Supporting Information

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

J. Schmalisch and O. Seitz

Institut für Chemie, Humboldt-Universität zu Berlin, Brook-Taylor-Straße 2, D-12489 Berlin, Germany.
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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 % H₂O, 1 % acetonitrile, 0.1 % TFA
B: 98.9 % acetonitrile, 1 % H₂O, 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 % H₂O, 1 % acetonitrile, 0.1 % formic...
acid) and B (98.9 % acetonitrile, 1 % H2O, 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 % Ac₂O 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 CH₂Cl₂ before Boc cleavage.

Boc cleavage: 5 % m-cresol in TFA was added for 5 min. The resin was washed ten times with CH₂Cl₂ 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 CH₂Cl₂ 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 Et₂O and dissolved in H₂O/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 % Ac₂O 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-NH₂  1aCYG
The synthesis of X = Gly (1aG_CYG), Ala (1aA_CYG), Asn (1aN_CYG), Ser (1aS_CYG), Leu (1aCYG), Val (1aV_CYG), Pro (1aP_CYG) was performed by using 15 µmol resin-bound Fmoc-Gly.

1aG_CYG: 5 µmol (35 %), ε₂₇₈ = 1400 L/mol · cm, C₄₄H₇₁N₁₃O₁₃S₂, 1054.2 g/mol, tᵣ = 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<sub>CYG</sub>.

1aA<sub>CYG</sub>: 4 µmol (30 %), $\varepsilon_{278} = 1400 \text{ L/mol \cdot cm}$, C<sub>43</sub>H<sub>73</sub>N<sub>13</sub>O<sub>13</sub>S<sub>2</sub>, 1068.3 g/mol, $t_R = 1.4 \text{ min}$ (UPLC), ESI-MS (m/z): calcd. 1068.5 [M+H]<sup>+</sup>, 534.8 [M+2H]<sup>2+</sup>, found 1068.5 [M+H]<sup>+</sup>, 534.9 [M+2H]<sup>2+</sup>.

Figure S2: UPLC-trace (left) and ESI-MS (right) of purified 1aA<sub>CYG</sub>.

1aN<sub>CYG</sub>: 2 µmol (15 %), $\varepsilon_{278} = 1400 \text{ L/mol \cdot cm}$, C<sub>46</sub>H<sub>74</sub>N<sub>14</sub>O<sub>14</sub>S<sub>2</sub>, 1111.3 g/mol, $t_R = 1.3 \text{ min}$ (UPLC), ESI-MS (m/z): calcd. 1111.5 [M+H]<sup>+</sup>, 556.3 [M+2H]<sup>2+</sup>, found 1111.6 [M+H]<sup>+</sup>, 556.4 [M+2H]<sup>2+</sup>.

Figure S3: UPLC-trace (left) and ESI-MS (right) of purified 1aN<sub>CYG</sub>. 
1aS\textsubscript{CYG}: 3 µmol (20 %), \(\varepsilon_{278} = 1400 \text{ L/mol} \cdot \text{cm} \), C_{49}H_{73}N_{13}O_{14}S_{2}, 1084.3 g/mol, t\textsubscript{R} = 1.3 min (UPLC), ESI-MS (m/z): calcd. 1084.5 [M+H]\textsuperscript{+}, 542.7 [M+2H]\textsuperscript{2+}, found 1084.5 [M+H]\textsuperscript{+}, 542.9 [M+2H]\textsuperscript{2+}.

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

1a\textsubscript{CYG}: 3 µmol (20 %), \(\varepsilon_{278} = 1400 \text{ L/mol} \cdot \text{cm} \), C_{48}H_{79}N_{13}O_{13}S_{2}, 1110.4 g/mol, t\textsubscript{R} = 1.6 min (UPLC), ESI-MS (m/z): calcd. 1110.5 [M+H]\textsuperscript{+}, 555.8 [M+2H]\textsuperscript{2+}, found 1110.6 [M+H]\textsuperscript{+}, 555.9 [M+2H]\textsuperscript{2+}.

