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## General Methods

Fmoc-Gly-Wang resin (0.32 mmol/g substitution) was purchased from EMD Millipore (Gibbstown, NJ). 2-Chlorotrityl resin (1.5 mmol/g substitution) was purchased from AnaSpec (Fremont, CA). Fmoc-L-amino acids were purchased from AnaSpec (Fremont, CA), AGTC Bioproducts (Wilmington, MA), Creosalus (Louisville, KY), and Sigma Aldrich (St. Louis, MO). Other chemical reagents were purchased from either Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich Chemical Company (St. Louis, MO). DNA synthesis was performed by Integrated DNA Technologies (Coralville, IA). Gene sequencing was performed by Genewiz (South Plainfield, NJ). Plasmid mini-prep, PCR purification and gel extraction kits were purchased from QIAGEN (Valencia, CA). Reversed-phase HPLC (RP-HPLC) was performed on either a Varian (Palo Alto, CA) ProStar HPLC or an Agilent (Santa Clara, CA) 1260 Infinity II HPLC using an Agilent Poroshell 120 SB-C18 analytical column (2.7 micron, 100 x 4.6 mm) at a flow rate of 1 ml/min, a Grace-Vydac (Deerfield, IL) analytical C18 column (5 micron, 150 x 4.6 mm) at a flow rate of 1 ml/min, or a Grace-Vydac preparative C18 column (10 micron, 250 x 22 mm) at a flow rate of 9 ml/min. Mobile phases were Buffer A containing 0.1% trifluoroacetic acid (TFA) in water, and Buffer B containing 90% acetonitrile, 0.1% TFA in water. Solid phase peptide synthesis was performed on a Liberty Blue Automated Microwave Peptide Synthesizer (CEM Corporation, Matthews, NC). Mass spectrometric analysis was conducted on a Bruker (Billerica, MA) Esquire, or a Thermo Scientific (Waltham, MA) LTQ Orbitrap ESI-MS. Analytical RP-HPLC-mass spectrometry (LC-ESI-MS) was performed on a Hewlett-Packard (Palo Alto, CA) 1100-series LC linked to the Bruker Esquire ESI-MS with an Agilent (Santa Clara, CA) Zorbax C18 column (3.5 micron, 100 x 2.1 mm) using Buffer C containing 5% acetonitrile, 1% acetic acid in water, and Buffer D containing acetonitrile and 1% acetic acid as the mobile phases. All NMR spectra were recorded on Bruker Avance AV-300, AV-301, or AV-500 instruments.

## Molecular Cloning

A plasmid containing full-length human SUMO-3(1-92)-AvaDnaE intein was subjected to site-directed mutagenesis in order to obtain the cysteineless (C47S) mutant as previously reported.<sup>1</sup> Truncation of the SUMO-3(1-92) sequence to generate SUMO-3(1-87)-AvaDnaE intein was accomplished by Q5 mutagenesis with the following primers.

Primer	DNA Sequence (5'- to -3')
SUMO-3(1-87) Forward Primer	TGCCTGAGCTATGATACCGAAGTGCTGACCG
SUMO-3(1-87) Reverse Primer	CTGGAACACGTCGATGGTGTCTCGTCCTCC
SUMO-3(1-92)C47S Forward Primer <sup>1</sup>	AGCAAGCTGATGAAGGCCTACTCTGAGAGGCAGGGCTTGTCAATG
SUMO-3(1-92)C47S Reverse Primer <sup>1</sup>	CATTGACAAGCCCTGCCTCTCAGAGTAGGCCTTCATCAGCTTGCT

The Q5 mutagenesis products were transformed into chemically competent *E. coli* DH5 $\alpha$  cells. A single colony was isolated and grown at 37 °C overnight in 5 mL LB supplemented with Ampicillin (100  $\mu$ g/mL). Plasmids were isolated using a Miniprep kit (QIAGEN) and sequenced to confirm the desired gene product (Genewiz). Only sequenced plasmids with the desired truncant were used to transform chemically competent *E. coli* BL21(DE3) cells for subsequent protein production.

