Supporting information for

Total Chemical Synthesis of All SUMO-2/3 Dimer Combinations

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I. General Methods

A. Reagents and solvents

N-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-b]pyridin-1-ylmethylene]-*N* methylmethanaminium hexafluorophosphate *N*-oxide (HATU) and *N*α-Fmoc protected amino acids were obtained from Iris Biotech GmbH. Side-chain protecting groups used for the amino acids were Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Asp(O*t*Bu)[Dmb-Gly]-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(O*t*Bu)-OH, Fmoc-Thr(O*t*Bu)-OH, Fmoc-Tyr(O*t*Bu)-OH, Fmoc-Val-OH, Fmoc-Cys(S*t*Bu)-OH or Fmoc-Cys(Trt)-OH.

Synthesis of *bis*(2-sulfanylethyl)aminotrityl polystyrene (SEA PS) resin was carried out as described elsewhere.[1, 2] 4-mercaptophenylacetic acid (MPAA) and *tris*(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich. All other reagents were purchased from Acros Organics or Merck and were of the purest grade available. Peptide synthesis grade *N,N*-dimethylformamide (DMF), dichloromethane (CH_2Cl_2), diethylether (Et_2O), acetonitrile (CH_3CN), heptane, LC–MS-grade acetonitrile (CH_3CN, 0.1% TFA and CH_3CN, 0.1% formic acid), LC–MS-grade water (H_2O, 0.1% TFA and H_2O, 0.1% formic acid), *N,N*-diisopropylethylamine (DIEA), acetic anhydride (Ac_2O) were purchased from Biosolve and Fisher-Chemical. Trifluoroacetic acid (TFA) was obtained from Biosolve. Water was purified with a Milli-Q Ultra Pure Water Purification System.

B. HPLC analysis & purification

The reactions were monitored by analytical UPLC–MS (Dionex UltiMate 3000 LC/ LCQ Fleet Ion Trap) on a reverse phase column. The column, eluent system and gradient used are indicated in the figure legends. The column eluate was monitored with a Dionex DA detector (215 nm, 254 nm) and Corona Veo charged aerosol detector. The peptide masses were measured by on-line UPLC–MS: Ionization mode: ES+, m/z range 300–2000, capillary voltage 3.5 kV, cone voltage 10 V, tube lens 75 V, capillary voltage temperature 350 °C.

Ligations were analyzed by quenching aliquots (1.5-2 µL) of the reaction mixtures with 100 µL of 10% aqueous acetic acid. The mixture was extracted with Et_2O to remove MPAA before analysis.

The peptides were purified by semi-preparative HPLC (Waters 600 controller, UV 2487 Detector, 215 nm, TL 105 HPLC column heater) on a reverse phase column (Waters XBridge BEH300 C18, 20 x 100 mm; pore size 300 Å; particle size : 5 µm). The eluent system and gradient used are indicated in the figure legends.

C. MALDI-TOF analysis

MALDI-TOF mass spectra were recorded with a BrukerAutoflex Speed mass spectrometer. The matrix used for the analysis is indicated in the figure legends.

II. Peptide synthesis

Peptides **4**, **8a-b** and **9** were produced as described elsewhere.[3]

A. Synthesis of peptides 1a-b and 5

Peptide **1a**: ADEKPKEGVK(C)TENNDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAY-SEA^{on}

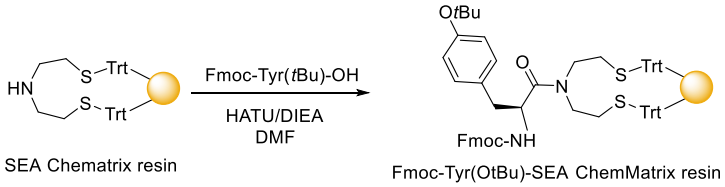
Peptide **1b**: SEEKPKEGVK(C)TENDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAY-SEA^{on}

Peptide **5**: CERQGLSMRQIRFRFDGQPINETDTPAQLEMEDEDTIDVFQQQTGG-NHNH$_2$

Coupling of the first amino acid residue to the SEA ChemMatrix resin

The first amino acid was coupled manually to the SEA ChemMatrix® resin for SEA peptides (Scheme S 1) or to the HMPA ChemMatrix® resin for peptide acids using HATU/DIEA activation in DMF.

Scheme S 1. Loading of the first amino acid on the resin. Loading on SEA ChemMatrix® resin



SEA ChemMatrix resin (0.220 mmol/g, 454 mg, 100 µmol) was conditioned in CH_2Cl_2 (3 × 2 min, 3 mL) and then in DMF (3 × 2 min, 3 mL) in a manual SPPS glass reactor.

Fmoc-Tyr(O*t*Bu)-OH (460 mg, 1.00 mmol) and HATU (376 mg, 0.950 mmol) were dissolved in the minimal volume of DMF (2 mL). DIEA (348 µL, 2.00 mmol) was added to the above solution to start the activation of the amino acid. This solution was agitated for 1 min and then added to the resin which was shaken during 1 h 30 min at room temperature. The resin was subsequently washed with DMF (5 × 2 min, 3 mL). The chloranil assay was negative. The resin was then acetylated with a mixture of acetic anhydride/DIEA/DMF: 10/5/85 by vol (2 × 3 mL, 2 min and then 20 min) before being washed successively with DMF (3× 2 min, 3 mL), CH_2Cl_2 (3× 2 min, 3 mL) and diethylether (3 ×2 min, 3 mL). The resin was finally dried in vacuo for ~2 h.

Preparation of hydrazine PS resin

Commercial 2-chloro-4'-polystyryl triphenylmethyl chloride resin (0.600 mmol/g, 667 mg, 400 µmol) was swollen for 15 min in DMF (150 mL) and cooled at 0 °C. A mixture of trimethylamine (6 eq, 240 mmole, 334.5 µL) and hydrazine hydrate (4 eq, 160 mmole, 102.4 µL) in DMF (4 mL) was added dropwise and the suspension was stirred for 60 min at room temperature. Methanol (107 µL) was then added and stirring continued for 10 min in order to quench the excess of reactive Cl. The resin was filtered, washed with DMF (2 x 5 mL), water (2 x 5 mL), DMF (2 x 5 mL), methanol (2 x 5 mL) and ether and dried in vacuo for 2 h at room temperature (protocol adapted from ref [4]).

Coupling of the first amino acid residue to the hydrazide PS resin

Fmoc-Gly-OH (297 mg, 1.00 mmol) and HATU (376 mg, 0.950 mmol) were dissolved in the minimal volume of DMF (2 mL). DIEA (348 µL, 2.00 mmol) was added to the above solution to start the activation of the amino acid. This solution was agitated for 1 min and then added to the hydrazine resin prepared above (1.00 mmole) which was shaken during 1 h 30 min at room temperature. The resin was subsequently washed with DMF (5 × 2 min, 3 mL). The picrylsulfonic acid assay was negative. The resin was then acetylated with a mixture of acetic anhydride/DIEA/DMF: 10/5/85 by vol (2 × 3 mL, 2 min and then 20 min) before being washed successively with DMF (3× 2 min, 3 mL), CH_2Cl_2 (3× 2 min, 3 mL) and diethylether (3 ×2 min, 3 mL). The resin was finally dried in vacuo for ~2 h.

Determination of resin loading

The loading of the resins was determined by UV quantification at 290 nm of the dibenzofulvene-piperidine adduct formed by treating aliquots of the resin with piperidine (20% by vol in DMF). We found 0.18 mmol/g for Fmoc-Tyr(O*t*Bu)-SEA ChemMatrix resin and 0.18 mmol/g for Fmoc-Gly-hydrazine PS resin.

Automated solid phase peptide synthesis

The peptide elongation step was performed using an automated column peptide synthesizer and standard Fmoc-SPPS protocols (0.1 mmol scale). The amino acids (10 equiv) were activated using HATU (9.9 equiv)/DIEA (20 equiv) in DMF. The peptidyl resin was acetylated with Ac_2O/DIEA/DMF 10/5/85 by vol after each coupling step.

Coupling of the dipeptide unit Fmoc-Asp(OtBu)-(Dmb)Gly-OH

Fmoc-Asp(O*t*Bu)-(Dmb)Gly-OH (186 mg, 0.300 mmol, 3 equiv) and HATU (108 mg, 0.284 mmol, 2.85 equiv) were dissolved in the minimal volume of DMF (2 mL). DIEA (104 µL, 0.600 mmol, 6 equiv) was added to the above solution to start the activation of the amino acid. This solution was agitated for 1 min and then added to the resin which was shaken for 1 h 30 min at room temperature. The resin was subsequently washed with DMF (5 × 2 min, 3 mL).

The chloranil assay was negative. An aliquot of the peptidyl resin was treated with piperidine in DMF (20 % by vol) and cleaved for 1 h 30 min in a mixture of TFA/triisopropylsilane (TIS)//thioanisole/H_2O/thiophenol: 87.5/5/2.5/2.5/2.5 by vol (1 mL) for SEA ChemMatrix® resin, or TFA/TIS/H_2O/ethanedithiol (EDT): 90/5/2.5/2.5 by vol (1 mL) for hydrazide PS resin 1 µL of the cleavage mixture was diluted with water (50 µL) and analyzed by MALDI-TOF to confirm the successful coupling of the dipeptide unit.

Deprotection and cleavage step

The peptidyl resins (0.1 mmol scale) were deprotected and cleaved in a mixture of TFA/TIS/thioanisole/H_2O/thiophenol: 87.5/5/2.5/2.5/2.5 by vol (10 mL) for 1 h 30 min (twice) for peptides **1a-b** and in a mixture of TFA/TIS/EDT/H_2O: 90/5/2.5/2.5 by vol (10 mL) for 1 h 30 min (twice) for peptide **5**. The crude peptides were precipitated in an ice-cold mixture of Et_2O/*n*-heptane : 1/1 by vol (200 mL) to give 280.5 mg (53% crude) of peptide **1a**, 276.8 mg (51% crude) of peptide **1b**, 197.1 mg (37% crude) peptide **5**.

Purification of SEA^{off} peptide segments

The crude peptide segments **1a-b**, **5** were dissolved in phosphate buffer/6 M guanidine hydrochloride (final peptide concentration ~0.25 mM).The mixture was then immediately filtered and purified by reversed-phase HPLC.

Gradient used for the HPLC purification: eluent A water containing 0.1% of TFA, eluent B CH_3CN/water : 4/1 by vol containing 0.1% of TFA, gradient: 0-25% B in 5 min, then 25-45% B in 50 min, flow rate 6 mL/min, 65 °C, UV detection at 215 nm.
Yield for peptide **1a**: 34.4 mg of crude product furnished (11.2 mg, 17% global yield) of peptide **1a**.

Gradient used for the HPLC purification: eluent A water containing 0.1% of TFA, eluent B CH_3CN/water : 4/1 by vol containing 0.1% of TFA, gradient: 0-25% B in 5 min, then 25-45% B in 50 min, flow rate 6 mL/min, 65 °C, UV detection at 215 nm.
Yield for peptide **1b**: 30.8 mg of crude product furnished (11.2 mg, 18% global yield) of peptide **1b**.

Gradient used for the HPLC purification: eluent C water containing 0.1% of FA, eluent D CH_3CN/water : 4/1 by vol containing 0.1% of FA, gradient: 0-10% D in 10 min, then 10-30% D in 50 min, flow rate 6 mL/min, 50 °C, UV detection at 215 nm, ZORBAX 300SB-C3 (5 µm, 9.4 x 250 mm).
Yield for peptide **5**: 36.3 mg of crude product furnished (6.2 mg, 6.3% global yield) of peptide **5**.

1) Characterization of peptide 1a

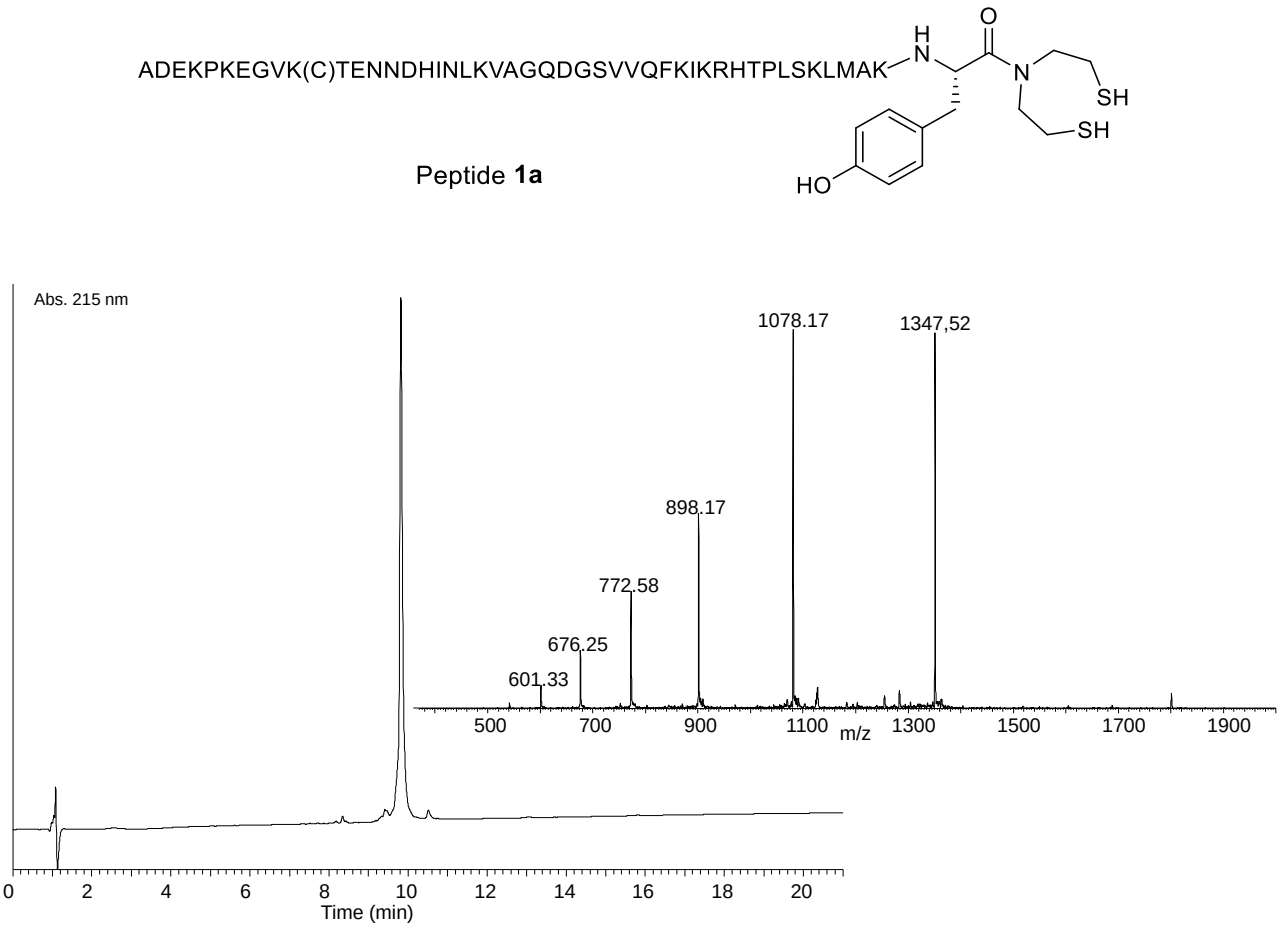
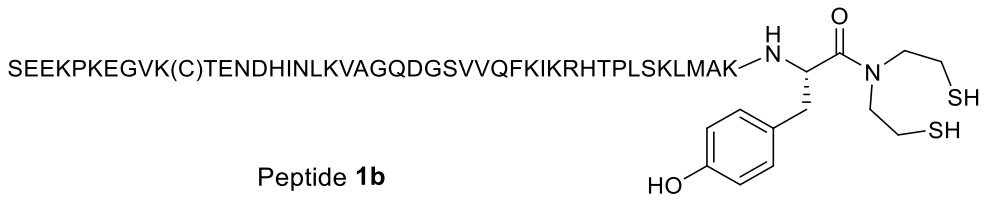


Figure S 1 Analysis of peptide **1a**. UPLC-MS analysis ACQUITY UPLC peptide BEH C18 300 Å 1.7 µm 2.1 mm × 150 mm, 50 °C. Flow 0.400 mL/min, eluent A 0.1% trifluoroacetic acid in water, eluent B 0.1% trifluoroacetic acid in 100% acetonitrile. Gradient from 0% eluent B to 70% eluent B in 20 min. HPLC trace (UV detection) and MS trace. Calculated for M (average mass) 5386.33 , observed 5385.97 after deconvolution.

