

Supporting Information

Fast protein modification in the nanomolar concentration range using an oxalyl amide as latent thioester

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1 General information and methods

1.1. Reagents for synthetic amino acid derivatives and peptide synthesis

1-[*bis*(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU), 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium fluorophosphates (HBTU) and *N*-Fmoc protected amino acids were obtained from Iris Biotech GmbH. Piperidine, diisopropylcarbodiimide (DIC), 4-Mercaptophenylacetic acid (97%, MPAA), 3-mercaptopropionic acid (MPA), tris(2-carboxyethyl)phosphine hydrochloride ($\geq 98\%$, TCEP·HCl), thiophenol, triisopropylsilane (TIS), guanidine hydrochloride ($\geq 99\%$, Gn·HCl), hydrochloric acid (reagent grade, 37%) and sodium hydroxide (pellets, 97%) as well as other reagents were purchased from Sigma-Aldrich, Acros Organics or Merck and were of the purest grade available. Peptide synthesis grade *N,N*-dimethylformamide (DMF), dichloromethane, diethyl ether, acetonitrile, heptane, LC-MS-grade acetonitrile (0.1% TFA), LC-MS grade water (0.1% TFA), *N,N*-diisopropylethylamine (DIEA), acetic anhydride (Ac₂O) and trifluoroacetic acid (TFA) were purchased from Biosolve and Fisher-Chemical. NovaSyn TGR solid support was purchased from Sigma-Aldrich. *Bis*(2-sulfenylethyl)amino trityl 1% divinylbenzene cross-linked polystyrene solid support (SEA PS) was produced on demand by Roowin. Solvents and reagents were used as received.

1.2. Instrumentation

The following instrumentation was used to purify and to characterize the amino acids and peptides synthesized in this work.

¹H and ¹³C NMR spectra were recorded on a Bruker Advance-300 spectrometer operating at 300 MHz and 75 MHz respectively. The spectra are reported as parts per million (ppm) down field shift using tetramethylsilane as internal reference. The data are reported as chemical shift (δ), multiplicity, relative integral, coupling constant (J Hz) and assignment where possible.

Analytical HPLC as well as micro preparative HPLC were performed with a Thermofisher system on a reverse phase column XBridge BEH300 C18 (3.5 μ m, 300 Å, 4.6 \times 150 mm) using a linear gradient of increasing concentration of eluent B in eluent A (eluent A: 0.1% by vol. of TFA in water; eluent B: 0.1% vol. of TFA in acetonitrile or acetonitrile/water: 4/1 v/v). The column eluant were monitored by UV at 215 nm or 280 nm. Preparative HPLC Waters or Gilson systems equipped with a C18 reverse-phase column and a UV detector at 215 nm were used for the purification of peptides on larger scale.

Purified products as well as reaction mixtures were characterized 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) using a linear gradient of increasing concentration of eluent B in eluent A. The column eluate was monitored by UV at 215 nm, evaporative light scattering (ELS, waters 2424) and electrospray ionization mass spectrometry (ESI-MS). Using similar eluants, analytical UPLC-MS analyses were performed on System Ultimate 3000 UPLC (Thermofisher) equipped with a column Acquity peptide BEH300C18, 1.7 μ m, 2.1 \times 100 mm (Waters), a diode array detector, a charged aerosol detector (CAD) and a mass spectrometer (Ion trap LCQfleet).

MALDI-TOF mass spectra were recorded with a Bruker Autoflex Speed using alpha cyano 4-hydroxycinnamic acid, sinapinic acid or 2,5-dihydroxybenzoic acid (DHB) as matrix. For HRMS analyses, this work has benefited from the facilities and expertise of « CUMA » (Université de Lille, France).

Optical rotations were recorded at 20 °C on a Perkin–Elmer 343 digital polarimeter at 589 nm with a cell path length of 10 cm.

The IR spectra were recorded on a Bruker FT-IR spectrometer ALPHA.

1.3. Amino acid purification and characterization

The Fmoc-Lys(^{oxo}SEA)-OH amino acid was prepared using classical instrumentation and methods used in synthetic organic chemistry. Structural characterization and purity were determined by ¹H and ¹³C NMR, MS, HRMS, IR and polarimetric analyses.

1.4. Peptide purifications and characterizations

The peptides were purified by reversed-phase HPLC and the fractions containing the target peptide were then combined, frozen, and lyophilized. Synthesized peptides were characterized by LC-MS and MALDI-TOF analyses. Sequence or disulfide bridge pattern of key peptides were confirmed using MALDI-TOF MS-MS analysis or proteomic analysis (enzymatic digestion followed by MS characterization of the fragments). For more information about the instrumentation used, see the corresponding section.

2. Synthesis of Fmoc-Lys(^{oxo}SEA)-OH derivative **1**

2.1. General synthetic scheme

Fmoc-protected Lys(^{oxo}SEA)-OH amino acid **1** was synthesized in 6 steps from commercial Boc-Lys(2-CIZ)-OH **14** as described in Figure S1. The *bis*(2-tritylsulfanylethyl)amine **11** was prepared as described previously by reacting *bis*(2-chloroethyl)amine hydrochloride with triphenylmethyl mercaptan in the presence of DBU.¹ Both *bis*(2-chloroethyl)amine hydrochloride and triphenylmethyl mercaptan are commercially available. In brief, the carboxyl group of Boc-Lys(2-CIZ)-OH **11** was first protected as a *tert*butyl ester and the 2-chloro-benzyl carbamate sidechain protecting group was removed by hydrogenation to yield known intermediate **15**. Coupling of the oxoSEA moiety on lysine sidechain was achieved using classical enthalpic activation protocols to provide **16**. After I₂-promoted thiol deprotection and oxidation of the SEA group to form **17**, the Boc carbamate and the O-*t*Bu ester were removed at once upon exposure to HCl in dioxane. The resulting free aminoacid **18** was finally reprotected as a Fmoc group on its N^α position to yield the targeted amino acid **1**.

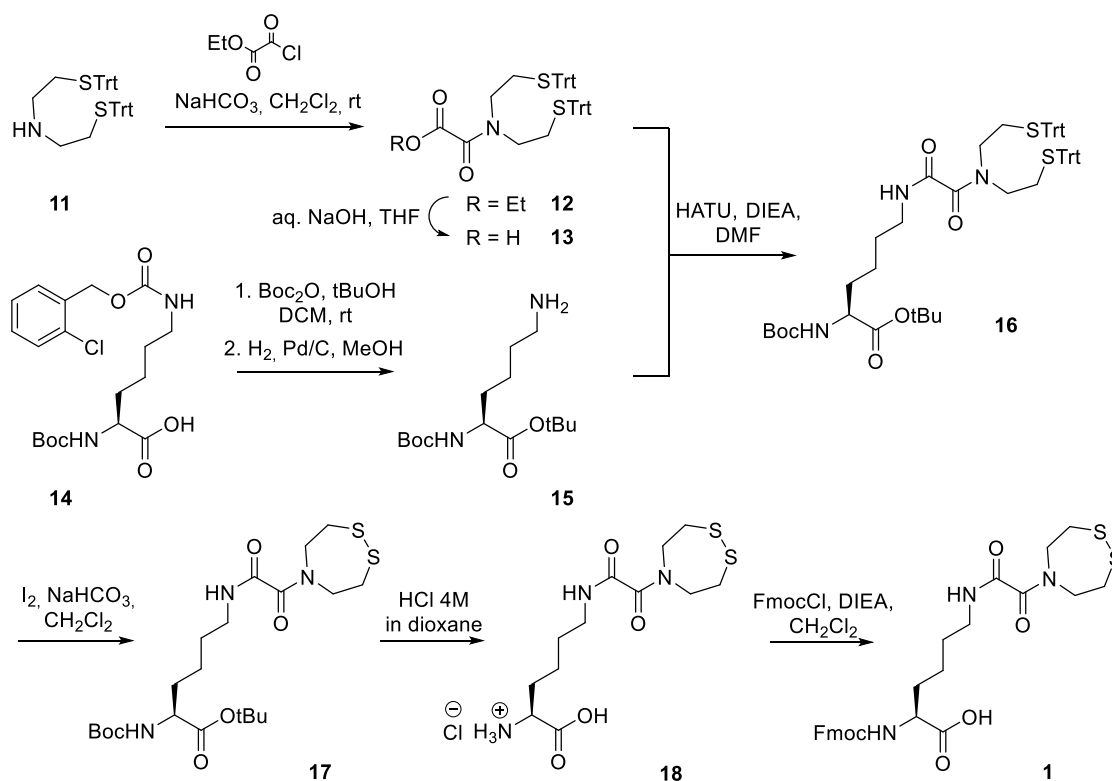


Figure S1. General synthetic scheme for the preparation of Fmoc-Lys(^{oxo}SEA)-OH **1** in 6 steps from commercially available Boc-Lys(2CIZ)-OH **14**.

¹ OL SEA 2010

2.2. Protocols

Ethyl 2-(bis(2-((triphenylmethyl)sulfonyl)ethyl)amino)-2-oxoacetate **12**

To a solution of *bis*[(2-(triphenylmethyl)sulfonyl)ethyl]amine **11** (6.22 g, 10 mmol) in CH₂Cl₂ (100 mL) was added sodium bicarbonate NaHCO₃ (1.69 g, 20 mmol, 20 equiv.). The resulting suspension was cooled to 0 °C under argon and ethyl chlorooxoacetate (2.1 mL, 19 mmol, 1.9 equiv) was added dropwise. The ice bath was removed and the reaction was left to proceed at rt. TLC monitoring indicated complete consumption of the starting material after 2 h. Water (50 mL) was added. The aqueous layer was extracted with CH₂Cl₂ (2 × 20 mL). The organic extracts were then combined and washed with water (20 mL), with a saturated bicarbonate aqueous solution (20 mL) and brine (20 mL). The organic layer was dried over MgSO₄ and concentrated to dryness under reduced pressure to afford a white amorphous solid (6.63 g, 93 %). The resulting compound **12** was used in the next step without further purification.

R_f 0.65 in EtOAc/cyclohexane (20:80). **Mp** (°C) 76. **¹H NMR** (300 MHz, CDCl₃, Figure S2) δ 7.20-7.41 (m, 30H, H_{Ar}), 4.24 (q, *J* = 7.2 Hz, 2H), 2.89 (t, *J* = 7.8 Hz, 2H), 2.78 (t, *J* = 7.8 Hz, 2H), 2.24-2.34 (m, 4H), 1.29 (t, *J* = 7.2 Hz, 3H). **¹³C APT NMR** (75 MHz, CDCl₃, Figure S3) δ 162.2 (Cq), 161.3 (Cq), 144.5 (Cq), 144.3 (Cq), 129.5 (CH), 129.4 (CH), 128.0 (CH), 127.9 (CH), 126.9 (CH), 126.7 (CH), 67.0 (Cq), 66.9 (Cq), 62.1 (CH₂), 47.5 (CH₂), 44.6 (CH₂), 30.5 (CH₂), 29.0 (CH₂), 13.9 (CH). **IR** (ATR, cm⁻¹) 3054, 2925, 1732, 1659, 1487, 1442, 1150. **MALDI-TOF** (matrix 2,5-dihydroxybenzoic acid, positive detection mode) Calcd. for [M+H]⁺ (monoisotopic) 721.3, found 721.3.

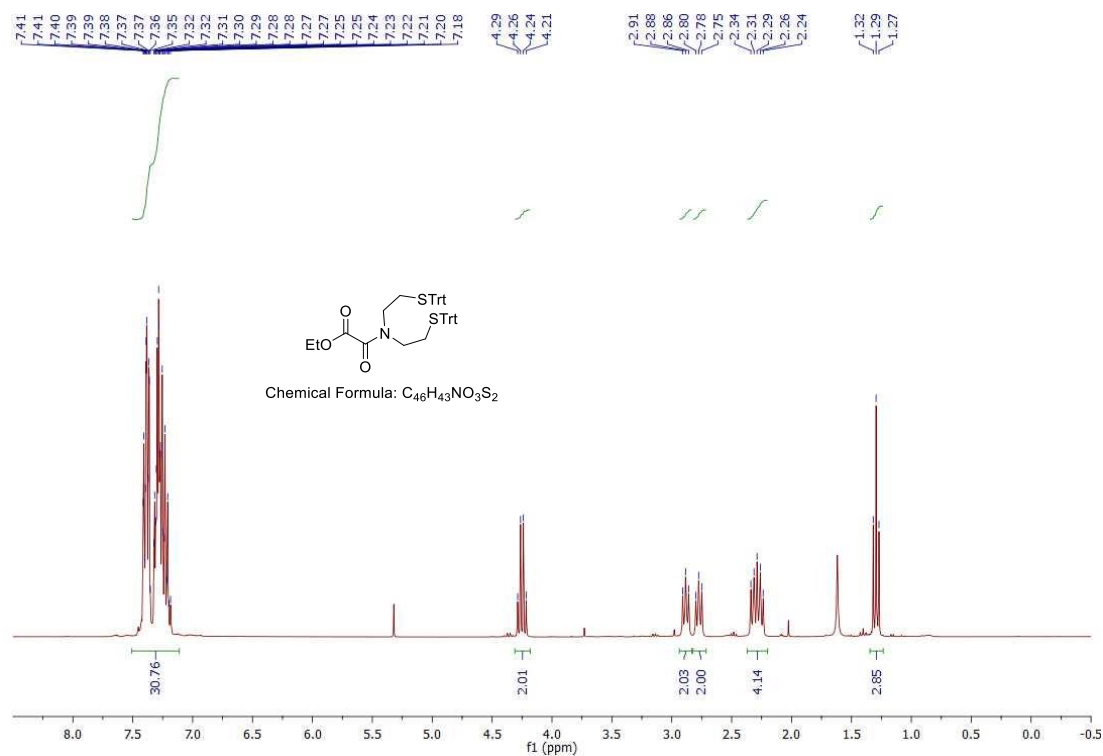


Figure S2. ¹H NMR (300 MHz) spectrum of compound **12** (CDCl₃, 298 K).

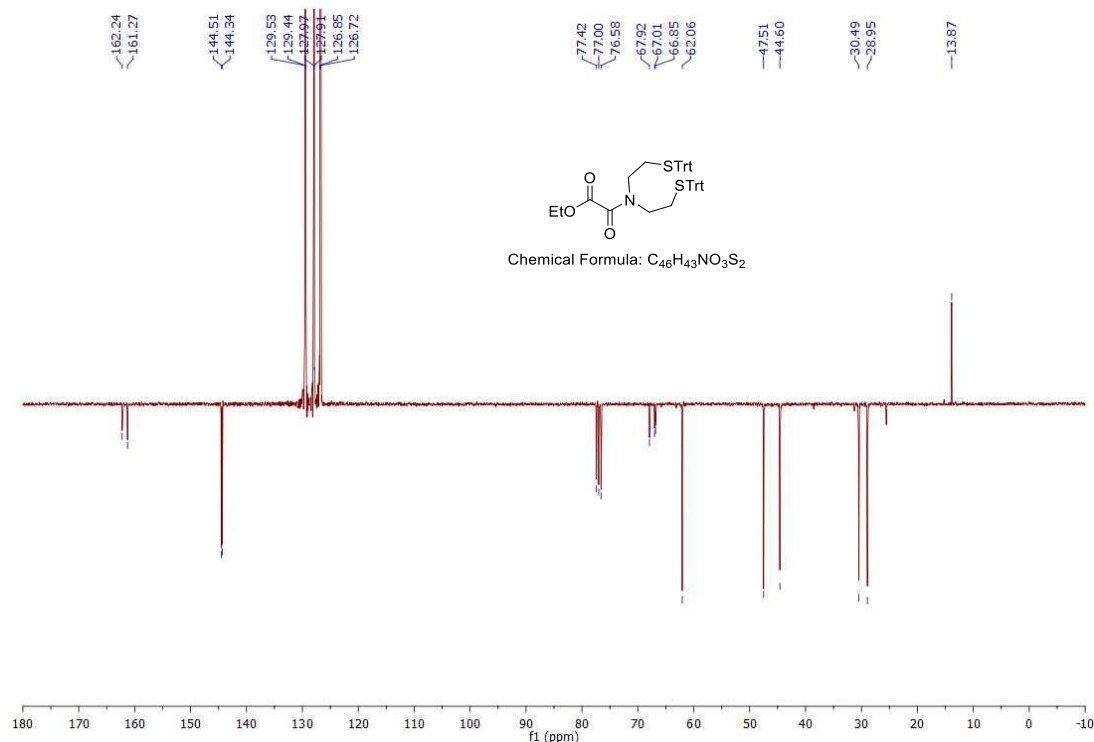


Figure S3. ^{13}C APT NMR (75 MHz) spectrum of compound **12** (CDCl_3 , 298 K).

2-(bis(2-((triphenylmethyl)sulfonyl)ethyl)amino)-2-oxoacetic acid **13**

To a solution of intermediate **12** (5.26 g, 7.3 mmol) in THF (100 mL) was added an aqueous solution of sodium hydroxide (0.43 M, 100 mL, 43 mmol, 5.9 equiv.). The resulting solution was stirred at rt until complete conversion of starting material (generally about 4-5 h according to TLC). The crude mixture was acidified to pH 2 by addition of 1 M HCl. Layers were separated. The aqueous layer was extracted with ethyl acetate (3×50 mL). The organic extracts were combined, washed with water (3×20 mL), brine (20 mL), dried over MgSO_4 and evaporated under reduced pressure to provide oxalic acid derivative **13** as a powdery white solid (4.46 g, 88 %).

Mp ($^\circ\text{C}$) 172. ^1H NMR (300 MHz, CDCl_3 , Figure S4) δ 7.16-7.37 (m, 30H, H_{Ar}), 3.36 (t, $J = 7.2$ Hz, 2H), 2.84 (q, $J = 7.2$ Hz, 2H), 2.31 (t, $J = 7.2$ Hz, 4H). ^{13}C APT NMR (75 MHz, CDCl_3 , Figure S5) δ 144.6 (Cq), 144.5 (Cq), 129.6 (CH), 129.5 (CH), 127.93 (CH), 127.91 (CH), 126.7 (CH), 66.8 (CH_2), 48.7 (CH_2), 30.8 (CH_2), 28.4 (CH_2). **IR** (ATR, cm^{-1}) above 2200 (large band, low intensity), 1660, 1594, 1486, 1440.

Acid **13** decomposes during mass analysis (ESI or MALDI-TOF).

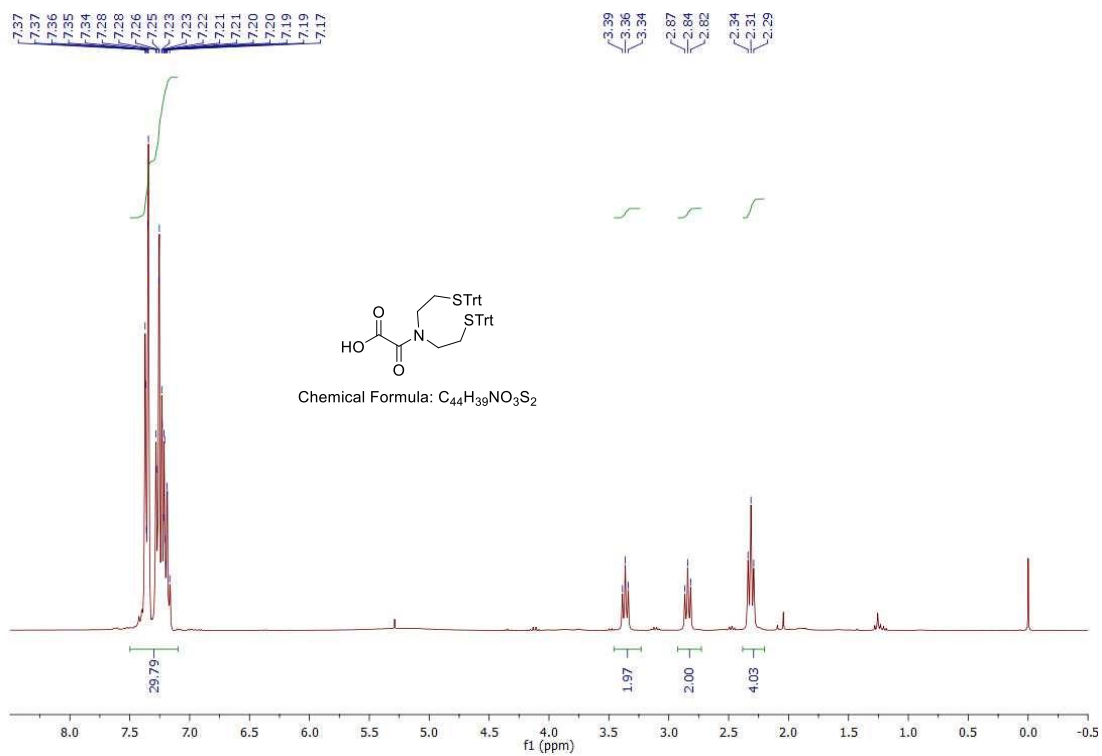


Figure S4. 1H NMR (300 MHz) spectrum of compound **13** ($CDCl_3$, 298 K).

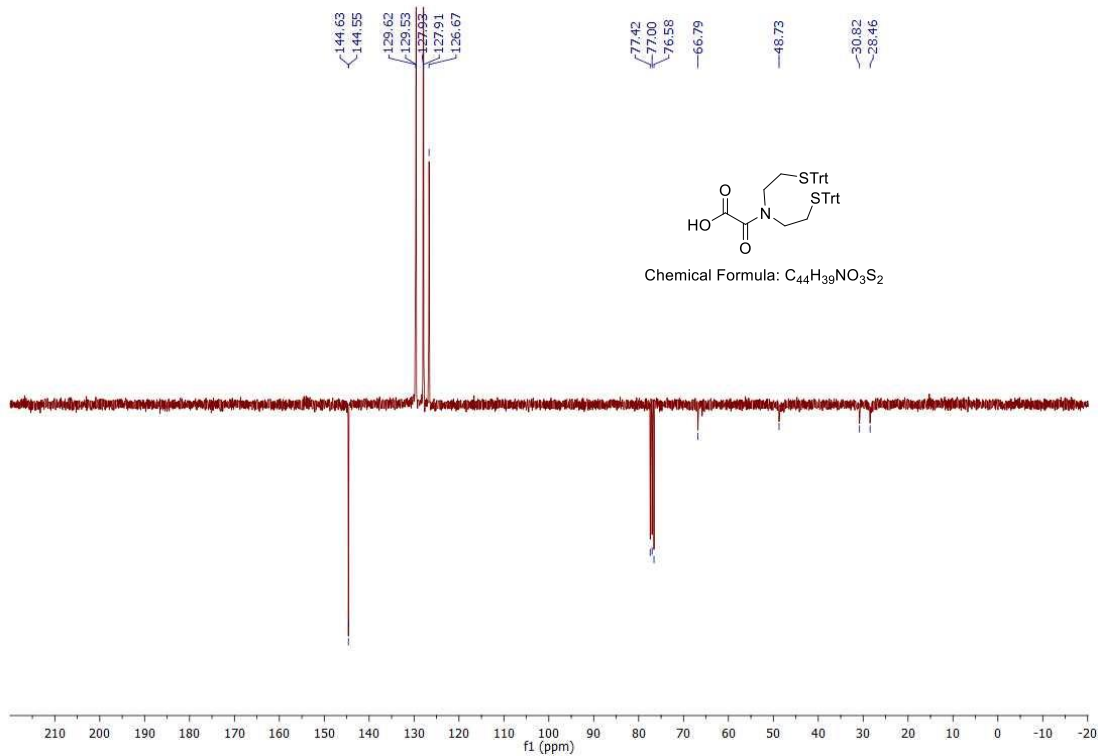


Figure S5. ^{13}C APT NMR (75 MHz) spectrum of compound **13** ($CDCl_3$, 298 K).

Boc-Lys-O^tBu intermediate **15** was prepared in two steps from commercial Boc-Lys(2-Cl-Z)-OH **14** (CAS: 54613-99-9) according to reported protocols.^{2,3}

Boc-Lys(2-ClZ)-O^tBu intermediate

Commercial Boc-Lys(2-ClZ)-OH **14** (6.5 g, 15.7 mmol) was dissolved in a *tert*-butanol/CH₂Cl₂ mixture (3:1, 50 mL). To this solution were successively added Boc₂O (4.8 g, 22.04 mmol, 1.4 equiv.) and DMAP (600 mg, 3.75 mmol, 0.24 equiv.) and the reaction was stirred at rt overnight. The crude mixture was evaporated under reduced pressure and the residue was purified by silica gel column chromatography to afford the *tert*butyl ester as a white solid (6.72 g, 91%).

R_f 0.4 in EtOAc/cyclohexane (25:75). **Mp** (°C) 64. **¹H NMR** (300 MHz, CDCl₃, Figure S6) δ 7.36-7.43 (m, 2H), 7.24-7.27 (m, 2H), 5.21 (s, 2H), 5.07 (d, *J* = 6.7 Hz, 1H), 4.93 (m, 1H), 4.14 (m, 1H), 3.20 (q, *J* = 6.7 Hz, 2H) 1.19-1.76 (m, 35H, contaminated with residual *tert*-butanol). **¹³C NMR** (75 MHz, CDCl₃, Figure S7) δ 171.9 (Cq), 156.2 (Cq), 155.5 (Cq), 134.3 (Cq), 133.5 (Cq), 129.7 (CH), 129.4 (CH), 129.3 (CH), 126.8 (CH), 81.8 (Cq), 79.7 (Cq), 63.8 (CH₂), 53.6 (CH), 40.8 (CH₂), 32.6 (CH₂), 29.3 (CH₂), 28.3 (CH₃), 28.0 (CH₃), 26.9 (CH₂), 22.3 (CH₂). **IR** (ATR, cm⁻¹) 3322, 2978, 2929, 2860, 1686 (shoulder), 1599, 1531, 1348, 1282, 1244. **ESI** (positive detection mode, Figure S8): [M+Na]⁺ *m/z* calcd. (monoisotopic mass) 493.2, found 493.3. **[α]_D²⁰** (c 1.0, CH₃OH): + 13.2 °.

² Shetty, D.; Jeong, J. M.; Ju, C. H.; Kim, Y. J.; Lee, J.-Y.; Lee, Y.-S.; Lee, D. S.; Chung, J.-K.; Lee, M. C. Synthesis and evaluation of macrocyclic amino acid derivatives for tumor imaging by gallium-68 positron emission tomography. *Bioorg. Med. Chem.* **2010**, *18*, 7338-7347.

³ MacKenzie, D. A.; Sherratt, A. R.; Chigrinova, M.; Kell, A. J.; Pezacki, J. P. Bioorthogonal labelling of living bacteria using unnatural amino acids containing nitron derivative of vancomycin. *Chem. Commun.* **2015**, *51*, 12501-12504.

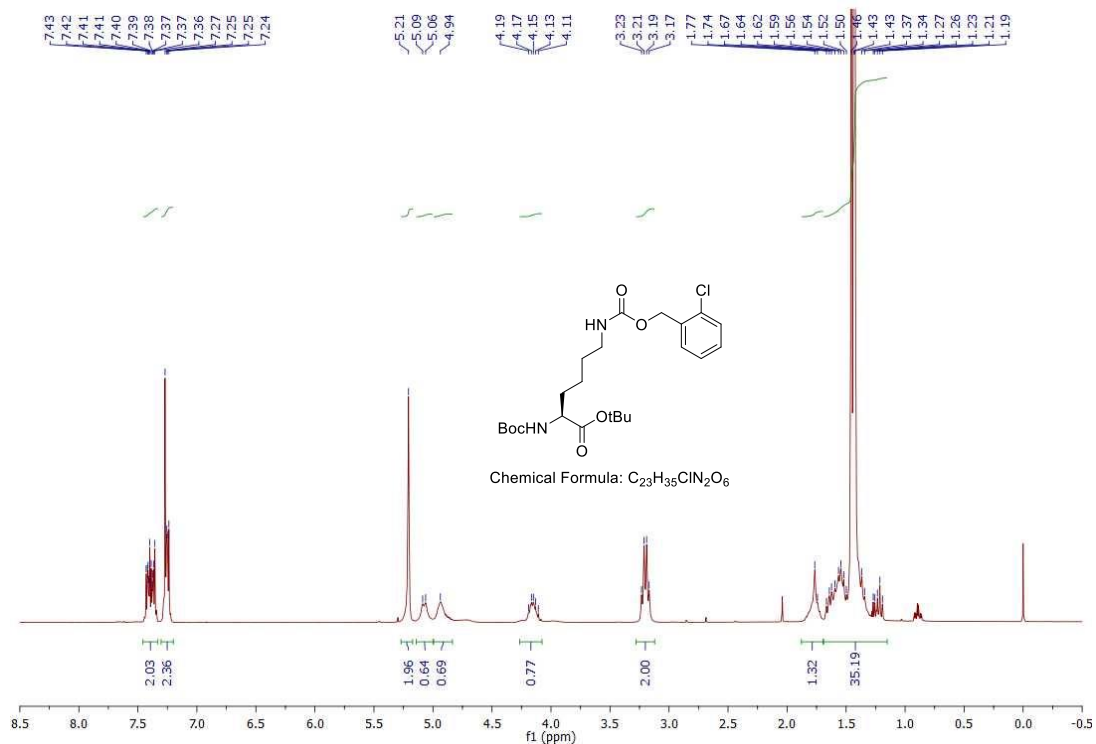


Figure S6. 1H NMR (300 MHz) spectrum ($CDCl_3$, 298 K).

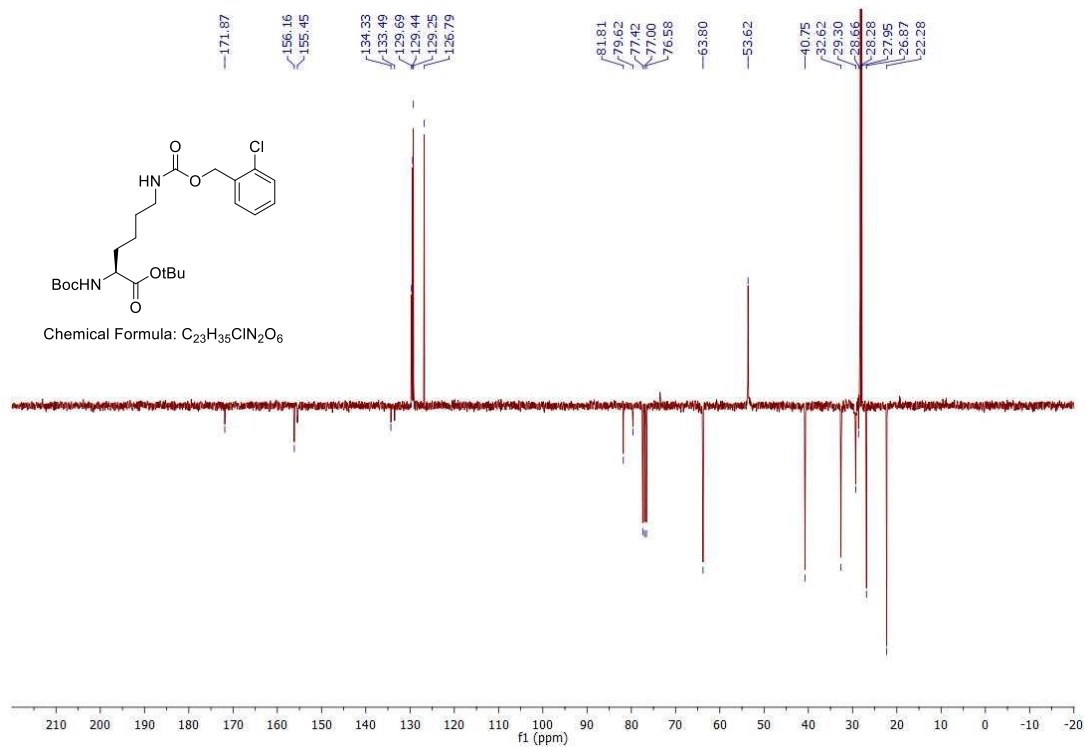


Figure S7. ^{13}C APT NMR (75 MHz) spectrum ($CDCl_3$, 298 K).