## Protein overexpression and purification

Overexpression of both cysteineless SUMO-3(2-87)-AvaDnaE and SUMO-3(2-92)-AvaDnaE proteins in *E. coli* BL21(DE3) cells was achieved by growth in LB medium supplemented with Ampicillin (100  $\mu$ g/mL) at 37 °C with continuous shaking at 200 rpm. *We note that Met1 in SUMO-3 is completely processed by endogenous methionine endopeptidases in E. coli leaving Ser2 as the N-terminal amino acid.* When cultures reached an OD<sub>600</sub> ~0.35 they were cooled to 16 °C and protein overexpression induced at OD<sub>600</sub> ~0.4 by the addition of 300  $\mu$ M Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG), and cells were grown for a further 16 h at 16 °C. After 16 h, the cells were harvested by centrifugation at 6,000  $\times$ g for 15 min at 4 °C, affording ~8 g of cells from 2 L of culture. The cell pellet was resuspended in 16 mL of Lysis Buffer containing 50 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 8.0, 300 mM NaCl, 5 mM Imidazole and lysed by sonication. The lysate was centrifuged at 20,000  $\times$ g for 30 min at 4 °C. The lysate supernatant was filtered through a 0.45  $\mu$ m membrane then bound to 5 mL Ni-NTA resin, pre-equilibrated in Lysis Buffer, for 1 h at 4 °C. The resin was then drained and washed with 20 column volumes (CVs) of Lysis Buffer followed by 20 CVs of Lysis Buffer with 20 mM Imidazole and then 5 CVs of Lysis Buffer with 50 mM Imidazole. Finally, bound proteins were eluted with 5 successive washes with 1 CV of Lysis Buffer containing 250 mM Imidazole. The eluted fractions were checked for the presence of protein by 15% SDS-PAGE. Fractions containing the desired protein were combined and dialyzed twice against 1L of 100 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, 150 mM NaCl, 1 mM EDTA, 1 mM MESNa at 4 °C for 3 h. To the dialyzed eluate the sodium salt of 2-mercaptoethanesulfonic acid (MESNa) was added to a final concentration of 500 mM at pH 7.2, and the solution was gently shaken overnight at 30 °C. The cysteineless SUMO-3(2-87)- $\alpha$ -thioester was isolated from the overnight thiolysis mixture by lyophilization of the solution followed by resuspension in 6 M Gn-HCl, and purification by C18 preparative RP-HPLC using a gradient of 30-70% B over 60 min. This yielded 28.0 mg SUMO-3(2-87)- $\alpha$ -thioester from 4 L of cell culture. Similarly, the C-terminal  $\alpha$ -carboxylic acid full-length SUMO-3(2-92)-CO<sub>2</sub>H, was isolated following MESNa-thiolysis of the SUMO-3(2-92)-AvaDnaE

intein fusion by increasing the pH of the thiolysis reaction mixture from pH 7.2 to 8.0 with NaOH, followed by overnight nutation at 25 °C. Following hydrolysis, the solution was lyophilized and SUMO-3(2-92)-CO<sub>2</sub>H purified by C18 preparative RP-HPLC employing a gradient of 30-60% B over 60 min.

