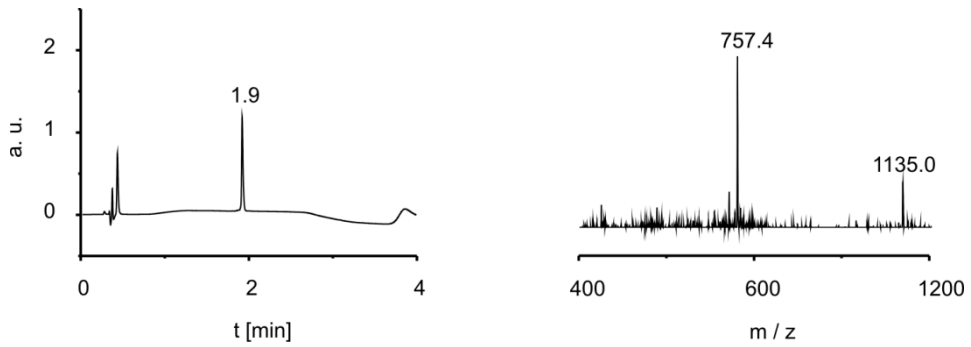


Figure S26: UPLC-trace (left) and ESI-MS (right) of purified **9**.

H-CFKKGDVITIlKKSDSQNDWWTGRTNGKEGIFPANYVRVS-NH_2 10

The synthesis was performed in a 10 µmol scale. The couplings of the pseudoproline dipeptides Fmoc-Trp(Boc)-Thr[Ψ(Me, Me)Pro]-OH and Fmoc-Lys(Boc)-Ser[Ψ(Me, Me)Pro]-OH was done manually using 4 eq. of the pseudoproline diepeptide, 3.9 eq. HATU and 8 eq. NMM in DMF for twice 30 min. 4.2 µmol (42 %), ε_{278} = 12600 L/mol · cm, $C_{204}H_{318}N_{58}O_{59}S$, 4559.1 g/mol, t_R = 2.7 min (UPLC-MS), ESI-MS

(m/z): calcd. 1519.8 $[M+3H]^{3+}$, 1140.1 $[M+4H]^{4+}$, 912.3 $[M+5H]^{5+}$, 760.4 $[M+6H]^{6+}$, 651.9 $[M+7H]^{7+}$, 570.6 $[M+8H]^{8+}$, found 1520.2 $[M+3H]^{3+}$, 1140.6 $[M+4H]^{4+}$, 912.8 $[M+5H]^{5+}$, 760.8 $[M+6H]^{6+}$, 652.4 $[M+7H]^{7+}$, 571.0 $[M+8H]^{8+}$.

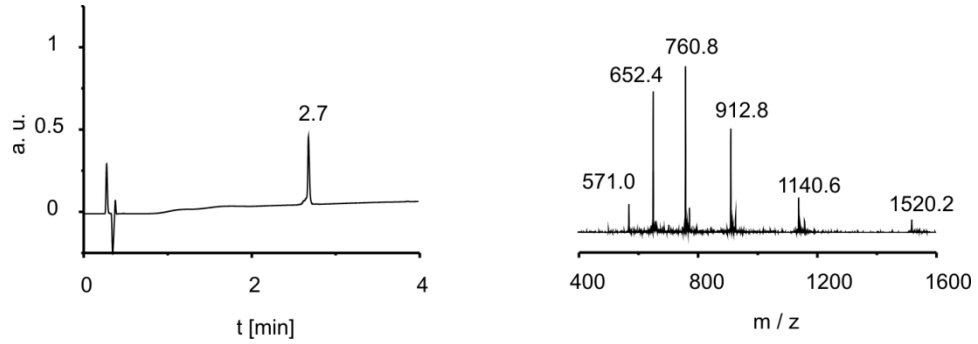


Figure S27: UPLC-trace (left) and ESI-MS (right) of purified **10**.

Ligation: The lyophilized peptides (150 nmol **9**, 225 nmol **10**) were dissolved in 75 µL degassed buffer (6 M GnHCl, 100 mM NaH_2PO_4, 20 mM TCEP, pH 7.3, 2 mM concentration of thioester) and incubated at 37°C. After 48 h the conversion to the ligation product was determined to 88 %.