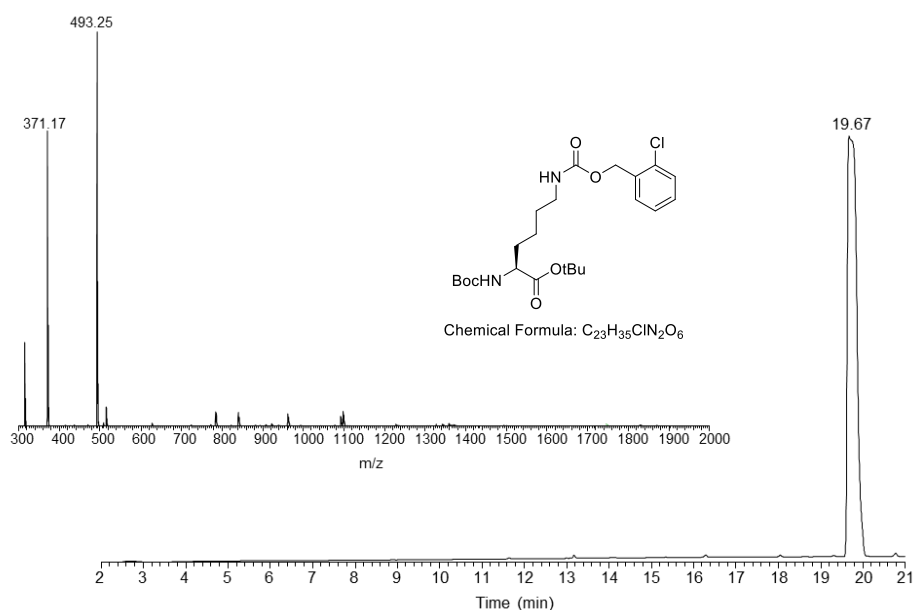


Figure S8. UPLC-MS analysis. a) LC trace. Eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in CH₃CN, ACQUITY UPLC Peptide BEH 300 Å 1.7 μm (2.1 × 150 mm) column, 50 °C, gradient 0-70% B in 20 min, 0.4 mL/min, UV detection at 215 nm. b) MS trace. Calcd. for [M+Na]⁺ (monoisotopic): 493.2, found: 493.3.

Boc-Lys-O^tBu intermediate 15

The above intermediate (6.37 g, 13.5 mmol) was dissolved in MeOH (100 mL) and Pd/C (0.45 g, 7 mass %) was added in small portions to the solution. The round bottom flask was closed, purged three times (vacuum/hydrogen gas cycles). The reaction was stirred overnight. After completion, the crude mixture was filtered through a plug of Celite®. The filtrate was concentrated under reduced pressure to afford compound **15** as a colorless glass (4.05 g, quantitative).

¹H NMR (300 MHz, CDCl₃, Figure S9) δ 5.19 and 6.12 (m, 1H), 3.90 and 4.12 (m, 1H), 3.00 (m, 2H), 1.44-2.01 (m, 24H). (¹H NMR of Boc-Lys-O^tBu **15** is poorly resolved likely due to the presence of conformers). ¹³C APT NMR (75 MHz, CDCl₃, Figure S10) δ 171.8 (Cq), 155.5 (Cq), 82.0 (Cq), 79.8 (Cq), 53.8 (CH), 39.8 (CH₂), 32.4 (CH₂), 28.3 (CH₃), 28.0 (CH₃), 27.3 (CH₂), 22.5 (CH₂). IR (ATR, cm⁻¹) 2976, 2933, 2864, 1710 (shoulders), 1508, 1365, 1149. MS (ESI, positive detection mode, Figure S 11): [M+H]⁺ m/z calcd. (monoisotopic mass) 303.2, found 302.9. [α]_D²⁰ (c 1.0, CH₃OH): + 9.2 °.

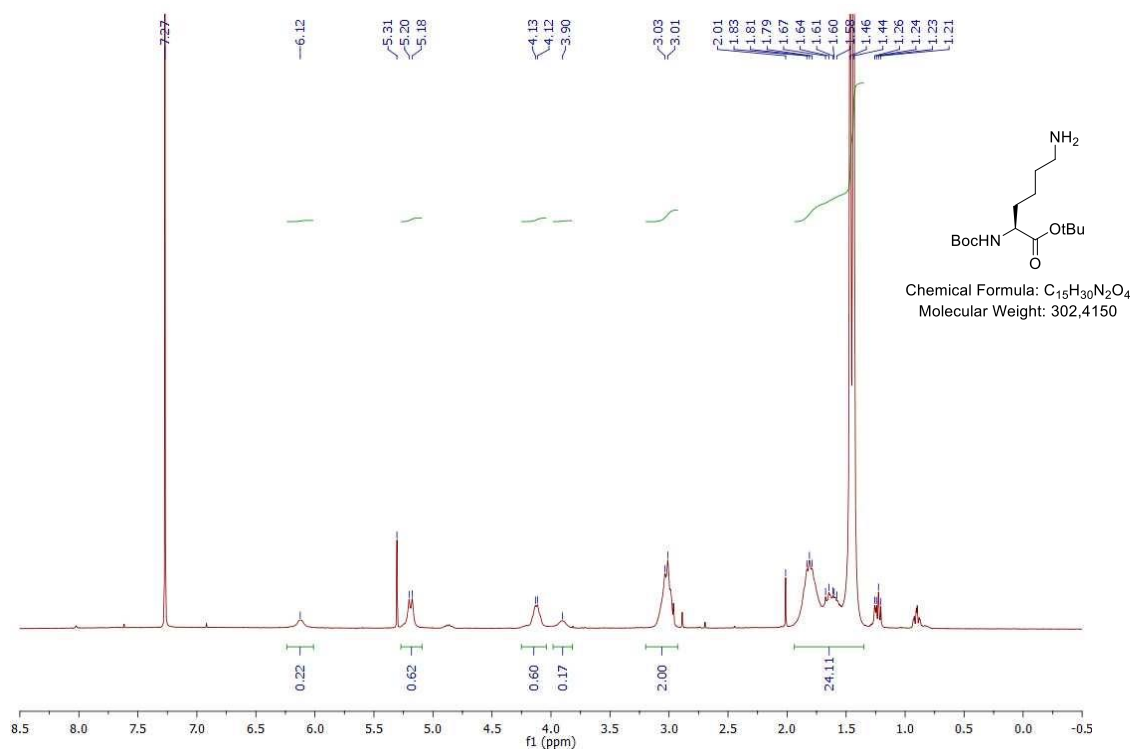


Figure S9. 1H NMR (300 MHz) spectrum of compound **15** ($CDCl_3$, 298 K).

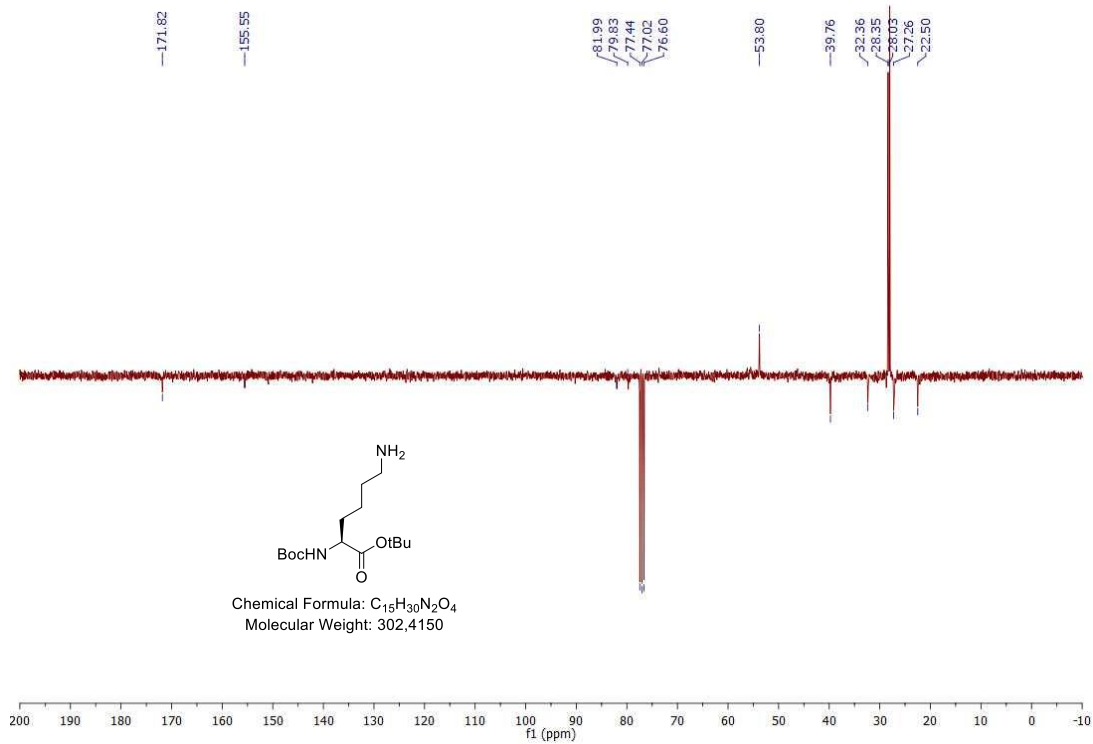


Figure S10. ^{13}C APT NMR (75 MHz) spectrum of compound **15** ($CDCl_3$, 298 K).

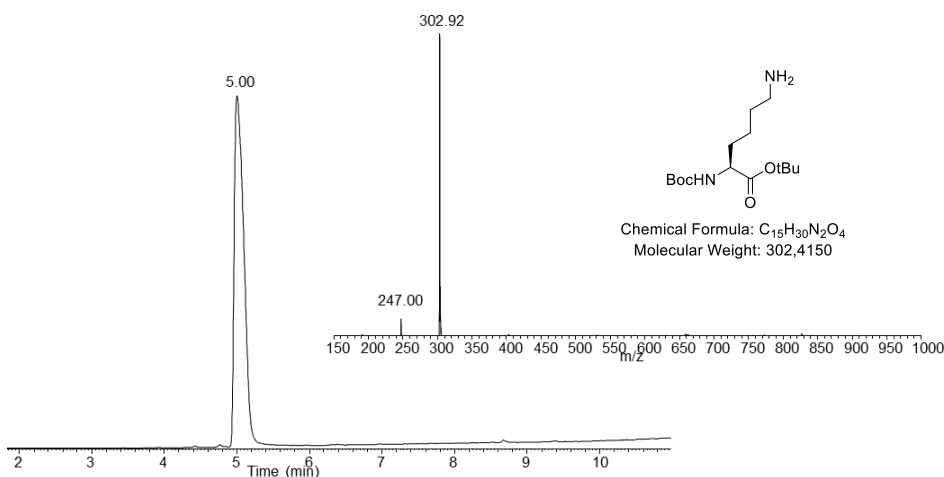


Figure S 11. UPLC-MS analysis of compound **15**. a) LC trace. Eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in CH₃CN, ACQUITY UPLC Peptide BEH 300 Å 1.7 μm (2.1 × 150 mm) column, 50 °C, gradient 0-50% B in 15 min, 0.4 mL/min, UV detection at 215 nm. b) MS trace. Calcd. for [M+H]⁺ (monoisotopic): 303.2, found: 302.9.

Boc-Lys(^{oxo}SEAdiTrt)-O'Bu intermediate **16**

The coupling reaction of the oxalic acid derivative **13** involves highly reactive species which might decompose upon prolonged activation times. Therefore, all the reagents were predissolved in dry DMF as follows:

- Solution 1: acid **13** (2.0 g, 2.88 mmol, 1.03 equiv.) and *N,N*-diisopropylethylamine (1.33 mL, 7.66 mmol, 2.75 equiv.) were dissolved in DMF (9 mL).
- Solution 2: HATU (1.057 g, 2.79 mmol, 1 equiv.) was dissolved in DMF (4.5 mL)
- Solution 3: Boc-Lys-O'Bu intermediate **15** (843 mg, 2.79 mmol) was dissolved in DMF (4.5 mL)

Activation of the acid was performed by adding solution 2 to solution 1. The mixture was stirred 25 sec at room temperature (turns orange) and was then immediately added to solution 3. After 1 h at room temperature, the crude was analyzed by TLC showing complete consumption of the starting materials.

An aqueous saturated solution of ammonium chloride (50 mL) and EtOAc (50 mL) were added to the reaction. The two layers were separated. The aqueous layer was extracted with EtOAc (2 × 20 mL). The organic extracts were combined, washed with saturated NH₄Cl (6 × 20 mL), dried over MgSO₄ and concentrated under reduced pressure. The crude mixture was purified by silica gel column chromatography (eluent Cyclohexane / EtOAc 70:30) to yield compound **16** as a white solid (2.51 g, 92%).

R_f 0.31 in EtOAc/cyclohexane (30:70). **Mp** (°C) 77. **¹H NMR** (300 MHz, CDCl₃, Figure S12) δ 7.08-7.31 (m, 31H), 4.94 (d, *J* = 8.1 Hz, 1H), 4.08 (m, 1H), 3.27 (t, *J* = 6.9 Hz, 2H), 3.11 (q, *J* = 6.9 Hz, 2H), 2.74 (t, *J* = 6.9 Hz, 2H), 2.21 (m, 4H), 1.11-1.68 (m, 25H). **¹³C NMR** (75 MHz, CDCl₃, Figure S13) δ 171.9, 161.6, 160.6, 155.6, 144.6, 129.6, 127.9, 126.8, 81.8, 79.7, 66.7, 53.7, 47.9, 47.2, 39.1, 32.6, 31.5, 29.7, 29.0, 28.9, 28.3, 28.0, 22.6. **IR** (ATR, cm⁻¹) 3329, 2973, 2929, 2864, 1712 (shoulder), 1683, 1488, 1442, 1364, 1150. **MALDI-TOF** (matrix 2,5-dihydroxybenzoic acid, positive detection mode) Calcd. for [M+Na]⁺ (monoisotopic) 1000.4, found 1000.5. **MS** (ESI, positive detection mode, Figure S14): [M+Na]⁺ *m/z* calcd. (monoisotopic mass) 1000.4, found 1000.5. **HRMS** (ES⁺) Calcd. for C₅₉H₆₈N₃O₆S₂: 978.4544, found: 978.4526 (δ_{ppm} -1.81). **[α]_D²⁰** (c 1.0, CH₃OH): + 5.1 °.

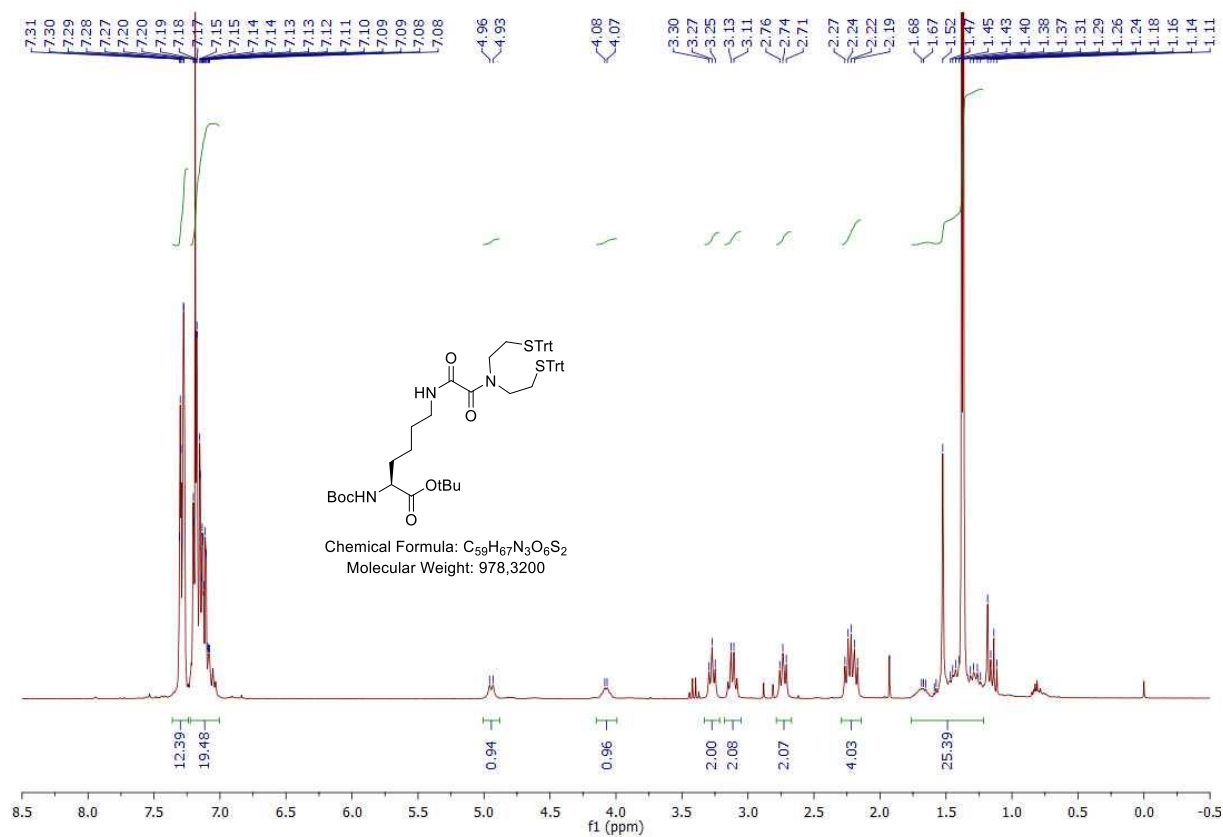


Figure S12. 1H NMR (300 MHz) spectrum of compound **16** ($CDCl_3$, 298 K).

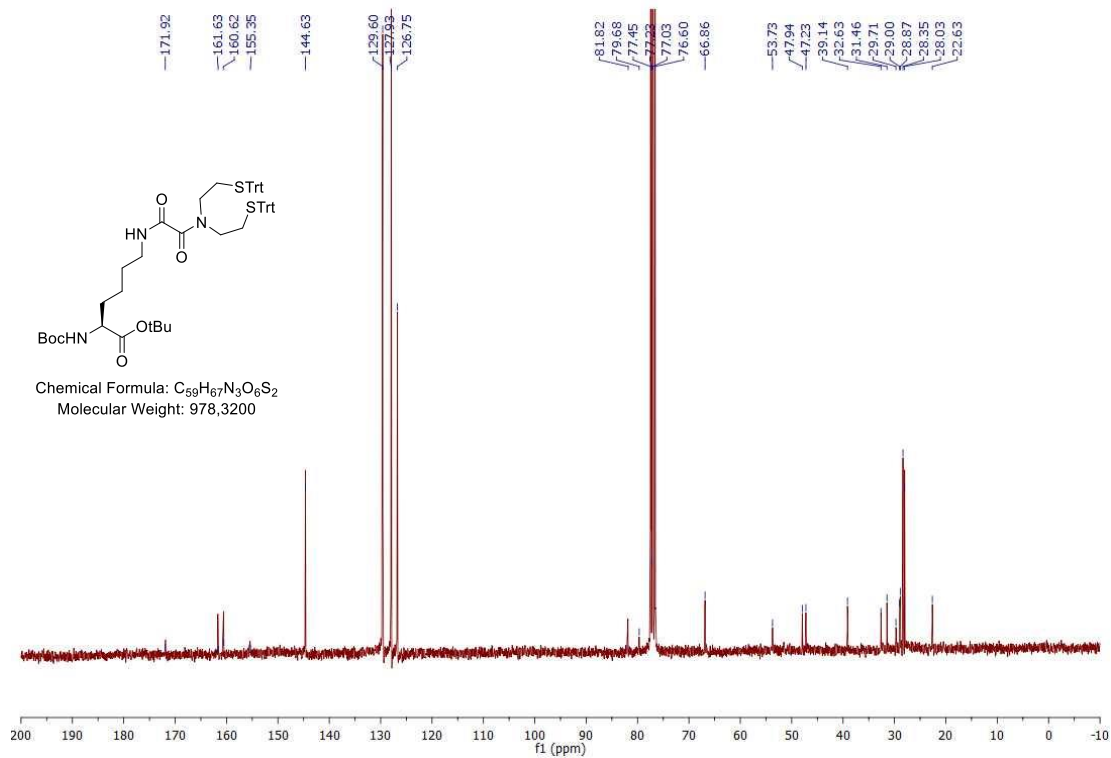


Figure S13. ^{13}C NMR (75 MHz) spectrum of compound **16** ($CDCl_3$, 298 K).

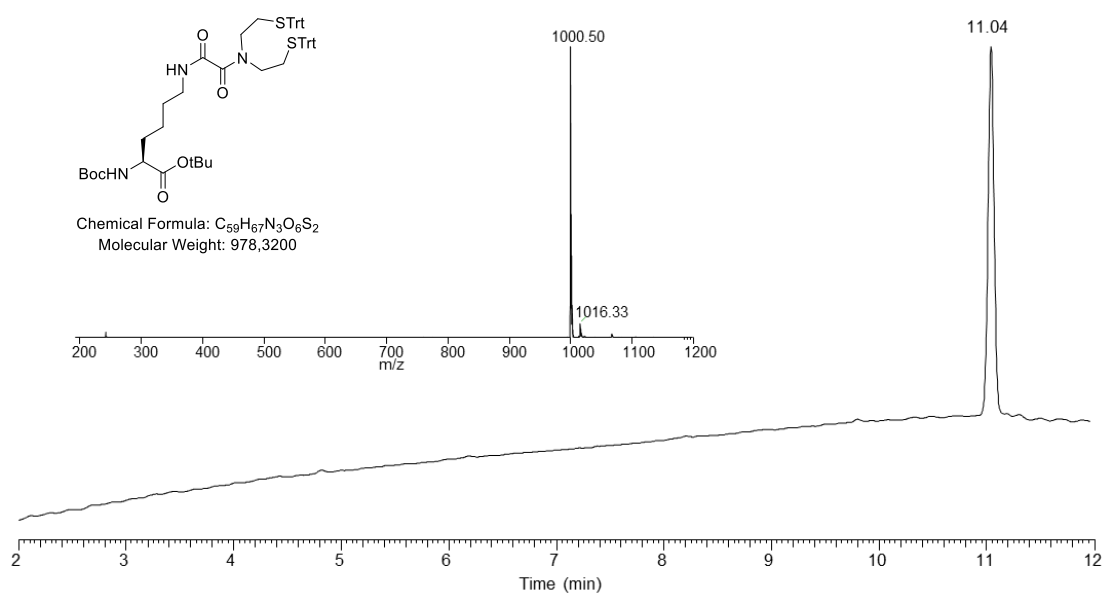


Figure S14. UPLC-MS analysis of compound **16**. a) LC trace. Eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in CH₃CN, ACQUITY UPLC Peptide BEH 300 Å 1.7 μm (2.1 × 150 mm) column, 50 °C, gradient 0-50% B in 15 min, 0.4 mL/min, UV detection at 215 nm. b) MS trace. Calcd. for [M+Na]⁺ (monoisotopic): 1000.4, found: 1000.5.

Boc-Lys(^{oxo}SEA)-O^tBu intermediate **17**

To a solution of intermediate **16** (5.16 g, 5.27 mmol) in CH₂Cl₂ (140 mL) was added sodium bicarbonate NaHCO₃ (1.33 g, 15.9 mmol, 3 equiv.). To the resulting suspension was added a solution of iodine (4.01 g, 15.9 mmol, 3 equiv.) in CH₂Cl₂ (140 mL) at rt. Completion of the reaction was checked by TLC after 30 min. An aqueous solution of sodium bisulfite (1 M, 200 mL, 200 mmol) was poured in the crude mixture resulting in a colorless biphasic mixture. Layers were separated. The aqueous layer was extracted with CH₂Cl₂ (3 * 30 mL). The organic extracts were combined, washed with water (50 mL) and brine (50 mL), dried over MgSO₄ and concentrated under reduced pressure. Note that the crude reaction was not evaporated to dryness (ideally to a ~10-15 mL remaining volume or until the remaining solution starts to turn yellowish). The crude mixture was purified by silica gel column chromatography (eluent Cyclohexane / EtOAc / CH₂Cl₂ 70:30:10 to 60:40:0) to yield compound **17** as a colorless glass (2.04 g, 79%).

R_f 0.17 in EtOAc/cyclohexane (30:70). **¹H NMR** (300 MHz, CDCl₃, Figure S15) δ 7.46 (m, 1H), 5.05 (d, *J* = 8.1 Hz, 1H), 4.28 (m, 2H), 4.11 (m, 1H), 3.96 (t, *J* = 7.2 Hz, 2H), 3.24 (q, *J* = 7.2 Hz, 2H), 3.04 (m, 4H), 1.19-1.62 (m, 24H). **¹³C APT NMR** (75 MHz, CDCl₃, Figure S16) δ 171.8 (Cq), 161.8 (Cq), 160.4 (Cq), 155.4 (Cq), 81.8 (Cq), 79.6 (Cq), 54.2 (CH₂), 53.7 (CH), 52.1 (CH₂), 39.7 (CH₂), 39.2 (CH₂), 34.3 (CH₂), 32.5 (CH₂), 28.9 (CH₂), 28.3 (CH₃), 28.0 (CH₃), 22.6 (CH₂). **IR** (ATR, cm⁻¹) 3338, 2975, 2929, 2864, 1708 (shoulder), 1685, 1363, 1148. **MALDI-TOF** (matrix 2,5-dihydroxybenzoic acid, positive detection mode) Calcd. for [M+Na]⁺ (monoisotopic) 514.2, found 514.2. **MS** (ESI, positive detection mode, Figure S17): [M+Na]⁺ *m/z* calcd. (monoisotopic mass) 514.2, found 514.2. **HRMS** (ES⁺) Calcd. for C₂₁H₃₈N₃O₆S₂: 492.2197, found: 492.2188 (δ_{ppm} -1.84). **[α]_D²⁰** (c 1.0, CHCl₃): +14.9 °.

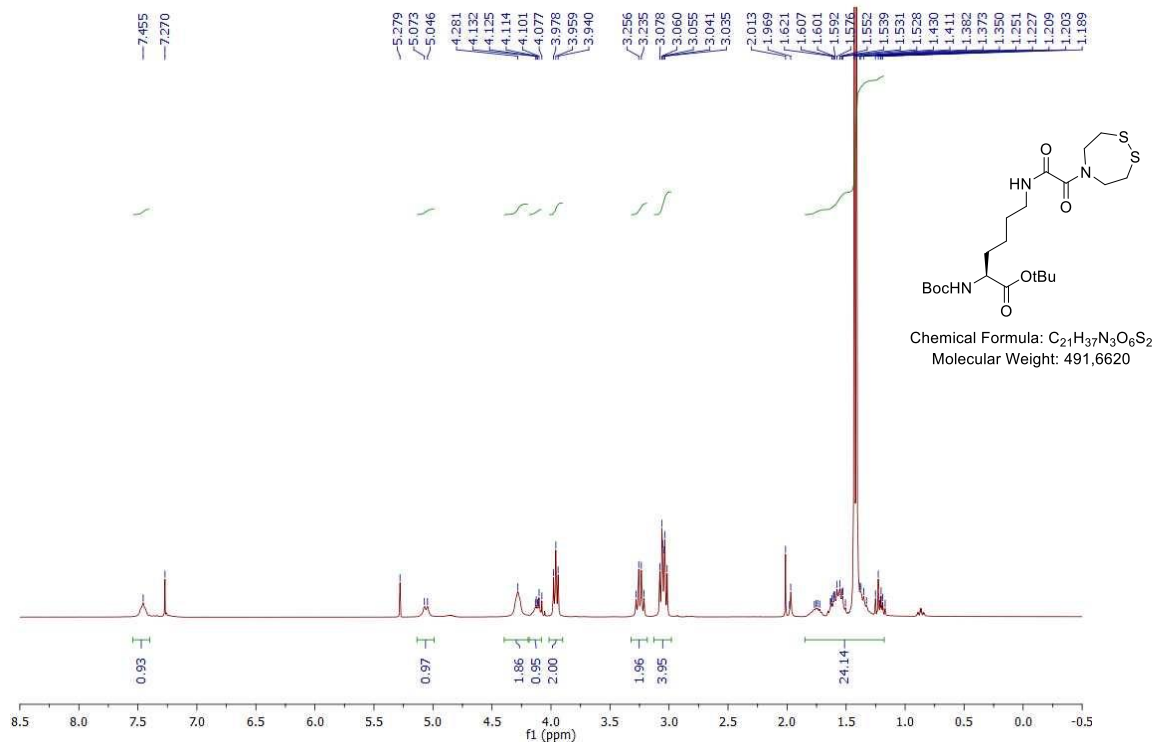


Figure S15. ^1H NMR (300 MHz) spectrum of compound **17** (CDCl_3 , 298 K).

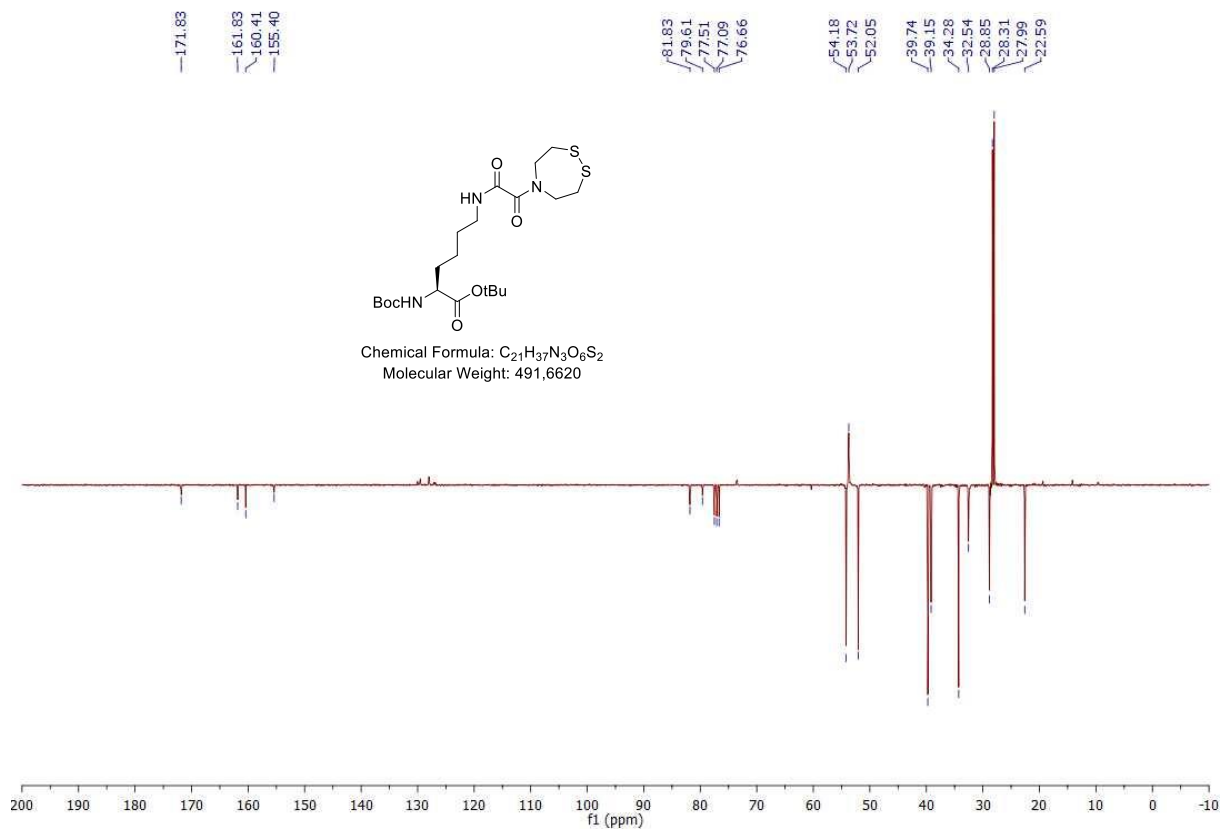


Figure S16. ^{13}C APT NMR (75 MHz) spectrum of compound **17** (CDCl_3 , 298 K).

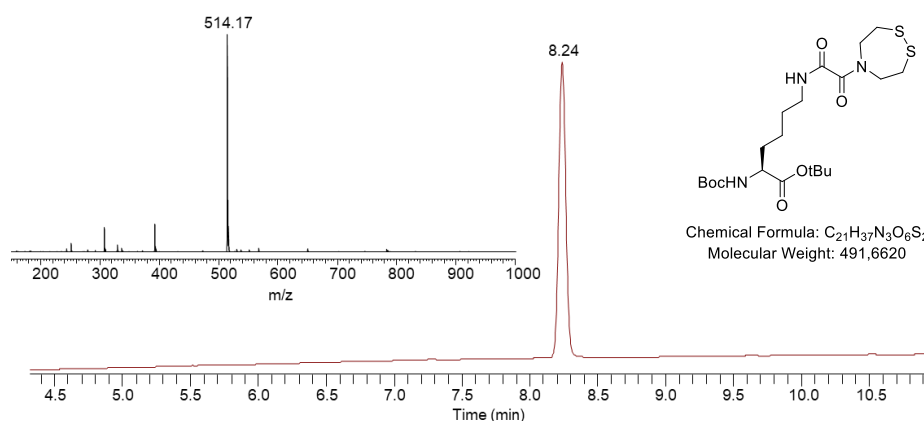


Figure S17. UPLC-MS analysis of compound **17**. a) LC trace. Eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in CH₃CN, ACQUITY UPLC Peptide BEH 300 Å 1.7 μm (2.1 × 150 mm) column, 50 °C, gradient 0-50% B in 15 min, 0.4 mL/min, UV detection at 215 nm. b) MS trace. Calcd. for [M+Na]⁺ (monoisotopic): 514.2, found: 514.2.