## Solid Phase Peptide Synthesis

### *H<sub>2</sub>N-CQ(N-(O-2-propyne))TGG-CO<sub>2</sub>H synthesis*

Fmoc-Gly Wang resin was pre-swollen in dry DMF for 5 min with microwave heating to 75 °C. Fmoc- deprotections were undertaken with 20% (v/v) Piperazine in DMF for 65 s at 75 °C. All peptide deprotections conducted after incorporation of the alkyne handle employed 5% (v/v) Piperazine and 0.1 M HOBt in DMF for 10 min at 25 °C. Fmoc-Gly-OH, Fmoc-Thr(tBu)-OH, and Fmoc-Glu(OAll)-OH were each coupled onto the solid-phase by using 5.25 eq of amino acid, 5.1 eq *N,N*-diisopropylcarbodiimide (DIC), and 5.1 eq ethyl cyano(hydroxyimino)acetate (Oxyma) for 2 min at 75 °C. Boc-Cys(Trt)-OH was coupled at 50 °C as per manufacturer recommendations. Deprotection of the Glu(OAll) ester was undertaken as previously reported.<sup>2</sup> Briefly, 20 mM Pd(PPh<sub>3</sub>)<sub>4</sub> in DCM (4 mL per 0.1 mmol resin) and 0.4 M Phenylsilane in DCM (2 mL per 0.1 mmol resin) were added to the peptidyl resin and the solution was agitated by bubbling nitrogen for 1 h. Following allyl ester deprotection, the resin was extensively washed with dry DMF then 0.4 M HATU in DMF (2 mL per 0.1 mmol resin) and DIEA (200 μL per 0.1 mmol resin) were added to the resin and allowed to react for 5 min. To the activated Glu side-chain was then added a solution of 0.6 M O-2-propynylhydroxylamine in DMF (2 mL per 0.1 mmol resin) and the solution was agitated with nitrogen bubbling for 40 min. After 40 min of the coupling reaction, the resin was washed extensively with DMF, and side chain functionalization repeated once more to generate the clickable Gln (CliQ).

### *Azidoacetyl-FLAG synthesis*

2-Chlorotrityl resin (1.5 meq/g resin-loading, 167 mg, 0.25 mmol) was pre-swollen in dry DCM at 25 °C for 1 h. The resin was drained and reacted with Fmoc-Lys(Boc)-OH (312 mg, 4 eq) and 2,4,6-Collidine (750 μL, 5.6 mmol) in 20 mL of dry DCM. The resin was gently shaken overnight at 25 °C then drained, washed, and capped for 1 h by the addition of 0.5 mL methanol in 9 mL dry DCM with 1 mL DIEA. The remaining amino acids in the sequence, namely H<sub>2</sub>N-DYKDDDD, were coupled to the Lys-loaded resin on a Liberty Blue peptide synthesizer using the coupling conditions described above. Azidoacetylation of the N-terminal Asp was undertaken with azidoacetic acid (4 eq, prepared as previously described<sup>3</sup>), DIC (4 eq), Oxyma (4 eq), and DIEA (4 eq) for 1 h at 25 °C. The resin was washed with DMF, then DCM, and the fully protected peptide was cleaved with a 1:2:7 (v/v) mixture of acetic acid/trifluoroethanol (TFE)/DCM (10 mL per 1.0 g resin). After 1 h of cleavage, the resin was drained and washed twice with 1:5 (v/v) TFE/DCM (10 mL per 1.0 g resin). The combined organics were washed with a solution of saturated sodium bicarbonate, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. This yielded the fully side-chain protected FLAG peptide with an N-terminal azide, which was directly applied to click reactions without further purification.

### *Copper Azide Alkyne Cycloaddition*

The resin-bound N $\alpha$ -Boc-C(CliQ)TGG peptide (50 μmol) was allowed to swell in a minimal volume of dry DCM that was sufficient to cover the resin. The resin was then agitated for 5 min in a solution of Cu(I)Cl (25 mg, 0.25 mmol) and Ascorbic acid (44 mg, 0.28 mmol) in nitrogen sparged 20% (v/v) piperidine in DMF (1 mL) under an atmosphere of nitrogen.<sup>4</sup> The FLAG-peptidyl azide

(0.1 mmol) was taken up in nitrogen sparged 20% (v/v) piperidine in DMF (500  $\mu$ L) and transferred to the resin container. The peptidyl azide vial was washed with additional nitrogen sparged 20% (v/v) piperidine in DMF (500  $\mu$ l), and the wash was also added to the resin. The click reaction mixture was allowed to proceed for 24 h under argon. After reaction, the resin was drained and washed extensively with 20% (v/v) piperidine in DMF supplemented with 20 mM Ascorbic acid, then washed successively with dry DMF, dry DCM, and finally MeOH. The final click peptide product was cleaved from the resin and side-chain deprotected with Reagent K (82.5% TFA, 5% Phenol, 5% H<sub>2</sub>O, 5% Thioanisole, 2.5% 1,2-Ethanedithiol (v/v)) prior to purification by RP-HPLC. The desired peptide **2** was purified by C18 semi-preparative HPLC on a gradient of 0-20% B over 45 min to yield 10 mg (6%) pure peptide. Peptide **3** was purified by C18 semi-preparative HPLC on a gradient of 5-40% B over 45 min to yield 15.8 mg (33% yield) pure peptide.