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

1aV\textsubscript{CYG}: 4 µmol (30 %), \(\varepsilon_{278} = 1400 \text{ L/mol} \cdot \text{cm} \), C_{47}H_{77}N_{13}O_{13}S_{2}, 1096.3 g/mol, t\textsubscript{R} = 1.5 min (UPLC), ESI-MS (m/z): calcd. 1096.5 [M+H]\textsuperscript{+}, 548.8 [M+2H]\textsuperscript{2+}, found 1096.5 [M+H]\textsuperscript{+}, 548.9 [M+2H]\textsuperscript{2+}.
1a\textsubscript{V\textsubscript{CYG}}: 2 \textmu mol (15 \%), \epsilon_{278} = 1400 \text{ L/mol} \cdot \text{cm}, C_{47}H_{75}N_{13}O_{13}S_{2}, 1094.3 \text{ g/mol}, t_{R} = 1.3 \text{ 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+}.

1a\textsubscript{P\textsubscript{CYG}}: 2 \textmu mol (15 \%), \epsilon_{278} = 1400 \text{ L/mol} \cdot \text{cm}, C_{47}H_{75}N_{13}O_{13}S_{2}, 1094.3 \text{ g/mol}, t_{R} = 1.3 \text{ 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+}.

H-RIGELX-MPA-AYG-NH\textsubscript{2} 1a\textsubscript{AYG}

The synthesis of X = Leu (1a\textsubscript{AYG}) was performed by using 10 \textmu mol resin-bound Fmoc-Gly.

1a\textsubscript{AYG}: 1.5 \textmu mol (15 \%), \epsilon_{278} = 1400 \text{ L/mol} \cdot \text{cm}, C_{48}H_{79}N_{13}O_{13}S, 1078.28 \text{ g/mol}, t_{R} = 1.4 \text{ 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+}.
The synthesis of $X = \text{Val}\ (1aV_{AYG})$ or $X = \text{Pro}\ (1aP_{AYG})$ was performed by using 7 µmol resin-bound Fmoc-Gly.

$1aV_{AYG}$: 1.8 µmol (26 %), $\varepsilon_{278} = 1400 \ L/mol \cdot cm$, C$_{47}$H$_{77}$N$_{13}$O$_{13}$S, 1064.26 g/mol, $t_R = 1.4 \text{ min (UPLC)}$, ESI-MS (m/z): calcd. 1177.6 [M+TFA]$^+$, found 1176.5 [M+TFA]$^+$.

$1aP_{AYG}$: 2.2 µmol (2.3 mg, 31 %), $\varepsilon_{278} = 1400 \ L/mol \cdot cm$, C$_{47}$H$_{75}$N$_{13}$O$_{13}$S, 1062.24 g/mol, $t_R = 1.4 \text{ min (UPLC)}$, 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 S8: UPLC-trace (left) and ESI-MS (right) of purified $1a_{AYG}$.

Figure S9: UPLC-trace (left) and ESI-MS (right) of purified $1a_{VAYG}$.

Figure S10: UPLC-trace (left) and ESI-MS (right) of purified $1a_{PAYG}$. 
H-RIGELX-MPA-CCCY-NH$_2$ 1a$_{CCCY}$

The synthesis of X = Gly (1a$_G_{CCCY}$), Ala (1a$_A_{CCCY}$), Asn (1a$_N_{CCCY}$) or Ser (1a$_S_{CCCY}$) was performed by using 10 µmol resin-bound Fmoc-Gly.