H-Lys^{(oxo)SEA}-OH·HCl intermediate **18**

Intermediate **17** (1.82 g, 3.70 mmol) was dissolved in a 4 M solution of HCl in dioxane (32 mL) at 0 °C under argon. The reaction was left to proceed overnight at rt (shorter reaction times lead to partial *O*^tBu ester deprotection). The crude mixture was then concentrated under reduced pressure and the residue was triturated in diethyl ether (2 × 10 mL). The white pasty and sticky solid was taken up in a deionized water/acetonitrile (95:5) mixture and lyophilized to provide a hygroscopic solid (1.35 g). Analysis of the crude showed the presence of an impurity corresponding to partial deprotection of the *tert*-butyl ester (~10% mol). One part of the crude product (700 mg) was purified by semi-preparative RP-HPLC (eluent A: H₂O, HCl (1/1000); eluent B: MeCN, HCl (1/1000); 0 to 30 % eluent B in 15 min, 30% to 100% eluent B in 5 min). The fractions were lyophilized and the hydrochloride salt of compound **18** was obtained as a white solid (480 mg, 67% yield based on the purified fraction).

Mp (°C) 74. **¹H NMR** (300 MHz, D₂O, **Erreur ! Source du renvoi introuvable.**) δ 3.99 (t, *J* = 6.3 Hz, 1H), 3.92 (t, *J* = 5.7 Hz, 2H), 3.83 (t, *J* = 5.7 Hz, 2H), , 3.25 (t, *J* = 6.6 Hz, 2H), 3.00 (t, *J* = 5.7 Hz, 2H), 2.94 (t, *J* = 5.7 Hz, 2H), 1.90 (m, 2H), 1.56 (m, 2H), 1.41 (m, 2H). **¹³C APT NMR** (75 MHz, D₂O, **Erreur ! Source du renvoi introuvable.**) δ 172.9 (Cq), 166.7 (Cq), 165.2 (Cq), 53.7 (CH₂), 53.5 (CH), 50.0 (CH₂), 39.9 (CH₂), 39.2 (CH₂), 34.1 (CH₂), 30.0 (CH₂), 28.3 (CH₂), 22.3 (CH₂). **IR** (ATR, cm⁻¹) 2923 (br.), 1734, 1621, 1475, 1420, 1361, 1210, 1191. **MS** (ESI, positive detection mode, **Erreur ! Source du renvoi introuvable.**) Calcd. for [M+H]⁺ (monoisotopic) 336.2, found 336.4. **HRMS** (ES⁺) Calcd. for C₁₂H₂₂N₃O₄S₂: 336.1046, found: 336.1057 (δ_{ppm} 3.06). **[α]_D²⁰** (c 1.0, CH₃OH): +13.7 °.

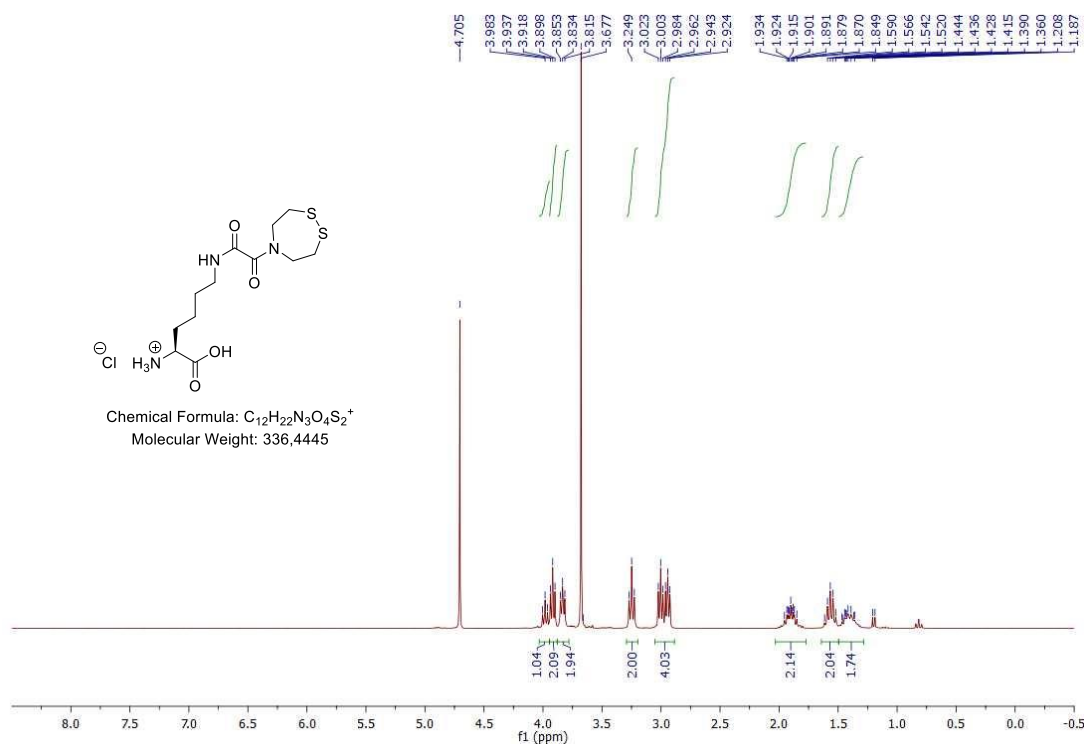


Figure S18. 1H NMR (300 MHz) spectrum of compound **18** (D_2O , 298 K).

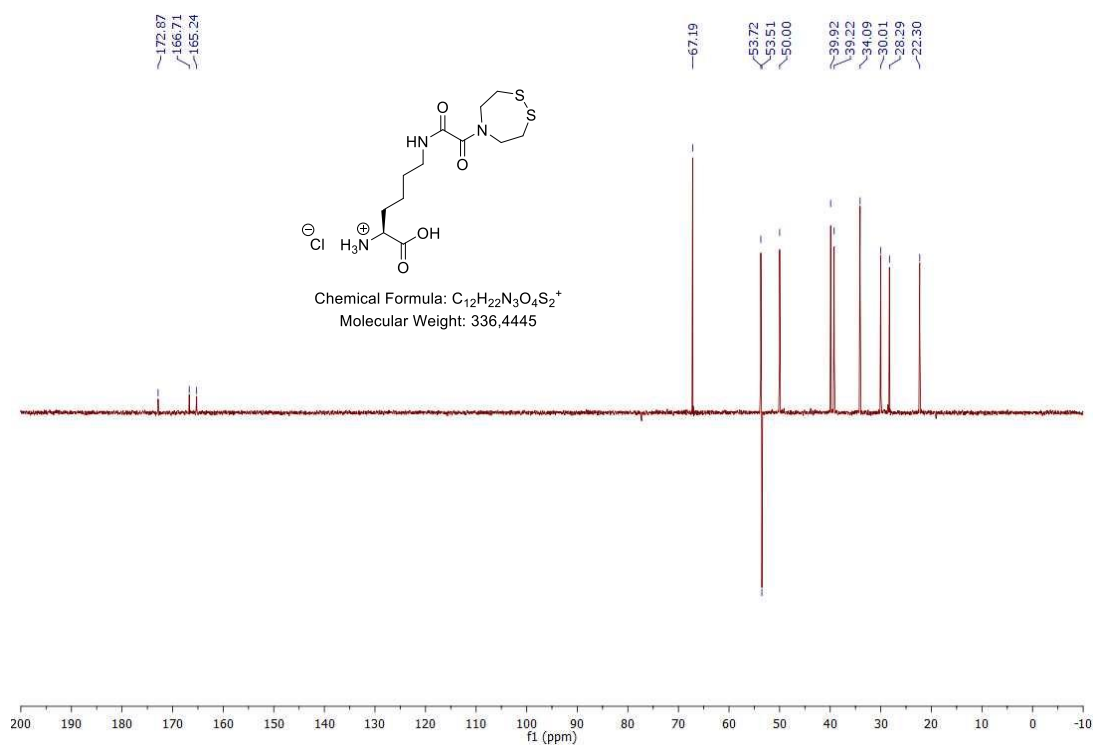


Figure S19. ^{13}C APT NMR (75 MHz) spectrum of compound **18** (D_2O , 298 K).

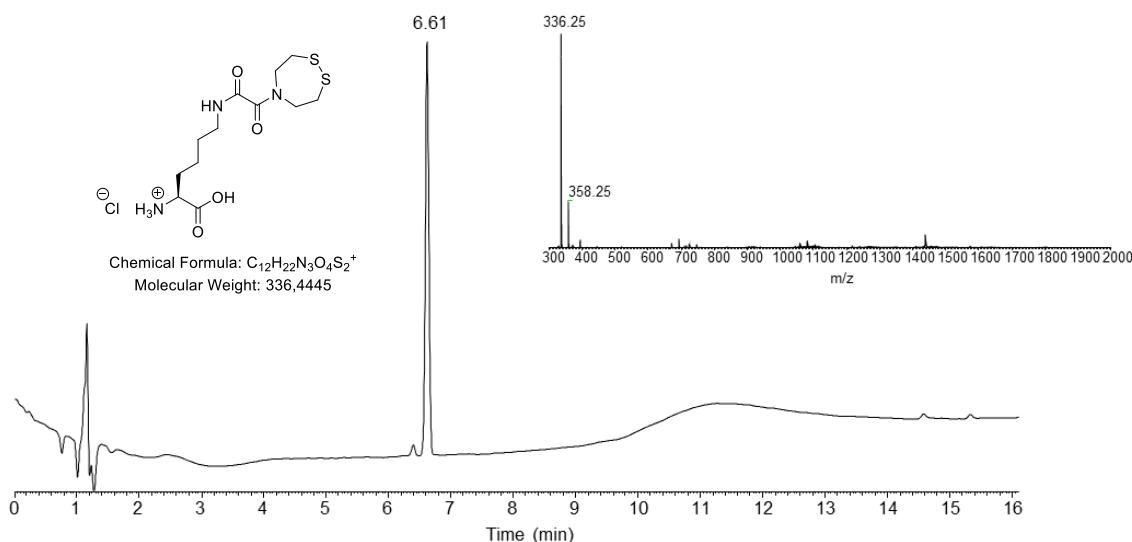


Figure S20. UPLC-MS analysis of compound **18**. a) LC trace. Eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in CH₃CN, ACQUITY UPLC Peptide BEH 300 Å 1.7 μm (2.1 × 150 mm) column, 50 °C, gradient 0-50% B in 15 min, 0.4 mL/min, UV detection at 215 nm. b) MS trace. Calcd. for [M+H]⁺ (monoisotopic): 336.2, found: 336.3.

Fmoc-Lys^(oxoSEA)-OH **1**

Intermediate **18** (628 mg, 1.86 mmol) was suspended in a mixture of dioxane (2 mL) and a 10% w/v sodium carbonate solution (6 mL). To this solution was added a solution of Fmoc chloroformate (508 mg, 1.93 mmol, 1.05 equiv.) in dioxane (6 mL) at 0 °C. The reaction was stirred at room temperature during 7 hours and was acidified to pH 2 by addition of 6 M HCl. Water was added (20 mL) and the crude mixture was extracted with ethyl acetate (3 × 10 mL). Organic layers were combined, washed with brine, dried over MgSO₄ and evaporated to dryness. Purification by silica gel column chromatography (Cyclohexane/EtOAc 80:20 to remove fluorenylmethanol and then pure EtOAc) provided compound **1** as a white amorphous solid (228 mg, 22%).

Mp (°C) 76. **¹H NMR** (300 MHz, CDCl₃, Figure S21) δ 7.76 (d, *J* = 7.3 Hz, 2H), 7.67 (t, *J* = 5.6 Hz, 1H), 7.60 (m, 2H), 7.40 (t, *J* = 7.3 Hz, 2H), 7.30 (t, *J* = 7.3 Hz, 2H), 5.66 (d, *J* = 8.0 Hz, 1H), 4.39-4.49 (m, 2H), 4.20-4.32 (m, 3H), 3.96 (t, *J* = 5.6 Hz, 2H), 3.92 (t, *J* = 5.7 Hz, 2H), 3.20-3.37 (m, 2H), 3.02-3.09 (m, 4H), 1.22-2.02 (m, 6H). **¹³C APT NMR** (75 MHz, CDCl₃, Figure S22) δ 175.4 (Cq), 162.1 (Cq), 160.5 (Cq), 156.2 (Cq), 143.7 (Cq), 141.3 (Cq), 127.7 (CH), 127.1 (CH), 125.1 (CH), 120.0 (CH), 67.1 (CH₂), 54.3 (CH₂), 53.4 (CH), 52.1 (CH₂), 47.1 (CH), 39.6 (CH₂), 39.0 (CH₂), 34.1 (CH₂), 31.7 (CH₂), 28.5 (CH₂), 22.3 (CH₂). **IR** (ATR, cm⁻¹) 3313, 2930, 2861, 1714, 1679, 1629, 1525, 1475, 1449, 1419, 1212, 1191. **MS** (ESI, positive detection mode, Figure S23) Calcd. for [M+H]⁺ (monoisotopic) 558.2, found 558.2. **MALDI-TOF** (matrix 2,5-dihydroxybenzoic acid, positive detection mode, Figure S24) Calcd. for [M+Na]⁺ (monoisotopic) 580.2, found 580.2. **HRMS** (ES⁺) Calcd. for C₂₇H₃₂N₃O₆S₂: 558.1727, found: 558.1723 (δ_{ppm} -0.72). **[α]_D²⁰** (c 1.0, CH₃OH): +3.7 °.

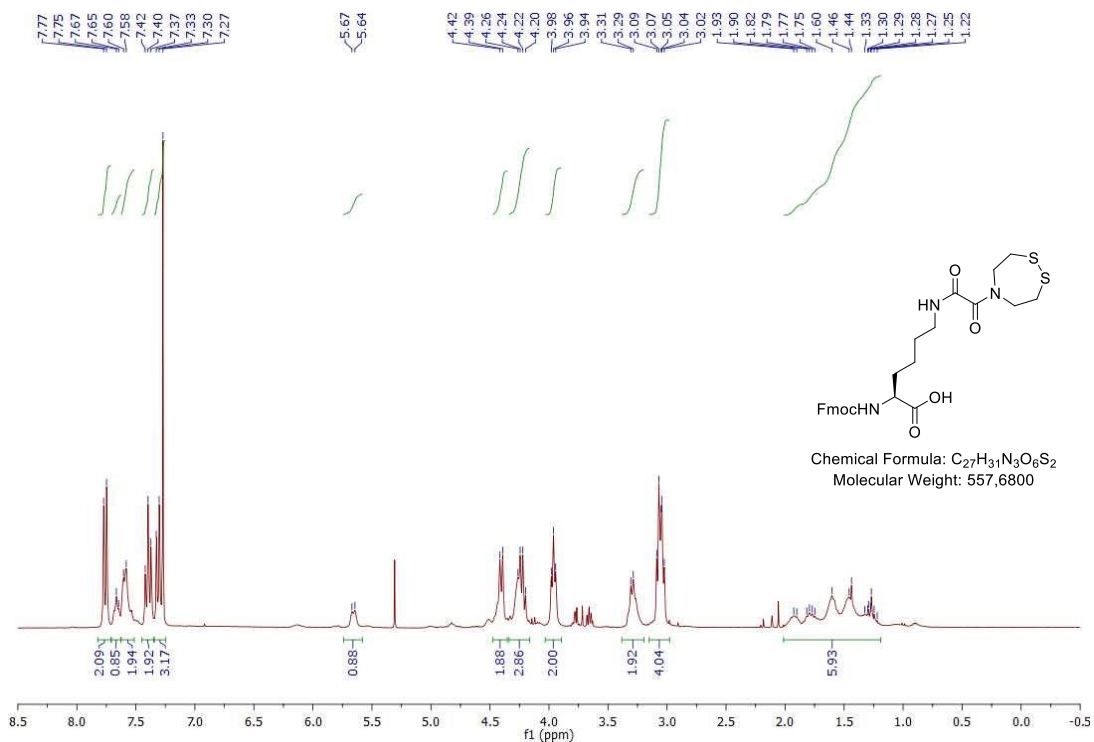


Figure S21. 1H NMR (300 MHz) spectrum of compound **1** ($CDCl_3$, 298 K).

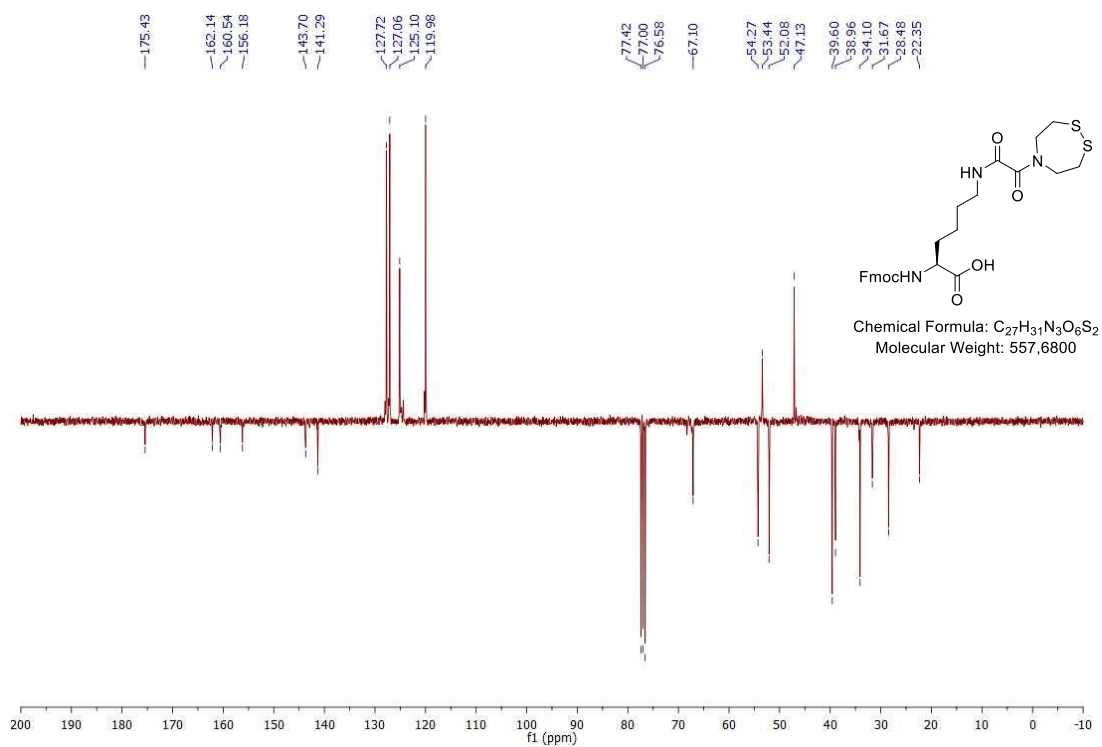


Figure S22. ^{13}C APT NMR (75 MHz) spectrum of compound **1** ($CDCl_3$, 298 K).

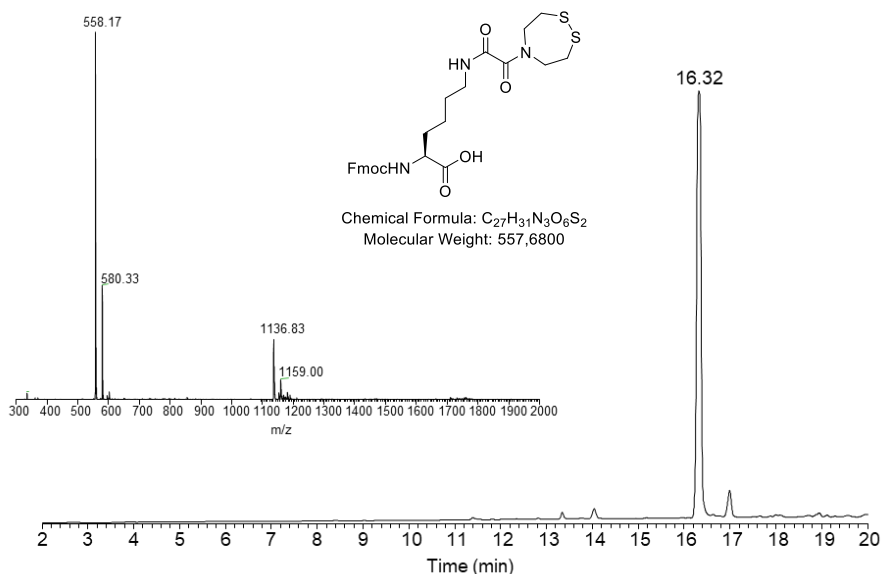


Figure S23. UPLC-MS analysis of compound **1**. a) LC trace. Eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in CH₃CN, ACQUITY UPLC Peptide BEH 300 Å 1.7 µm (2.1 × 150 mm) column, 50 °C, gradient 0-70% B in 20 min, 0.4 mL/min, UV detection at 215 nm. b) MS trace. Calcd. for [M+H]⁺ (monoisotopic): 558.3, found: 558.2.

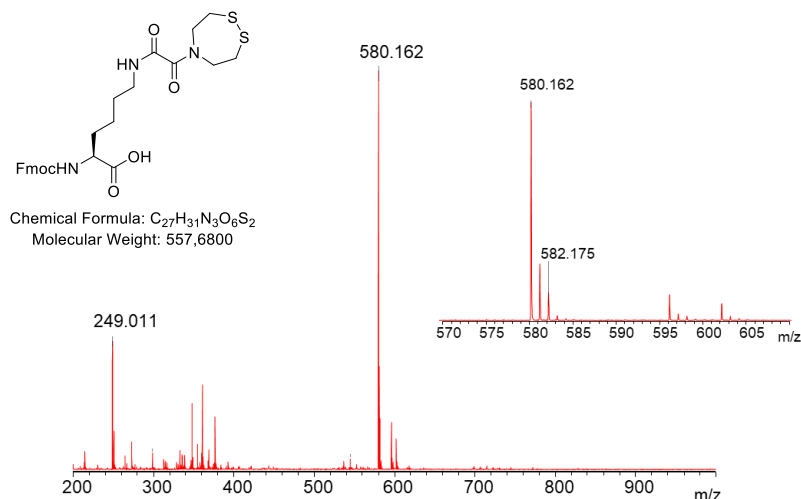


Figure S24. MALDI-TOF analysis of compound **1**. Matrix 2,5-dihydroxybenzoic acid, positive detection mode, [M+Na]⁺ calcd. (monoisotopic) 580.2, found 580.2.

2.3. Crystal structure of Fmoc-Lys(^{oxo}SEA)-OH **1**

Product **1** was recrystallized in isopropanol. The crystals were dried under a gentle stream of nitrogen.

The X-ray crystallographic coordinates for the structures reported in this study have been deposited at the Cambridge Crystallographic Data Centre (CCDC) under deposition number CCDC 2164270. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Table S 1. Experimental details

Chemical formula	C ₂₇ H ₃₁ N ₃ O ₆ S ₂ ·C ₃ H ₈ O
$M_r = 617.76$	
Crystal system, space group	Monoclinic, $P2_1$
Temperature (K)	296
a, b, c (Å)	16.0538 (14), 5.1260 (5), 20.698 (2)
β (°)	112.301 (4)
V (Å ³)	1575.9 (3)
Z	2
Radiation type	Cu $K\alpha$
μ (mm ⁻¹)	1.94
Crystal size (mm)	0.33 × 0.24 × 0.07
Data collection	
Diffractometer : Bruker APEX-II CCD	
Absorption correction : Multi-scan TWINABS-2012/1	

For component 1: $wR2(\text{int})$ was 0.1084 before and 0.0701 after correction.

For component 2: $wR2(\text{int})$ was 0.1249 before and 0.0740 after correction. The Ratio of minimum to maximum transmission is 0.65.

Final HKLF 4 output contains 63430 reflections, $R_{\text{int}} = 0.0619$ (44193 with $I > 3\sigma(I)$, $R_{\text{int}} = 0.0560$)

$T_{\text{min}}, T_{\text{max}}$	0.488, 0.753
No. of measured, independent and observed [$I > 2\sigma(I)$] reflections	5524, 5524, 5023
R_{int}	0.062
$(\sin \theta/\lambda)_{\text{max}}$ (Å ⁻¹)	0.602
Refinement	
$R[F^2 > 2\sigma(F^2)], wR(F^2), S$	0.051, 0.155, 1.08
No. of reflections	5524
No. of parameters	391
No. of restraints	1
H-atom treatment	H atoms treated by a mixture of independent and constrained refinement
$\Delta\rho_{\text{max}}, \Delta\rho_{\text{min}}$ (e Å ⁻³)	0.24, -0.46
Absolute structure	Flack x determined using 1961 quotients $[(I^+)-(I^-)]/[(I^+)+(I^-)]$ (Parsons, Flack and Wagner, Acta Cryst. B69 (2013) 249-259).

Absolute structure parameter	0.018 (18)
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Computer programs: APEX3 Ver. 2019.11-0 (Bruker-AXS, 2019), SAINT V8.40B (Bruker-AXS, 2016), ShelXT (Sheldrick, 2015), SHELXL (Sheldrick, 2015), Olex2 1.5 (Dolomanov *et al.*).

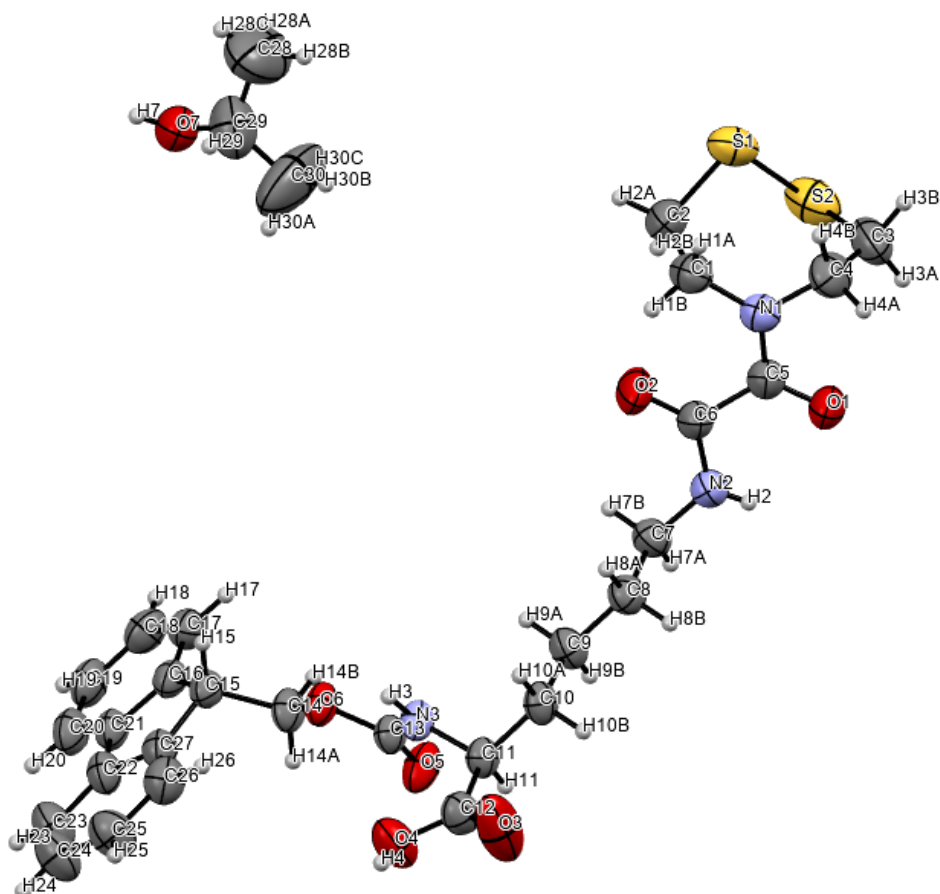


Figure S 25. ORTEP diagram of the X-ray structure of compound **1** with ellipsoids shown at the 50% probability level (H atoms omitted for clarity).

Table S 2. Atomic coordinates

Number	Label	Charge	SybylType	Xfrac + ESD	Yfrac + ESD	Zfrac + ESD	Symm. op.
1	S1	0	S.3	0.58170(8)	1.1690(3)	0.00994(6)	x,y,z
2	S2	0	S.3	0.66387(12)	0.8727(3)	0.00735(8)	x,y,z
3	O1	0	O.2	0.8751(2)	0.8438(9)	0.1822(2)	x,y,z
4	O2	0	O.2	0.7075(3)	0.9247(10)	0.2484(2)	x,y,z
5	O3	0	O.2	1.0159(3)	0.8607(12)	0.6835(2)	x,y,z
6	O4	0	O.3	0.9213(3)	0.7031(9)	0.72706(19)	x,y,z
7	H4	0	H	0.944462	0.812175	0.75762	x,y,z
8	O5	0	O.2	0.79301(19)	0.1367(6)	0.6112(2)	x,y,z

9	O6	0	O.3	0.68270(17)	0.4061(5)	0.61282(16)	x,y,z
10	N1	0	N.am	0.7671(2)	1.1506(7)	0.14574(18)	x,y,z
11	N2	0	N.am	0.8366(2)	0.6981(9)	0.2901(2)	x,y,z
12	H2	0	H	0.875(5)	0.64(2)	0.283(5)	x,y,z
13	N3	0	N.am	0.8027(2)	0.5774(6)	0.60154(18)	x,y,z
14	H3	0	H	0.776(3)	0.735(10)	0.602(2)	x,y,z
15	C1	0	C.3	0.6892(3)	1.2970(9)	0.1474(3)	x,y,z
16	H1A	0	H	0.692337	1.474635	0.132307	x,y,z
17	H1B	0	H	0.692566	1.303887	0.195148	x,y,z
18	C2	0	C.3	0.6002(3)	1.1810(13)	0.1020(3)	x,y,z
19	H2A	0	H	0.552361	1.28246	0.107562	x,y,z
20	H2B	0	H	0.596468	1.005179	0.118043	x,y,z
21	C3	0	C.3	0.7725(4)	1.0240(14)	0.0299(3)	x,y,z
22	H3A	0	H	0.817945	0.888025	0.042213	x,y,z
23	H3B	0	H	0.773956	1.112237	-0.011131	x,y,z
24	C4	0	C.3	0.7976(3)	1.2170(11)	0.0889(3)	x,y,z
25	H4A	0	H	0.862636	1.234506	0.108632	x,y,z
26	H4B	0	H	0.772711	1.385578	0.07002	x,y,z
27	C5	0	C.2	0.8109(3)	0.9556(9)	0.1885(2)	x,y,z
28	C6	0	C.2	0.7790(3)	0.8627(9)	0.2463(2)	x,y,z
29	C7	0	C.3	0.8207(3)	0.5801(10)	0.3483(2)	x,y,z
30	H7A	0	H	0.837084	0.397041	0.351189	x,y,z
31	H7B	0	H	0.757007	0.590277	0.339449	x,y,z
32	C8	0	C.3	0.8729(3)	0.7084(9)	0.4171(2)	x,y,z
33	H8A	0	H	0.854855	0.889748	0.414951	x,y,z
34	H8B	0	H	0.936537	0.704243	0.425217	x,y,z
35	C9	0	C.3	0.8586(3)	0.5763(9)	0.4783(2)	x,y,z
36	H9A	0	H	0.794768	0.573309	0.469283	x,y,z
37	H9B	0	H	0.879346	0.397097	0.48188	x,y,z
38	C10	0	C.3	0.9082(2)	0.7142(8)	0.5470(2)	x,y,z
39	H10A	0	H	0.887257	0.893286	0.543133	x,y,z
40	H10B	0	H	0.971873	0.718139	0.555556	x,y,z
41	C11	0	C.3	0.8959(2)	0.5878(7)	0.6094(2)	x,y,z
42	H11	0	H	0.918436	0.408428	0.613444	x,y,z
43	C12	0	C.2	0.9515(3)	0.7331(9)	0.6773(2)	x,y,z
44	C13	0	C.2	0.7636(2)	0.3553(7)	0.60921(19)	x,y,z
45	C14	0	C.3	0.6306(2)	0.1804(8)	0.6158(2)	x,y,z
46	H14A	0	H	0.66767	0.057765	0.650439	x,y,z
47	H14B	0	H	0.607091	0.093582	0.570771	x,y,z
48	C15	0	C.3	0.5535(2)	0.2783(8)	0.6354(2)	x,y,z
49	H15	0	H	0.524868	0.429726	0.606668	x,y,z
50	C16	0	C.2	0.4842(2)	0.0659(8)	0.6263(2)	x,y,z
51	C17	0	C.2	0.4317(3)	-0.0670(9)	0.5666(3)	x,y,z
52	H17	0	H	0.43546	-0.029277	0.523812	x,y,z
53	C18	0	C.2	0.3729(3)	-0.2586(9)	0.5717(3)	x,y,z
54	H18	0	H	0.336333	-0.347928	0.531992	x,y,z

55	C19	0	C.2	0.3688(3)	-0.3164(10)	0.6356(3)	x,y,z
56	H19	0	H	0.330816	-0.449112	0.638378	x,y,z
57	C20	0	C.2	0.4194(3)	-0.1827(10)	0.6950(3)	x,y,z
58	H20	0	H	0.415047	-0.221777	0.737476	x,y,z
59	C21	0	C.2	0.4780(2)	0.0141(8)	0.6906(2)	x,y,z
60	C22	0	C.2	0.5379(3)	0.1881(9)	0.7436(2)	x,y,z
61	C23	0	C.2	0.5525(4)	0.2215(12)	0.8133(3)	x,y,z
62	H23	0	H	0.523338	0.115988	0.834692	x,y,z
63	C24	0	C.2	0.6112(4)	0.4145(12)	0.8507(3)	x,y,z
64	H24	0	H	0.620277	0.44108	0.897356	x,y,z
65	C25	0	C.2	0.6563(4)	0.5674(12)	0.8203(3)	x,y,z
66	H25	0	H	0.696117	0.694027	0.846835	x,y,z
67	C26	0	C.2	0.6433(3)	0.5357(9)	0.7505(3)	x,y,z
68	H26	0	H	0.674306	0.638796	0.730136	x,y,z
69	C27	0	C.2	0.5832(3)	0.3473(8)	0.7120(2)	x,y,z
70	O7	0	O.3	0.0100(3)	0.5340(9)	0.1684(2)	x,y,z
71	H7	0	H	-0.023278	0.630069	0.179465	x,y,z
72	C28	0	C.3	0.0428(8)	0.816(4)	0.0900(8)	x,y,z
73	H28A	0	H	0.005757	0.697729	0.055014	x,y,z
74	H28B	0	H	0.092023	0.873674	0.078007	x,y,z
75	H28C	0	H	0.007605	0.964171	0.092715	x,y,z
76	C29	0	C.3	0.0770(5)	0.6879(16)	0.1557(4)	x,y,z
77	H29	0	H	0.094265	0.825952	0.191076	x,y,z
78	C30	0	C.3	0.1568(6)	0.531(3)	0.1693(9)	x,y,z
79	H30A	0	H	0.175423	0.456918	0.215233	x,y,z
80	H30B	0	H	0.204159	0.638171	0.166277	x,y,z
81	H30C	0	H	0.143595	0.39332	0.13539	x,y,z

3. Synthesis and characterization of model peptides

3.1. General procedures

Peptides were synthesized using standard Fmoc solid phase peptide synthesis methods. Peptide amides were prepared on a NovaSyn TGR solid support (0.25 mmol g^{-1}), whereas the sequences of the peptide hydrazides were assembled on hydrazine 2-chlorotriptyl 1% divinylbenzene cross-linked polystyrene ($\sim 0.2 \text{ mmol g}^{-1}$). SEA peptides were assembled on a SEA-PS solid support ($\sim 0.15 \text{ mmol g}^{-1}$) as described elsewhere.⁴

Preparation of hydrazine solid support and coupling of the first amino acid

Hydrazine solid support was prepared as described elsewhere.⁵

For a 0.1 mmol scale synthesis, 0.3 mmol of 2-CTC solid support (0.6 mmol g^{-1}) was swelled in DMF (2.5 mL). After 15 min , DMF was drained and the solid support was cooled at $0 \text{ }^\circ\text{C}$. A solution of hydrazine (4.00 equiv , 1.20 mmol , $76.8 \text{ }\mu\text{L}$) and triethylamine (6.00 equiv , 1.80 mmol , $251 \text{ }\mu\text{L}$) in DMF (1 mL) was added slowly to the beads at $0 \text{ }^\circ\text{C}$. The bead suspension was agitated for 1 h at rt. The reaction was stopped by adding methanol ($80.3 \text{ }\mu\text{L}$) to the bead suspension which was further agitated for 10 min . The beads were then washed with DMF ($2 \times 2 \text{ min}$), water ($2 \times 2 \text{ min}$) and DMF ($3 \times 2 \text{ min}$).