2) Characterization of peptide **1b**



Peptide **1b**

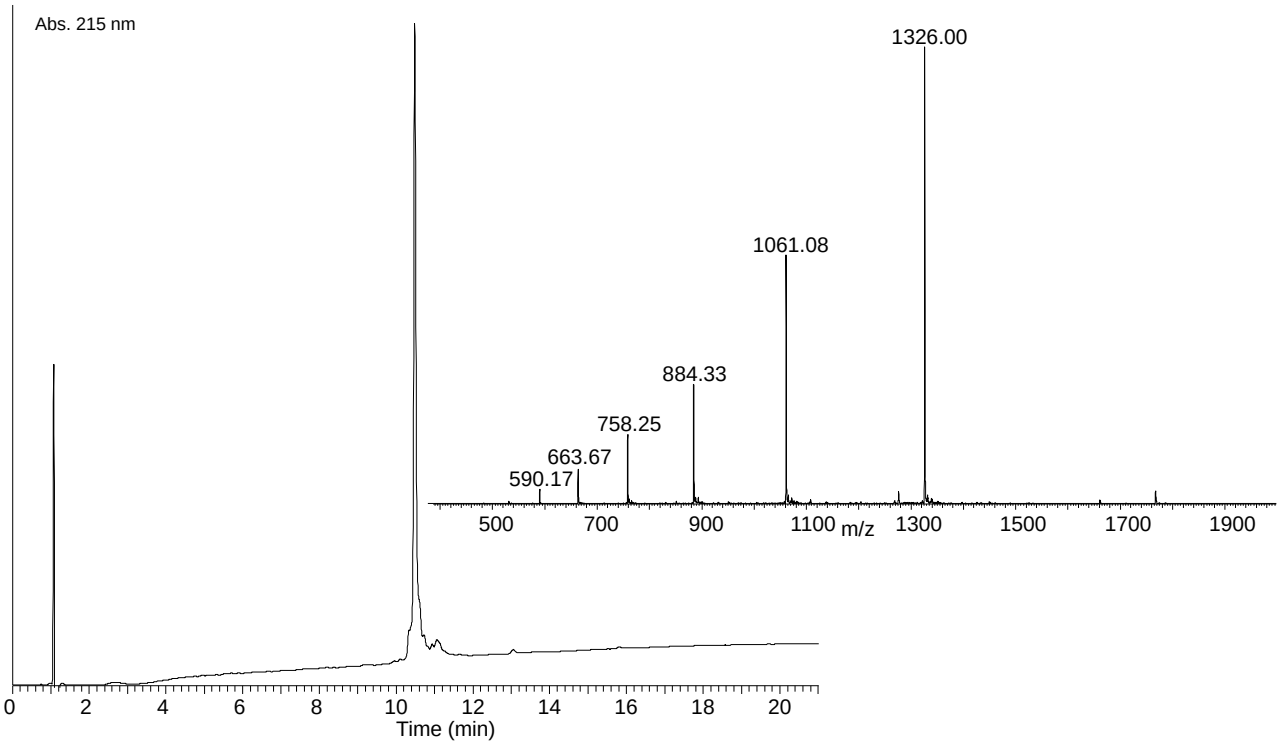


Figure S 2 Analysis of peptide 1b. UPLC-MS analysis ACQUITY UPLC peptide BEH C18 300 Å 1.7 µm 2.1 mm × 150 mm, 50 °C. Flow 0.400 mL/min, eluent A 0.1% trifluoroacetic acid in water, eluent B 0.1% trifluoroacetic acid in 100% acetonitrile. Gradient from 0% eluent B to 70% eluent B in 20 min. HPLC trace (UV detection) and MS trace. Calculated for M (average mass) 5302.25 , observed 5302.20 after deconvolution.

3) Characterization of peptide 5

CERQGLSMRQIRFRFDGQPINETDTPAGLEMEDEDTIDVFQQQTGG-NHNH$_2$

Peptide **5**

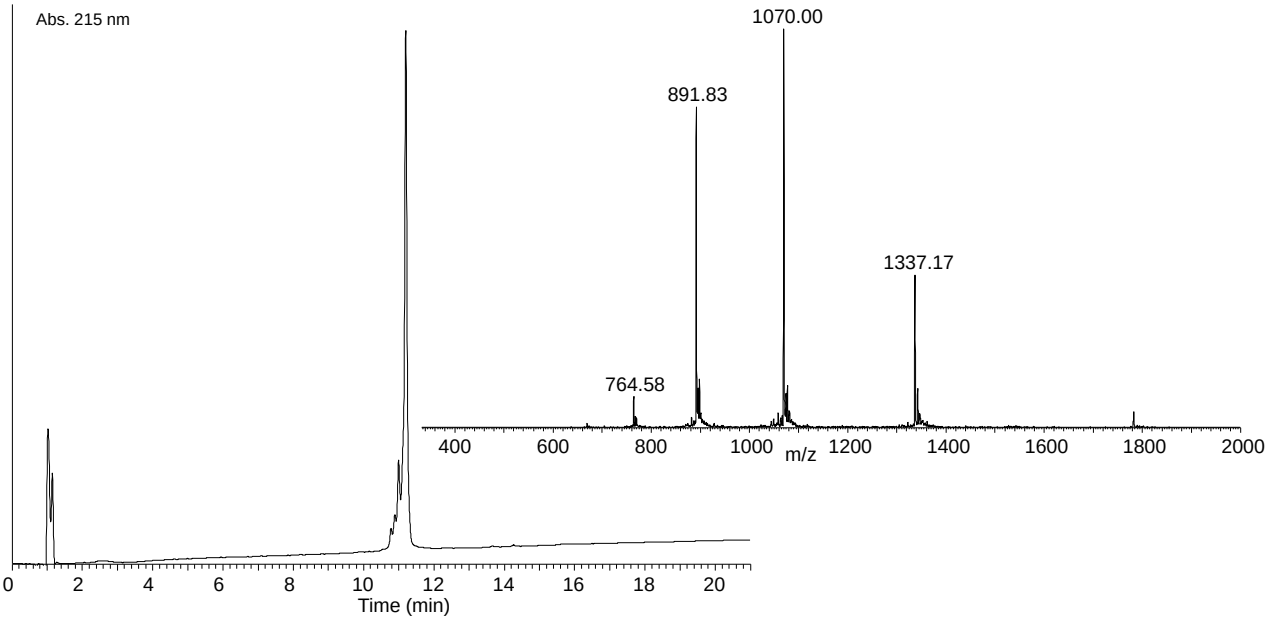


Figure S 3. Analysis of peptide **5**. UPLC-MS analysis ACQUITY UPLC peptide BEH C18 300 Å 1.7 µm 2.1 mm × 150 mm, 50 °C. Flow 0.400 mL/min, eluent A 0.1% trifluoroacetic acid in water, eluent B 0.1% trifluoroacetic acid in 100% acetonitrile. Gradient from 0% eluent B to 70% eluent B in 20 min. HPLC trace (UV detection) and MS trace. Calculated for M (average mass) 5345.85 , observed 5344.85 after deconvolution.

III. Proteins synthesis

A. Chemical synthesis of SUMO-2/3 Lys(Cys) proteins

A typical procedure is illustrated with the preparation of peptide **6a**.

1) One-pot synthesis and characterization of SUMO-2 Lys(Cys) 6a

Oxidation of SEA dithiol into SEA cyclic disulfide

A solution of *N*-octylglucoside (6.90 mg, 20 mM) in 6 M guanidinium chloride/0.1 M pH 7.0 sodium phosphate buffer (1.180 mL) was prepared (solution A). Solution A (501 µL) was then used to dissolve 4-mercaptophenylacetic acid (MPAA, 16.85 mg, 200 mM) to give solution B. Then 4-mercaptophenylacetic acid disulfide (MPAA ox, 0.183 mg, 5 mM) was dissolved in solution B (109 µL) and the pH was adjusted to 7.2 by addition of aqueous NaOH 6 N to give solution C. Peptide **1a** (2.45 mg, 0.364 µmol, 5 mM) was solubilized in solution C (72 µL) and the mixture was stirred for 60 min to give peptide **2a**.

Protection of Lys(Cys) with AcA

Protection of Lys(Cys) residue was performed by adding acetoacetyl-methylthioglycolate (AcA-MTG, 1.00 eq, 0.364 µmol, 7 µL of a 10 mg/mL solution in 6 M guanidinium chloride/0.1 M pH 7.0 sodium phosphate buffer) to the above peptide solution. The reaction was shaken for 23 h to give solution D.

Ligation

TCEP (29.02 mg, 101.2 µmol) and MPAA (16.43 mg, 97.68 µmol) were dissolved in 6 M guanidinium chloride/0.1 M pH 7.0 sodium phosphate buffer (506 µL). Peptide **4** (2.14 mg, 0.364 µmol, 20 mM) was dissolved in the TCEP/MPAA solution (68 µL) and the pH of was adjusted to 5.5. Then, this solution was added to solution D. The pH of the resulting mixture was adjusted again to 5.5 by addition of aqueous NaOH 6 N (3 µL).The final peptide concentration was 2.1 mM. The reaction was shaken for 90 h to give solution E.

AcA removal

To remove AcA protecting group, a 10 mg/mL hydroxylamine hydrochloride ($NH_2OH.HCl$) solution in 6 M guanidinium chloride/0.1 M pH 7.0 sodium phosphate buffer was prepared. The solution is acidic due to the presence of $NH_2OH.HCl$. This solution (0.73 µmol, 2.0 eq, 5.0 µL) was added to solution E. The deprotection reaction was achieved in 30 min.

Purification

The crude mixture was purified by semi-preparative HPLC using XBridge BEH300 C18 column (5 µm, 300 Å, 10 × 250 mm), eluent A water containing 0.1% of TFA, eluent B CH_3CN/water : 4/1 by vol containing 0.1% of TFA, gradient: 0-25 % B in 5 min, 25-45 % B in 50 min, flow rate 6 mL/min, UV detection at 215 nm, 65 °C to give **6a** (1.58 mg, 60% overall isolated).

Peptide 6a: ADEKPKEGVK(C)TENNDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQGLSMRQIRF RFDGQPINETDTPAQLEMEDEDTIDVFQQQTGG

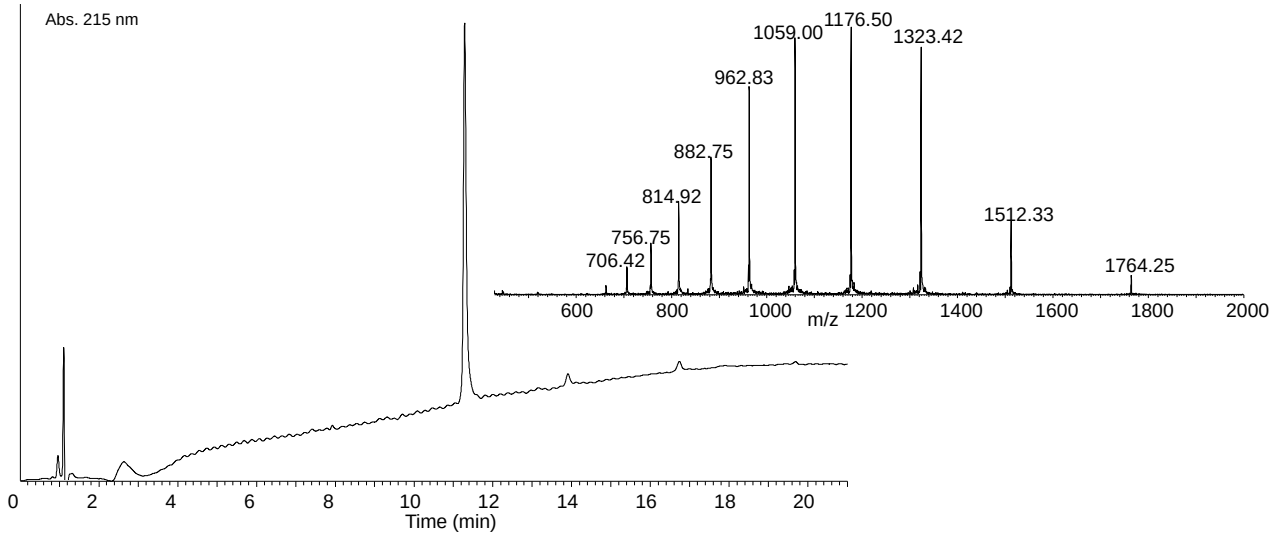


Figure S 4. Analysis of SUMO-2 Lys(Cys) **6a**. UPLC-MS analysis ACQUITY UPLC peptide BEH C18 300 Å 1.7 µm 2.1 mm × 150 mm, 50 °C. Flow 0.400 mL/min, eluent A 0.1% trifluoroacetic acid in water, eluent B 0.1% trifluoroacetic acid in 100% acetonitrile. Gradient from 0% eluent B to 70% eluent B in 20 min. HPLC trace (UV detection) and MS trace. Calculated for M (average mass) 10580.88, observed 10579.75 after deconvolution.