1a$_G_{CCCY}$: 900 nmol (15 %), $\varepsilon_{278} = 1400$ L/mol $\cdot$ 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 1a$_G_{CCCY}$](image1)

1a$_A_{CCCY}$: 1.0 µmol (17 %), $\varepsilon_{278} = 1400$ L/mol $\cdot$ 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 1a$_A_{CCCY}$](image2)

1a$_N_{CCCY}$: 550 nmol (10 %), $\varepsilon_{278} = 1400$ L/mol $\cdot$ 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]$^{2+}$.
**1aS_{CCCY}**: 660 nmol (9 %), $\varepsilon_{278} = 1400 \text{ L/mol} \cdot \text{cm}$, $C_{49}H_{80}N_{14}O_{15}S_{4}$, 1233.50 g/mol, $t_R = 1.4 \text{ min}$ (UPLC), ESI-MS (m/z): calcd. 1231.5 [2M+H]$^+$, 616.2 [2M+2H]$^{2+}$, found 1231.4 [2M+H]$^+$, 616.3 [2M+2H]$^{2+}$.

**1aL_{CCCY}**: 900 nmol (15 %), $\varepsilon_{278} = 1400 \text{ L/mol} \cdot \text{cm}$, $C_{52}H_{86}N_{14}O_{14}S_{4}$, 1259.58 g/mol, $t_R = 1.6 \text{ min}$ (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 S13: UPLC-trace (left) and ESI-MS (right) of purified 1aN_{CCCY}.

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

Figure S15: UPLC-trace (left) and ESI-MS (right) of purified 1aL_{CCCY}.
**H-YIGELX-MPA-G-NH₂ 1bG**

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

1bG: 3.2 µmol (32 %), \( \varepsilon_{278} = 1400 \text{ L/mol} \cdot \text{cm} \), \( C_{35}H_{54}N_{8}O_{11}S \), 794.92 g/mol, \( t_R = 1.8 \text{ min} \) (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 1bGₜ.

1bA: 3.2 µmol (32 %), \( \varepsilon_{278} = 1400 \text{ L/mol} \cdot \text{cm} \), \( C_{36}H_{56}N_{8}O_{11}S \), 808.94 g/mol, \( t_R = 1.9 \text{ min} \) (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 1bAₜ.

1bN: 1.9 µmol (20 %), \( \varepsilon_{278} = 1400 \text{ L/mol} \cdot \text{cm} \), \( C_{37}H_{57}N_{9}O_{12}S \), 851.97 g/mol, \( t_R = 1.7 \text{ min} \) (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⁺.
\textbf{1bSG}: 2.4 \mu mol (24 \%), \varepsilon_{278} = 1400 \text{ L/mol} \cdot \text{cm}, C_{36}H_{56}N_{8}O_{12}S, 824.94 \text{ g/mol}, t_R = 1.7 \text{ 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+}.}

\textbf{1bG}: 2.2 \mu mol (22 \%), \varepsilon_{278} = 1400 \text{ L/mol} \cdot \text{cm}, C_{39}H_{62}N_{8}O_{11}S, 851.02 \text{ g/mol}, t_R = 2.3 \text{ 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+}.}
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 NaH₂PO₄, 20 mM TCEP, pH 7.5, 2 mM thioester concentration) and the reaction was gently agitated at room temperature. 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: C₆₂H₁₀₈N₂₁O₁₉S, 1480.69 g/mol, tᵣ = 18.6 min (analyt. HPLC), ESI-MS (m/z): calcd. 1480.8 [M+H]⁺, found 1480.6 [M+H]⁺.

YIGELGCRAEYSK 3bG: C₆₅H₁₀₂N₁₈O₂₀S, 1487.68 g/mol, tᵣ = 20.2 min (analyt. HPLC), ESI-MS (m/z): calcd. 2972.4 [2M+H]⁺, found 2972.6 [2M+H]⁺.

RIGELACRAEYSK 3aA: C₆₃H₁₀₇N₂₁O₁₉S, 1494.72 g/mol, tᵣ = 18.9 min (analyt. HPLC), ESI-MS (m/z): calcd. 1494.8 [M+H]⁺, found 1494.6 [M+H]⁺.