The first amino acid (4 equiv) was coupled using HBTU (3.8 equiv)/DIEA (8 equiv) activation in DMF. The amino acid was pre-activated for 30 s at rt before being added to the beads. The bead suspension was agitated for 1 h , and then washed with DMF ($2 \times 2 \text{ min}$). The coupling was repeated, and the beads were washed with DMF ($2 \times 2 \text{ min}$), DCM ($6 \times 2 \text{ min}$) and MeOH ($2 \times 2 \text{ min}$). The beads were finally dried *in vacuo* for 2 h . The loading was determined by treating aliquots with piperidine in DMF ($20\% \text{ v/v}$) and measuring the absorbance of the dibenzofulvene-piperidine adduct at 280 nm . Loading 0.20 mmol g^{-1} .

Peptide elongation and cleavage

Unless otherwise stated, peptide elongation was performed at room temperature (rt) using an automated peptide synthesizer. In brief, amino acids (10 equiv) were activated using HBTU (9.5 equiv)/DIEA (10 equiv) in DMF. The peptidyl solid support was acetylated after each coupling step using Ac_2O /DIEA/DMF $10/5/85 \text{ v/v/v}$. The removal of the Fmoc group was performed by treating the peptidyl solid support with DMF/piperidine $80/20 \text{ v/v}$. After elongation, the peptidyl solid support was washed with DMF ($3 \times 1 \text{ min}$), DCM ($3 \times 1 \text{ min}$) and Et_2O ($2 \times 1 \text{ min}$). The solid support was finally dried *in vacuo*. The experimental conditions used for the final cleavage and deprotection step are indicated for each peptide.

⁴ Boll, E.; Drobecq, H.; Ollivier, N.; Blanpain, A.; Raibaut, L.; Desmet, R.; Vicogne, J.; Melnyk, O. One-pot chemical synthesis of small ubiquitin-like modifier protein-peptide conjugates using *bis*(2-sulfanylethyl)amido peptide latent thioester surrogates. *Nat. Protocols* **2015**, *10*, 269-292.

⁵ Zheng, J.-S.; Tang, S.; Qi, Y.-K.; Wang, Z.-P.; Liu, L. Chemical synthesis of proteins using peptide hydrazides as thioester surrogates. *Nat. Protocols* **2013**, *8*, 2483-2495.

Analyses

Products were characterized by analytical UPLC–MS using a System Ultimate 3000 UPLC (ThermoFisher) equipped with an Acquity peptide BEH300 C18 column (1.7 μm , 2.1 \times 100 mm) or an Agilent SB C3 column (1.8 μm , 3.0 \times 100 mm), a diode array detector, a charged aerosol detector (CAD) and a mass spectrometer (Ion trap LCQfleet). Analyses were performed at 50 °C using a linear gradient of 0-40% of eluent B in eluent A over 20 min at a flow rate of 0.4 mL min⁻¹ (eluent A = 0.1% TFA in H₂O; eluent B = 0.1% TFA in CH₃CN) or 0-70% of eluent B in eluent A over 20 min at a flow rate of 0.4 mL min⁻¹ (eluent A = 0.1% FA in H₂O; eluent B = 0.1% FA in CH₃CN). The column eluate was monitored by UV at 215 nm and CAD. The peptide masses were measured by on-line UPLC–MS (LCQ Fleet Ion Trap Mass Spectrometer, ThermoFisherScientific): heat temperature 350 °C, spray voltage 2.8 kV, capillary temperature 350 °C, capillary voltage 10 V, tube lens voltage 75 V.

Products were sometimes characterized by analytical LC–MS (Waters 2695 LC/ZQ 2000 quadrupole) on a reverse phase column XBridge BEH300 C18 (3.5 μm , 300 Å, 4.6 \times 150 mm) at 30 °C using a linear gradient of 0-100% of eluent B in eluent A over 30 min at a flow rate of 1 mL/min (eluent A = 0.1% TFA in H₂O; eluent B = 0.1% TFA in CH₃CN/H₂O: 4/1 by vol) or on a Zorbax 300SB-C3 (3.5 μm , 300 Å, 4.6 \times 150 mm) at 50 °C using a linear gradient of 0-50% of eluent D in eluent C in 30 min at a flow rate of 1 mL/min (eluent C = 0.1% formic acid in H₂O; eluent D = 0.1% formic acid 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: 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.

MALDI-TOF mass spectra were recorded with a Bruker Autoflex Speed using alpha cyano 4-hydroxycinnamic acid, sinapinic acid or 2,5-dihydroxybenzoic acid (DHB) as matrix. The observed m/z corresponded to the monoisotopic ions, unless otherwise stated.

Purification

Preparative reverse phase HPLC of crude peptides were performed with a preparative HPLC Waters system using a reverse phase XBridge BEH300 Prep C18 column (5 μm , 300 Å, 10 \times 250 mm) or C3Zorbax column (300SB-C3, 5 μm , 9.4 \times 250 mm) and appropriate linear gradient of increasing concentration of eluent B in eluent A (flow rate of 6 mL min⁻¹, detection at 215 nm). Selected fractions were then combined and lyophilized.

3.2. Amide peptides

Synthesis and characterization of CILKEPVHGA-NH₂ peptide 4a

Peptide **4a** (CILKEPVHGA-NH₂) was synthesized on a 0.1 mmol scale as described in the general procedure presented in the Methods section. TFA/H₂O/TIS/ethanedithiol 90/2.5/5/2.5 v/v/v/v (10 mL) was used as the cleavage cocktail. Purification of the crude by preparative HPLC (eluent A 0.1% TFA in water, eluent B 0.1% TFA in CH₃CN/water 4/1 v/v, 0-15% eluent B in 5 min then 15-22% in 30 min,

50 °C, detection at 215 nm, 6 mL min⁻¹, C18 Xbridge 5 μm (19 × 100 mm) column) provided the title peptide as a white solid after lyophilisation (93.6 mg, 67%).

MS (ESI, positive detection mode, Figure S26) m/z = 1066.42 ([M+H]⁺), 533.58 ([M+2H]²⁺). Calcd. for M (average) 1065.30, found: 1065.29.

MALDI-TOF (matrix α-cyano-4-hydroxycinnamic acid, positive detection mode, Figure S27) calcd. for [M+H]⁺(monoisotopic): 1065.58, found: 1065.63.

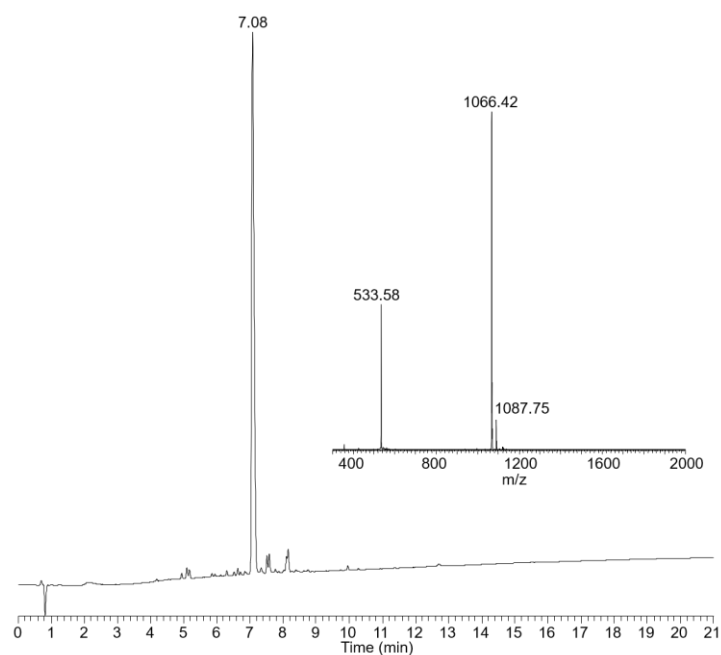


Figure S26. UPLC-MS analysis of peptide **4a**. a) LC trace. Eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN, ACQUITY UPLC Peptide BEH 300Å 1.7 μm (2.1 × 150 mm) column, 50 °C, gradient 0-70% B in 20 min, 0.4 mL/min, UV detection at 215 nm. b) MS trace: m/z = 1066.42 ([M+H]⁺), 533.58 ([M+2H]²⁺); calcd. for M (average): 1065.30, found: 1065.29.

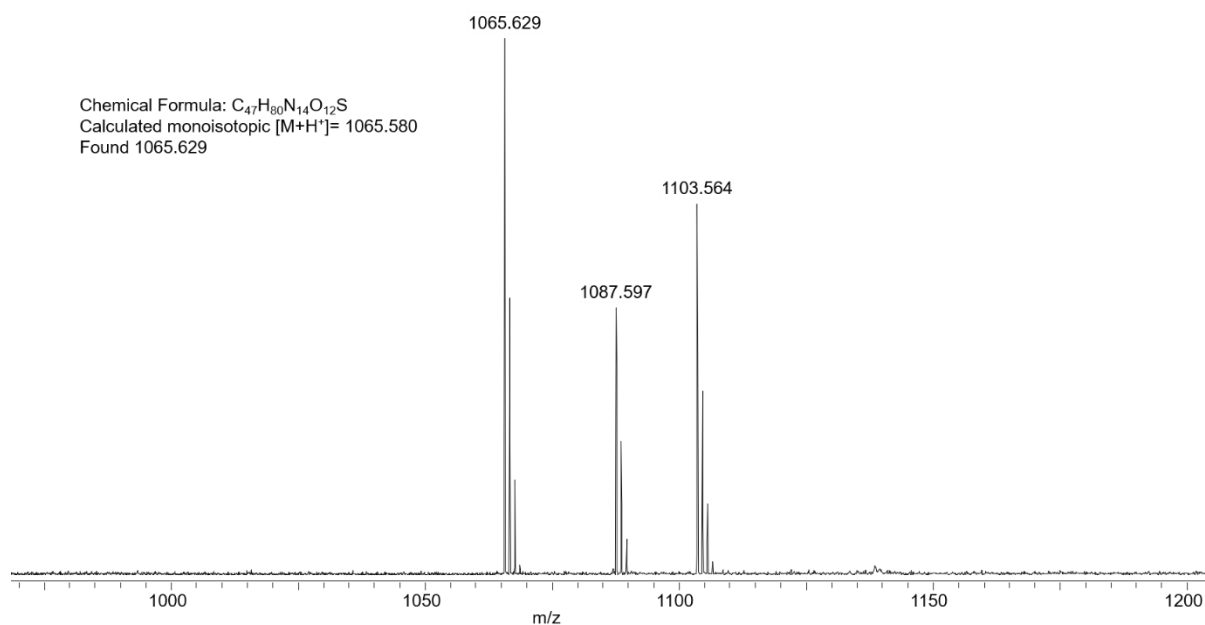


Figure S27. MALDI-TOF analysis of peptide **4a** α -cyano-4-hydroxycinnamic acid, positive detection mode, [M+H]⁺ (calculated): 1065.58, found: 1065.63.

Synthesis and characterization of Ac-ALK*EPVHGW- NH₂ peptide **3a** (K* = Lys^(ox)SEA))

Peptide **3a** (Ac-ALK*EPVHGW- NH₂) was synthesized on a 0.1 mmol scale as described in the general procedure presented in the Methods section. TFA/H₂O/TIS 92.5/2.5/5 v/v/v (10 mL) was used as the cleavage cocktail. Purification of the crude by preparative HPLC (eluent A 0.1% TFA in water, eluent B 0.1% TFA in CH₃CN/water 4/1 v/v, 0-10% eluent B in 2 min then 10-30% in 40 min, 25 °C, detection at 215 nm, 6 mL min⁻¹, C18 Xbridge 5 μ m (19 \times 100 mm) column) provided the title peptide as a white solid after lyophilisation (54.8 mg, 40%).

MS (ESI, positive detection mode, Figure S28) m/z = 1267.67 ([M+H]⁺), 634.25 ([M+2H]²⁺). Calcd. for M (average) 1266.502, found: 1266.58.

MALDI-TOF (matrix α -cyano-4-hydroxycinnamic acid, positive detection mode, Figure S29) calcd. for [M+H]⁺(monoisotopic): 1266.57, found: 1266.58.

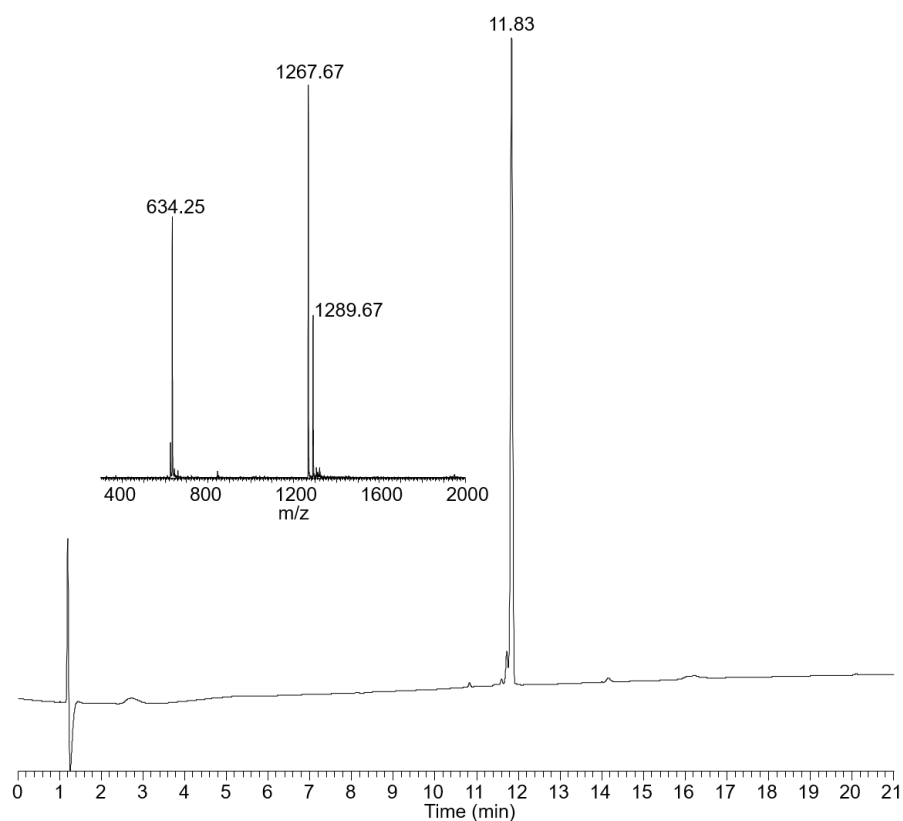


Figure S28. LC-MS analysis of peptide **3a**. a) LC trace. Eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN, ACQUITY UPLC Peptide BEH 300Å 1.7 μm (2.1 × 150 mm) column, 50 °C, gradient 0-70% B in 20 min, 0.4 mL/min, UV detection at 215 nm. b) MS trace: m/z = 1267.67 ([M+H]⁺), 634.25 ([M+2H]²⁺); calcd. for M (average): 1266.502, found: 1266.58.

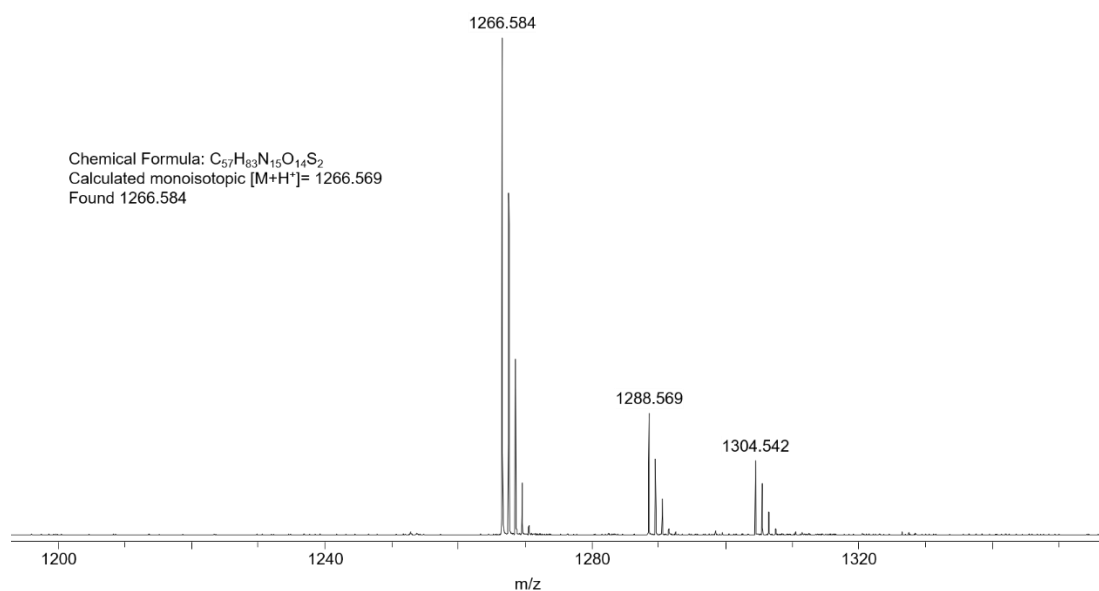


Figure S29. MALDI-TOF analysis of peptide **3a** α-cyano-4-hydroxycinnamic acid, positive detection mode, [M+H]⁺ (calculated): 1266.57, found: 1266.58.

Synthesis and characterization of Ac-K*GSGSGSGKPIP NPLLGLDSTGSGSGSA- NH₂ peptide **3b**

Peptide **3b** (Ac-K*GSGSGSGKPIP NPLLGLDSTGSGSGSA- NH₂) was synthesized on a 0.1 mmol scale as described in the general procedure presented in the Methods section. TFA/H₂O/TIS 92.5/5/2.5 v/v/v (10 mL) was used as the cleavage cocktail. Purification of the crude by preparative HPLC (eluent A 0.1% TFA in water, eluent B 0.1% TFA in CH₃CN, 0-10% eluent B in 2 min then 10-30% in 40 min, 25 °C, detection at 215 nm, 6 mL min⁻¹, C18 Xbridge 5 μm (19 × 100 mm) column) provided the title peptide as a white solid after lyophilisation (56.4 mg, 20%).

MS (ESI, positive detection mode, Figure S30) m/z = 1358.42 ([M+2H]²⁺), 906.00 ([M+3H]³⁺). Calcd. for M (average) 2715.99, found: 2714.92.

MALDI-TOF (matrix α-cyano-4-hydroxycinnamic acid, positive detection mode, Figure S31) calcd. for [M+H]⁺(monoisotopic): 2715.26, found: 2715.06.

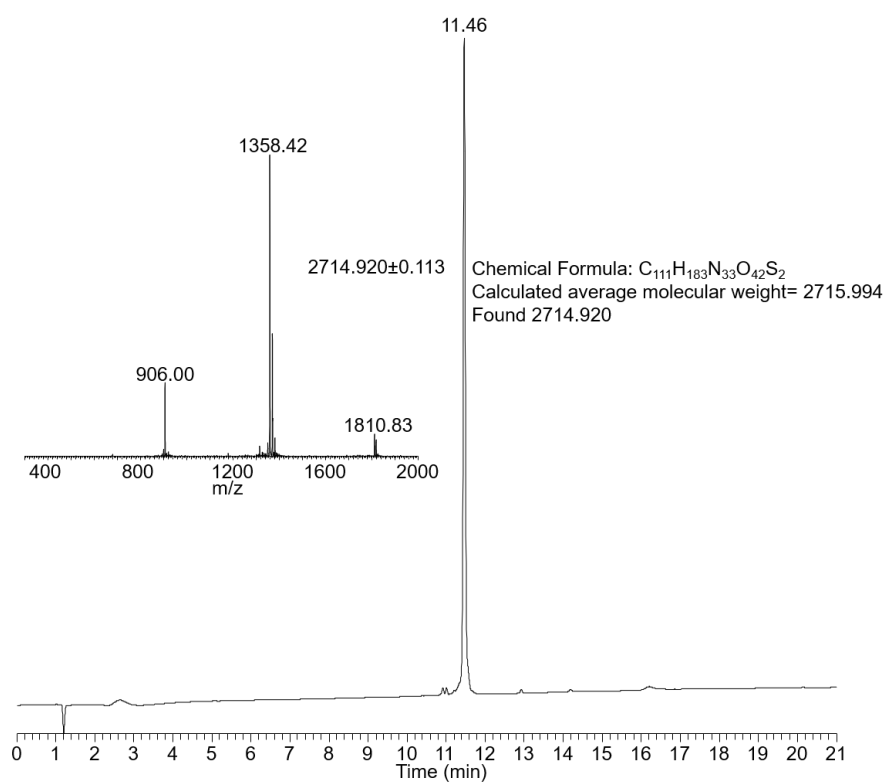


Figure S30. LC-MS analysis of peptide **3b**. a) LC trace. Eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN, ACQUITY UPLC Peptide BEH 300Å 1.7 μm (2.1 × 150 mm) column, 50 °C, gradient 0-70% B in 20 min, 0.4 mL/min, UV detection at 215 nm. b) MS trace: m/z = 1358.42 ([M+2H]²⁺), 906.00 ([M+3H]³⁺); calcd. for M (average): 2715.99, found: 2714.92.

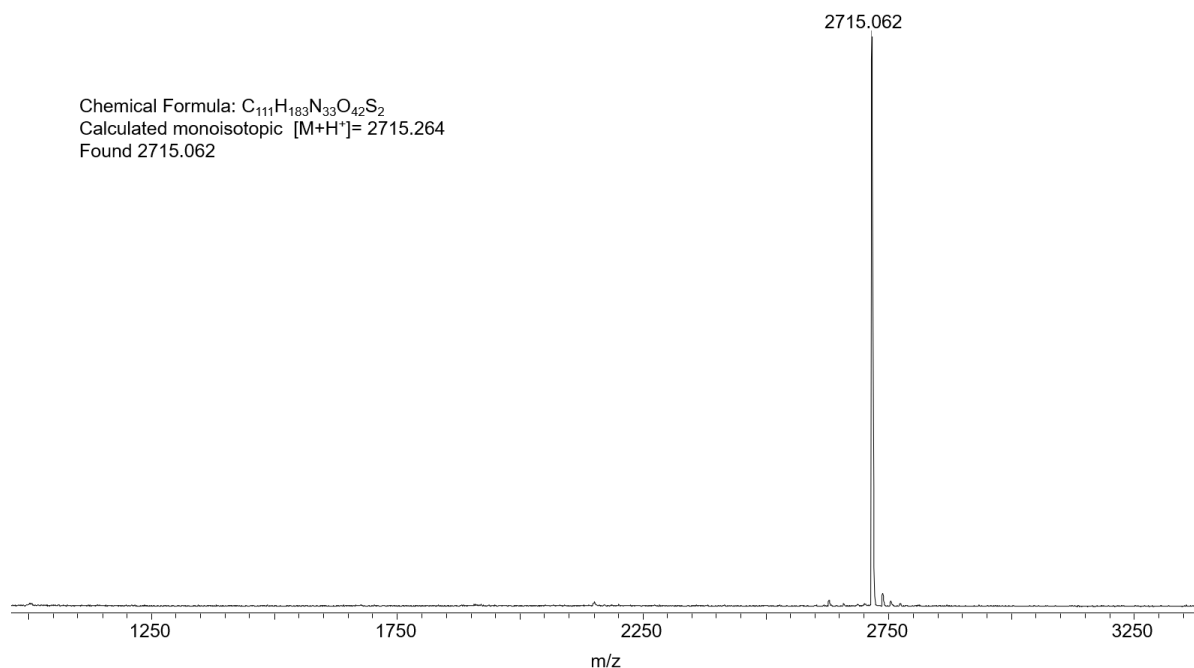


Figure S31. MALDI-TOF analysis of peptide **3b** α -cyano-4-hydroxycinnamic acid, positive detection mode, [M+H]⁺ (calculated): 2715.26, found: 2715.06.

Synthesis and characterization of Ac-K*GSGSGSGKPIPPELLGLDSTGSGSGSAK(biotin)-NH₂ peptide **3c**

Peptide **3c** (Ac-K*GSGSGSGKPIPPELLGLDSTGSGSGSAK(biotin)-NH₂) was synthesized on a 0.025 mmol scale as described in the general procedure presented in the Methods section. TFA/H₂O/TIS 92.5/5/2.5 v/v/v (10 mL) was used as the cleavage cocktail. Purification of the crude by preparative HPLC (eluent A 0.1% TFA in water, eluent B 0.1% TFA in CH₃CN, 0-20% eluent B in 5 min then 20-40% in 45 min, 50 °C, detection at 215 nm, 20 mL min⁻¹, C18 Xbridge 5 μ m (19 \times 100 mm) column) provided the title peptide as a white solid after lyophilisation (7.1 mg, 10%).

UPLC-MS (ESI, positive detection mode,) m/z = 1500.25 ([M+2H]²⁺), 1000.58 ([M+3H]³⁺). Calcd. for M (average) 2999.38, found: 2998.62.

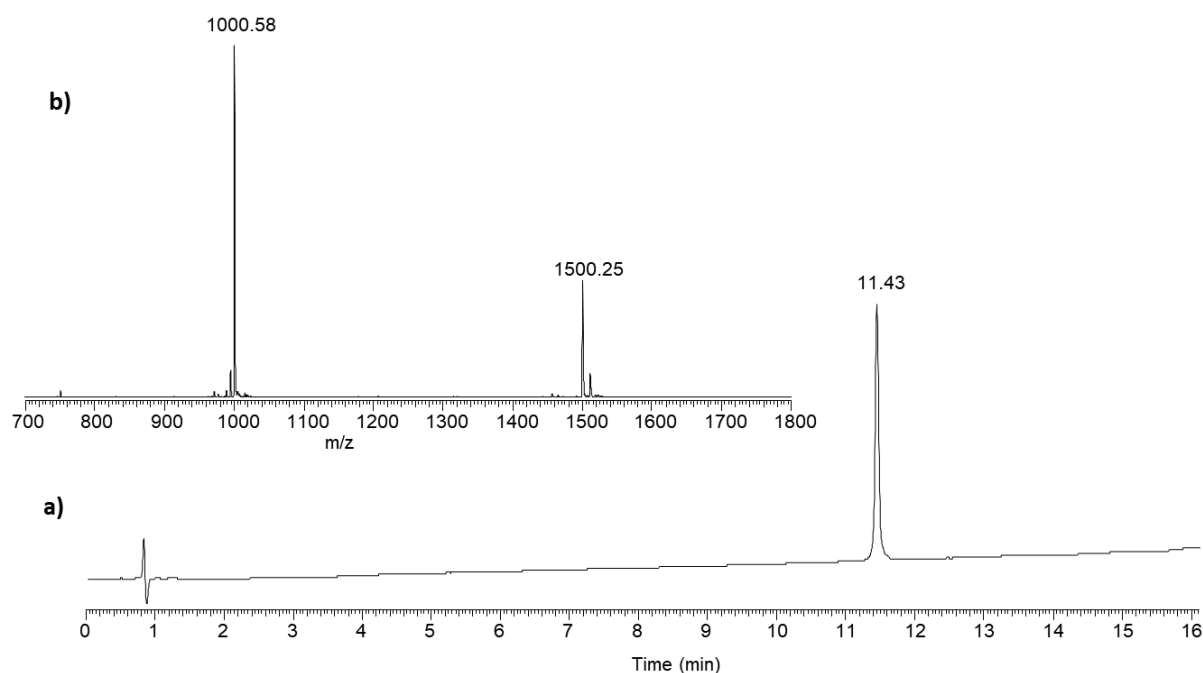


Figure S 32. LC-MS analysis of peptide **3c**. a) LC trace. Eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN, ACQUITY UPLC Peptide BEH 300Å 1.7 μm (2.1 × 150 mm) column, 50 °C, gradient 0-70% B in 20 min, 0.4 mL/min, UV detection at 215 nm. b) MS trace: m/z = 1500.25 ([M+2H]²⁺), 1000.58 ([M+3H]³⁺); calcd. for M (average): 2999.38, found: 2998.62.

3.3. MPA-thioester peptides

MPA-thioester peptides were produced according to reported procedures (Figure S33).⁶ Briefly, the targeted peptide sequence was elongated on a SEA-PS solid support following the general protocol described above for Fmoc-SPPS. After cleavage, the resulting SEA-peptide was engaged in a thiol-thioester exchange in the presence of 3-mercaptopropionic acid to afford the MPA-thioester derivative.