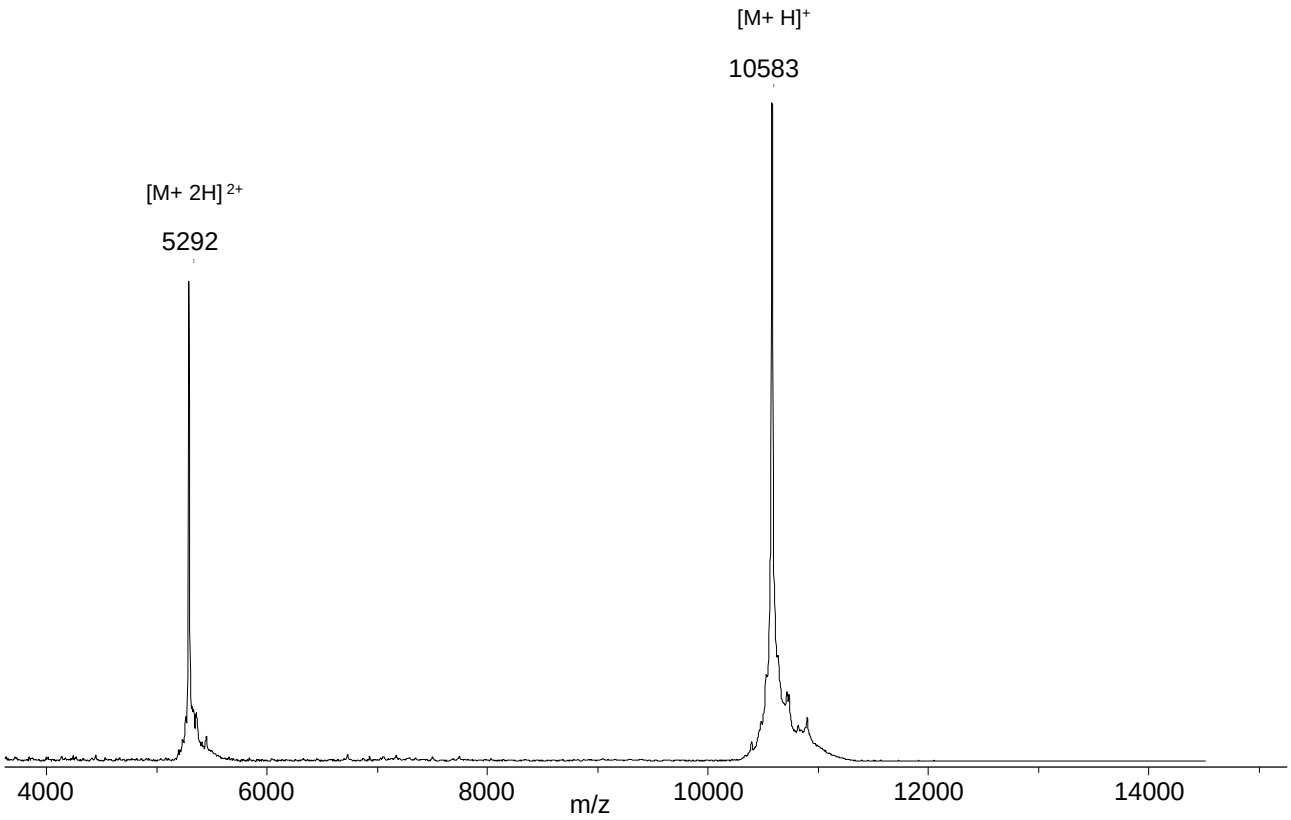


Figure S 5 MALDI-TOF analysis peptide **6a**. Matrix sinapinic acid, positive detection mode, linear mode $[M+H]^+$ calcd. (mean) 10582, found 10583.

2) One-pot synthesis and characterization of SUMO-3 Lys(Cys) 6b

Peptide **6b** was prepared similarly using peptide **1b** (2.57 mg, 0.386 µmol) and peptide **4** (2.27 mg, 0.386 µmol).

The crude mixture was purified by semi-preparative HPLC using XBridge BEH300 C18 column (5 µm, 300 Å, 10 × 250 mm), eluent A water containing 0.1% of TFA, eluent B CH_3CN/water : 4/1 by vol containing 0.1% of TFA, gradient: 0-25 % B in 5 min, 25-45 % B in 50 min, flow rate 6 mL/min, UV detection at 215 nm, 65 °C to give **6b** (1.65 mg, 64% overall isolated).

Peptide 6b: SEEKPKEGVK(C)TENDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQGLSMRQIRFR FDGQPINETDTPAQLEMEDEDTIDVFQQQTGG

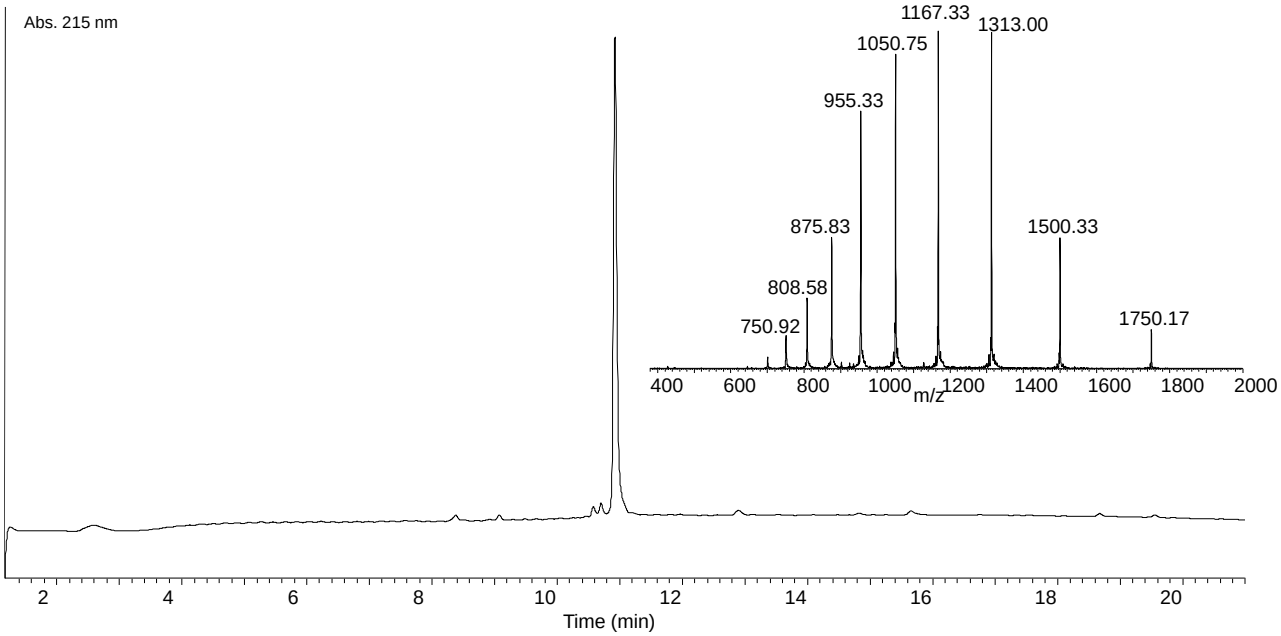


Figure S 6. Analysis of SUMO-3 Lys(Cys) **6b**. UPLC-MS analysis ACQUITY UPLC peptide BEH C18 300 Å 1.7 µm 2.1 mm × 150 mm, 50 °C. Flow 0.400 mL/min, eluent A 0.1% trifluoroacetic acid in water, eluent B 0.1% trifluoroacetic acid in 100% acetonitrile. Gradient from 0% eluent B to 70% eluent B in 20 min. HPLC trace (UV detection) and MS trace. Calculated for M (average mass) 10496.80, observed 10496.49 after deconvolution.

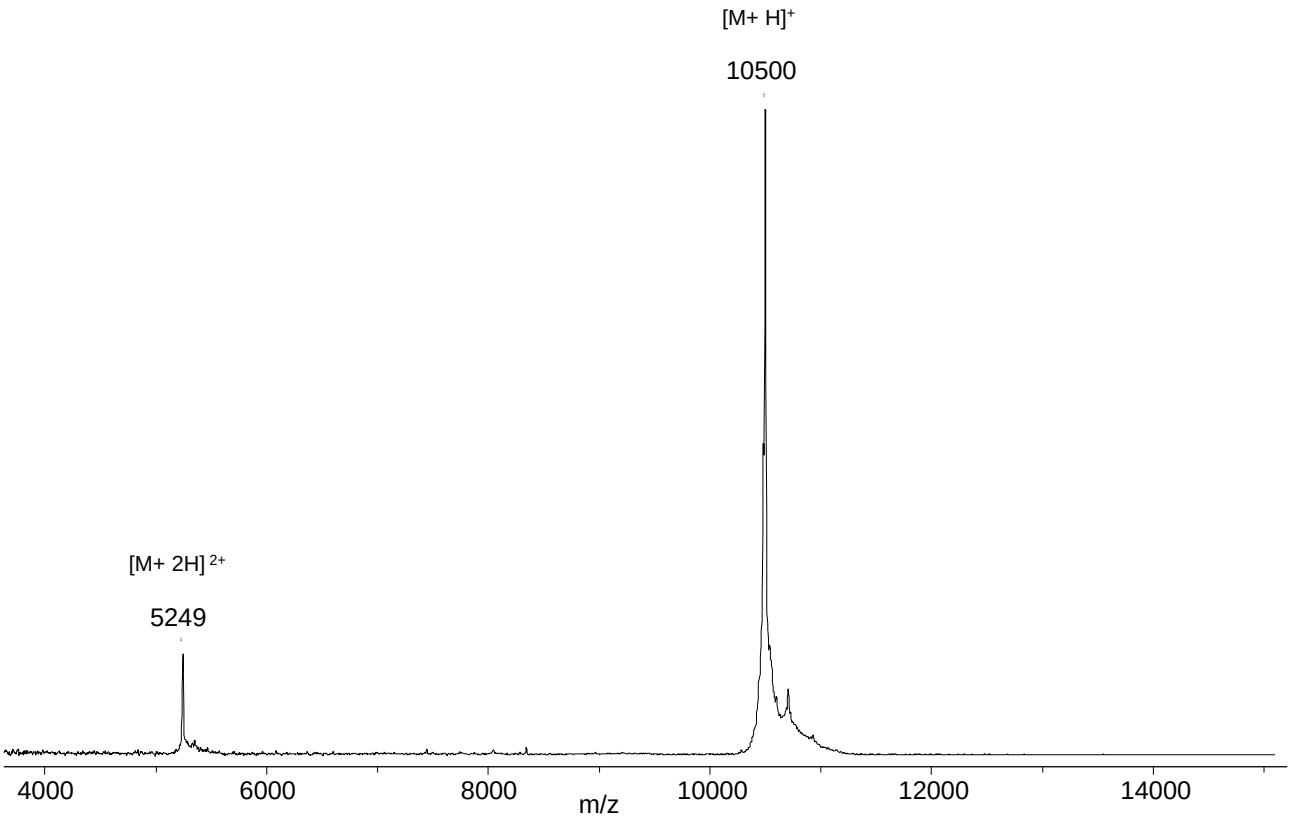


Figure S 7 MALDI-TOF analysis peptide **6b**. Matrix sinapinic acid, positive detection mode, linear mode $[M+H]^+$ calcd. (mean) 10498, found 10500.

3) One-pot synthesis and characterization of SUMO-3 Lys(Cys) hydrazide 7

Peptide **7** was prepared similarly using peptide **1b** (2.86 mg, 0.429 µmol) and peptide **5** (2.53 mg, 0.429 µmol).

The crude mixture was purified by semi-preparative HPLC using column XBridge BEH300 C18 (5 µm, 300 Å, 10 × 250 mm), eluent A water containing 0.1% of TFA, eluent B CH_3CN/water : 4/1 by vol containing 0.1% of TFA, gradient: 0-25 % B in 5 min, 25-45 % B in 50 min, flow rate 6 mL/min, UV detection at 215 nm, 65 °C to give **7** (1.67 mg, 58% overall isolated).

Peptide 7: SEEKPKEGVK(C)TENDHINLKVAGQDGSVVQFKIKRHTPLSKLMKA YCERQGLSMRQIRFRFDGQPINETDTPAQLEMEDEDTIDVFQQQTGG-NHNH$_2$

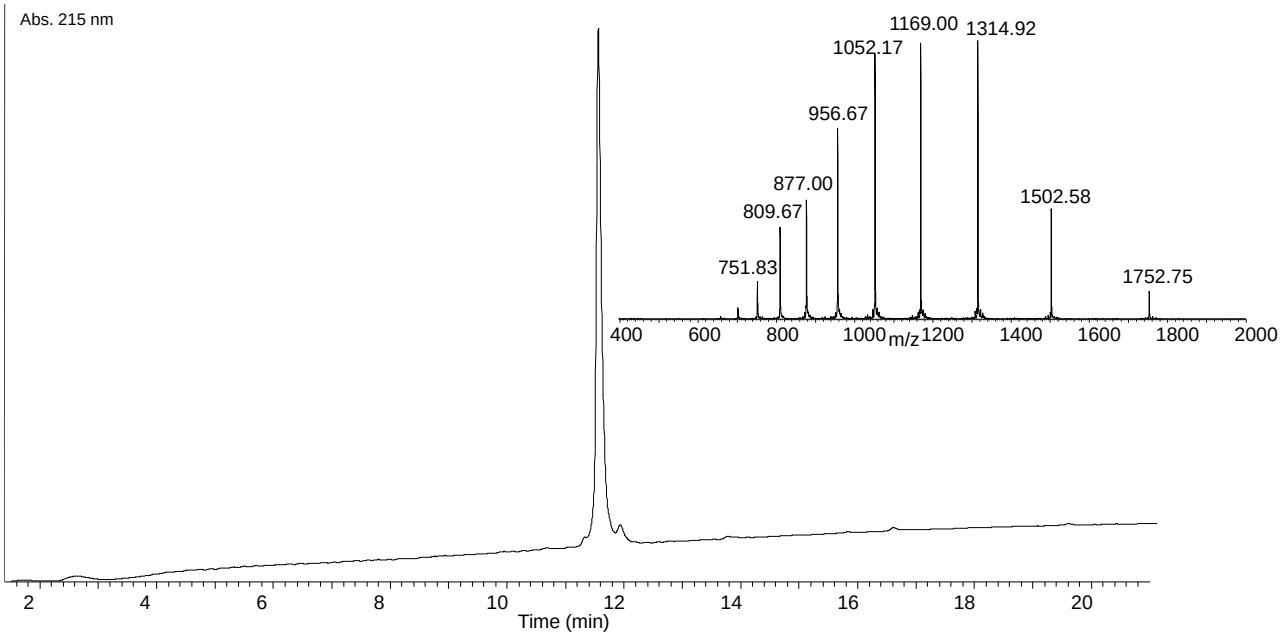


Figure S 8. Analysis of SUMO-3 Lys(Cys) hydrazide **7**. UPLC-MS analysis ACQUITY UPLC peptide BEH C18 300 Å 1.7 µm 2.1 mm × 150 mm, 50 °C. Flow 0.400 mL/min, eluent A 0.1% trifluoroacetic acid in water, eluent B 0.1% trifluoroacetic acid in 100% acetonitrile. Gradient from 0% eluent B to 70% eluent B in 20 min. HPLC trace (UV detection) and MS trace. Calculated for M (average mass) 10510.83, observed 10511.85 after deconvolution.