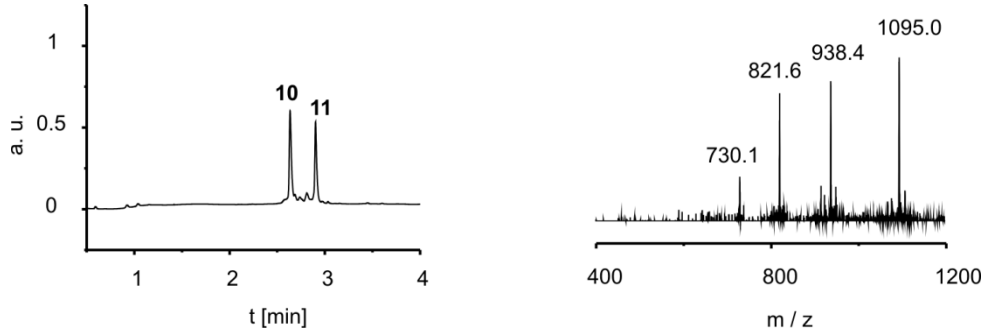


Figure S28: UPLC-trace (left) of the ligation between **9** and **10** after 48 h at λ = 210 nm (6 M GnHCl, 100 mM NaH_2PO_4, 20 mM TCEP, pH 7.3, 37°C) and ESI-MS (right) of the ligation product **11** (ESI-MS (m/z): calcd. 1094.2 $[M+6H]^{6+}$, 938.1 $[M+7H]^{7+}$, 820.9 $[M+8]^{8+}$, 729.8 $[M+9H]^{9+}$, found 1095.0 $[M+6H]^{6+}$, 938.4 $[M+7H]^{7+}$, 821.6 $[M+8]^{8+}$, 730.1 $[M+9H]^{9+}$).

Desulfurization: After the ligation, the reaction mixture was diluted (1 mM peptide concentration) by adding the same amount of degassed desulfurization buffer (100 mM NaH_2PO_4, 480 mM TCEP, pH 7.3). The radical starter VA-044 (final concentration: 200 mM) and *t*BuSH (final concentration: 80 mM) were added and the mixture was incubated at 65°C for 1 h. After this time the desulfurization reaction had reached completion as indicated by UPLC–MS analysis. Semipreparative HPLC (10-70 % B in 30 min) afforded 80 nmol **12** (53% yield over two steps, determined by UV-analysis, ε_{278} = 140000 L/mol · cm).

$C_{294}H_{452}N_{80}O_{89}$, 6531.2 g/mol, t_R = 1.4 min (UPLC), ESI-MS (m/z): calcd. 1088.9 $[M+6H]^{6+}$, 933.5 $[M+7H]^{7+}$, 816.9 $[M+8]^{8+}$, 726.3 $[M+9H]^{9+}$, 653.7 $[M+10H]^{10+}$, found 1089.5 $[M+6H]^{6+}$, 933.9 $[M+7H]^{7+}$, 817.4 $[M+8]^{8+}$, 726.7 $[M+9H]^{9+}$, 654.1 $[M+10H]^{10+}$.

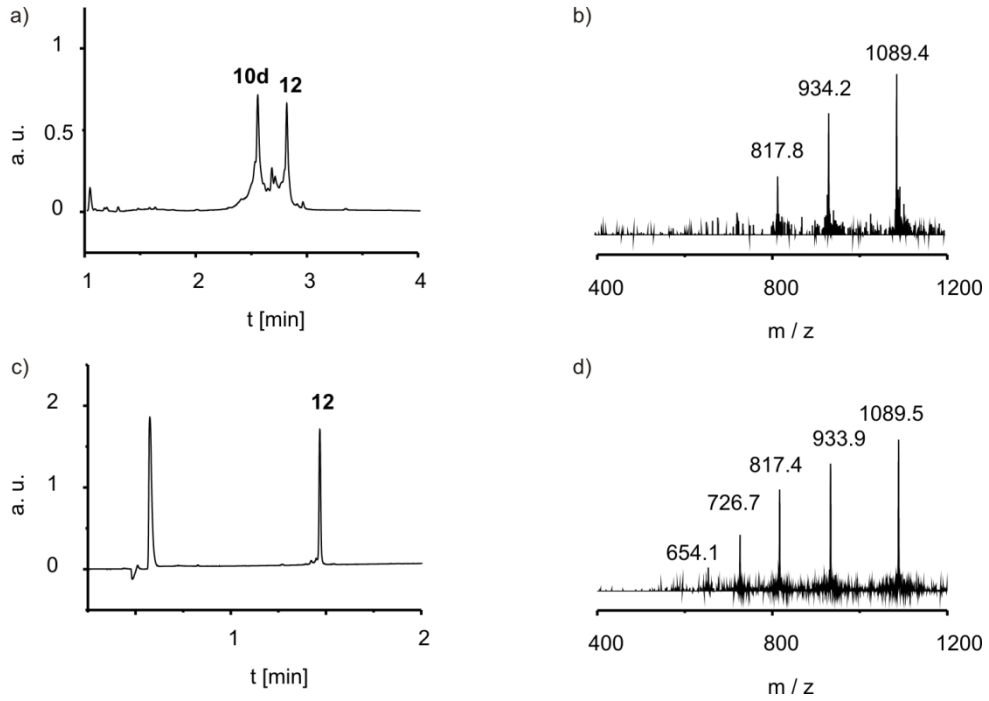


Figure S29: a) UPLC-MS trace of the desulfurization reaction after 1 h at 65°C, b) ESI-MS of the crude **12**, c) UPLC trace and d) ESI-MS of purified **12 (**ESI-MS (m/z): calcd. 1088.9 $[M+6H]^{6+}$, 933.5 $[M+7H]^{7+}$, 816.9 $[M+8]^{8+}$, 726.3 $[M+9H]^{9+}$, 653.7 $[M+10H]^{10+}$, found 1089.5 $[M+6H]^{6+}$, 933.9 $[M+7H]^{7+}$, 817.4 $[M+8]^{8+}$, 726.7 $[M+9H]^{9+}$, 654.1 $[M+10H]^{10+}$).