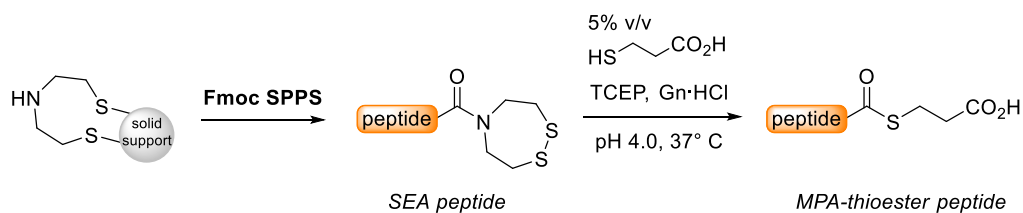


Figure S33. General approach for the production of MPA-thioesters in this work

Synthesis and characterization of ILKEPWHGA-SEA peptide

⁶ Boll, E.; Drobecq, H.; Ollivier, N.; Blanpain, A.; Raibaut, L.; Desmet, R.; Vicogne, J.; Melnyk, O. One-pot chemical synthesis of small ubiquitin-like modifier protein-peptide conjugates using bis(2-sulfanylethyl)amido peptide latent thioester surrogates. *Nat. Protocols* **2015**, *10*, 269-292.

Peptide (ILKEPWHGA-SEA) was synthesized on a 0.1 mmol scale as described in the general procedure presented in the Methods section. TFA/H₂O/TIS/ethanedithiol 90/2.5/5/2.5/2.5 v/v/v/v (10 mL) was used as the cleavage cocktail. Purification of the crude by preparative HPLC (eluent A 0.1% TFA in water, eluent B 0.1% TFA in CH₃CN/water 4/1 v/v, 0-10% eluent B in 2 min then 10-30% in 40 min, 25 °C, detection at 215 nm, 6 mL min⁻¹, C18 Xbridge 5 μm (19 × 100 mm) column) provided the title peptide as a white solid after lyophilisation (56.6 mg, 38%).

MS (ESI, positive detection mode, Figure S34) $m/z = 1168.33$ ($[M+H]^+$), 584.75 ($[M+2H]^{2+}$). Calcd. for M (average) 1167.45, found: 1167.41.

MALDI-TOF (matrix α -cyano-4-hydroxycinnamic acid, positive detection mode, Figure S35) calcd. for $[M+H]^+$ (monoisotopic): 1167.57, found: 1167.70.

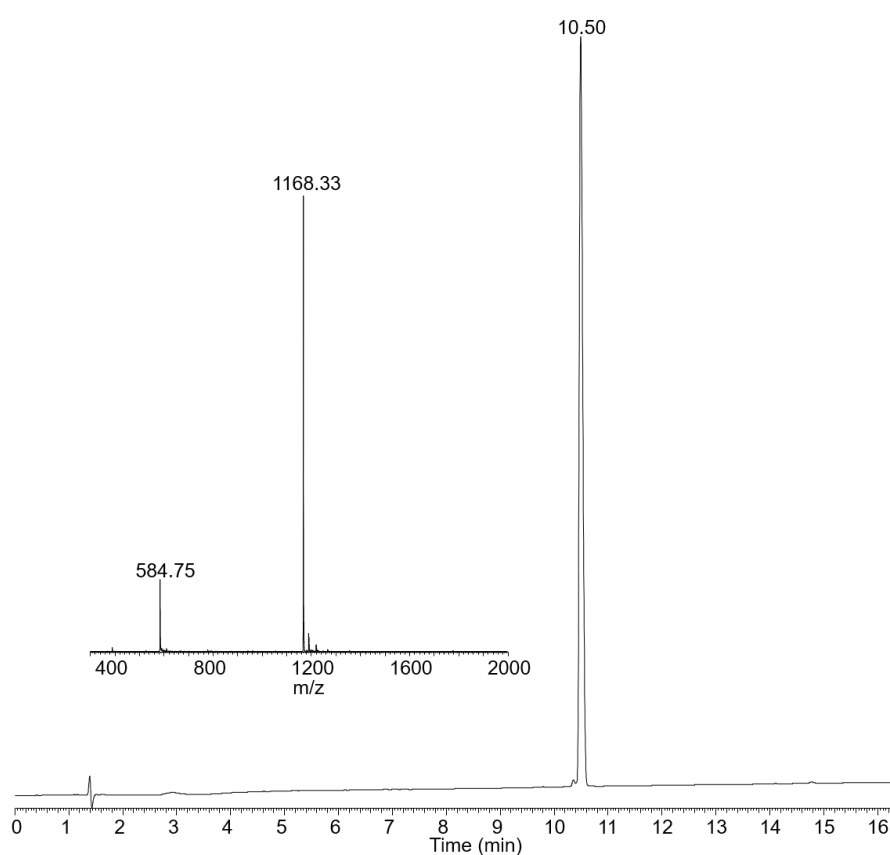


Figure S34. LC-MS analysis of peptide ILKEPWHGA-SEA. a) LC trace. Eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN, ACQUITY UPLC Peptide BEH 300Å 1.7 μm (2.1 × 150 mm) column, 50 °C, gradient 0-70% B in 20 min, 0.4 mL/min, UV detection at 215 nm. b) MS trace: $m/z = 1168.33$ ($[M+H]^+$), 584.75 ($[M+2H]^{2+}$); calcd. for M (average): 1167.45, found: 1167.41.

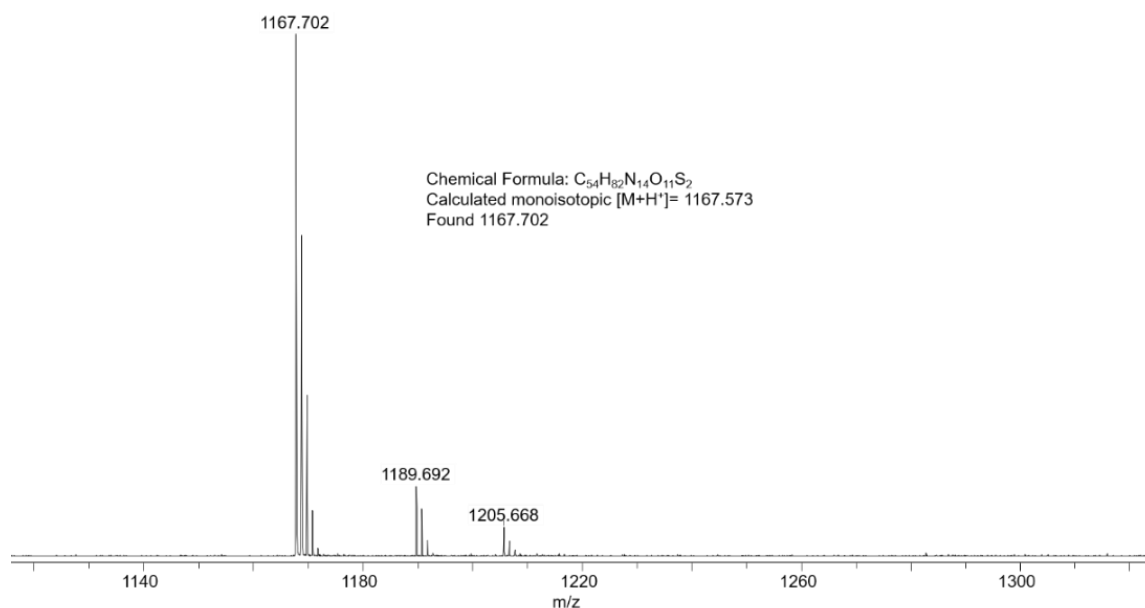


Figure S35. MALDI-TOF analysis of peptide ILKEPWHGA-SEA α -cyano-4-hydroxycinnamic acid, positive detection mode, [M+H]⁺ (calculated): 1167.57, found: 1167.70.

Synthesis and characterization of ILKEPWHGA-MPA peptide

The above SEA peptide (33.12 mg) was dissolved in a 0.1 M TCEP aqueous solution containing 5% v/v 3-mercaptopropionic acid (MPA) of pH 4 (7.1 mL, 4 mM). The reaction was left to proceed at 37 °C overnight (12 – 15 h). The mixture was quenched by addition of glacial acetic acid (1 mL). Purification of the crude by preparative HPLC (eluent A 0.1% TFA in water, eluent B 0.1% TFA in CH₃CN, 0-20% eluent B in 30 min, 50 °C, detection at 215 nm, 6 mL min⁻¹, C18 Xbridge 5 μ m (19 \times 100 mm) column) provided the title peptide as a white solid after lyophilisation (17.9 mg, 55%).

MS (ESI, positive detection mode, Figure S36) m/z = 1138.92 ([M+H]⁺), 570.08 ([M+2H]²⁺). Calcd. for M (average) 1138.35, found: 1138.04.

MALDI-TOF (matrix α -cyano-4-hydroxycinnamic acid, positive detection mode, Figure S37) calcd. for [M+H]⁺(monoisotopic): 1138.56, found: 1138.62.

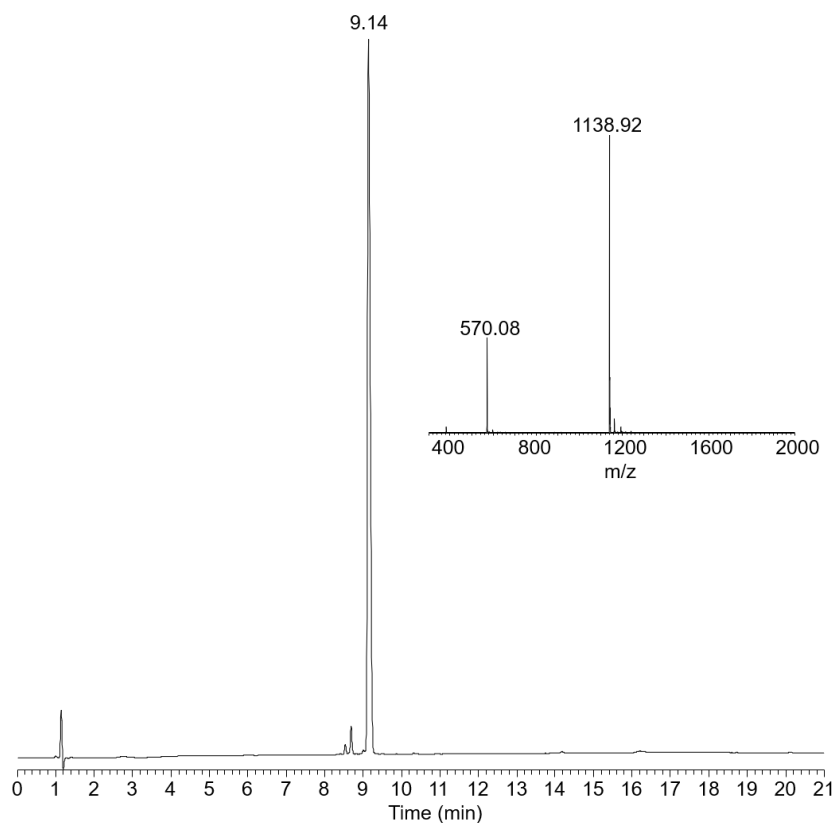


Figure S36. LC-MS analysis of peptide ILKEPWHGA-MPA. a) LC trace. Eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH_3CN , ACQUITY UPLC Peptide BEH 300Å 1.7 μm (2.1×150 mm) column, 50 °C, gradient 0-70% B in 20 min, 0.4 mL/min, UV detection at 215 nm. b) MS trace: $m/z = 1138.92$ ($[\text{M}+\text{H}]^+$), 570.08 ($[\text{M}+2\text{H}]^{2+}$); calcd. for M (average): 1138.353, found: 1138.04.

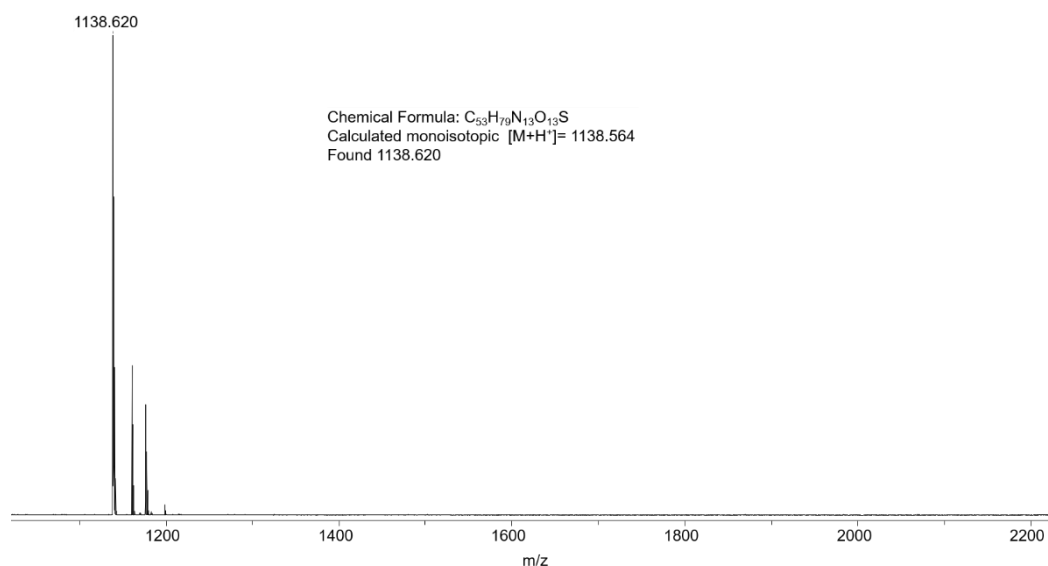


Figure S37. MALDI-TOF analysis of peptide ILKEPWHGA-MPA α -cyano-4-hydroxycinnamic acid, positive detection mode, $[\text{M}+\text{H}]^+$ (calculated): 1138.56, found: 1138.62.

3.4. MPAA-thioester peptides

MPAA-thioester peptides were produced according to reported procedures (Figure S33).⁷ Briefly, the targeted peptide sequence was elongated on a hydrazine-functionalized resin following the general protocol described above for Fmoc-SPPS. After cleavage, the resulting peptide hydrazide was converted to the corresponding MPAA-thioester derivative.

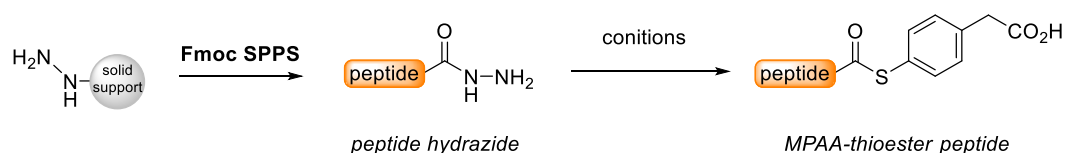


Figure S38. General approach for the production of MPAA-thioesters in this work

Synthesis and characterization of ILKEPWHGA-NHNH₂ peptide

Peptide ILKEPWHGA- NHNH₂ was synthesized on a 0.1 mmol scale as described in the general procedure presented in the Methods section. TFA/H₂O/TIS 92.5/5/2.5 v/v/v (10 mL) was used as the cleavage cocktail. Purification of the crude by preparative HPLC (eluent A 0.1% TFA in water, eluent B 0.1% TFA in CH₃CN, 0-10% eluent B in 2 min then 10-30% in 40 min, 25 °C, detection at 215 nm, 6 mL min⁻¹, C18 Xbridge 5 μm (19 × 100 mm) column) provided the title peptide as a white solid after lyophilisation (24.2 mg, 16%).

MS (ESI, positive detection mode, Figure S36) $m/z = 1064.58$ ($[\text{M}+\text{H}]^+$), 535.92 ($[\text{M}+2\text{H}]^{2+}$). Calcd. for M (average) 1064.26, found: 1063.71.

MALDI-TOF (matrix α -cyano-4-hydroxycinnamic acid, positive detection mode, Figure S37) calcd. for $[\text{M}+\text{H}]^+$ (monoisotopic): 1064.59, found: 1064.58.

⁷ Boll, E.; Drobecq, H.; Ollivier, N.; Blanpain, A.; Raibaut, L.; Desmet, R.; Vicogne, J.; Melnyk, O. One-pot chemical synthesis of small ubiquitin-like modifier protein-peptide conjugates using *bis*(2-sulfanylethyl)amido peptide latent thioester surrogates. *Nat. Protocols* **2015**, *10*, 269-292.

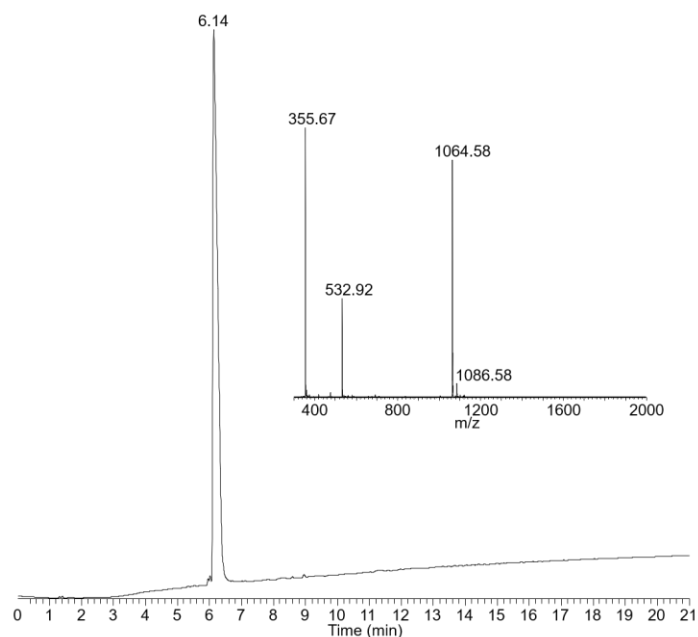


Figure S39. LC-MS analysis of peptide ILKEPWHGA- NHNH₂. a) LC trace. Eluent A 0.10% FA in water, eluent B 0.10% FA in CH₃CN, ZORBAX RRHT StableBond C3 80Å 1.8 μm (3.0 × 100 mm) column, 50 °C, gradient 0-70% B in 20 min, 0.4 mL/min, UV detection at 215 nm. b) MS trace: m/z = 1064.58 ([M+H]⁺), 532.92 ([M+2H]²⁺); calcd. for M (average): 1064.26, found: 1063.71.

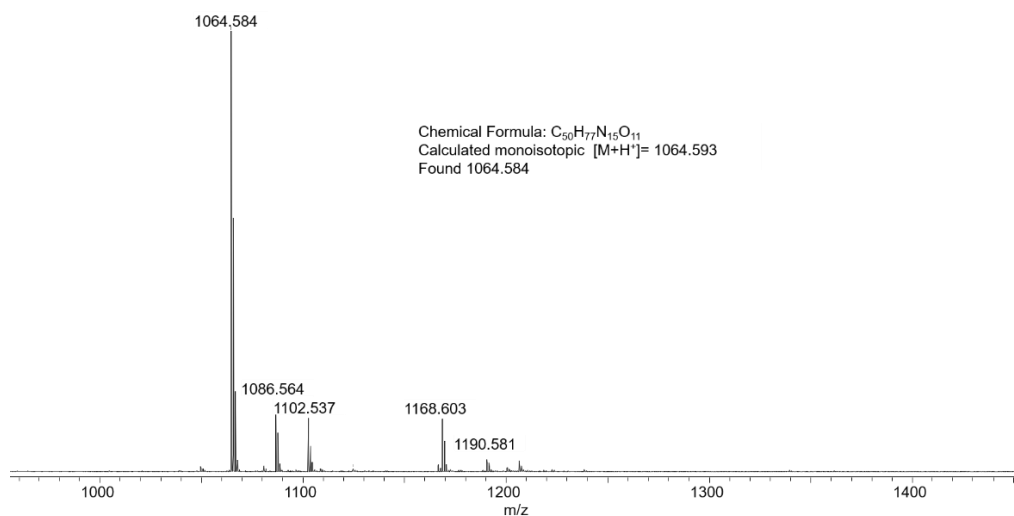


Figure S40. MALDI-TOF analysis of peptide ILKEPWHGA- NHNH₂ α-cyano-4-hydroxycinnamic acid, positive detection mode, [M+H]⁺ (calculated): 1064.59, found: 1064.58.

Synthesis and characterization of ILKEPWHGA-MPAA peptide

ILKEPWHGA-MPAA peptide was synthesized from the corresponding peptide hydrazide using Liu's method (20.05 mg, 82%).

MS (ESI, positive detection mode, Figure S41) m/z = 1200.67 ([M+H]⁺), 600.92 ([M+2H]²⁺). Calcd. for M (average) 1200.424, found: 1199.755.

MALDI-TOF (matrix α -cyano-4-hydroxycinnamic acid, positive detection mode, Figure S42) calcd. for $[M+H]^+$ (monoisotopic): 1200.58, found: 1200.69.

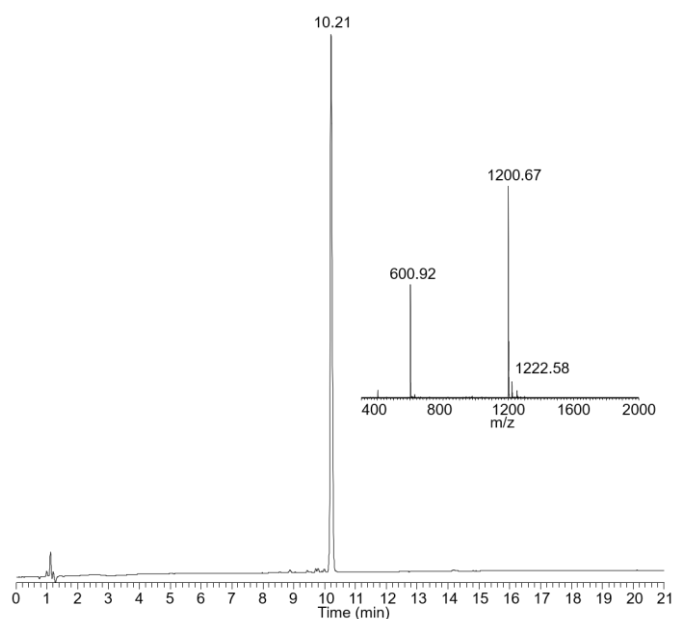


Figure S41. LC-MS analysis of peptide ILKEPWHGA-MPAA. a) LC trace. Eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH_3CN , ACQUITY UPLC Peptide BEH 300Å 1.7 μm (2.1×150 mm) column, 50 °C, gradient 0-70% B in 20 min, 0.4 mL/min, UV detection at 215 nm. b) MS trace: $m/z = 1200.67$ ($[M+H]^+$), 600.92 ($[M+2H]^{2+}$); calcd. for M (average): 1200.424, found: 1199.755.

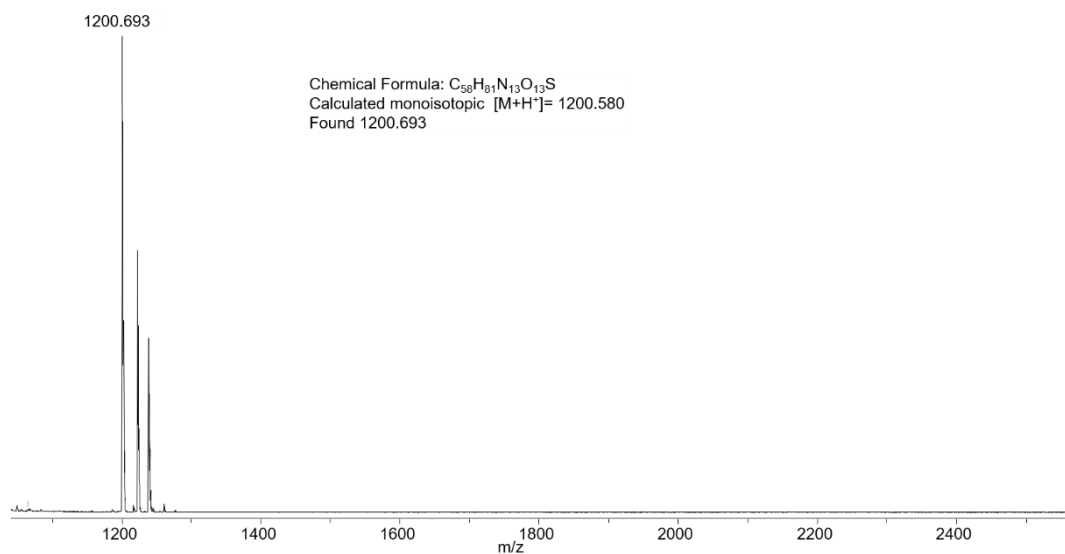


Figure S42. MALDI-TOF analysis of peptide ILKEPWHGA-MPAA. α -cyano-4-hydroxycinnamic acid, positive detection mode, $[M+H]^+$ (calculated): 1200.58, found: 1200.69.

4. Lys(^{oxo}SEA)-mediated ligation with Cys peptides

4.1. General methods and procedures

4.1.1. General experimental procedure for the ^{oxo}SEA-mediated ligation with Cys peptides

A typical procedure for the ^{oxo}SEA-mediated ligation is described below in the presence of peptide partners concentrated at ~1 mM, 200 mM MPAA and 100 mM TCEP (Figure S43). During our screening campaign, the concentration of peptides and additives, the pH, the temperature and the nature of the buffer were modified. Unless otherwise specified, the experiments were conducted according to the typical procedure detailed hereafter.

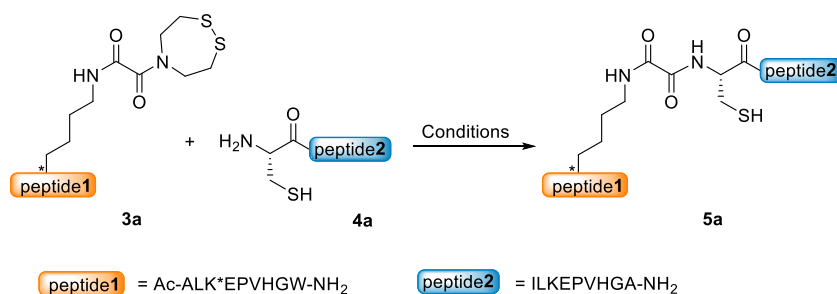


Figure S43. General principle of the ^{oxo}SEA-mediated ligation with Cys peptides

Analytical scale

To a solution of Gn·HCl (574 mg) in 0.1 M, pH 7.2 phosphate buffer (600 μL) were added TCEP (33.65 mg, 100 mM) and MPAA (33.4 mg, 200 mM). The pH of the mixture was adjusted to 5.49 by addition of 6 M NaOH (79 μL). The Cys peptide **4a** (1.099 mg, 0.522 μmol) was dissolved in the buffer solution containing MPAA and TCEP (651 μL, 1.2 mM). The resulting solution (476 μL) was added on the ^{oxo}SEA peptide **3a** (603 μg, 0.436 μmol, 0.92 mM) and the reaction was left to proceed at 37 °C.

The progress of the reaction was monitored by HPLC. Typically, a 10 μL aliquot was withdrawn from the reaction mixture at regular time intervals and added to 100 μL of a 10% AcOH aqueous solution. The resulting solution was immediately extracted three times with Et₂O to remove the MPAA, flushed with a gentle stream of nitrogen gas and analyzed by HPLC (eluent A 0.1% TFA in water, eluent B 0.1% TFA in acetonitrile, C18 column, 50°C, UV detection at 215 and 280 nm). Conversions were determined from the chromatograms by integration of the signals at 280 nm (Trp residue).

Preparative scale

The same reaction was repeated at the multimilligram scale in the exact same conditions of peptide and additive concentrations, pH and temperature (Cys peptide **3a** 6.215 mg, 4.4 μmol; ^{oxo}SEA peptide **4a** 4.710 mg, 3.41 μmol). After completion of the reaction (~10 min), the crude mixture was quenched by

glacial AcOH (400 μ L), extracted three times with Et₂O to remove MPAA, flushed with nitrogen and purified on a semipreparative HPLC system (eluent A 0.1% TFA in water, eluent B 0.1% TFA in acetonitrile, 50°C, detection at 215 nm, 6 mL min⁻¹, 0-20% eluent B in 10 min, then 20-35% eluent B in 30 min, C18 column) to give ligated peptide **5a** as a white solid after lyophilisation (3.75 mg, 43%).

UPLC-MS (ESI, positive detection mode, Figure S 44) m/z = 1100.00 ([M+2H]²⁺), 733.25 ([M+3H]³⁺); calcd. for M: 2196.56, found: 2197.38.

MALDI-TOF (matrix 2,5-dihydroxybenzoic acid (DHB), positive detection mode, Figure S45) calcd. for [M+H]⁺(average) 2197.57, found 2197.54.

4.1.2. Characterization of Ac-ALK(C(O)C(O)CILKEPVHGA-NH₂)EPVHGW-NH₂

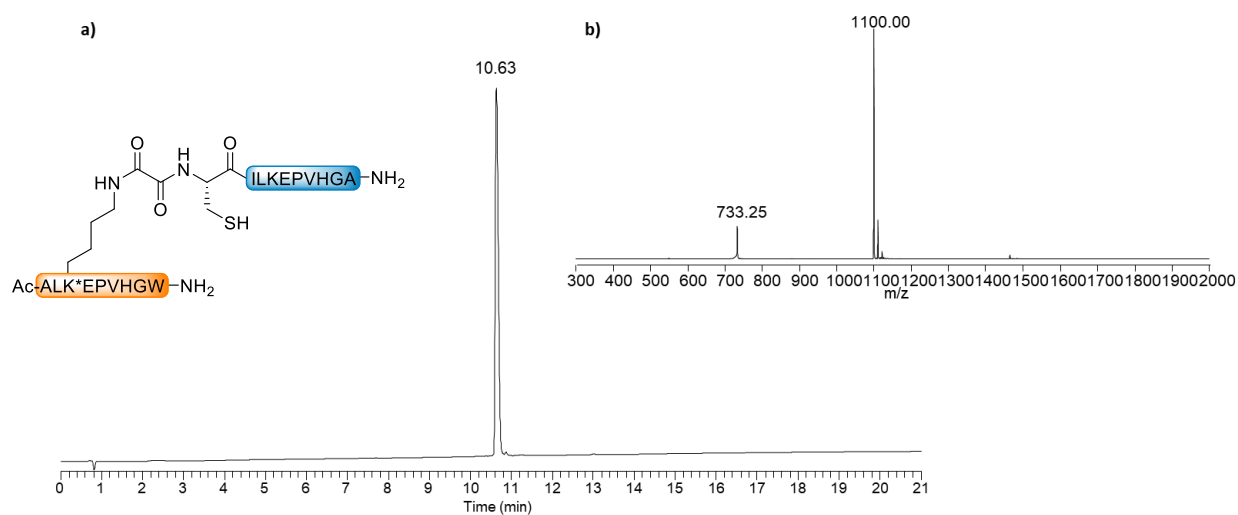


Figure S 44. LC-MS analysis of peptide **5a**. a) LC trace. Eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN, ACQUITY UPLC Peptide BEH 300Å 1.7 μ m (2.1 \times 150 mm) column, 50 °C, gradient 0-70% B in 20 min, 0.4 mL/min, UV detection at 215 nm. b) MS trace: m/z = 1100.00 ([M+2H]²⁺), 733.25 ([M+3H]³⁺); calcd. for M (average): 2196.56, found: 2197.38.

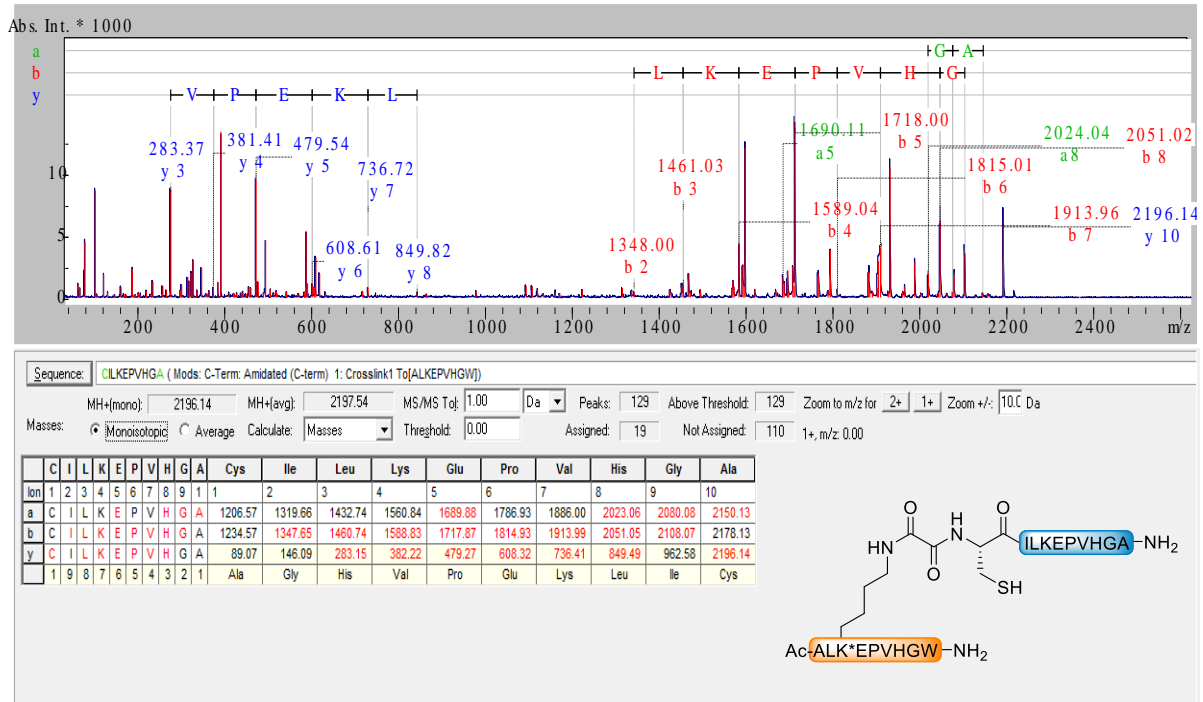


Figure S45. MALDI-TOF-TOF sequencing of Ac-ALK(C(O)C(O)CILKEPVGHA-NH₂)EPVHGW-NH₂ peptide **5a**. Matrix α -cyano-4-hydroxycinnamic acid, positive detection mode.