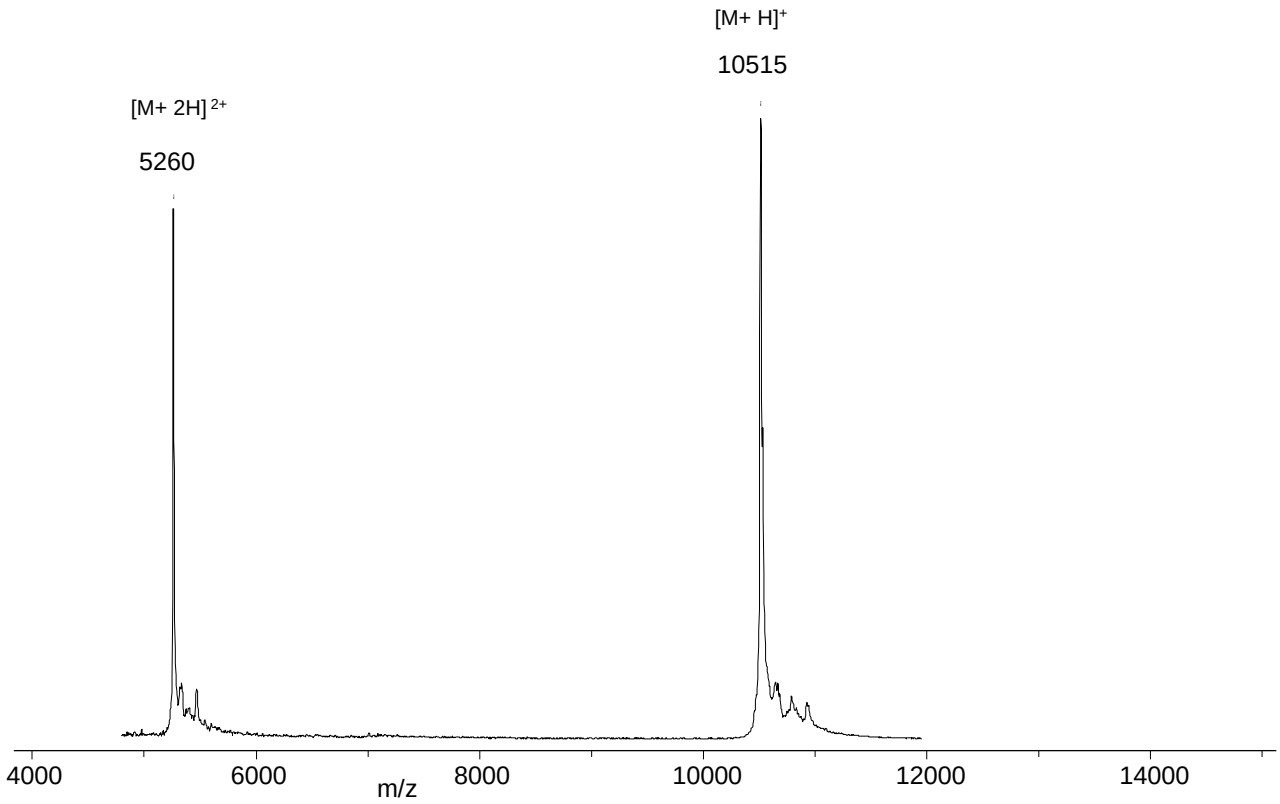


Figure S 9 MALDI-TOF analysis of peptide **7**. Matrix sinapinic acid, positive detection mode, linear mode $[M+H]^+$ calcd. (mean) 10512, found 10515.

B. Chemical synthesis of SUMO-2/3 dimers

A typical procedure is illustrated with the synthesis of SUMO-2/3 dimer **11**.

1) Synthesis of SUMO dimer 11

Step 1. Synthesis of SUMO-2 SEA^{off} ***10a***

A solution of *N*-octylglucoside (6.90 mg, 20 mM) in 6 M guanidinium chloride/0.1 M pH 7.0 sodium phosphate buffer (1.180 mL) was prepared (solution A). Solution A (501 µL) was used to dissolve 4-mercaptophenylacetic acid (MPAA, 16.85 mg, 200 mM) and the pH of the mixture was then adjusted to 7.2 with 6 N NaOH to give solution B.

Thioester peptide **8a** (2.04 mg, 0.308 µmol, 5 mM) and peptide **9** (1.85 mg, 0.308 µmol, 5 mM, 1 equiv) were dissolved in solution B (62 µL) and the reaction mixture was stirred at 25 °C for 25 h under nitrogen atmosphere to give the solution C.

The progress of the reaction was monitored by HPLC. For each analysis, a 1.5 µL aliquot was taken from the reaction mixture and quenched by adding 100 µL of 10 % acetic acid. The sample was then extracted with Et_2O to remove MPAA prior to HPLC analysis.

Step 2. Assembly of the dimer

A fresh solution of *N*-octylglucoside (6.90 mg, 20 mM) in 6 M guanidinium chloride/0.1 M pH 7.0 sodium phosphate buffer (1.180 mL) was prepared again (solution D). Solution D (421 µL) was used to dissolve TCEP (24.14 mg, 84.20 µmol, 200 mM) to give the solution E. Then MPAA (14.16 mg, 84.20 µmol, 200 mM) was dissolved in solution E (421 µL) and the pH of the solution was adjusted to 5.5 with 6 N NaOH to give solution F. Peptide **6a** (3.8 mg, 0.307 µmol, 5 mM) was dissolved in solution F (60 µL) to give solution G.

Solutions C and G were mixed and the pH was adjusted to 5.5 by addition of 6 N NaOH. The final peptide concentration was 2.5 mM. The reaction was agitated for 96 h and then acidified with glacial acetic acid (10 µL) to quench the reaction. The solution was extracted with diethyl ether (3 × 2 mL) to remove the excess of MPAA. The mixture was immediately purified by HPLC to yield 1.85 mg (36% overall isolated) of SUMO dimer **11**.

2) Synthesis of SUMO dimer 12

SUMO-3 SEA^{off} **10b** was prepared using the thioester peptide **8b** (2.36 mg, 0.362 µmol, 5 mM) and peptide **9** (2.17 mg, 0.362 µmol) and ligated with SUMO-3 Lys(Cys) **6b** (4.50 mg, 0.360 µmol) as described for dimer **11**.

The reaction was acidified with glacial acetic acid (10 µL) to quench the reaction. The solution was extracted with diethyl ether (3 × 2 mL) to remove the excess of MPAA. The mixture was immediately purified by HPLC to yield 0.836 mg (22% overall isolated) of SUMO dimer **12**.

3) Synthesis of SUMO dimer 13

SUMO-2 SEA^{off} **10a** was prepared using thioester peptide **8a** (2.04 mg, 0.308 µmol, 5 mM) and peptide **9** (1.85 mg, 0.308 µmol, 5 mM, 1 equiv) and ligated with SUMO-3 Lys(Cys) **6b** (3.80 mg, 0.303 µmol) as described for dimer **11**.

The reaction was acidified with glacial acetic acid (10 µL) to quench the reaction. The solution was extracted with diethyl ether (3 × 2 mL) to remove the excess of MPAA. The mixture was immediately purified by HPLC to yield 1.102 mg (29% overall isolated) of SUMO dimer **13**.

4) Synthesis of SUMO dimer 14

SUMO-3 SEA^{off} **10b** was prepared using thioester peptide **8b** (2.36 mg, 0.362 µmol, 5 mM) and peptide **9** (2.17 mg, 0.362 µmol, 5 mM, 1.0 equiv) and ligated with SUMO-2 Lys(Cys) **6a** (4.40 mg, 0.363 µmol) as described for dimer **11**.

The reaction was acidified with glacial acetic acid (10 µL) to quench the reaction. The solution was extracted with diethyl ether (3 × 2 mL) to remove the excess of MPAA. The mixture was immediately purified by HPLC to yield 0.874 mg (23% overall isolated) of SUMO dimer **14**.

5) Synthesis and characterization of SUMO dimer 15

SUMO-2 SEA^{off} **10a** was prepared using thioester peptide **8a** (2.04 mg, 0.308 µmol, 5 mM) and peptide **9** (1.85 mg, 0.308 µmol, 5 mM, 1 equiv) and ligated with SUMO-3 Lys(Cys) (4.50 mg, 0.360 µmol) as described for dimer **11**.

The reaction was acidified with glacial acetic acid (10 µL) to quench the reaction. The solution was extracted with diethyl ether (3 × 2 mL) to remove the excess of MPAA. The mixture was immediately purified by HPLC to yield 0.555 mg (13% overall isolated) of SUMO dimer **15**.

D. Desulfurization of SUMO-2/3 dimers

The desulfurization protocols in native or denaturing conditions were optimized and validated using SUMO-2/3 dimer **13**.

- ### Desulfurization of SUMO-2/3 dimer 13 in denaturing conditions

SUMO-2/3 dimer **13** (0.130 mg, 6.20 µmol, 0.048 mM final concentration) was dissolved in a solution of TCEP (57.3 mg/mL, 200 mM final concentration), 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044, 6.50 mg/mL, 20 mM final concentration), methionine (Met, 2.98 mg/mL, 20 mM final concentration) and reduced glutathione (GSH, 15.40 mg/mL, 50 mM final concentration) in 6 M guanidinium chloride/ 0.1 M pH 7.2 ammonium phosphate buffer (3492 µL). The desulfurization was carried out at 25 °C and monitored by UPLC-MS. The reaction product was SUMO-2/3 dimer **20**.

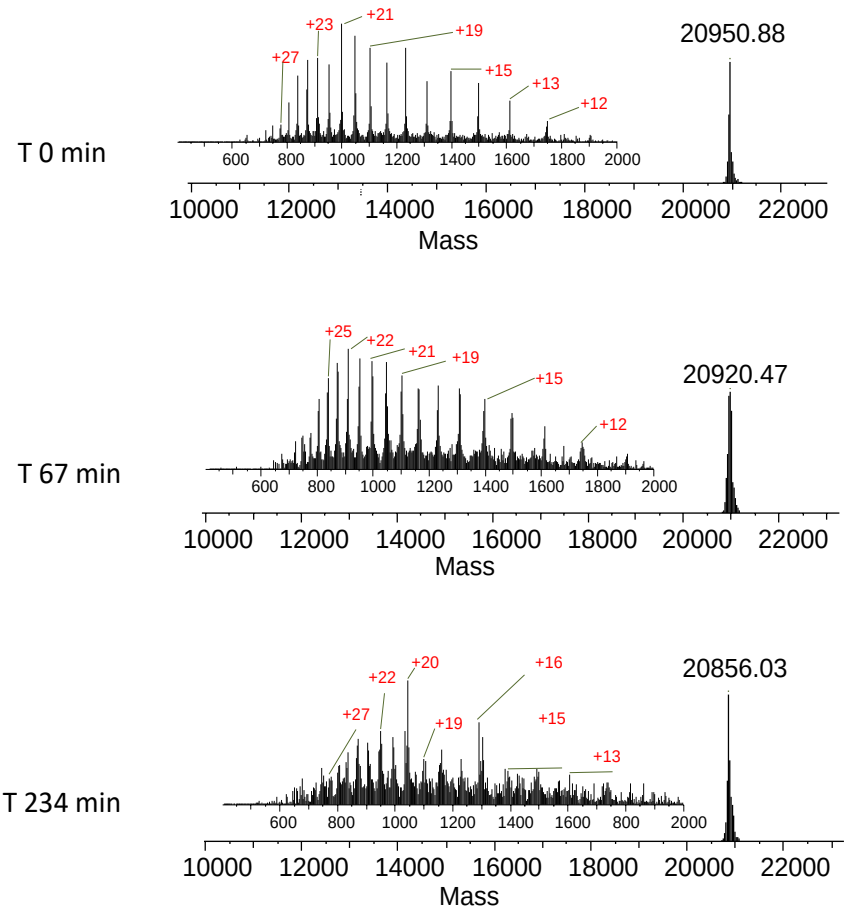


Figure S 10. Analysis of the desulfurization reaction in denaturing condition and leading to the production of SUMO-2/3 dimer **20**. UPLC-MS analysis using ATLANTIS waters T3column (3 µm 2.1 mm × 50 mm). 50 °C, flow 0.400 mL/min, eluent A 0.1% trifluoroacetic acid in water, eluent B 0.1% trifluoroacetic acid in 100% aqueous acetonitrile. Gradient from 0% eluent B to 70% eluent B in 4 min. MS trace.

- Desulfurization of SUMO-2/3 dimer 13 in native conditions

SUMO-2/3 dimer **13** (0.136 mg, 6.49 µmol, 0.048 mM final concentration) was dissolved in a solution of TCEP (57.3 mg/mL, 200 mM final concentration), 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044, 6.50 mg/mL, 20 mM final concentration), methionine (Met, 2.98 mg/mL, 20 mM final concentration) and reduced glutathione (GSH, 15.40 mg/mL, 50 mM final concentration) in 0.1 M pH 7.2 ammonium phosphate buffer (3492 µL). The desulfurization was carried out at 25 °C and monitored by UPLC-MS. The reaction yielded selectively SUMO-2/3 dimer **18**.

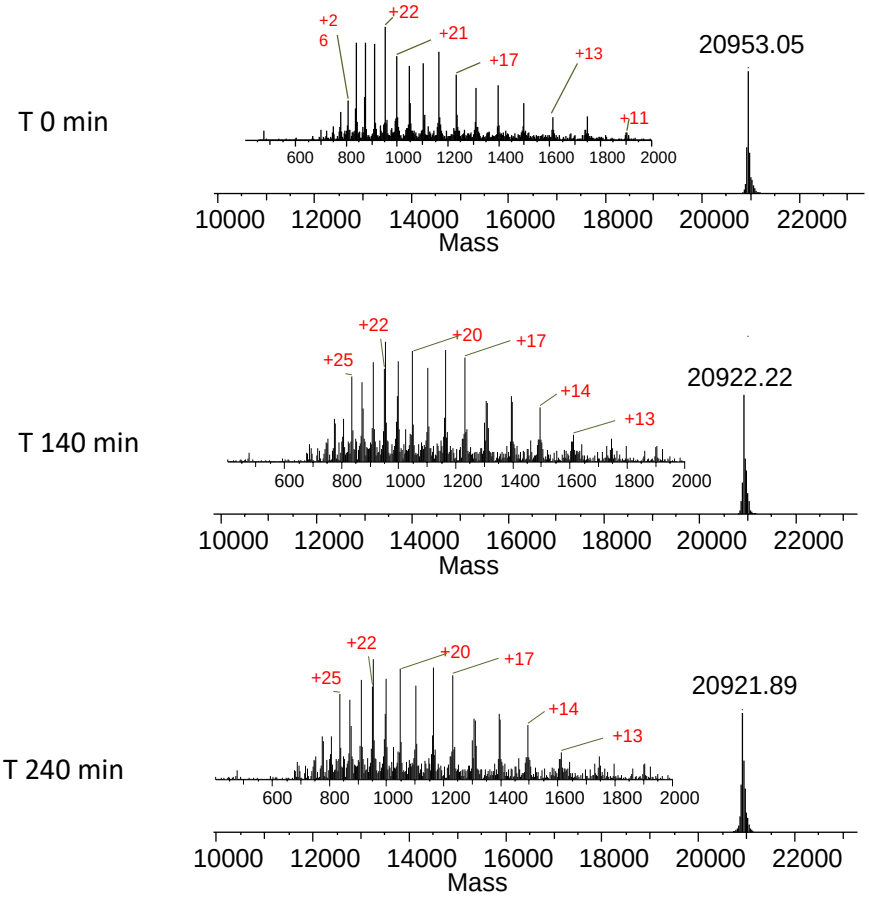


Figure S 11. Analysis of the desulfurization reaction in native conditions. UPLC-MS analysis using ATLANTIS waters T3column (3 µm, 2.1 mm × 50 mm), 50 °C. Flow 0.400 mL/min, eluent A 0.1% trifluoroacetic acid in water, eluent B 0.1% trifluoroacetic acid in 100% aqueous acetonitrile. Gradient from 0% eluent B to 70% eluent B in 4 min. MS trace.

This protocol was applied to the selective desulfurization of SUMO-2/3 dimers **11**-**14**.

SUMO-2/3 dimer **16** was prepared by desulfurization of SUMO-2/3 dimer **11** (0.050 mg, 2.37 µmol, 0.048 mM final concentration).