YIGELACRAEYSK₂ 3bA: C₆₆H₁₀₄N₁₈O₂₀S, 1501.71 g/mol, tᵣ = 20. min (analyt. HPLC), ESI-MS (m/z): calcd. 3000.5 [2M+H]⁺, found 3000.9 [2M+H]⁺.

RIGELNCRAEYSK 3aN: C₆₄H₁₀₈N₂₂O₂₀S, 1537.72 g/mol, tᵣ = 18.8 min (analyt. HPLC), ESI-MS (m/z): calcd. 1537.8 [M+H]⁺, found 1538.0 [M+H]⁺.

YIGELNCRAEYSK 3bN: C₆₇H₁₀₅N₁₉O₁₉S, 1544.73 g/mol, tᵣ = 19.2 min (analyt. HPLC), ESI-MS (m/z): calcd. 1543.7 [2M+2H]²⁺, found 1543.9 [2M+2H]²⁺.

RIGELSCRAEYSK 3aS: C₆₃H₁₀₇N₂₁O₂₀S, 1510.72 g/mol, tᵣ = 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+.

RIGELL_CRAEYSK 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]^+.

YIGELL_CRAEYSK 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](image)

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

The ligation buffer (100 mM NaH2PO4, 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 concentration) and the reaction mixture was gently agitated at room temperature. 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

H-YKRLEKRG-MPA-CG-NH₂ 4

The synthesis was performed via Boc/Cbz-strategy by using 10 µmol resin-bound Fmoc-Gly. 1.8 µmol (18 %), ε₂₇₈ = 1400 L/mol · cm, C₅₄H₉₉N₁₉O₁₄S₂, 1296.56 g/mol, tᵣ = 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⁺.

H-CGTKFLSYKFSNSGRIT-MPA-G-NH₂ 5

The synthesis was performed via Boc/Cbz-strategy by using 20 µmol resin-bound Fmoc-Gly. 2.3 µmol (12 %), ε₂₇₈ = 1400 L/mol · cm, C₉₃H₁₄₆N₂₆O₂₈S₂, 2140.44 g/mol, tᵣ = 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⁺,
**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]$^{2+}$.

**First ligation:** The lyophilized peptides (40 nmol 4, 60 nmol 5) were dissolved in 12 µL degassed buffer (6 M GnHCl, 100 mM NaH$_2$PO$_4$, 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_{2}PO_{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-SATPTAVLYFAGEQPGDL-MPA-CG-NH₂ 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%), C₉₈H₁₄₉N₂₅O₃₃S₂, 2269.51 g/mol, $\varepsilon_{278} = 1400$ 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⁺.

**H-CFKKGDVITIIKKSQNDWWTGRTNGKEGIFPANYVRVS-NH₂ 10**

The synthesis was performed in a 10 µmol scale. The couplings of the pseudoproline dipeptides Fmoc-Trp(Boc)-Thr[$\Psi$(Me, Me)Pro]-OH and Fmoc-Lys(Boc)-Ser[$\Psi$(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 %), $\varepsilon_{278} = 12600$ L/mol · cm, C₂₀₄H₃₁₈N₅₈O₅₉S, 4559.1 g/mol, $t_R = 2.7$ min (UPLC-MS), ESI-MS
Ligation: The lyophilized peptides (150 nmol 9, 225 nmol 10) were dissolved in 75 µL degassed buffer (6 M GlnHCl, 100 mM NaH₂PO₄, 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%.

Desulfurization: After the ligation, the reaction mixture was diluted (1 mM peptide concentration) by adding the same amount of degassed desulfurization buffer (100 mM NaH₂PO₄, 480 mM TCEP, pH 7.3). The radical starter VA-044 (final concentration: 200 mM) and tBuSH (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, $\varepsilon_{278} = 140000 \text{ L/mol} \cdot \text{cm}$).
C_{294}H_{452}N_{80}O_{89}, 6531.2 \text{ g/mol}, t_R = 1.4 \text{ min (UPLC)}, \text{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+}, \text{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+}).