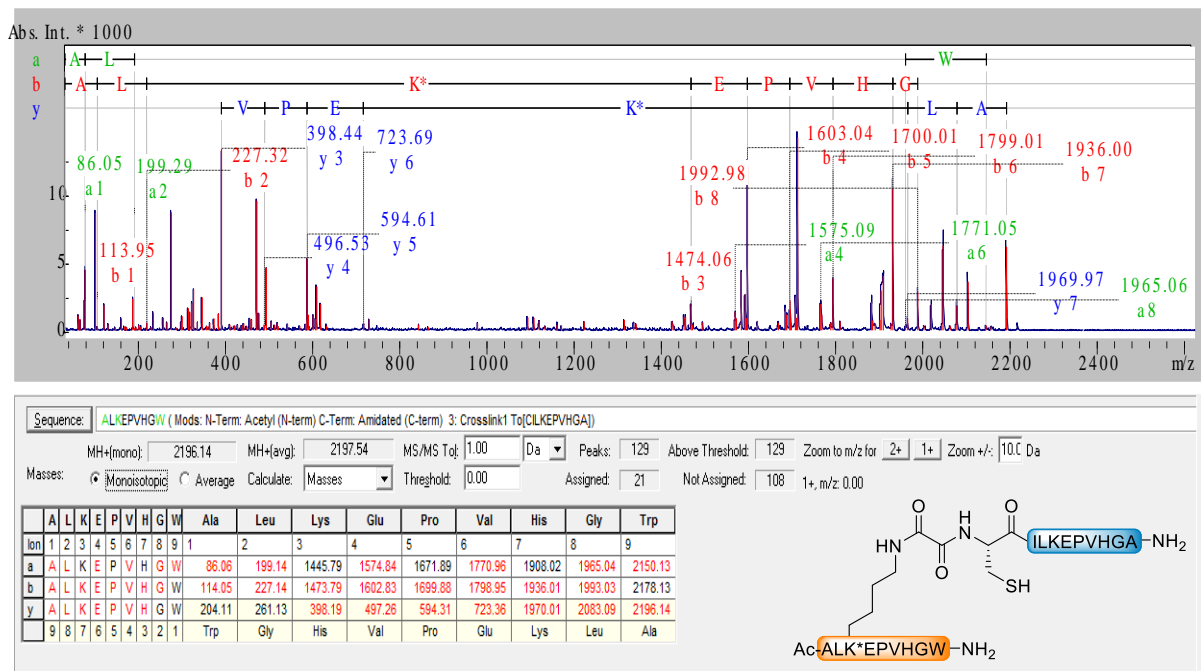


Figure S46. MALDI-TOF-TOF sequencing of Ac-ALK(C(O)C(O)CILKEPVGHA-NH₂)EPVHGW-NH₂ peptide **5a**. Matrix α -cyano-4-hydroxycinnamic acid, positive detection mode. The MS-MS analysis confirms the presence of an oxalyl crosslink on the lysine sidechain (K* on the figure).

4.1.3. Exploitation of kinetic data

General data fitting procedure

Unless otherwise specified, conversion to ligated product was calculated from the UV trace at 280 nm (Trp residue absorption). Prior to kinetic data analysis and fitting, conversions were transformed into concentrations of the product based on the starting Lys(^{OXO}SEA) peptide **4a** concentration.

Kintek Global Kinetic Explorer Software, Version 10.0.200514, was used for kinetic modeling. The model reaction used for numerical fitting of the ^{OXO}SEA-mediated ligation corresponds to a second-order reaction in the form of $A + B \rightarrow P$. The standard deviation for each trace was first estimated upon fitting the experimental dataset with an analytical function (3-exponential) so as to determine an average sigma value, further used for numerical data fitting. The subsequent numerical fit allowed determining the apparent second order rate constant k_{app} for each experiment. Fitting to a given model was achieved by nonlinear regression analysis based upon an iterative search to find a set of reaction parameters that gives a minimum χ^2 . The process was completed by careful visual examination of the fits.

4.2. Quantification of post-quench drifts

While great care has been taken to ensure maximum accuracy of the data, we wondered if the amount of ligated product **5a** formed in the reaction could be subject to small variations after quench and thus, could bias its quantification. Indeed, even though the reaction mixture is substantially diluted and acidified upon quench, the ^{OXO}SEA-mediated ligation reaction involves intermediates prone to react at very low concentrations which could further enrich the mixture with the desired product.

In order to check whether a drift could occur, we performed a reaction in the following conditions: 6 M Gn.HCl, 0.1 M phosphate buffer, 80 mM sodium ascorbate, 100 mM TCEP, pH 5.5, [^{OXO}SEA peptide] = 1 mM, 1.2 equiv. Cys peptide. Note that the MPAA catalyst was removed to slow down the reaction and sodium ascorbate was added to minimize the desulfurization which could take place in the absence of MPAA. The reaction was left to proceed for 3 min 20 sec (until 6% conversion). An aliquot of 100 μ L of the crude mixture was withdrawn and added to a 10% aqueous solution of acetic acid (1 mL). The formation of the ligated product in the quenched aliquot was monitored by RP-HPLC over 5 hours (Figure S 47).

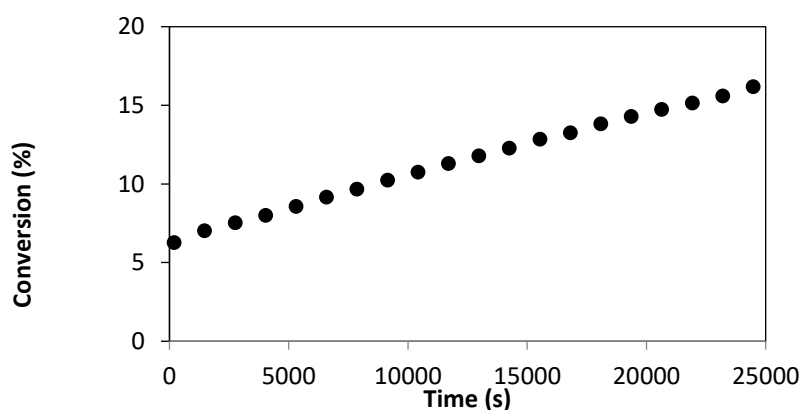


Figure S 47. RP-HPLC monitoring of the formation of the ligated product **5a** after quench

We noticed that the amount of product formed further evolved after the quench corresponding to 10% conversion over 5 hours. This result must however be put into perspective. It might be considered only for reactions achieved at the highest peptide concentrations (> 0.3 mM) and for those aliquots collected at the beginning of reactions (when the concentration of the starting material is at its highest). Even in this case and considering that one hour may elapse in between sample extraction and injection on the HPLC system, the expected drift would be about 1 to 2 %. In conclusion, no significant effect on the data is expected.

4.3. Comparison with classical alkyl and aryl thioesters-based NCL

The rate of the ^{oxo}SEA-mediated ligation was compared to that of classical alkyl and aryl thioesters-based NCL with a cysteinyl peptide in the conditions mentioned in Figure S 48. Alkyl and aryl thioester peptides were produced according to the protocols provided in the general procedures section.

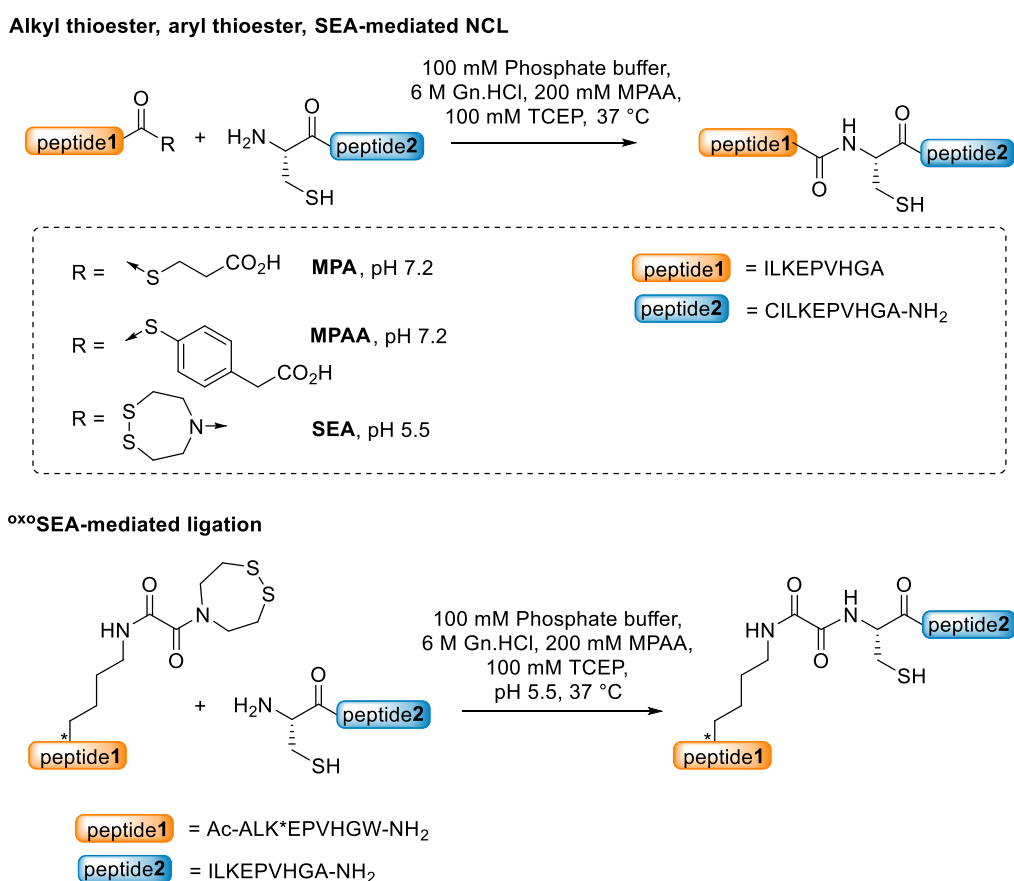


Figure S 48. Ligation of a Cys peptide with an alanyl MPA thioester, an alanyl MPAA thioester, an alanyl SEA peptide and an ^{oxo}SEA-containing peptide.

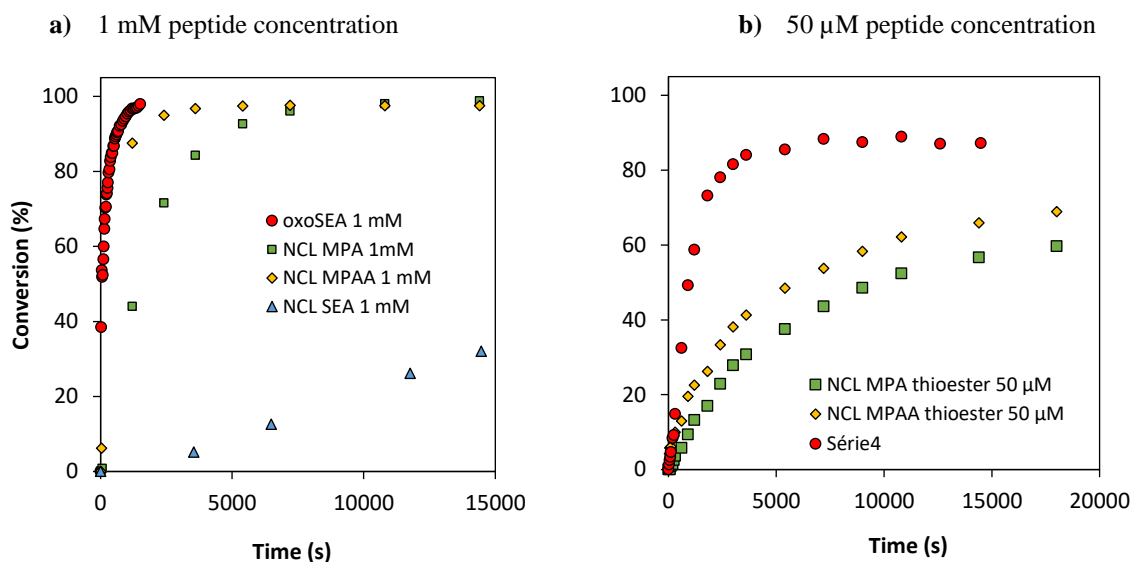


Figure S 49. Kinetic monitoring of the ^{oxo}SEA -mediated ligation vs NCL-related approaches at. a) 1 mM peptide concentration ; b) 50 μM peptide concentration.

4.4. Influence of peptide concentration on the ^{oxo}SEA -mediated ligation

4.4.1. Results

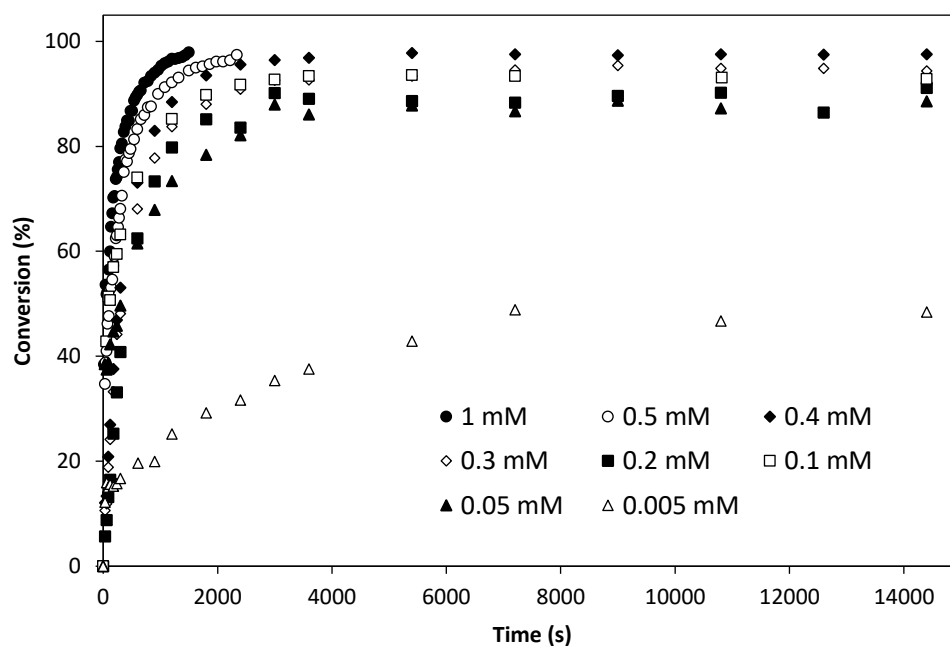
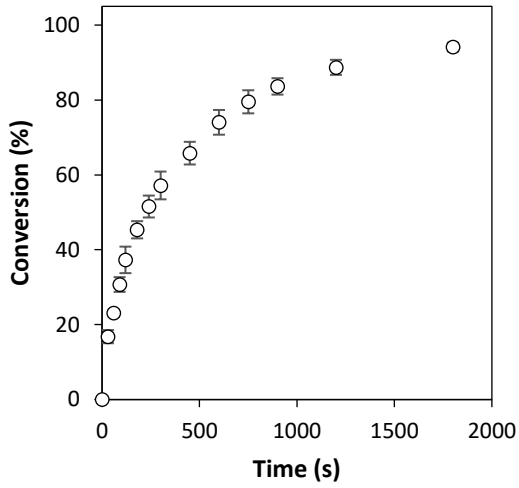
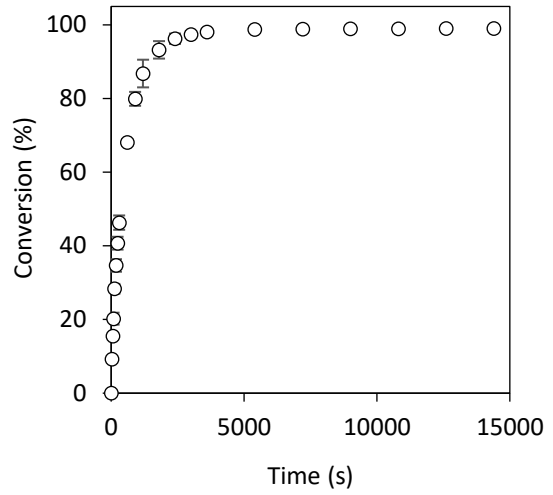


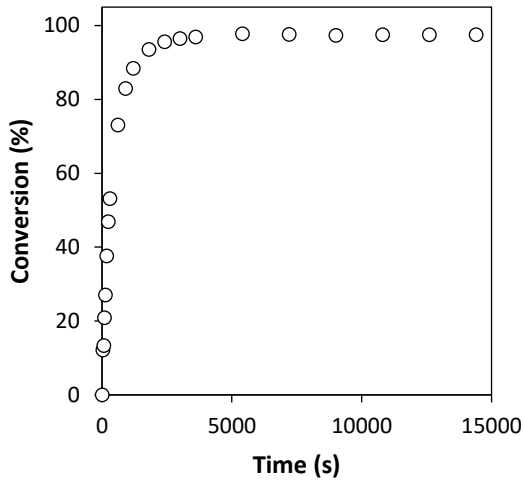
Figure S 50. Kinetic monitoring of the ^{oxo}SEA -mediated ligation presented in Figure S 48 at peptide concentrations **3a**: 1 mM, 500 μM , 400 μM , 300 μM , 200 μM , 150 μM , 100 μM , 50 μM , 5 μM (1.2 equiv. of Cys peptide, 6 M Gn.HCl, 100 mM phosphate buffer, 200 mM MPAA, 100 mM TCEP, pH 5.5, 37 $^{\circ}C$). One replicate is presented *per* concentration.



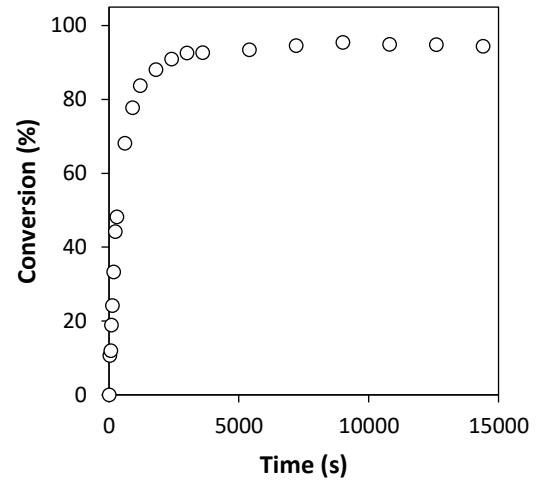
a) 1 mM



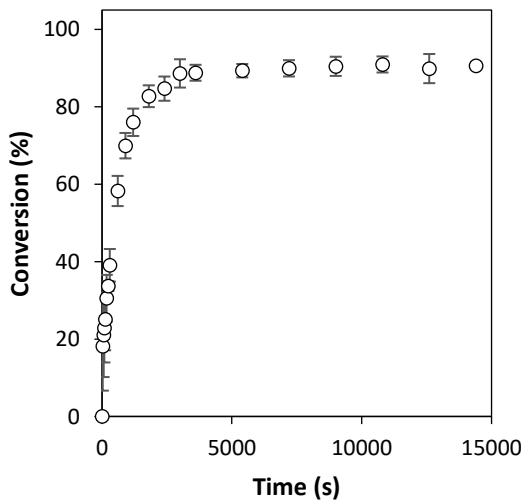
b) 500 μM



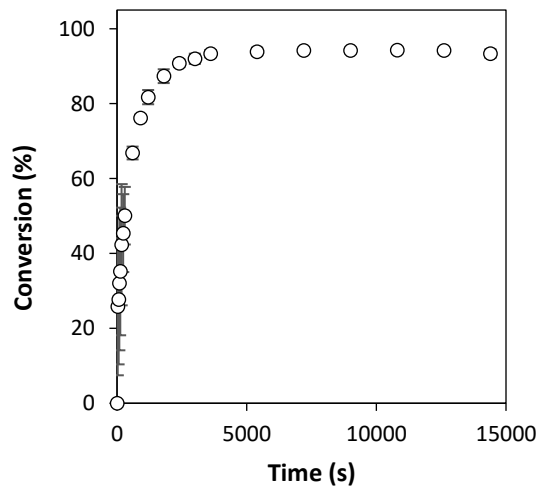
c) 400 μM



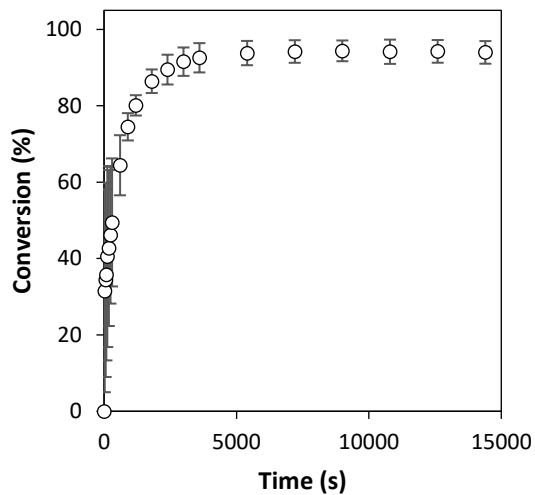
d) 300 μM



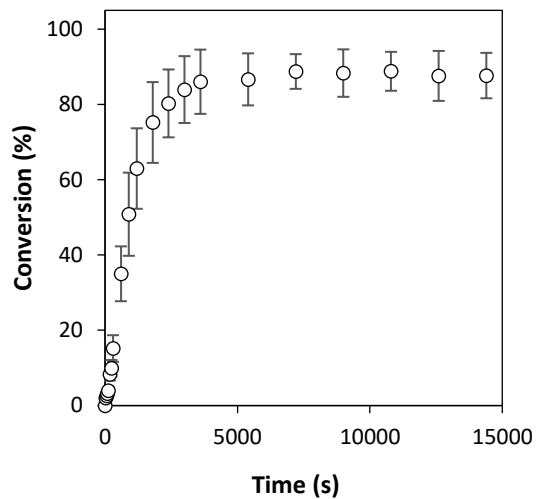
e) 200 μM



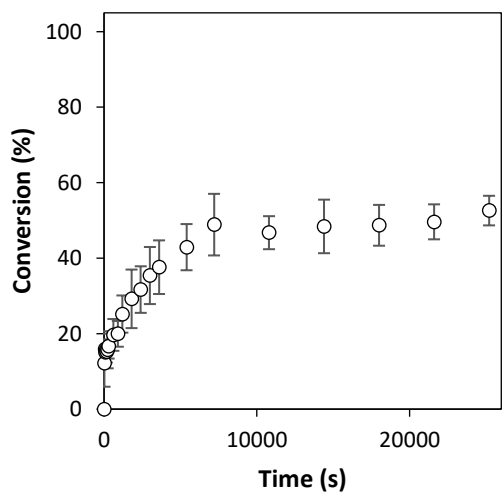
f) 150 μM



g) 100 μM



h) 50 μM



i) 5 μM, pH 5.5

Figure S 51. Kinetic monitoring of the ^{ox}SEA-mediated ligation between **3a** and **4a**. The traces at various working peptide concentration are presented with standard deviation. Each graphical representation is the result of three independent replicates (with the exception of measures done at 300 μM and 400 μM).

4.4.2. Example of kinetic monitoring of the ^{oxo}SEA-mediated ligation at 100 μ M peptide concentration

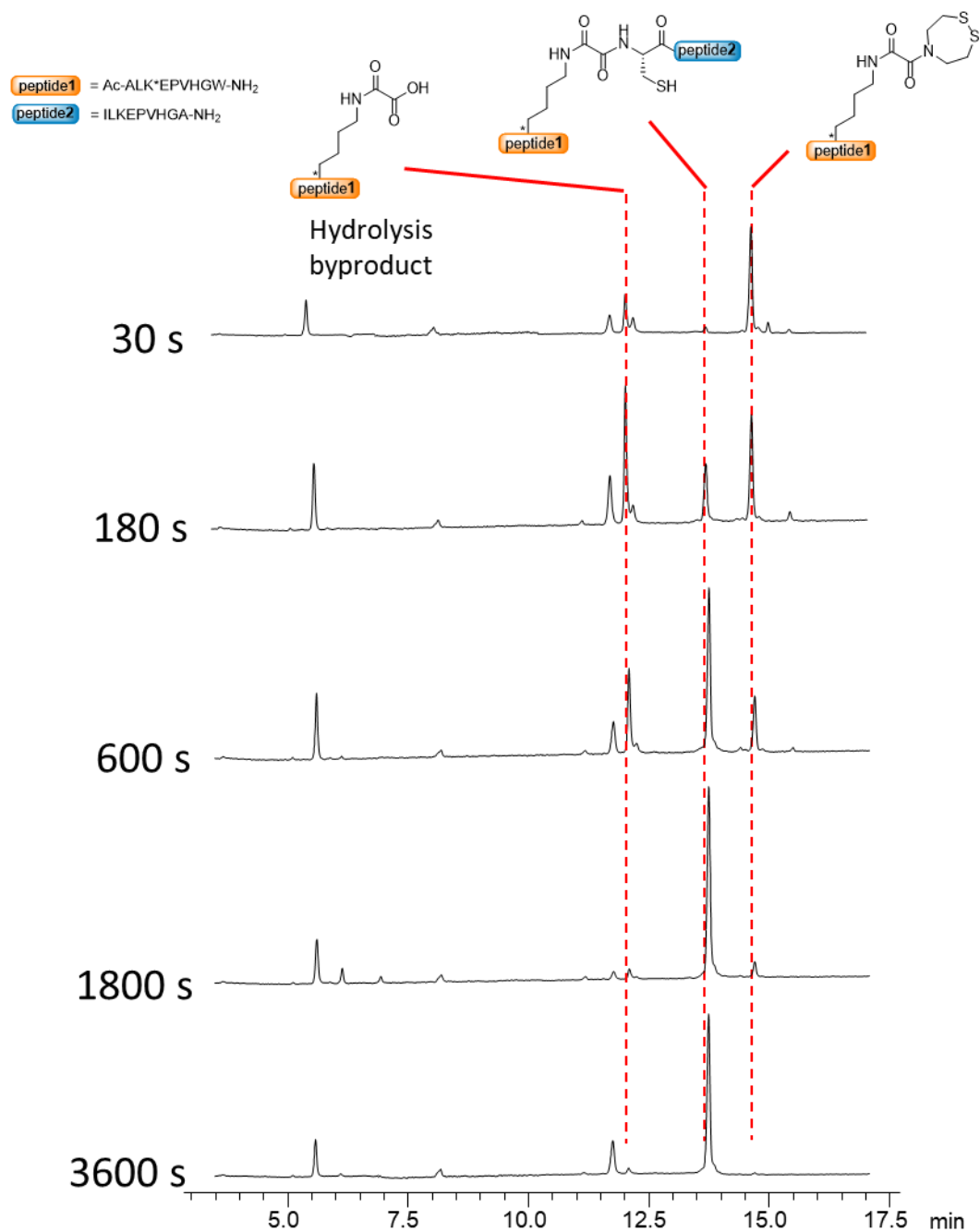


Figure S 52. RP-HPLC monitoring at 280 nm of the ^{oxo}SEA-mediated ligation reaction performed at 100 μ M **3a** concentration (100 μ M ^{oxo}SEA-containing peptide **3a**, 120 μ M Cys peptide **4a**, 6 M Gn.HCl in 0.1 M phosphate buffer, 200 mM MPAA, 100 mM TCEP, pH 5.5, 37 °C) .

Note that the important amount of hydrolysis byproduct observed at intermediate times originates from the hydrolysis of unreacted starting material in the HPLC vial and not from the reaction.

4.4.3. Data fitting and determination of rate constants

Below are provided the rate constants determined from each trace and presented as mean \pm standard error with a 95% confidence interval.

Table S1. Apparent second order rate constants for oxoSEA-mediated ligation product formation^a

Entry	oxoSEA-peptide concentration	k_{app} ($M^{-1}.s^{-1}$)	Standard Error ($M^{-1}.s^{-1}$)
1	1 mM	4.12	0.46
2	500 μ M	4.90	0.41
3	400 μ M	7.22	nd
4	300 μ M	8.70	nd
5	200 μ M	11.37	1.56
6	150 μ M	20.28	4.15
7	100 μ M	29.01	7.93
8	50 μ M	28.47	5.92

^a The reaction were performed in triplicate and data are presented as mean value \pm standard error with a 95% confidence interval.