### Synthesis of H3(1-28) peptide

2-Chlorotrityl chloride resin was swollen in 1:1 (v/v) dry DCM:DMF for 30 min at 25 °C. The swollen resin was washed with DMF then shaken in 10 mL 10% (v/v) hydrazine in DMF for 30 min at 30 °C. This was repeated a second time. Unreacted sites on the resin were capped with 10% (v/v) MeOH in DMF for 10 min at 30 °C twice. The 28<sup>th</sup> amino acid, Fmoc-Ser(OtBu)-OH (5 eq), was coupled to the freshly prepared hydrazide resin with HCTU (4.9 eq), HOBt (4.9 eq), and DIEA (10 eq) in DMF for 2.5 h at 25 °C. Any unreacted hydrazide was capped by reaction with acetic anhydride (20 eq) and DIEA (40 eq) in DMF for 45 min at 25 °C. All subsequent histone H3 tail residues (1-27) were coupled on a Liberty Blue microwave-assisted peptide synthesizer while substituting the Glu  $\gamma$ -allyl ester for Gln19. Deprotection of the Glu  $\gamma$ -allyl ester was undertaken as previously reported.<sup>3</sup> Briefly, 20 mM Pd(PPh<sub>3</sub>)<sub>4</sub> in DCM (4 mL per 0.1 mmol resin) and 0.4 M Phenylsilane in DCM (2 mL per 0.1 mmol resin) were added to the peptidyl resin and the solution was agitated by bubbling nitrogen for 1 h. Following allyl ester deprotection, the resin was extensively washed with dry DMF then 0.4 M HATU in DMF (2 mL per 0.1 mmol resin) and DIEA (200  $\mu$ L per 0.1 mmol resin) were added to the resin and allowed to react for 5 min. To the activated Glu side-chain was then added a solution of 0.6 M O-2-propynylhydroxylamine in DMF (2 mL per 0.1 mmol resin) and the solution was agitated with nitrogen bubbling for 40 min. After 40 min of the coupling reaction, the resin was washed extensively with DMF, and side chain functionalization repeated once more to generate the clickable Gln (CliQ) on the solid-phase.

The resin-bound H3 peptide (50  $\mu$ mol, based on Fmoc-Ser loading) was allowed to swell in a minimal volume of dry DCM to fully cover the resin. The resin was then agitated for 5 min in a solution of Cu(I)Cl (25 mg, 0.25 mmol) and Ascorbic acid (44 mg, 0.28 mmol) in nitrogen sparged 20% (v/v) piperidine in DMF (1 mL) under an atmosphere of nitrogen. The azide-PEG<sub>3</sub>-Biotin (0.1 mmol) was taken up in nitrogen sparged 20% (v/v) piperidine in DMF (500  $\mu$ L) and transferred to the resin container. The azide vial was washed with additional nitrogen sparged 20% (v/v) piperidine in DMF (500  $\mu$ l), and the wash was also added to the resin. The click reaction mixture was allowed to proceed for 24 h under argon. After reaction, the resin was drained and washed extensively with 20% (v/v) piperidine in DMF supplemented with 20 mM Ascorbic acid, then washed successively with dry DMF, dry DCM, and finally MeOH. The final click peptide product was cleaved from the resin and side-chain deprotected with Reagent K prior to purification by RP-HPLC. The desired H3(1-28)Q19(biotin) peptide was purified by C18 semi-preparative HPLC on a gradient of 0-30% B over 45 min to yield 6 mg of pure peptide (3% overall yield).