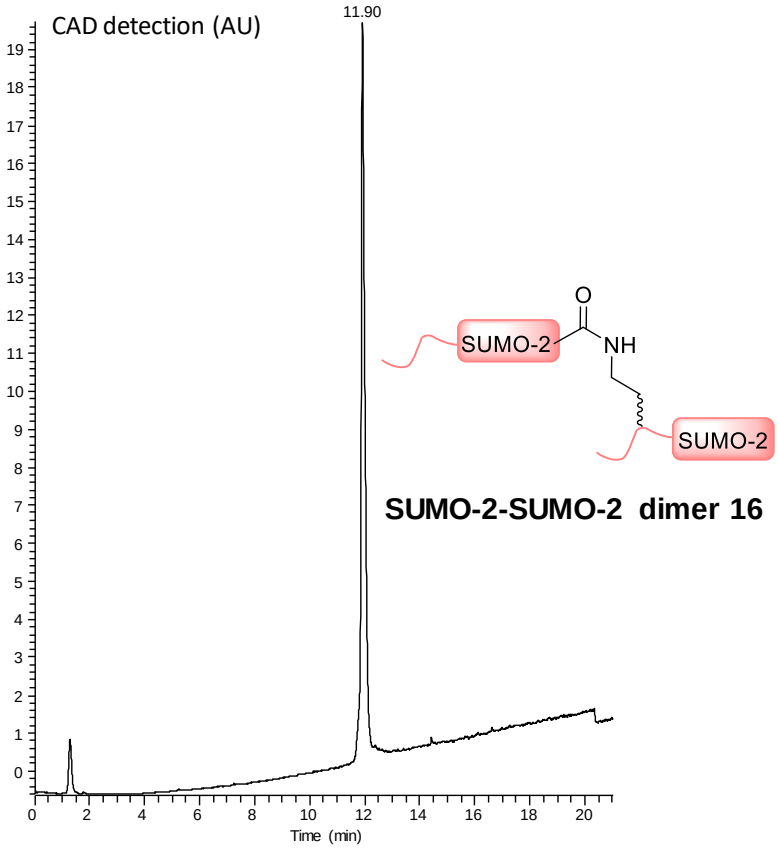
SUMO-2/3 dimer **17** was prepared by desulfurization of SUMO-2/3 dimer **12** (0.050 mg, 2.39 µmol, 0.048 mM final concentration).

SUMO-2/3 dimer **18** was prepared by desulfurization of SUMO-2/3 dimer **13** (0.61 mg, 0.46 µmol, 0.10 mM final concentration). The desulfurization was carried out at 25 °C and monitored by UPLC-MS. The mixture was purified by HPLC to yield 0.372 mg (61% isolated) of SUMO-2/3 dimer **18**.

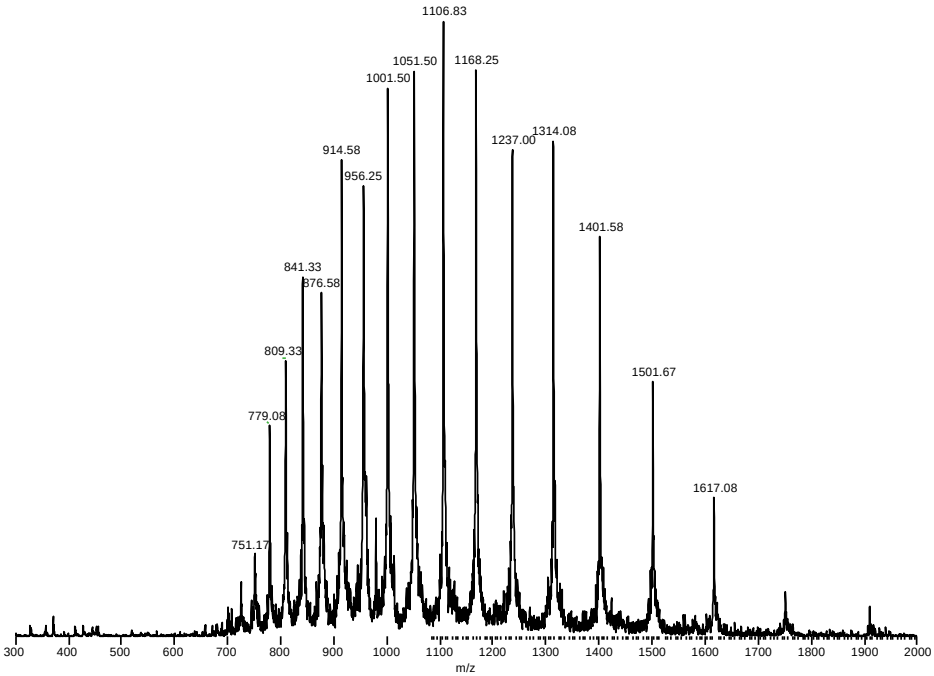
SUMO-2/3 dimer **19** was prepared by desulfurization of SUMO-2/3 dimer **14** (0.050 mg, 2.38 µmol, 0.048 mM final concentration).

- *UPLC-MS analysis*

A)


CAD detection (AU)
11.90
Time (min)
SUMO-2
NH
O
SUMO-2
SUMO-2-SUMO-2 dimer 16

B)


1106.83
1051.50
1168.25
1001.50
1237.00
1314.08
914.58
956.25
1401.58
841.33
876.58
809.33
1501.67
779.08
1617.08
751.17
m/z

C)

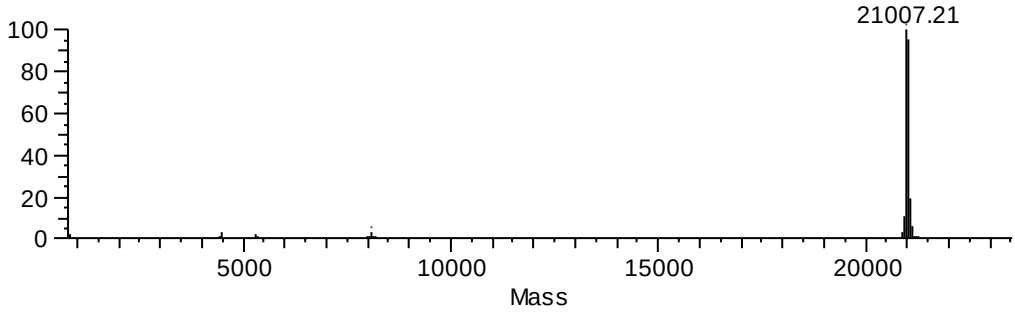
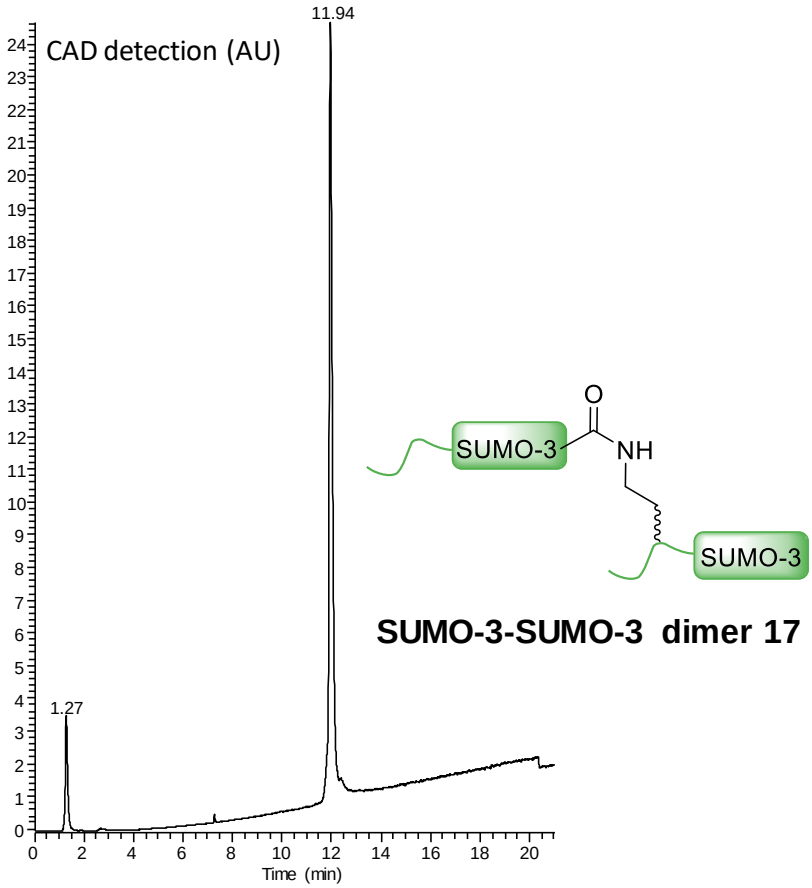
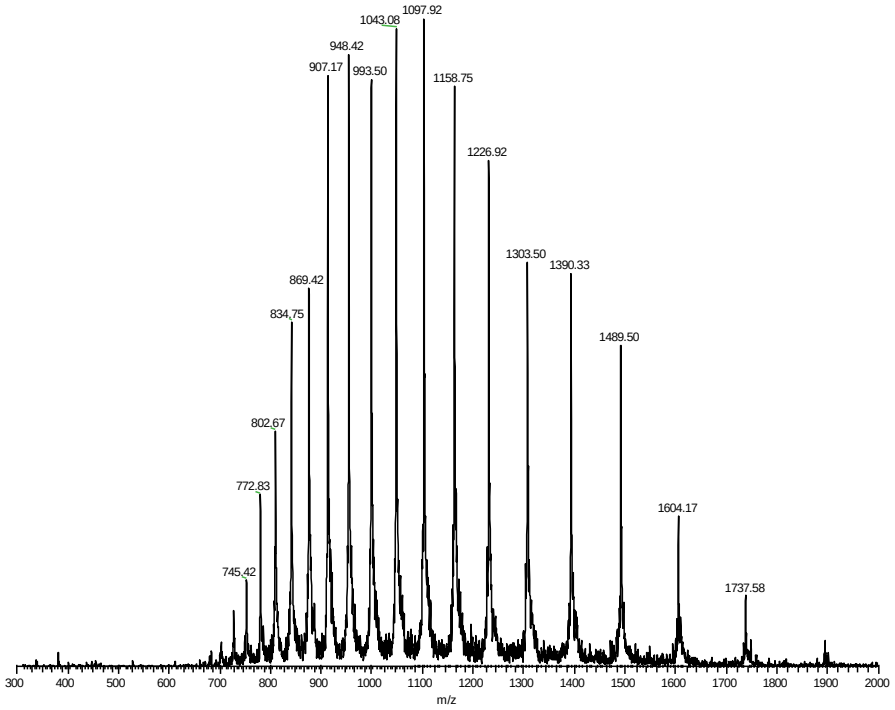


Figure S 12. LC-MS analysis of the desulfurized SUMO-2/3 dimers in native conditions. Formation of SUMO-2-SUMO-2 dimer **16**. A) LC-trace using charged aerosol detection (CAD, Dionex Corona Veo). B) MS trace. C) MS trace after deconvolution. UPLC-MS analysis ACQUITY UPLC peptide BEH C18 300 Å 1.7 µm (2.1 mm × 150 mm), 50 °C. Flow 0.400 mL/min, eluent A 0.1% trifluoroacetic acid in water, eluent B 0.1% trifluoroacetic acid in 100% acetonitrile. Gradient from 0% eluent B to 70% eluent B in 20 min.

A)



B)



C)

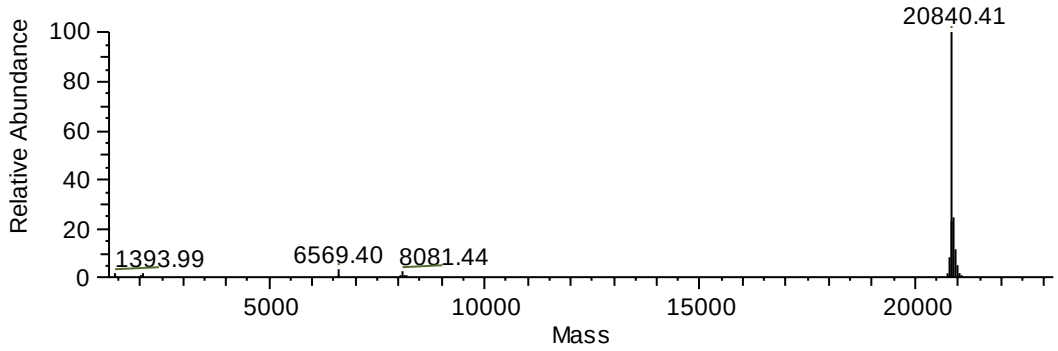
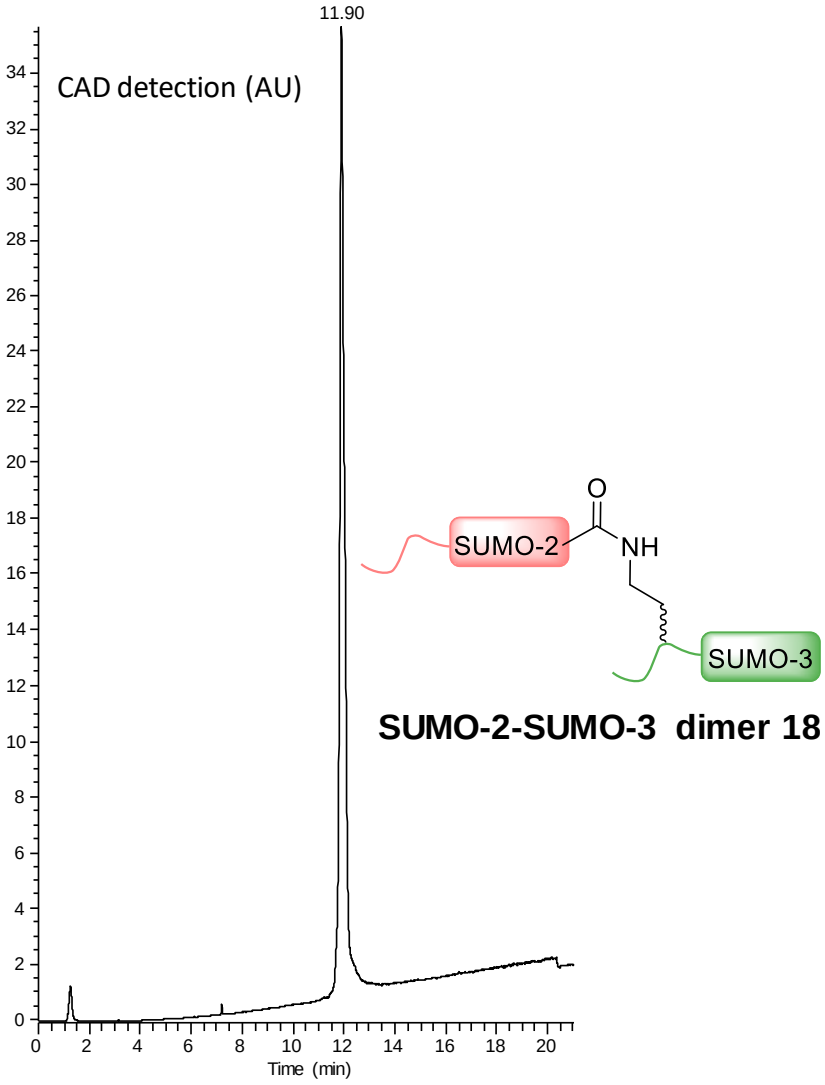
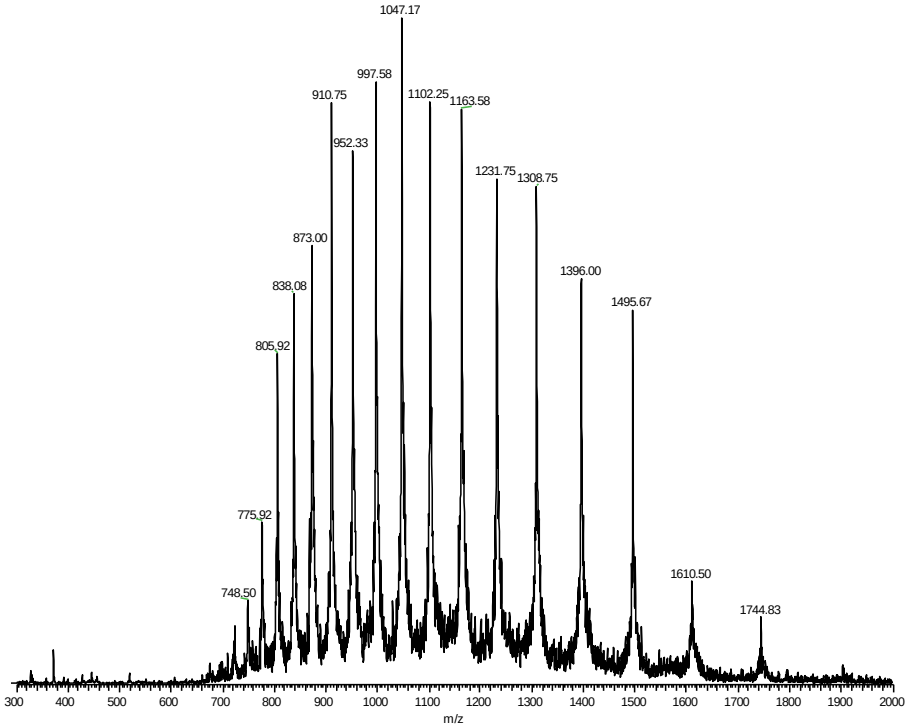


Figure S 13. LC-MS analysis of the desulfurized SUMO-2/3 dimers in native conditions. Formation of SUMO-3-SUMO-3 dimer **17**. A) LC-trace using charged aerosol detection (CAD, Dionex Corona Veo). B) MS trace. C) MS trace after deconvolution. UPLC-MS analysis ACQUITY UPLC peptide BEH C18 300 Å 1.7 µm (2.1 mm × 150 mm), 50 °C. Flow 0.400 mL/min, eluent A 0.1% trifluoroacetic acid in water, eluent B 0.1% trifluoroacetic acid in 100% acetonitrile. Gradient from 0% eluent B to 70% eluent B in 20 min.