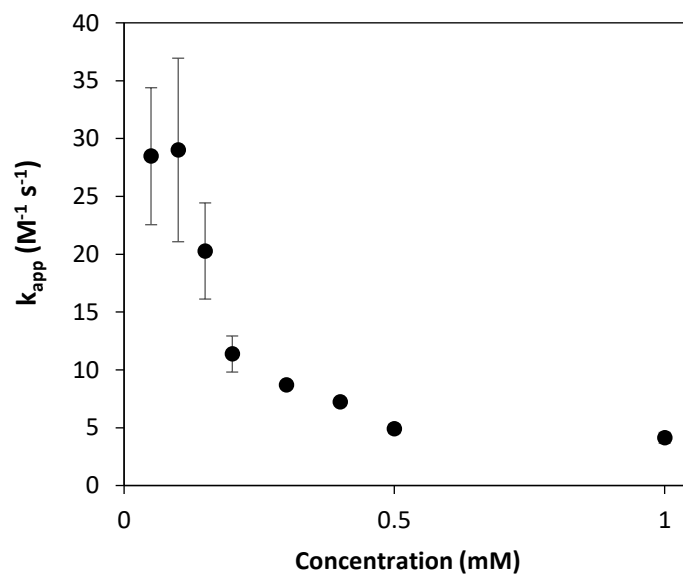
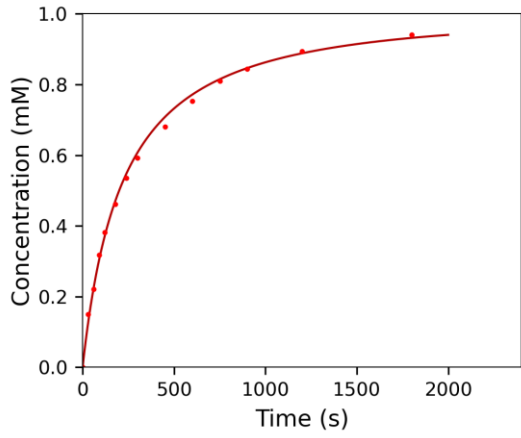
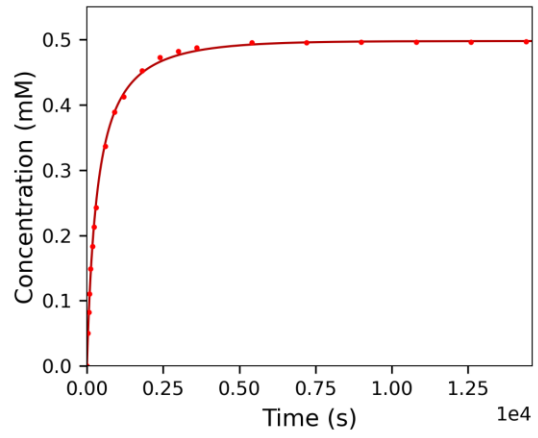


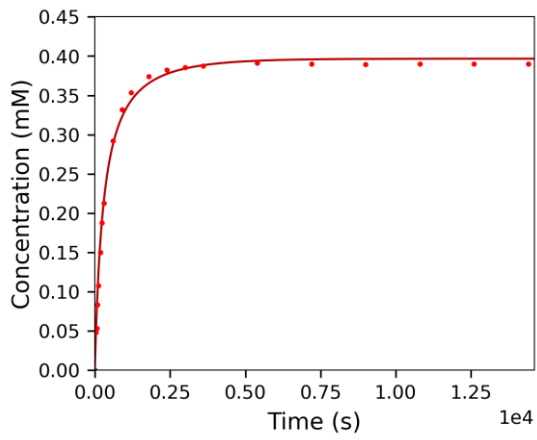
Figure S 53. Apparent second order rate of the oxoSEA-mediated ligation of **3a** and **4a** increases as peptide concentration decreases



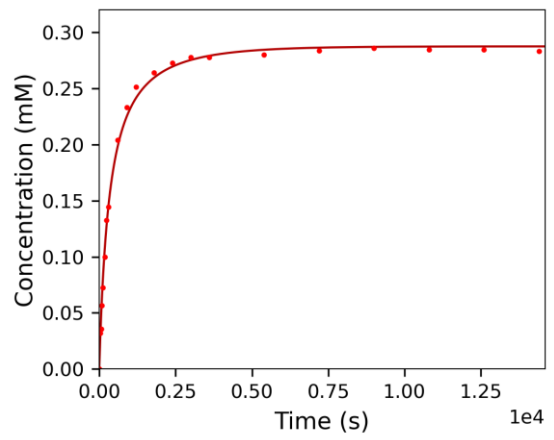
a) 1 mM



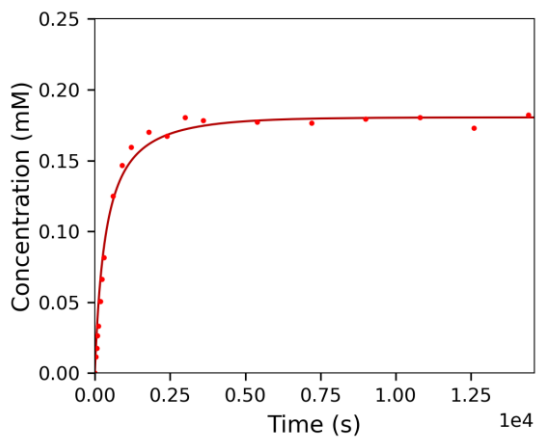
b) 500 μM



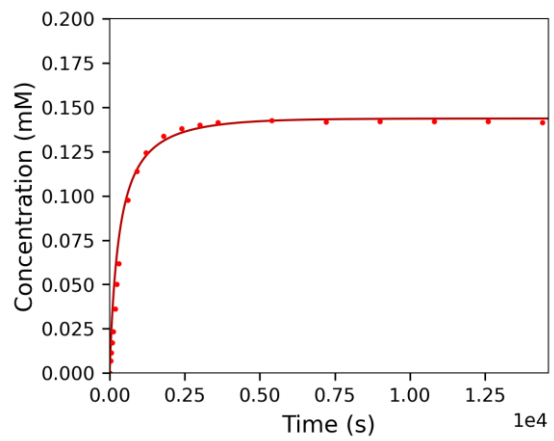
c) 400 μM



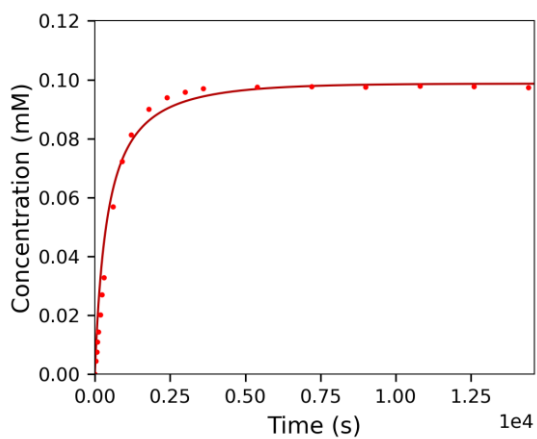
d) 300 μM



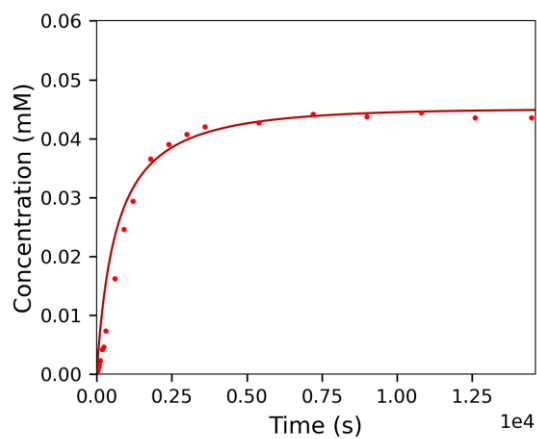
e) 200 μM



f) 150 μM



g) 100 μ M



h) 50 μ M

Figure S 54. Fitting of kinetic data presented in Table S1 (the fits are provided for one replicate for each concentration).

4.5. Influence of the pH

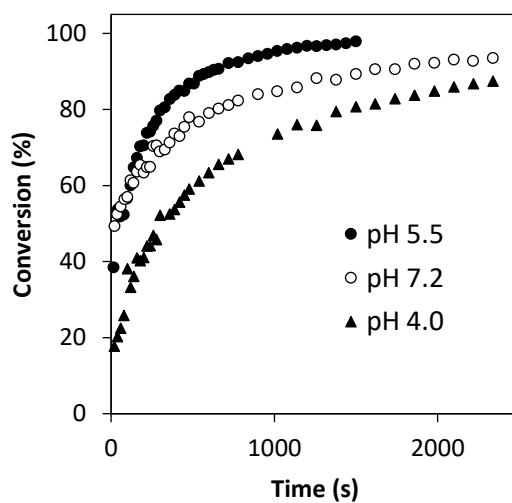


Figure S 55. Kinetic monitoring of the ^{OXO}SEA-mediated ligation presented in Figure S 48 at pH values of 4.0, 5.5 and 7.2. (1 mM **3a** in 0.1 M phosphate buffer, 1.2 equiv. **4a**, 6 M Gn.HCl, 200 mM MPAA, 100 mM TCEP, 37°C)

4.6. Influence of catalyst concentration

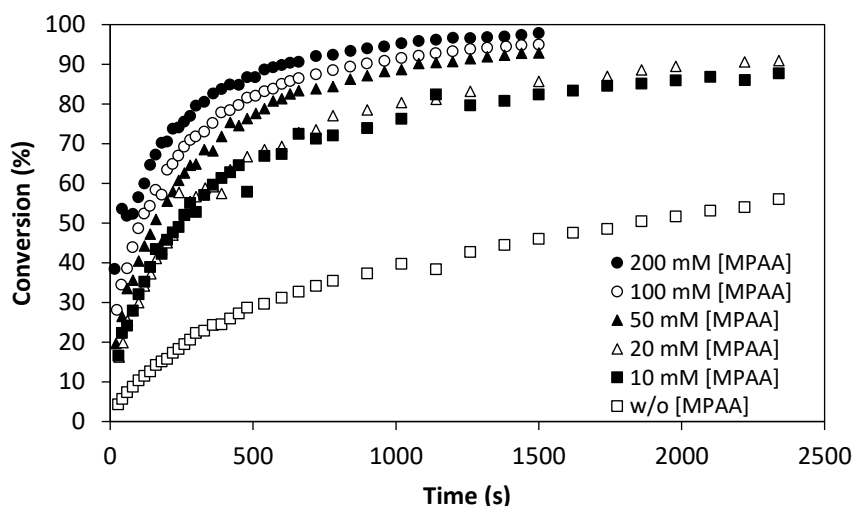


Figure S 56. Kinetic monitoring of the ^{oxo}SEA -mediated ligation presented in Figure S 48 at various concentrations of 4-mercaptophenylacetic acid (MPAA) : 200 mM, 100 mM, 50 mM, 20 mM, 10 mM, without catalyst (1 mM **3a** in 0.1 M phosphate buffer, 1.2 equiv. **4a**, 6 M Gn.HCl, x mM MPAA, 100 mM TCEP, pH 5.5, 37°C)

The reaction is efficient at low concentrations of catalyst (10 mM MPAA). The data also show that the reaction still operates in the absence of MPAA catalyst.

4.7. Tolerance of the ^{oxo}SEA group to reducing conditions

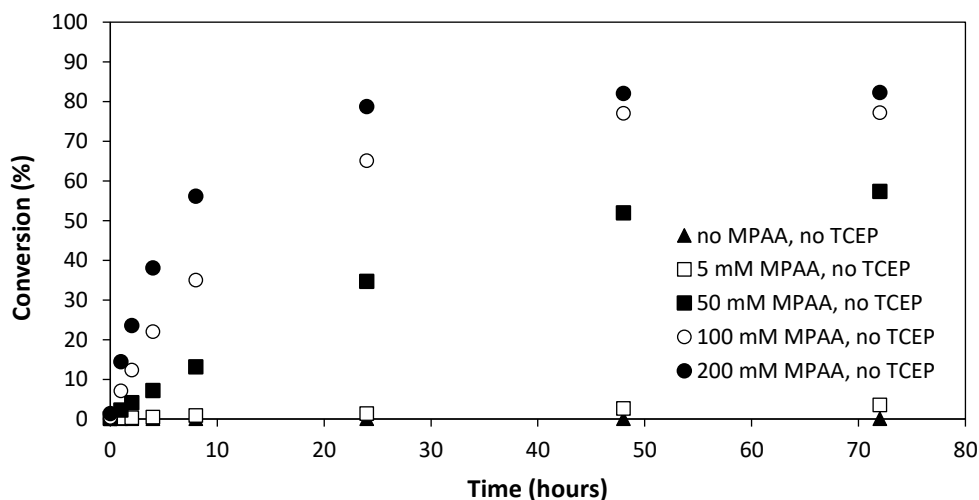


Figure S 57. Kinetic monitoring of the ^{oxo}SEA group stability in the presence of reducing agents (1 mM ^{oxo}SEA peptide **3a**, 1.2 equiv. of Cys peptide **4a**, 6 M Gn.HCl, 100 mM phosphate buffer, MPAA or TCEP additives, pH 5.5, 37 °C).

Note that the scale for the time axis is given in hours. In the absence of any reducing agents, the ^{oxo}SEA group is perfectly stable for days. The addition of increasing concentrations of MPAA lead to its

activation and to the formation of a ligation product (negligible at 5 mM MPAA - 5% over 3 days - and substantial at 200 mM MPAA - ~14% after 1 h).

4.8. Hydrolysis of the ^{oxo}SEA group

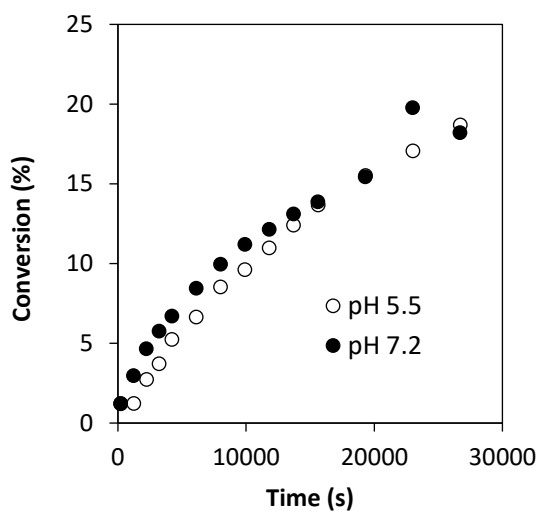


Figure S 58. Kinetic monitoring of ^{oxo}SEA group hydrolysis at pH 5.5 and pH 7.2 (100 μ M ^{oxo}SEA-containing peptide **3a**, 6 M Gn.HCl in 0.1 M phosphate buffer, 100 mM TCEP, 37 $^{\circ}$ C).

4.9. Influence of the pH in the low μ molar peptide concentration range

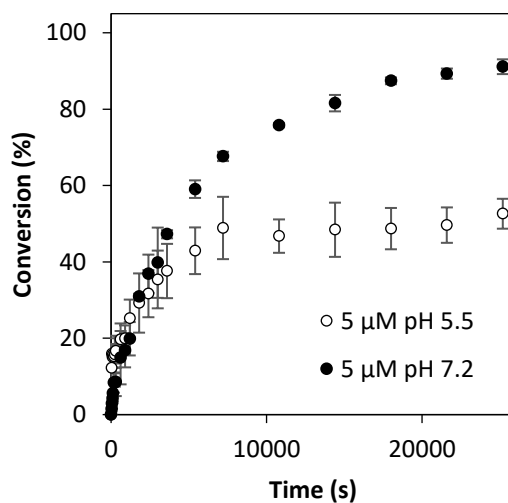


Figure S 59. Kinetic monitoring of ^{oxo}SEA-mediated ligation at pH 5.5 and pH 7.2 (5 μ M ^{oxo}SEA-containing peptide **3a**, 1.2 equiv. **4a**, 6 M Gn.HCl in 0.1 M phosphate buffer, 200 mM MPAA, 100 mM TCEP, 37 $^{\circ}$ C).

5. Ligation to Cys peptides mediated by N-terminal ^{oxo}SEA group

5.1. Preparation of N-terminal ^{oxo}SEA-peptides

N-terminal oxoSEA-peptides were prepared as described in Figure S 60.

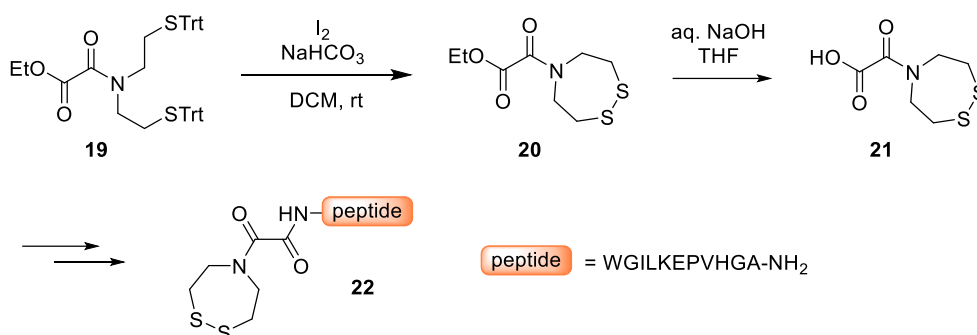


Figure S 60. Synthesis of N-terminal ^{oxo}SEA-functionalized peptides

Ethyl 2-(bis[2-(sulfenyl)ethyl]amino)-2-oxoacetate **20**

To a solution of intermediate **19** (7.2 g, 10 mmol) in CH_2Cl_2 (140 mL) is added sodium bicarbonate $NaHCO_3$ (2.5 g, 30 mmol). To the resulting suspension is added a solution of iodine (7.6 g, 30 mmol) in CH_2Cl_2 (140 mL) at room temperature. Completion of the reaction is checked by TLC after 30 minutes. An aqueous solution of sodium thiosulfate 1 M (400 mL) is then poured in the crude mixture, resulting in a colorless biphasic mixture. Layers are separated and the aqueous layer is extracted with CH_2Cl_2 (3 × 60 mL). The organic extracts are then combined, washed with water (100 mL) and brine (100 mL), dried over $MgSO_4$ and concentrated under reduced pressure until 10-15 mL remain. The crude mixture is purified by column chromatography (Eluent: Cyclohexane/Ethyl acetate/DCM from 75/15/10 to 60/40/0). Product **20** was isolated as a yellow oil with 87% yield.

R_f 0.22 in EtOAc/cyclohexane (20:80). **¹H NMR** (300 MHz, $CDCl_3$) δ 4.35 (q, 2H), 4.00 (t, 2H), 3.81 (t, 2H), 3.13 (t, 2H), 3.01 (t, 2H), 1.39 (t, 3H) ppm. **¹³C NMR** (75 MHz, $CDCl_3$) δ 162.21, 161.77, 62.19, 52.98, 48.97, 40.07, 34.27, 13.94 ppm.

2-(bis[2-(sulfenyl)ethyl]amino)-2-oxoacetic acid **21**

To a solution of intermediate **20** (2.0 g, 7.9 mmol) in THF (100 mL) is added an aqueous solution of sodium hydroxide (1.9 g, 46.6 mmol) in water (100 mL). The resulting solution is stirred at room temperature for 2 hours. The crude mixture is acidified to pH 2 by addition of 1 M HCl . The aqueous layer is extracted with ethyl acetate (3 × 50 mL). Organic extracts are combined, washed with water (3 × 20 mL), brine (20 mL), dried over $MgSO_4$ and concentrated under reduced pressure. Product **21** was isolated as a white amorphous solid with 37% yield.

R_f 0.22 in EtOAc/cyclohexane (20:80). **¹H NMR** (300 MHz, $CDCl_3$) δ 4.38 (s, 2H), 4.06 (t, 2H), 3.11-3.06 (m, 4H) ppm. **¹³C NMR** (75 MHz, $CDCl_3$) δ 54.88, 53.13, 38.94, 33.90 ppm.

Synthesis of ^{oxo}SEA-WGILKEPVHGA-NH₂ **22**

The H-WGILKEPVHGA sequence was elongated on a rink-amide functionalized solid support as described in the general procedure.

The activator HATU (38.0 mg, 0.10 mmol) was dissolved in DMF (1 mL) (solution 1). The ^{oxo}SEA derivative **21** (22.8 mg, 0.11 mmol) and DIEA (69.7 μ L, 0.4 mmol) were dissolved in DMF (2 mL) (solution 2). Solution 1 and 2 were mixed during 30 seconds and the resulting mixture was immediately poured on the solid support functionalized with 0.10 mmol of peptide. Reaction was stirred at room temperature during 30 minutes. The solid support was washed with DMF and the coupling was performed twice again. The peptide **22** was cleaved according to the protocol described in the general procedure and was isolated as a white amorphous solid with 25% yield.

UPLC-MS (positive detection mode, Figure S 61) [M+H]⁺ calcd. (monoisotopic): 1394.663, found: 1394.92.

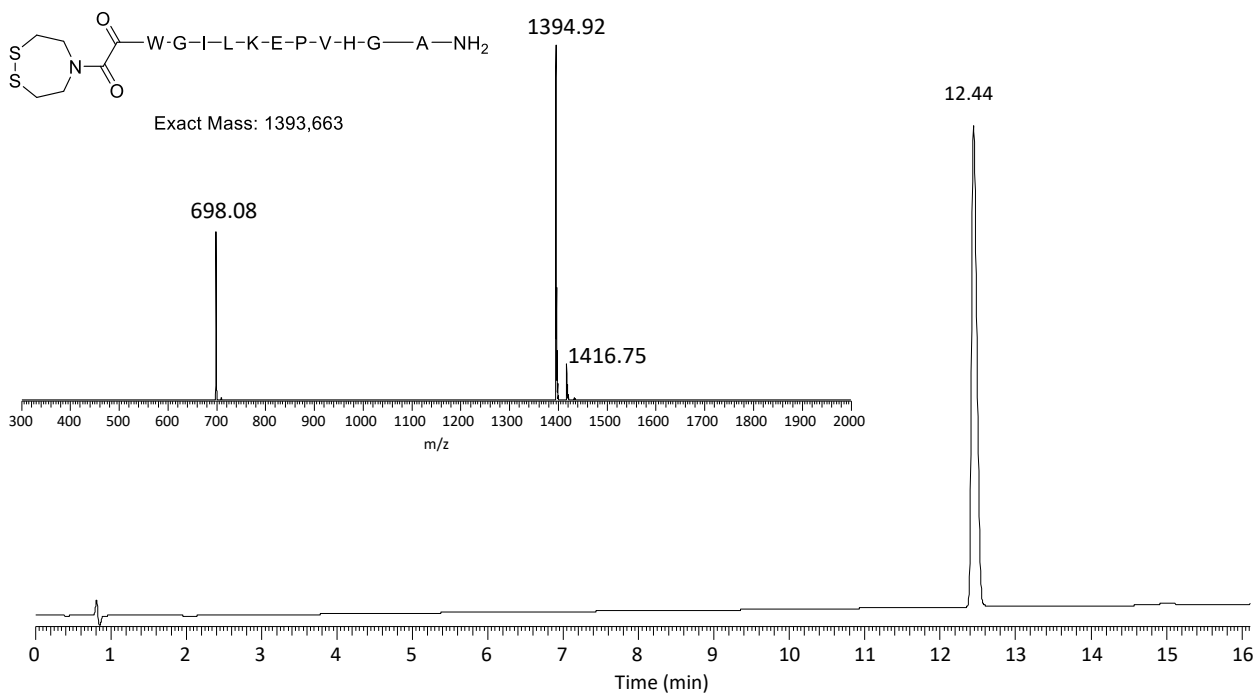


Figure S 61. UPLC-MS analysis of peptide **22**. a) LC trace. Eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN, ACQUITY UPLC Peptide BEH 300Å 1.7 μ m (2.1 \times 150 mm) column, 50 $^{\circ}$ C, gradient 0-70% B in 20 min, 0.4 mL/min, UV detection at 215 nm. b) MS trace: m/z = 1394.92 ([M+H]⁺), 698.08 ([M+2H]²⁺); calcd. for M (average): 1393.66, found: 1394.04.

5.2. Ligation to Cys peptides mediated by N-terminal ^{oxo}SEA group

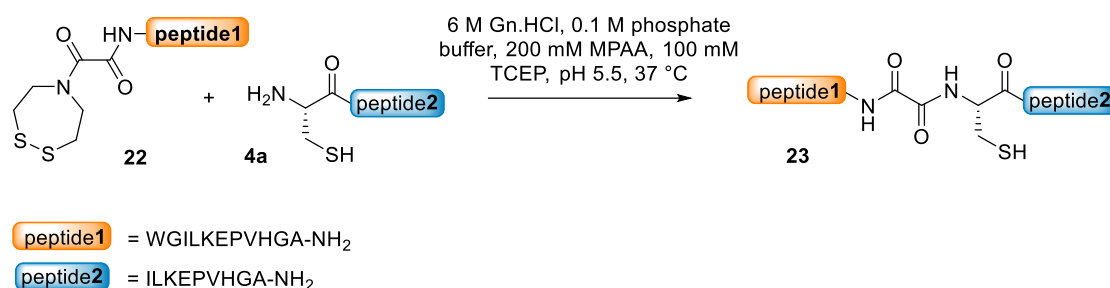


Figure S 62. ^{oxo}SEA-mediated ligation with a peptide featuring an ^{oxo}SEA group at its N-terminus

To a solution of Gn·HCl (574 mg) in 0.1 M, pH 7.2 phosphate buffer (600 μ L) were added TCEP (33.65 mg, 100 mM) and MPAA (33.4 mg, 200 mM). The pH of the mixture was adjusted to 5.5 by addition of 6 M NaOH. The Cys peptide **4a** (1.099 mg, 0.522 μ mol) was dissolved in the buffer solution containing MPAA and TCEP (651 μ L, 1.2 mM). The resulting solution (476 μ L) was added on the ^{oxo}SEA peptide **22** (707 μ g, 0.436 μ mol, 1 mM) and the reaction was left to proceed at 37 $^\circ$ C.

The progress of the reaction was monitored by HPLC. Typically, a 2 μ L aliquot was withdrawn from the reaction mixture at regular time intervals and added to 100 μ L of a 10% AcOH aqueous solution. The resulting solution was immediately extracted three times with Et₂O to remove the MPAA, flushed with a gentle stream of nitrogen gas and analyzed by HPLC (eluent A 0.1% TFA in water, eluent B 0.1% TFA in acetonitrile, C18 column, 50 $^\circ$ C, UV detection at 215 and 280 nm). Conversions were determined from the chromatograms by integration of the signals at 280 nm (Trp residue).

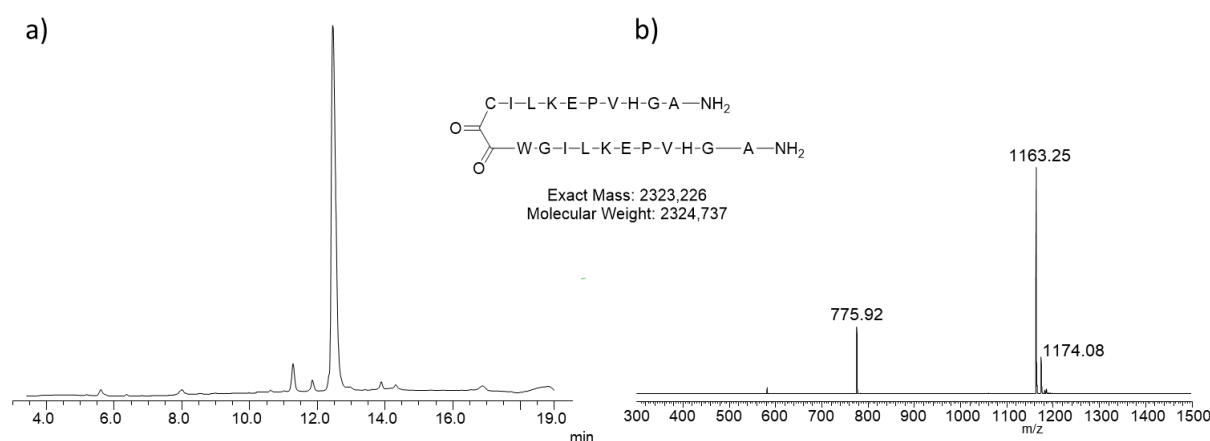


Figure S 63. Analysis of peptide **23**. a) LC trace from the aliquot at 5600 s after extraction (not purified). Eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN, ACQUITY UPLC Peptide BEH 300 \AA 1.7 μ m (2.1 \times 150 mm) column, 50 $^\circ$ C, gradient 0-70% B in 20 min, 0.4 mL/min, UV detection at 280 nm. b) MS trace: m/z = 1163.25 ([M+2H]²⁺), 775.92 ([M+3H]³⁺); calcd. for M (average): 2324.63, found: 2324.74.

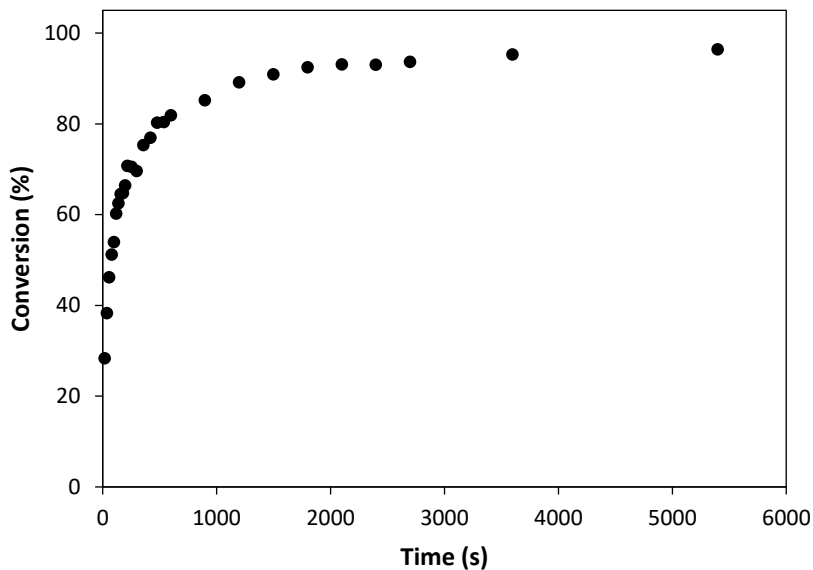


Figure S 64. RP-HPLC monitoring of the ^{oxo}SEA -mediated ligation in the presence of N-terminal ^{oxo}SEA functionalized peptide **23** described in **Figure S 62** (1 mM **22** in 0.1 M phosphate buffer, 1.2 equiv. **4a**, 6 M Gn.HCl, 200 mM MPAA, 100 mM TCEP, 37°C)

6. SDS-PAGE analysis of ^{oxo}SEA ligation with the Cys-K1Lys(Biot) polypeptide

6.1. Synthesis of Cys-K1Lys(Biot) polypeptide

The Cys-K1(Biot) polypeptide **4b** was assembled according to a procedure described elsewhere.⁸ UPLC-MS analysis of the sample produced for the study is provided below.

⁸ Ollivier, N.; Desmet, R.; Drobecq, H.; Blanpain, A.; Boll, E.; Leclercq, B.; Mougel, A.; Vicogne, J.; Melnyk, O. A simple and traceless solid phase method simplifies the assembly of large peptides and the access to challenging proteins. *Chem. Sci.* **2017**, 8, 5362-5370.

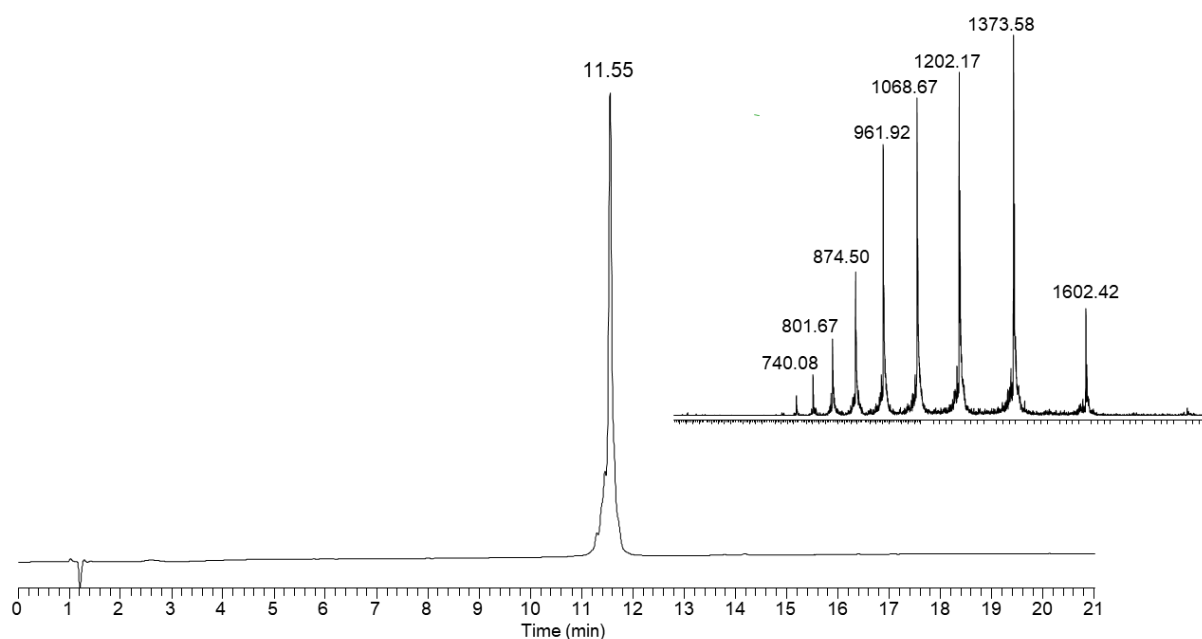


Figure S 65. UPLC-MS analysis. LC trace for Cys-K1(Biot) **4b**. Eluent A 0.1% TFA in water, eluent B 0.1% TFA in CH₃CN. XBridge BEH C18 (3.5 μm, 300 Å, 2.1 × 150 mm) column, gradient 0-70% B in 15 min (0.4 mL min⁻¹, detection UV 215 nm). MS trace: m/z = 1602.42 ([M+6H]⁶⁺), 1373.58 ([M+7H]⁷⁺), 1202.17 ([M+8H]⁸⁺), 1068.67 ([M+9H]⁹⁺), 961.92 ([M+10H]¹⁰⁺), 874.50 ([M+11H]¹¹⁺), 801.67 ([M+12H]¹²⁺), 740.08 ([M+13H]¹³⁺); calcd. for M (average): 9610.92, found: 9608.71.

6.2. Characterization of the ^{oxo}SEA-mediated ligation between Cys-K1Lys(Biot) polypeptide and Lys(^{oxo}SEA)-containing V5 peptidic tag by LCMS and Western blot

The production of the V5-CysK1(Biot) conjugate by reaction of Cys-K1Lys(Biot) **4b** with V5-Lys(^{oxo}SEA) peptide **3b** is depicted in Figure S 66.

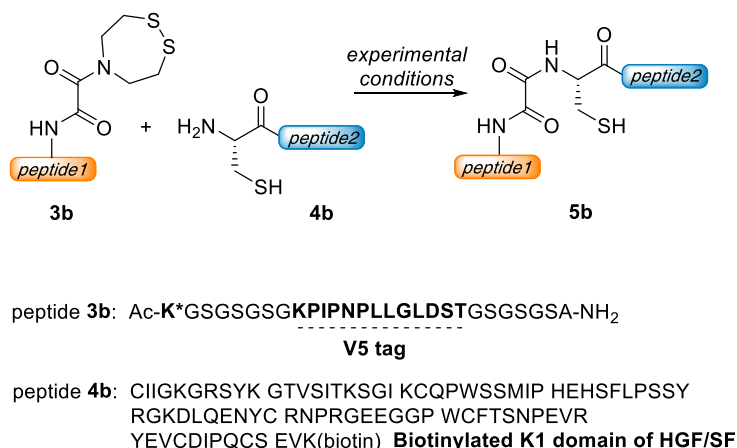


Figure S 66. Synthesis of V5-CysK1(Biot) conjugate by reaction of Cys-K1Lys(Biot) **4b** with V5-Lys(^{oxo}SEA) peptide **3b**.

6.2.1. Protocol: synthesis and characterization of the conjugate **5b**

In a 6 M Gn.HCl solution of 0.1 M phosphate buffer were successively added TCEP hydrochloride (28.63 mg, 100 mM) and MPAA (33.64 mg, 200 mM). The pH of the resulting mixtures was adjusted to 5.49 by addition of 75 μ L of 6 M NaOH.