A)


11.90
CAD detection (AU)
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
14
16
18
20
Time (min)
O
SUMO-2
NH
SUMO-3
SUMO-2-SUMO-3 dimer 18

B)



C)

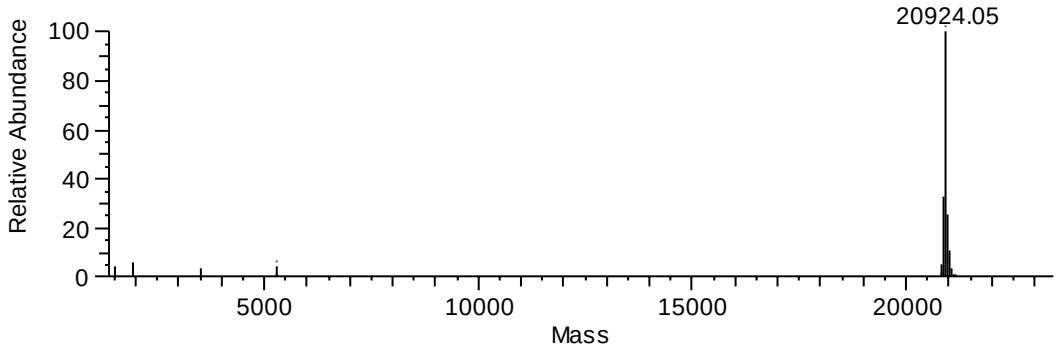
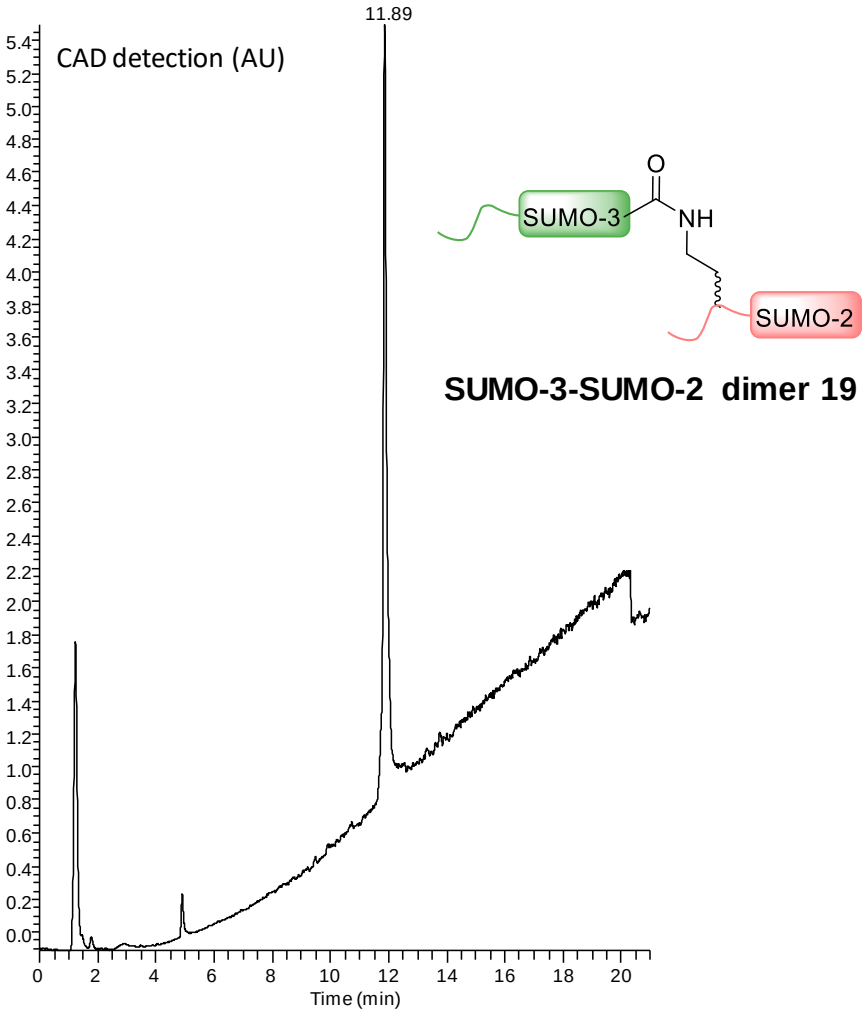
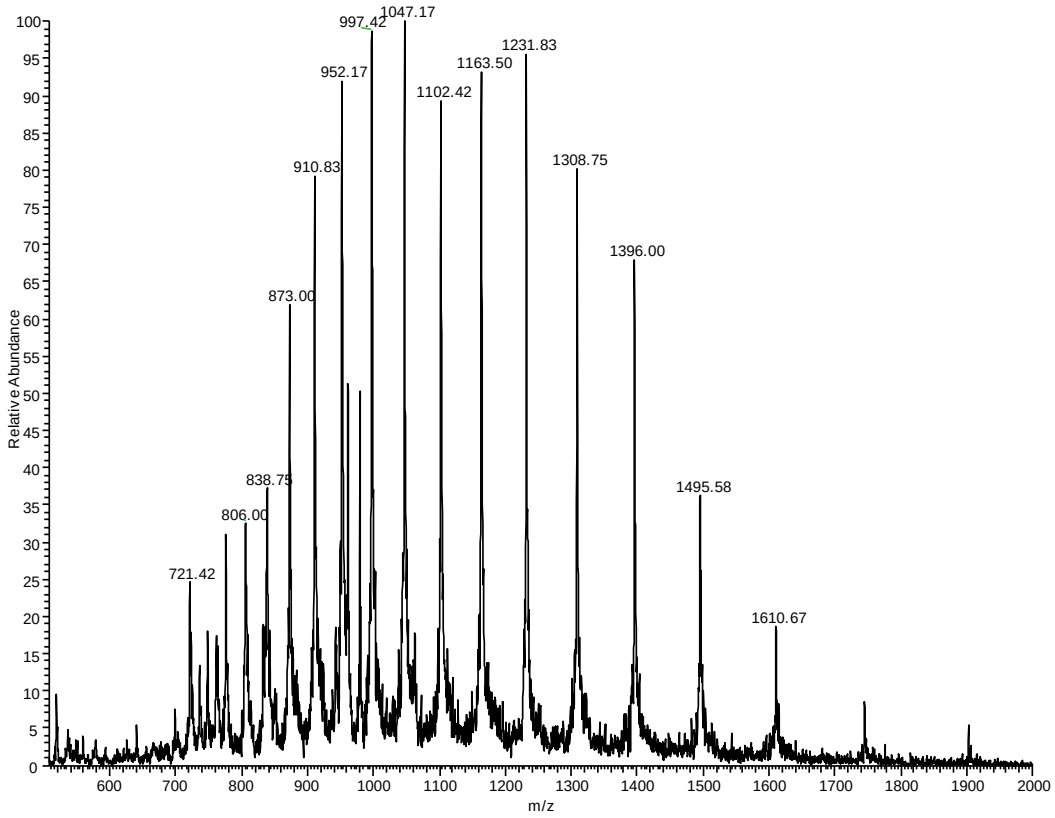


Figure S 14. LC-MS analysis of the desulfurized SUMO-2/3 dimers in native conditions. Formation of SUMO-2-SUMO-3 dimer **18**. A) LC-trace using charged aerosol detection (CAD, Dionex Corona Veo). B) MS trace. C) MS trace after deconvolution. UPLC-MS analysis ACQUITY UPLC peptide BEH C18 300 Å 1.7 µm (2.1 mm × 150 mm), 50 °C. Flow 0.400 mL/min, eluent A 0.1% trifluoroacetic acid in water, eluent B 0.1% trifluoroacetic acid in 100% acetonitrile. Gradient from 0% eluent B to 70% eluent B in 20 min.

A)


11.89
CAD detection (AU)
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
16
18
20
Time (min)
O
SUMO-3
NH
SUMO-2
SUMO-3-SUMO-2 dimer 19

B)



C)

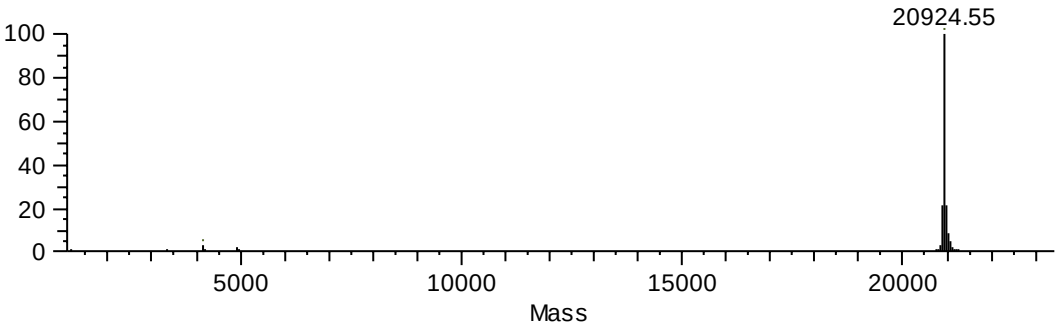


Figure S 15. LC-MS analysis of the desulfurized SUMO-2/3 dimers in native conditions. Formation of SUMO-3-SUMO-2 dimer **19**. A) LC-trace using charged aerosol detection (CAD, Dionex Corona Veo). B) MS trace. C) MS trace after deconvolution. UPLC-MS analysis ACQUITY UPLC peptide BEH C18 300 Å 1.7 µm (2.1 mm × 150 mm), 50 °C. Flow 0.400 mL/min, eluent A 0.1% trifluoroacetic acid in water, eluent B 0.1% trifluoroacetic acid in 100% acetonitrile. Gradient from 0% eluent B to 70% eluent B in 20 min.

C. Characterization of SUMO-2/3 dimers by proteomics analysis

Tryptic digestion

The proteins were dissolved in 25 mM ammonium bicarbonate pH 7.8 (1 mg/mL). The protein solution (5 µL, 5 µg) was mixed with DTT (5 mg/mL, 1 µL, 5 µg) and iodoacetamide (10 mg/mL, 5 µL, 50 µg). After 30 min, trypsin (1 µL, 500 ng) solution in 25 mM ammonium bicarbonate (0.5 mg/mL) was added the protein which was digested at 37 °C overnight.

Endopeptidase GluC digestion

The proteins were dissolved in 25 mM ammonium phosphate pH 7.5 (1 mg/mL). The protein solution (5 µL, 5 µg) was mixed with DTT (5 mg/mL, 1 µL, 5 µg) and iodoacetamide (10 mg/mL, 5 µL, 50 µg). After 30 min, endopeptidase GluC (1 µL, 500 ng) solution in 25 mM ammonium phosphate (0.5 mg/mL) was added on the above solution and was kept at room temperature overnight.

The digested protein solutions were directly spotted on a MALDI plate and mixed with the matrix (α-cyano-4-hydroxycinnamic acid or 2, 5-dihydroxybenzoic acid) before analysis.

1) Proteomic analysis of SUMO-2/3 dimer **16**

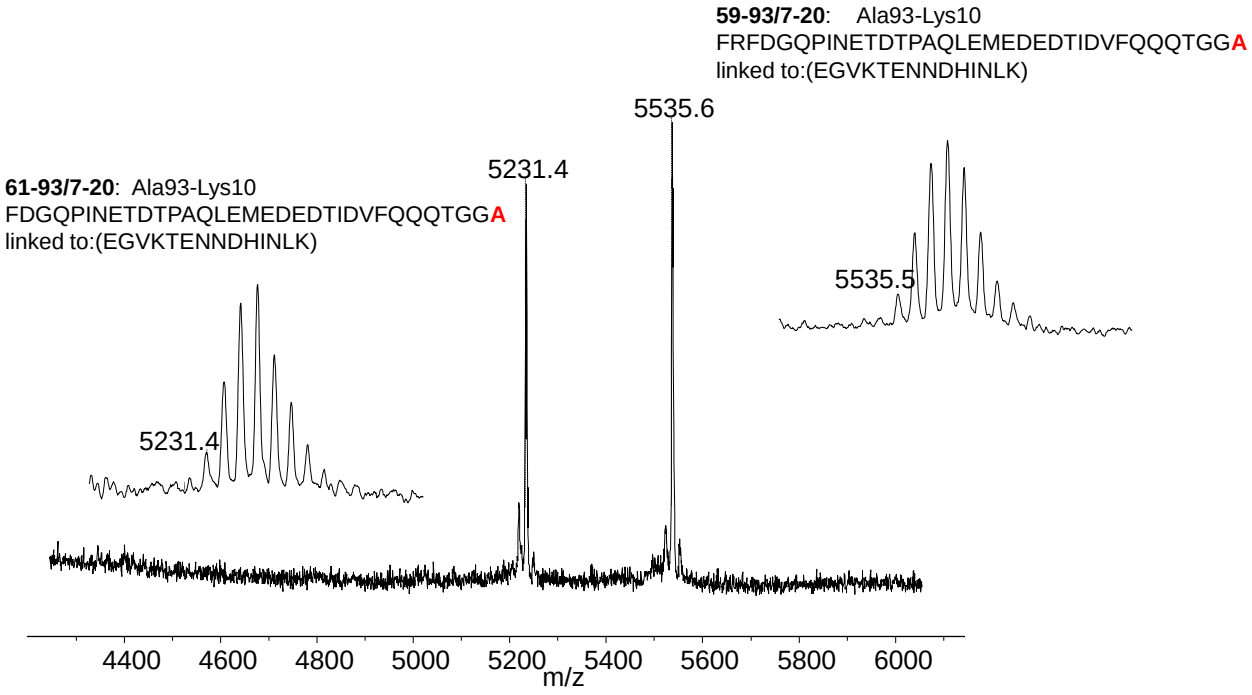


Figure S 16. MALDI-TOF spectrum of the fragments generated after reduction, alkylation and trypsin cleavage of SUMO-2/3 dimer **16**