V5-Lys(^{oxo}SEA) peptide **3b** (415 μ g) was dissolved into 147 μ L of the above solution (1 mM). 50 μ L of the latter solution was used to dissolve Cys-K1Lys(Biot) **4b** (549 μ g) and trigger the ligation reaction. After 30 minutes at 37 °C, the mixture was quenched with a 10% acetic acid aqueous solution (500 μ L) and extracted three times by Et₂O to remove MPAA, flushed with nitrogen and purified on an analytical HPLC system (eluent A 0.1% TFA in water, eluent B 0.1% TFA in CH₃CN/H₂O 4:1, 50°C, detection at 215 nm, 1 mL min⁻¹, 0-50% eluent B in 15 min, C18 column) to give ligated peptide **5b** as a white solid after lyophilisation (306 μ g, 45%).

UPLC-MS (ESI, positive detection mode, Figure S 67) m/z =1742.33 ([M+7H]⁷⁺), 1524.67 ([M+8H]⁸⁺), 1355.42 ([M+9H]⁹⁺), 1220.00 ([M+10H]¹⁰⁺), 1109.33 ([M+11H]¹¹⁺), 1016.92 ([M+12H]¹²⁺), 938.83 ([M+13H]¹³⁺), 871.92 ([M+14H]¹⁴⁺), 813.83 ([M+14H]¹⁴⁺), 763.25 ([M+15H]¹⁵⁺); calcd. for M: 12190.69 , found: 12189.57.

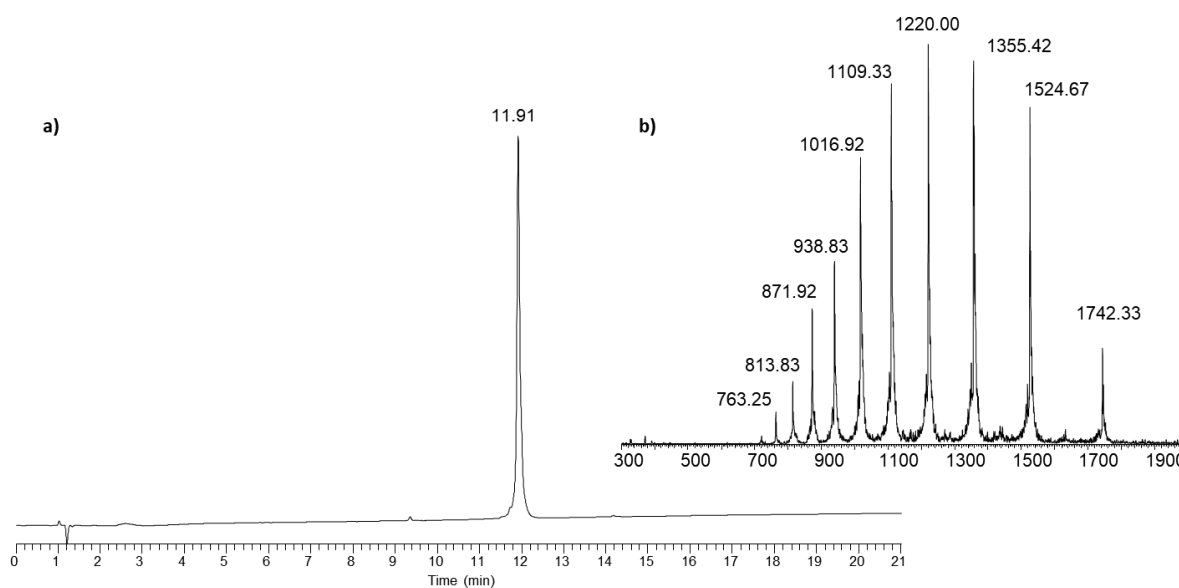


Figure S 67. UPLC-MS analysis. LC trace for ligation product **5b**. Eluent A 0.1% TFA in water, eluent B 0.1% TFA in CH₃CN. XBridge BEH C18 (3.5 μ m, 300 \AA , 2.1 \times 150 mm) column, gradient 0-70% B in 15 min (0.4 mL min⁻¹, detection UV 215 nm). MS trace: m/z =1742.33 ([M+7H]⁷⁺), 1524.67 ([M+8H]⁸⁺), 1355.42 ([M+9H]⁹⁺), 1220.00 ([M+10H]¹⁰⁺), 1109.33 ([M+11H]¹¹⁺), 1016.92 ([M+12H]¹²⁺), 938.83 ([M+13H]¹³⁺), 871.92 ([M+14H]¹⁴⁺), 813.83 ([M+14H]¹⁴⁺), 763.25 ([M+15H]¹⁵⁺); calcd. for M: 12190.69 , found: 12189.57.

6.2.2. SDS-Page (Western blot) elution controls

SDS-PAGE

Migration solution 1 was prepared by diluting NuPAGE 20X (NOVEX, Lifetechnologies, ref NP0002, MES SDS running buffer) 20 fold with water (40 mL NuPAGE 20X + 760 mL water). Migration solution 2 was prepared by adding an ascorbic acid solution (1 M in water, 2 mL) to 400 mL of migration solution 1.

SDS-PAGE was performed using NuPAGE 4-12% Bis-Tris Gel (Invitrogen, 1.5 mm, 10 wells).

The gel wells were washed three times with the migration solution and placed in the SDS-PAGE migration chamber. Migration solution 2 (200 mL) was introduced in negative electrode cell. Migration solution 1 (200 mL) was introduced in positive electrode cell.

PM NOVEX sharp pre-stained protein standard (INVITROGEN, ThermoFisher Scientific, ref LC5800, 8 μ L) was used to estimate the apparent molecular weight of the proteins.

Sample Buffer SB \times 1 solution was obtained by mixing water (650 μ L), a mixture of 2.45 M DTT and 2.45 M mercaptoethanol in water (RED10X, 100 μ L) and NuPAGE LDS Sample buffer 4X (INVITROGEN, ThermoFisher Scientific, ref NP0007, 250 μ L).

Western blot

The voltage applied to the gel was 80 V (concentration gel) and then 150 V for the migration of the proteins (separation gel).

After transfer onto PVDF membranes, membranes were washed with water (2 \times quickly then 3 \times 10 min under agitation), with PBS (GIBCO, ref 21600-069/Tween[®] 20 (SIGMA ALDRICH, ref P2287) (3 \times quickly then 2 \times 10 under agitation). Membranes were placed in 0.2% casein (VWR, ref E666-500G)/PBS 0.05% Tween[®] 20 during 30 min under agitation.

In the meantime, primary antibody is prepared: anti V5 MOUSE antibody, Invitrogen ref R960-25, 1/3000 (5 μ L in 15 mL 5% BSA 0.1% sodium azide in PBS)

Casein solution was removed and the antibody solutions (15 mL) were added to the corresponding membranes. One with anti V5 MOUSE antibody was gently agitated overnight at 4°C and the second was gently agitated overnight with 15 mL 5% BSA 0.1% sodium azide in PBS. Membranes were then washed quickly with PBS/ Tween[®] 20 (3 \times quickly then 3 \times 10 min).

Secondaries antibodies were prepared (anti mouse HRP-labelled JACKSON IMMUNORESEARCH ref 115035146, 1/15000, 2 μ L in 30 mL casein 0.2 %/0.05% Tween[®] 20 in PBS) and Streptavidin-peroxidase polymer, SIGMA ref S2438-250UG, 1/50000 (1 μ L in 50 mL casein (VWR, ref E666-500G)/PBS 0.05 % Tween[®]. The antibodies were added to the membranes (the one incubated with anti-V5 with anti-V5 HRP and the one with BSA with the streptavidin-HRP). After 45 min, membranes were washed with PBS/ Tween[®] 20 (3 \times quickly then 3 \times 10 min). PBS/ Tween[®] 20 solution was removed and luminol (700 μ L) and H₂O₂ (700 μ L) from Kit Dura (Luminol/H₂O₂ Kit Dura, Super Signal™ West Dura Extended Duration Substrate, Thermo Scientific, REF 34076) were added on the membranes.

The chemiluminescence of the membranes was read using a LAS 4000 instrument.

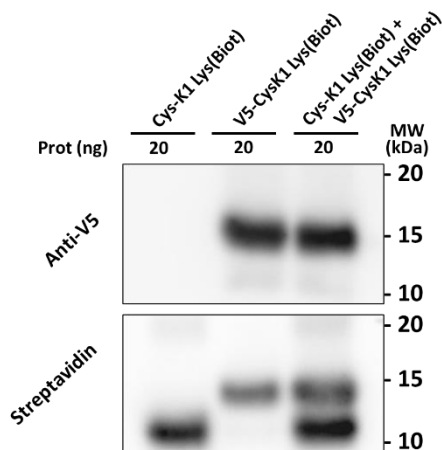


Figure S 68. SDS-PAGE analysis of the Cys-K1Lys(Biot) **4b**, Cys-K1Lys(Biot) **5b** and mixture of **4b** and **5b** (Western Blot). Solutions of **4b** and **5b** were prepared at a concentration of $5 \text{ ng } \mu\text{L}^{-1}$ by dilution in SB \times 1 sample buffer. $20 \text{ } \mu\text{L}$ of the corresponding solutions were loaded on the gel.

6.2.3. Optimization of reaction conditions for SDS-Page (Western blot)

At low concentration, the influence of various reaction parameters was reexamined with the aim of minimizing the large excesses of additives.

General procedure of the oxoSEA-mediated ligation monitored by SDS-PAGE (quantity of TCEP, MPAA or peptides is to be adjusted depending on the reaction conditions) :

To a 0.1 M phosphate buffer solution (1 mL) were added TCEP (14.34 mg , 50 mM) and MPAA (8.41 mg , 50 mM). The pH was adjusted to 7.2 by addition of 6 M NaOH .

Stock solutions of Cys-K1Lys(Biot) **4b**, V5-Lys(^{oxo}SEA) peptide **3b** in water at $1 \text{ } \mu\text{g } \mu\text{L}^{-1}$ were prepared. Protein concentration was determined from nanodrop measurements.

To $200 \text{ } \mu\text{L}$ of reaction buffer prepared above were added successively the solutions of Cys-K1Lys(Biot) **4b** ($4.8 \text{ } \mu\text{g}$, $4.8 \text{ } \mu\text{L}$, $2.16 \text{ } \mu\text{M}$) and V5-Lys(^{oxo}SEA) peptide **3b** ($7.07 \text{ } \mu\text{g}$, $7.07 \text{ } \mu\text{L}$, $12.48 \text{ } \mu\text{M}$, 5.8 equiv.) and the reaction was left to proceed at $37 \text{ } ^\circ\text{C}$.

Aliquots of $3 \text{ } \mu\text{L}$ were taken at the indicated time and were diluted in SB \times 1 sample buffer ($100 \text{ } \mu\text{L}$). Samples were immediately frozen in liquid nitrogen and stored at $-80 \text{ } ^\circ\text{C}$ until use for the Western blot analysis (Figure S 69 Figure S 73).

Study of pH and peptide stoichiometry

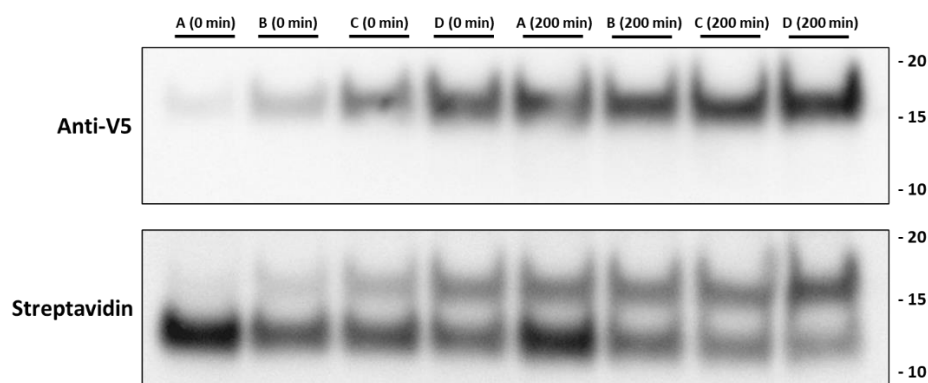


Figure S 69. SDS-PAGE analysis of the ligation of Cys-K1Lys(Biot) **4b** with V5-Lys(^{oxo}SEA) peptide **3b** to provide Cys-K1Lys(Biot) **5b** at t = 0 min and t = 200 min (Western Blot). Reaction conditions : (All lanes) 2.16 μ M Cys-K1Lys(Biot) **4b** in 0.1 M phosphate buffer, 50 mM MPAA, 50 mM TCEP. (A) 1.73 equiv. V5-Lys(^{oxo}SEA) peptide **3b**, pH 5.5 (B) 5.78 equiv. V5-Lys(^{oxo}SEA) peptide **3b**, pH 5.5, (C) 1.73 equiv. V5-Lys(^{oxo}SEA) peptide **3b**, pH 7.2, (D) 5.78 equiv. V5-Lys(^{oxo}SEA) peptide **3b**, pH 7.2.

Ligation reactions conducted at pH 7.2 in the presence of a larger excess of V5-Lys(^{oxo}SEA) peptide **3b** provide better conversions (experiment D).

Study of MPAA concentration

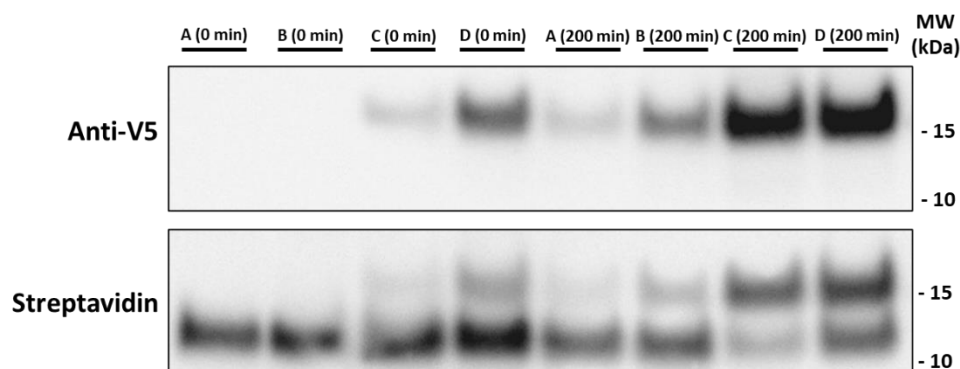


Figure S 70. SDS-PAGE analysis of the ligation of Cys-K1Lys(Biot) **4b** with V5-Lys(^{oxo}SEA) peptide **3b** to provide Cys-K1Lys(Biot) **5b** at t = 0 min and t = 200 min (Western Blot). Reaction conditions : (All lanes) 2.16 μ M Cys-K1Lys(Biot) **4b**, 5.78 equiv. V5-Lys(^{oxo}SEA) peptide **3b** in 0.1 M phosphate buffer, 50 mM TCEP, pH 7.2. (A) 0 mM MPAA, (B) 0.5 mM MPAA, (C) 5 mM MPAA, (D) 50 mM MPAA.

5 mM MPAA appear to be necessary to obtain the best conversions (experiment C).

Study of TCEP concentration

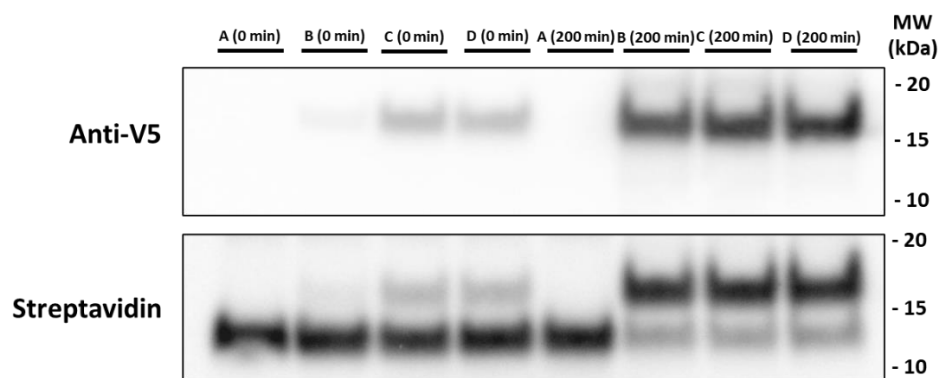


Figure S 71. SDS-PAGE analysis of the ligation of Cys-K1Lys(Biot) **4b** with V5-Lys(^{oxo}SEA) peptide **3b** to provide Cys-K1Lys(Biot) **5b** at t = 0 min and t = 200 min (Western Blot). Reaction conditions : (All wells) 2.16 μ M Cys-K1Lys(Biot) **4b**, 5.78 equiv. V5-Lys(^{oxo}SEA) peptide **3b** in 0.1 M phosphate buffer, 5 mM MPAA, pH 7.2. (A) 0 mM TCEP, (B) 0.5 mM TCEP, (C) 5 mM TCEP, (D) 50 mM TCEP.

0.5 mM TCEP are sufficient for the activation of the ^{oxo}SEA group (experiment B). Note the absence of reaction when TCEP is not added in the reaction mixture (experiment A).

6.3. Ligation between Cys-K1Lys(Biot) **4b** and V5-Lys(^{oxo}SEA) peptide **3b** in the low μ molar and nanomolar concentration range

6.3.1. SDS-PAGE analysis

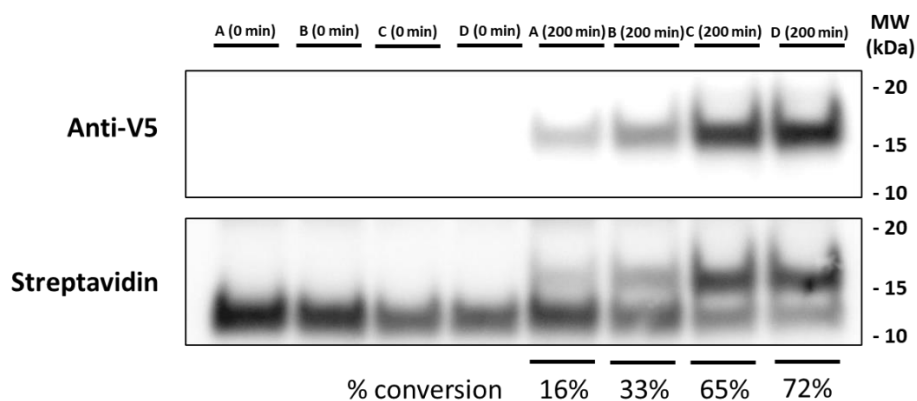


Figure S 72. SDS-PAGE analysis of the ligation of Cys-K1Lys(Biot) **4b** with V5-Lys(^{oxo}SEA) peptide **3b** to provide Cys-K1Lys(Biot) **5b** at t = 0 min and t = 200 min (Western Blot). Reaction conditions : (All wells) 5.78 equiv. V5-Lys(^{oxo}SEA) peptide **3b** in 0.1 M phosphate buffer, 5 mM MPAA, 0.5 mM TCEP, pH 7.2. (A) 270 nM μ M Cys-K1Lys(Biot) **4b** (B) 540 nM Cys-K1Lys(Biot) **4b** (C) 1.08 μ M Cys-K1Lys(Biot) **4b**, (D) 2.16 μ M Cys-K1Lys(Biot) **4b**. Conversions were calculated from relative band intensities based on streptavidin-HRP staining.

6.3.2. Control in the presence of a K1-derived polypeptide lacking the N-terminal Cys residue

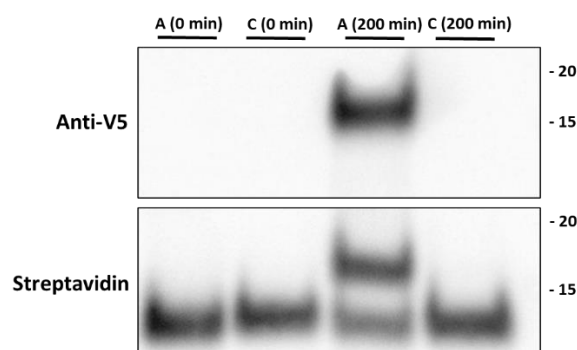


Figure S 73. SDS-PAGE analysis of the ligation of V5-Lys(^{oxo}SEA) peptide **3b** with Cys-K1Lys(Biot) **4b** (conditions A) or with K1Lys(Biot) (conditions C) at t = 0 min and t = 200 min (Western Blot). Reaction conditions : (All wells) 5.78 equiv. V5-Lys(^{oxo}SEA) peptide **3b** in 0.1 M phosphate buffer, 5 mM MPAA, 0.5 mM TCEP, pH 7.2. (A) 2.16 μ M Cys-K1Lys(Biot) **4b** (B) 2.16 μ M K1Lys(Biot).

No band corresponding to the formation of the ligation product is observed when the K1 domain lacks the N-terminal cysteine residue (experiment C) while a band is stained by anti-V5 antibody and streptavidin-HRP conjugate in the presence of Cys-K1Lys(Biot) **4b** (experiment A).

7. SDS-PAGE analysis of ^{oxo}SEA ligation at high dilution in non-purified media

7.1. Preparation of THP-1 protein extracts⁹

THP-1 cells stored at -80 °C in PBS buffers were defrosted and washed with PBS buffer (2 \times 5 mL). Cells were taken up in TRIzol reagent (Invitrogen, 1 mL) and vortexed 2 minutes at high speed. The resulting lysate was transferred in a 2 mL Eppendorf and chloroform was added (200 μ L). The heterogeneous mixture was vigorously shaken for 1 minute and left to stand for 5 min until complete separation of the layers. After centrifugation, a triphasic mixture was obtained. The upper layer containing the RNA was removed. To the lower layer and the solid at the interface was added absolute ethanol (300 μ L). The Eppendorf was gently agitated until complete dissolution of the solid. A solid corresponding to DNA precipitated. The mixture was centrifuged at 4 °C (2000 rpm, 5 min). The supernatant was divided into two equal volumes transferred in two eppendorf tubes.

After addition of isopropanol in each tube (750 μ L), the mixture was gently homogenized, was left to sit for 10 minutes at room temperature and was centrifuged at 4 °C (12000 rpm, 10 min). The supernatant was discarded. The pellet was taken up in a 0.3 M Gn.HCl solution in ethanol (1 mL), incubated 20

⁹ Hummon, A. B.; Lim, S. R.; Difilippantonio, M. J.; Ried, T. Isolation and solubilization of proteins after TRIzol® extraction of RNA and DNA from patient material following prolonged storage. *Biotechniques* **2007**, *42*, 467-472.

minutes at room temperature and centrifuged at 4 °C (7500 rpm, 5 min). The washing-centrifugation cycle was repeated twice. The supernatant was discarded and the pellet containing the proteins left to dry at room temperature.

Pellets were resolubilized in 1% SDS (300 µL) and frozen at -80 °C.

The protein content was estimated after a BCA assay to 3.2 µg µL⁻¹.

7.2. SDS-PAGE analysis (Western Blot) of the ligation of Cys-K1Lys(Biot) in a THP-1 protein extract

7.2.1. Protocol

The following solutions were prepared:

Solution A: 1% aqueous SDS

Solution B: protein extract in 1% SDS ([THP-1 proteins] = 3.2 µg µL⁻¹)

Solution C: Cys-K1Lys(Biot) **4b** at 20 ng mL⁻¹ in water

Solution D: V5-Lys^{(oxo)SEA} peptide **3c** at 0.467 mg mL⁻¹ in 50 mM Tris.HCl buffer.

Solution E: solution of MPAA (14.3 mM) in 128.6 mM Tris.HCl buffer. pH adjusted to 7.2 by addition of 6 M HCl

Solution F: solution of MPAA (14.3 mM) and TCEP (2.86 mM) in 128.6 mM Tris.HCl buffer. pH adjusted to 7.2 by addition of 6 M HCl

The reactions were performed in low-bind eppendorf tubes.

Lane 1 (Cys-K1Lys(Biot) w/o TCEP): 10 µL sol. A + 1 µL sol. C + 2 µL sol. D + 7 µL sol. E

Lane 2 (Cys-K1Lys(Biot) + TCEP): 10 µL sol. A + 1 µL sol. C + 2 µL sol. D + 7 µL sol. F

Lane 3 (THP-1 + Cys-K1Lys(Biot) w/o TCEP): 10 µL sol. B + 1 µL sol. C + 2 µL sol. D + 7 µL sol. E

Lane 4 (THP-1 + Cys-K1Lys(Biot) + TCEP): 10 µL sol. B + 1 µL sol. C + 2 µL sol. D + 7 µL sol. F

Lane 5 (THP-1 w/o TCEP) : 10 µL sol. B + 1 µL water + 2 µL sol. D + 7 µL sol. E

Lane 6 (THP-1 + TCEP) : 10 µL sol. B + 1 µL water + 2 µL sol. D + 7 µL sol. F

The reaction were left to proceed at 37 °C overnight, after which, 20 µL of SB×1 sample buffer were added. Samples were immediately frozen in liquid nitrogen and stored at -80°C until use for the Western blot analysis (Figure S 69).

Whenever present, the final concentration of Cys-K1Lys(Biot) **4b** corresponds to 100 nM.

7.2.2. Analysis

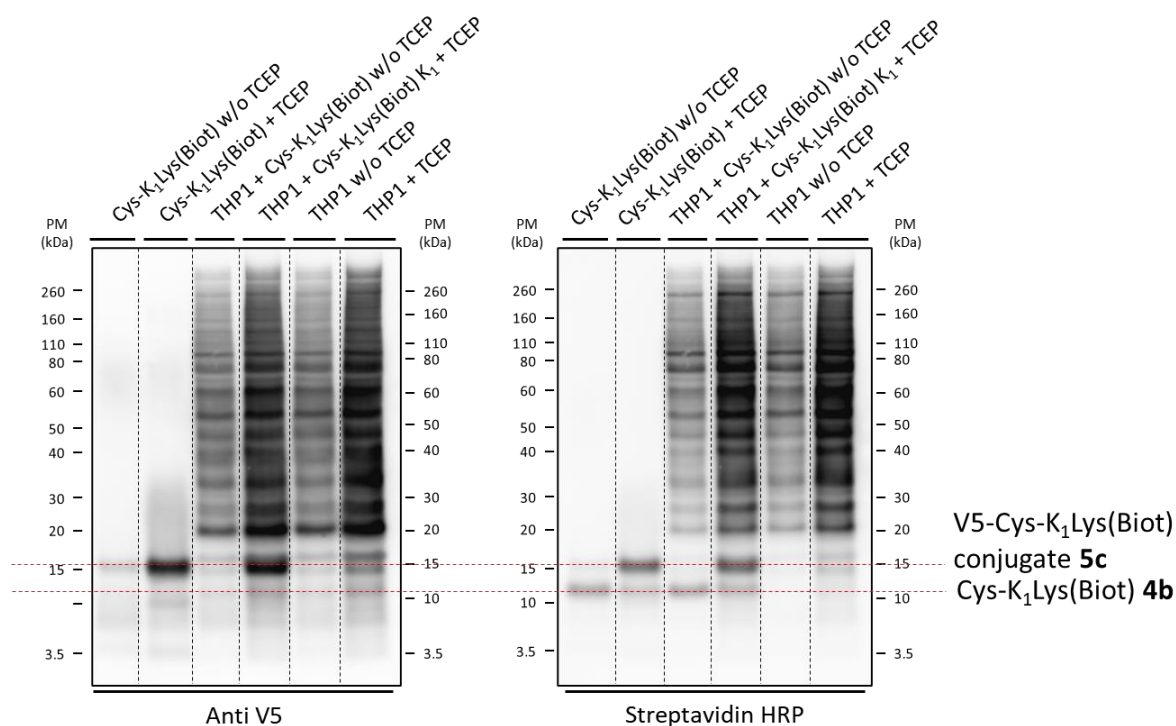


Figure S 74. SDS-PAGE analysis of the ligation of V5-Lys^(oxoSEA) peptide **3b** with Cys-K₁Lys(Biot) **4b** in a crude protein extract from THP-1 cells (Western Blot). Bands are revealed by anti-V5 antibodies or streptavidin-HRP conjugates.

In the presence of TCEP, the analysis shows the conversion of the Cys-K₁Lys(Biot) **4b** to a product of molecular weight ~15.5 kDa staining upon reaction with anti-V5 antibody and streptavidin-HRP conjugate (lanes 2 & 4). This suggests the formation of product **5c**. The control in the absence of TCEP (lanes 1 & 3) shows no reaction.

In all reactions performed within the protein extract (lanes 3 to 6), nonspecific bands are illuminated upon immunostaining. The control analyses below (Figure S 75, Figure S 76) indicate that this phenomenon occurs regardless of the presence of TCEP and as soon as the reaction starts.

7.2.3. Controls

With TCEP

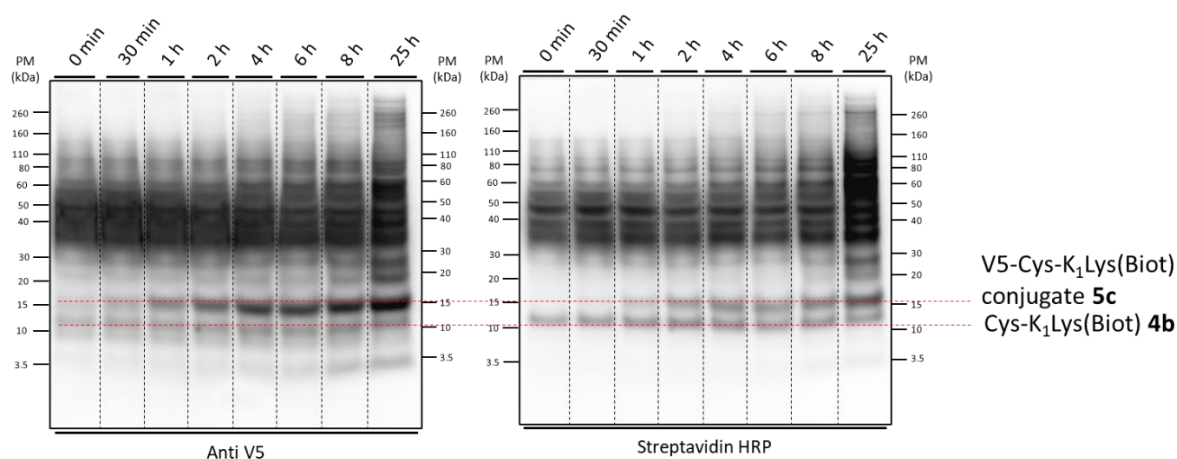


Figure S 75. Time course of the ligation reaction of V5-Lys(^{oxo}SEA) peptide **3b** with Cys-K1Lys(Biot) **4b** in the presence of a strong reducing agent (TCEP), in a crude protein extract from THP-1 cells (analyzed by SDS-PAGE - Western Blot). Bands are revealed by anti-V5 antibodies or streptavidin-HRP conjugates.

w/o TCEP

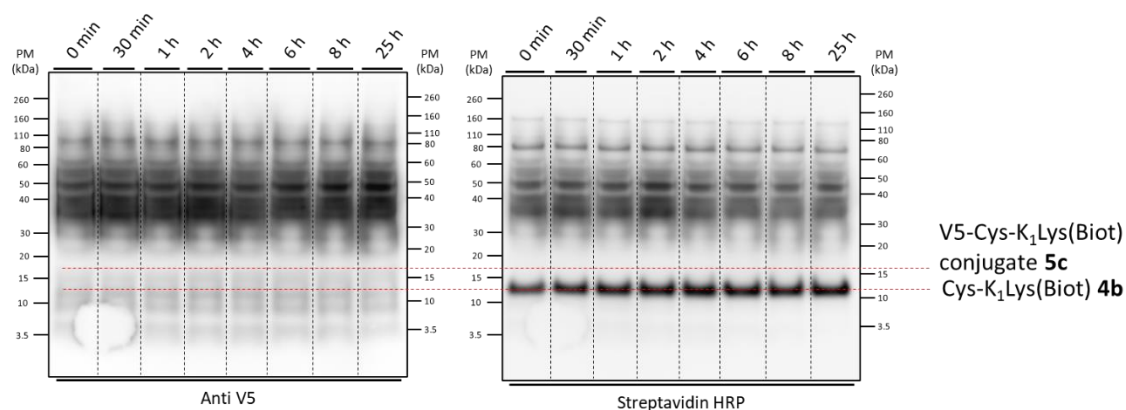


Figure S 76. Time course of the ligation reaction of V5-Lys(^{oxo}SEA) peptide **3b** with Cys-K1Lys(Biot) **4b** in the absence of a strong reducing agent (TCEP), in a crude protein extract from THP-1 cells (analyzed by SDS-PAGE - Western Blot). Bands are revealed by anti-V5 antibodies or streptavidin-HRP conjugates.

Note the formation of a ligation product in the presence of TCEP (Figure S 75) whereas the Cys-K1Lys(Biot) is recovered unreacted in the absence of TCEP (Figure S 76).