Internal cysteine state

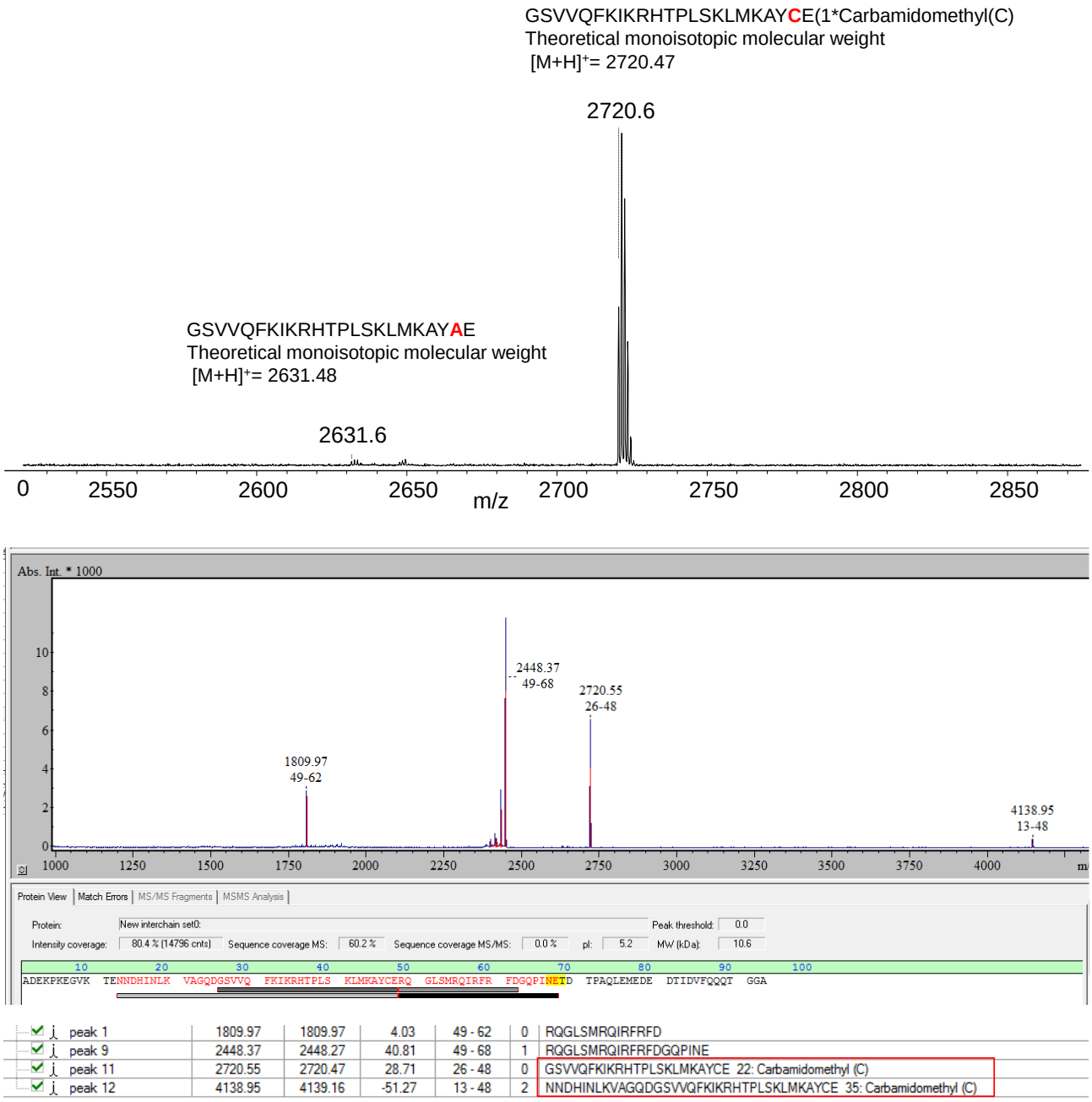


Figure S 17. MALDI-TOF spectrum of the fragments generated after reduction, alkylation and endopeptidase GluC cleavage of SUMO-2/3 dimer **16**.

2) Proteomic analysis of SUMO-2/3 dimer **17**

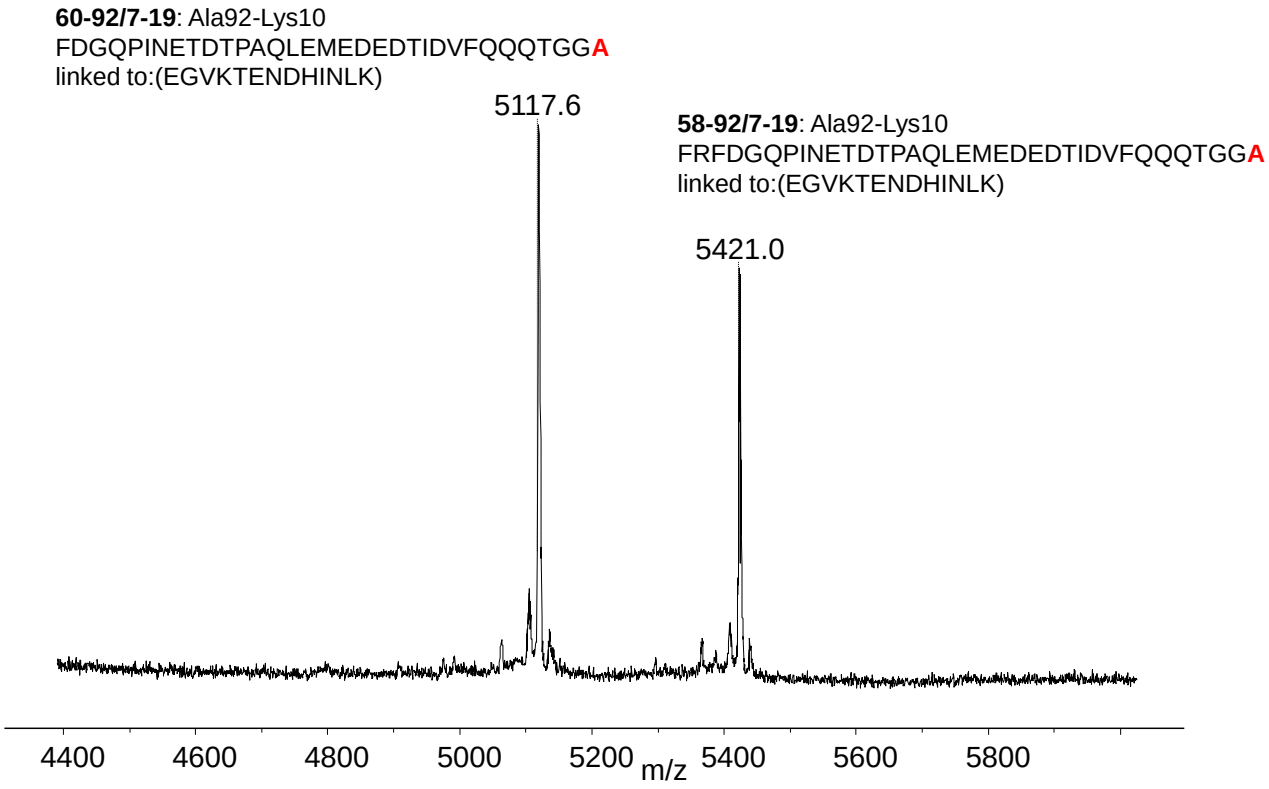


Figure S 18. MALDI-TOF spectrum of the fragments generated after reduction, alkylation and trypsin cleavage of SUMO-2/3 dimer **17**

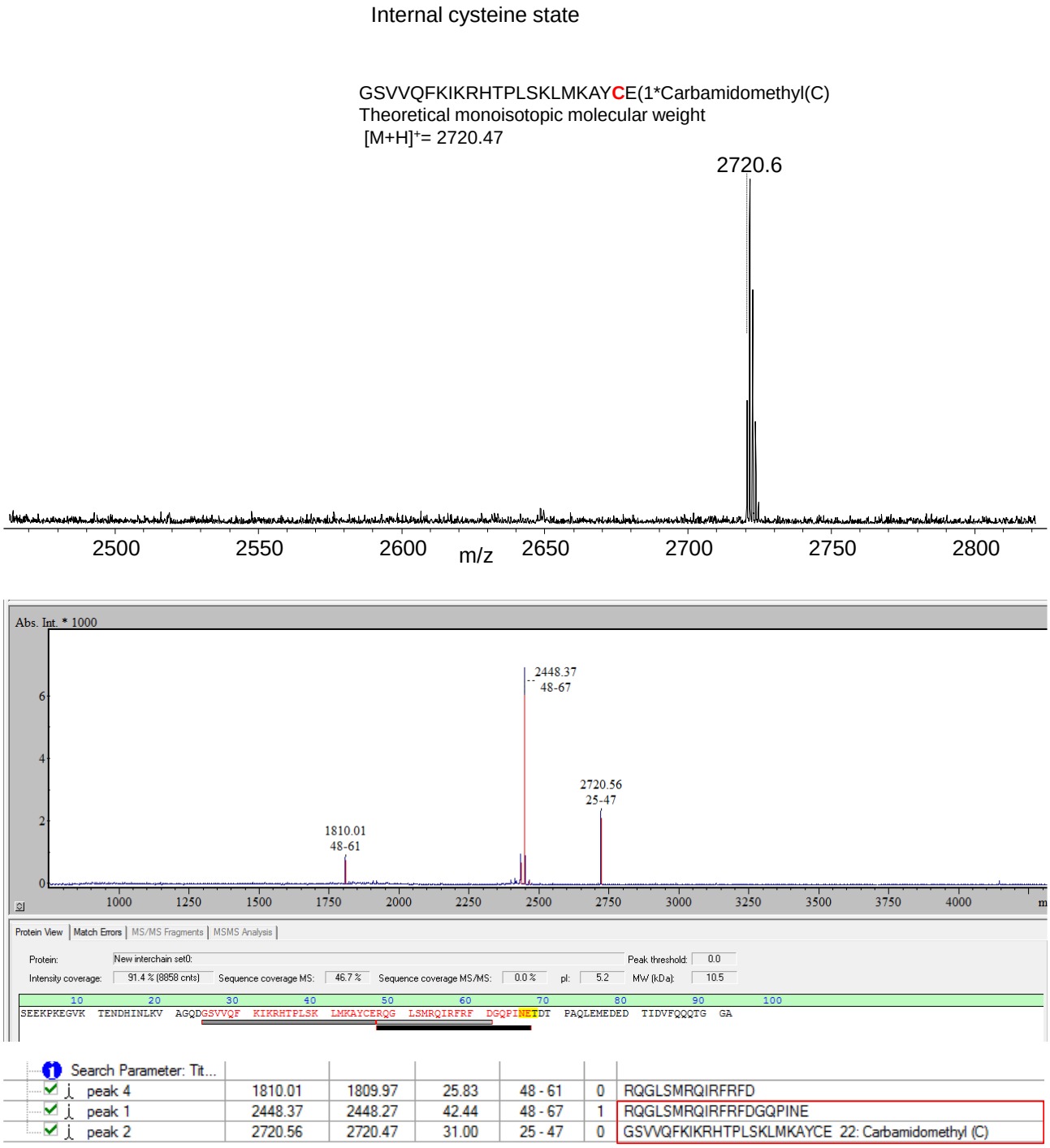


Figure S 19. MALDI-TOF spectrum of the fragments generated after reduction, alkylation and endopeptidase GluC cleavage of SUMO-2/3 dimer **17.**

3) Proteomic analysis of SUMO-2/3 dimer 18

Internal cysteine state

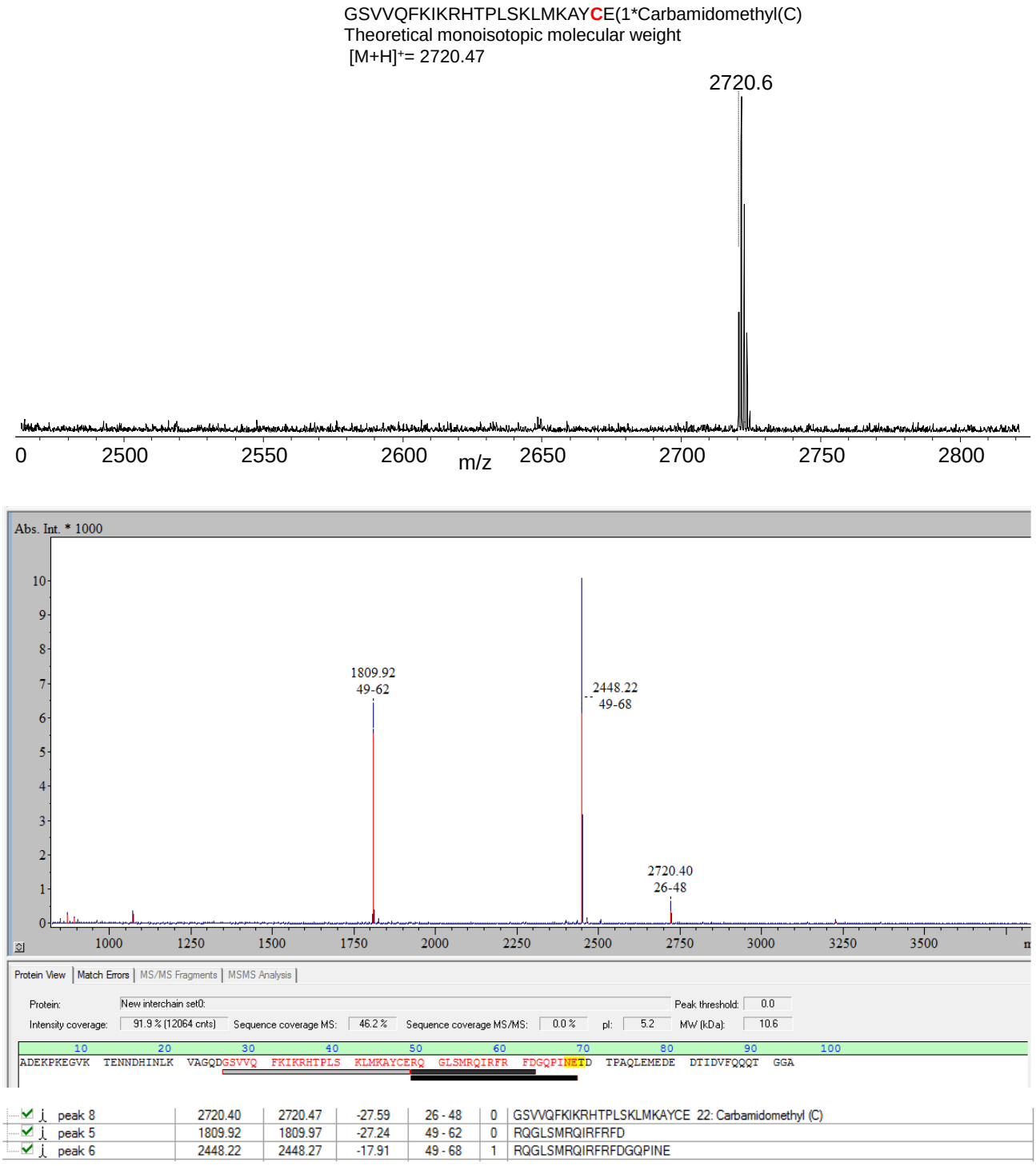


Figure S 20. MALDI-TOF spectrum of the fragments generated after reduction, alkylation and endopeptidase GluC cleavage of SUMO-2/3 dimer **18.**

4) Proteomic analysis of SUMO-2/3 dimer 19

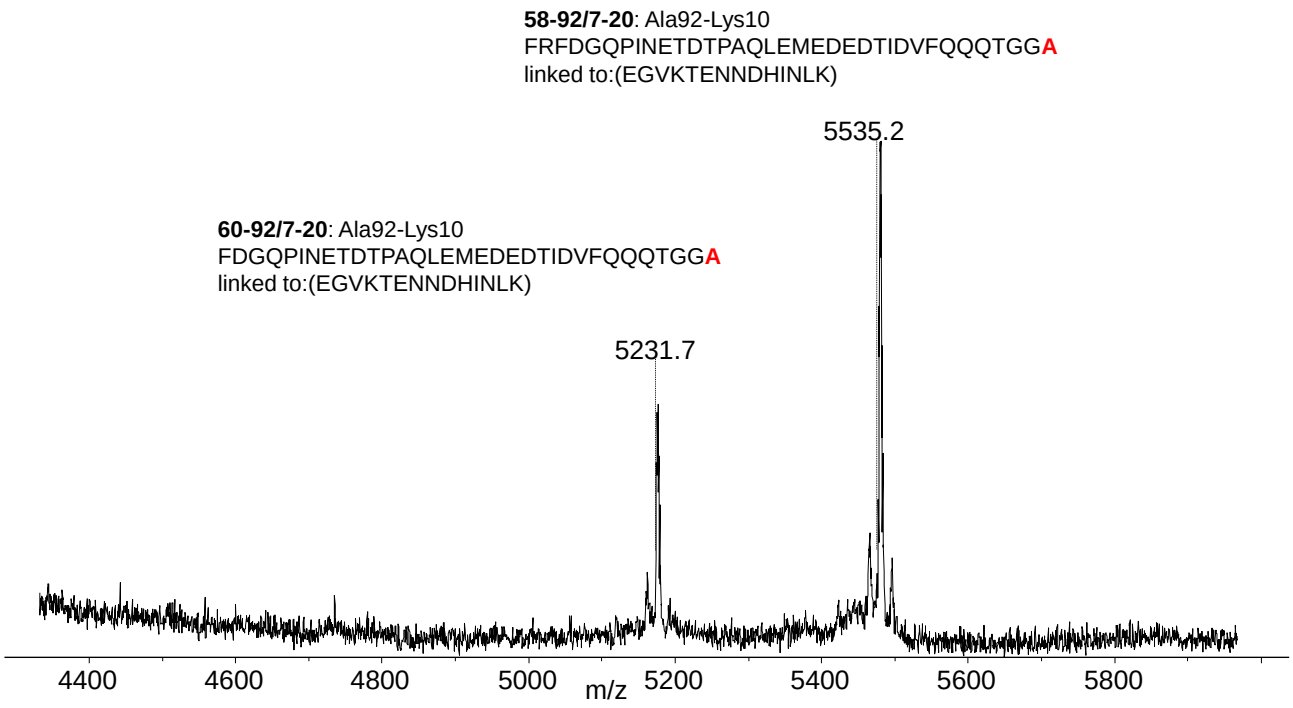


Figure S 21. MALDI-TOF spectrum of the fragments generated after reduction, alkylation and trypsin cleavage of SUMO-2/3 dimer **19**.

Internal cysteine state

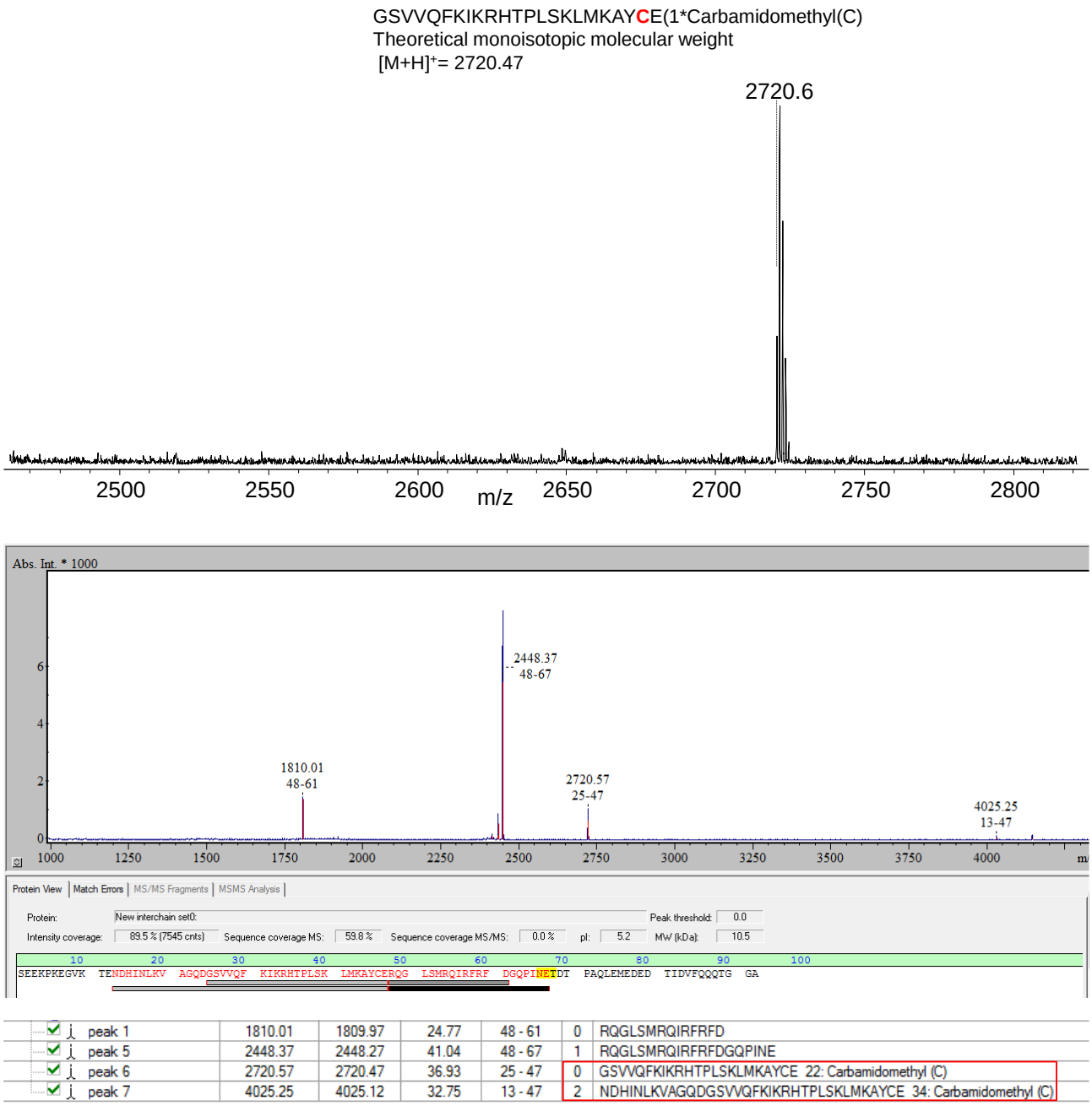


Figure S 22. MALDI-TOF spectrum of the fragments generated after reduction, alkylation and endopeptidase GluC cleavage of SUMO-2/3 dimer **19**.

5) Proteomic analysis of SUMO-2/3 dimer **20**

SUMO-2/3 is the product of the desulfurization of SUMO-2/3 dimer **13** in denaturing conditions.

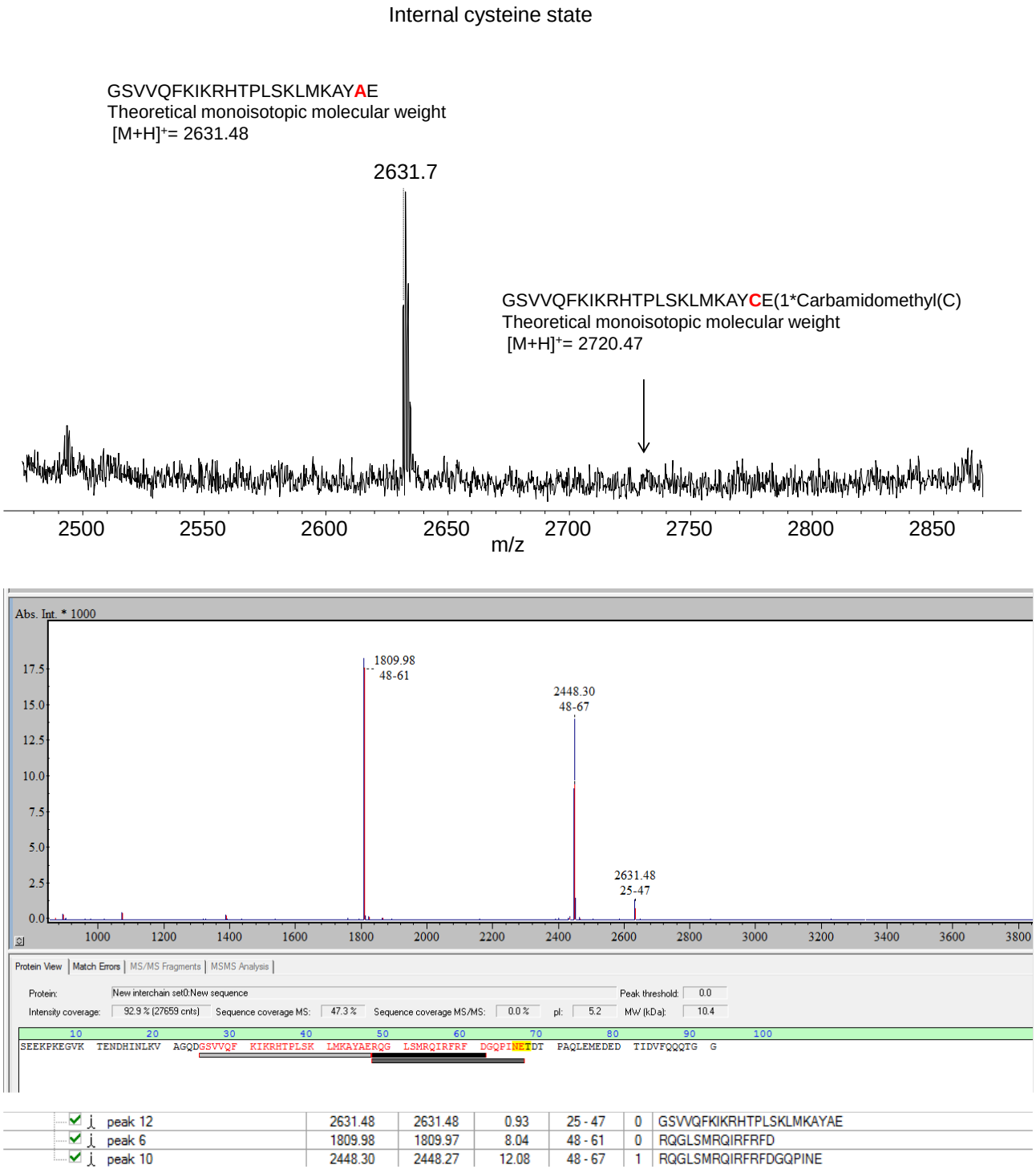


Figure S 23. MALDI-TOF spectrum of the fragments generated after reduction, alkylation and endopeptidase GluC cleavage of SUMO-2/3 dimer **20**

IV. Biophysical and biochemical characterizations

A. Circular dichroism analysis

The concentration of different SUMO proteins solubilized in 10 mM sodium phosphate buffer was determined by measuring the absorbance at 280 nm using a Nanodrop system. The stock solutions of SUMO proteins were diluted with sodium phosphate buffer (10 mM, pH 7.2) to a final concentration of 10-20 µM for analysis. The circular dichroism spectra were recorded with a CD6 spectropolarimeter (Jobin-Yvon).

CD spectra were measured at 25 °C over the range 185-260 nm using 0.1 cm path-length cell and averaging 40 scans. A 2 nm bandwidth, 1 nm data pitch were used for spectral acquisition.

The α-helical content of the proteins was estimated using the empirical equation of Green- field & Fasman (Greenfield, N.; Fasman, G. D. Biochemistry 1969, 8, 4108).

B. Cleavage of the SUMO-2/3 dimer **18** with SENP1 or SENP2

- ### Monitoring by SDS-PAGE and Coomassie staining (Figure 6)

The enzymatic reactions were carried out at 37°C and monitored by SDS-PAGE.
The concentration of the stock solution of the SUMO-2/3 dimer **18** was determined by measuring the absorbance at 280 nm using a Nanodrop system. The solution was diluted with HEPES buffer (50 mM, pH 8)/100 mM NaCl to a final concentration of 0.10 mg/mL (final volume 20 µL). The mixture was kept on ice until addition of SENP1 or SENP2. DTT (1.54 µL, 10 mg/mL) was added to the protein solutions to give solution A

The concentration of the stock solutions of SENP1 and SENP2 enzymes was 0.5 mg/mL and 10 units/µL respectively. The enzymes (2 µL) were first diluted with HEPES (50 mM, pH 8.0)/100 mM NaCl buffer (2000 µL). Then, 1 µL of SENP1 (0.5 ng) or SENP2 (0.02 units) solution was added to the protein solution A. The mixtures were immediately placed at 37 °C. Aliquots of the enzymatic reactions were quenched after 0, 2, 300 min for SENP1 and 0, 30 , 300 min for SENP2 by addition 9 µL of LDS NuPage Sample Buffer (Invitrogen, NP0007) supplemented with 50 mM DTT and 100 mM β-mercaptoethanol. Samples were heated at 70°C for 5 min. Next, 29 µL of each sample was loaded into a 4-12% gradient NuPage Midi Gel (Invitrogen, WG1402A) and eluted in a MES-SDS running buffer (Invitrogen, NP0002) at 150V for 1 h 30.

The gel was fixed and stained in Coomassie solution (water/MeOH/AcOH: 52/40/8 v/v/v, 0.01% Comassie R250, Sigma B-0149) overnight and then incubated in water/MeOH/AcOH: 72/20/8 v/v/v until obtaining clear background.

- ### Monitoring by western-blotting

The Western blotting was performed as proposed by the Sumoylation kit procedure using a 1/1000 anti-SUMO2/3 antibody and a 1/30000 anti-rabbit HRP conjugated secondary antibody dilution (Jackson Immunoresearch, #711-035-152).

VI. References

(1) Ollivier, N., Raibaut, L., Blanpain, A., Desmet, R., Dheur, J., Mhidia, R., Boll, E., Drobecq, H., Pira, S. L., and Melnyk, O. (2014) Tidbits for the Synthesis of *Bis*(2-Sulfanylethyl)Amido (SEA) Polystyrene Resin, SEA Peptides and Peptide Thioesters. *J. Pept. Sci. 20*, 92–97.

(2) Ollivier, N., Dheur, J., Mhidia, R., Blanpain, A., and Melnyk, O. (2010) *Bis*(2-Sulfanylethyl)Amino Native Peptide Ligation. *Org. Lett. 12*, 5238-5241.

(3) Bouchenna, J., Sénéchal, M., Drobecq, D., Stankovic-Valentin, N., Vicogne, J., and Melnyk, O. (2019) The Role of the Conserved SUMO-2/3 Cysteine Residue on Domain Structure Investigated Using Protein Chemical Synthesis. *Bioconjugate Chem. 30*, 2684-2696.

(4) Stavropoulos, G., Gatos, D., Magafa, V., and Barlos, K. (1996) Preparation of Polymer-Bound Trityl-Hydrazines and their Application in the Solid Phase Synthesis of Partially Protected Peptide Hydrazides. *Lett. Pept. Sci. 2*, 315-318.