### Expressed Protein Ligation

Cysteineless SUMO-3(2-87)-MESNa  $\alpha$ -thioester (1 mM) was reacted with either peptide **2** or **3** (1 mM) in a Ligation Buffer consisting of 6 M Gn-HCl, 150 mM NaCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM mercaptophenylacetic acid (MPAA), 10 mM EDTA, and 10 mM TCEP, pH 7.5 for 12 h at 25 °C. After 12 h the reaction was reduced for a further 30 min by the addition of a solution containing 250 mM TCEP, 500 mM Tris, pH 7.0 (1/4<sup>th</sup> of total reaction volume). The reduced products were acidified to pH 3.0 with phosphoric acid, and extracted thrice with 2 reaction volumes of diethyl ether to remove MPAA. After briefly evaporating the residual ether under a flow of nitrogen, the ligation mixture was dialyzed against 1 L of Tris-buffered saline, pH 7.5, supplemented with 1 mM TCEP at 4 °C. Reactions were then either directly subjected to affinity purification, or Cys S-alkylated with 2-iodoacetamide prior to affinity purification. Cys S-alkylation was accomplished by the addition of one volume of reducing buffer consisting of 100 mM TCEP, 1 M Tris, pH 8, and incubation for 30 min. Following this short period of reduction, 200 mM 2-iodoacetamide was added and S-alkylation allowed to proceed for 1 h. The alkylation reaction was quenched with 500 mM DTT for 30 min and the products first dialyzed against 2 L water and then against 2L of Tris-buffered saline, pH 7.0.

## Affinity Purification

### *$\alpha$ -FLAG purification*

$\alpha$ -FLAG resin (Anti-DYKDDDDK G1 Affinity Resin from Genscript, 1 mL resin per 2 mg SUMO- $\alpha$ -thioester) derived from a 4% cross-linked agarose matrix was equilibrated with Tris-buffered saline, pH 7.5. The dialyzed ligation reaction was bound to resin for 30 min at 4 °C with gentle agitation on a platform shaker. The resin was drained and the flow-through collected along with the first 2 mL of column washes. The column was washed a total of twelve times with single column volumes of Tris-buffered saline, pH 7.5. The FLAG-tagged ligation product was eluted over 5 washes with 3xFLAG peptide (one column volume per wash with 200  $\mu$ g 3xFLAG peptide). Protein containing eluted fractions were identified by 15% SDS-PAGE then combined and concentrated. The eluted resin was washed a further 5 times with 5 column volumes of Tris-buffered saline, pH 7.5. The concentrated eluate was purified to remove the 3xFLAG peptide and any residual **2** using C18 analytical HPLC on a gradient of 0-73% B over 30 min. The Cys-alkylated product **6** was obtained in 1.1 mg (24% yield). The non-alkylated product **6** was obtained in 1.4 mg (61% yield).

### *Anti-FLAG affinity resin reuse and regeneration*

The resin can be reused several times to purify the same protein by washing extensively (at least 10 column volumes) with Tris-buffered saline. To purify different proteins, the resin can be regenerated by washing with 5 column volumes of 0.1 M Tris HCl, 0.5 M NaCl, pH 8.0. followed by 5 column volumes of 0.1 M NaOAc, 0.5 M NaCl, pH 4.0. Finally, the resin is re-equilibrated in 10 column volumes of Tris-buffered saline.

### *Streptavidin purification*

Streptavidin-agarose resin derived from conjugating 1.2 mg/mL Streptavidin to a 4% cross-linked agarose matrix (EMD Millipore, 800  $\mu$ l per 2 mg SUMO- $\alpha$ -thioester) was equilibrated with 10 column volumes of Tris-buffered saline, pH 7.5. The dialyzed ligation reaction was passed over the equilibrated resin 5 times to bind biotinylated species. The resin was washed with 10 CVs of Tris-buffered saline, pH 7.5, followed by 20 CVs of water. The product was eluted with 20 CVs of 90% (v/v) DMF in H<sub>2</sub>O, followed by 20 CVs of water. The eluted protein was flash frozen and

concentrated by lyophilization. The Cys-alkylated product **7** was obtained in 1.6 mg (73% yield). The non-alkylated product **7** was obtained in 2.6 mg (85% yield).

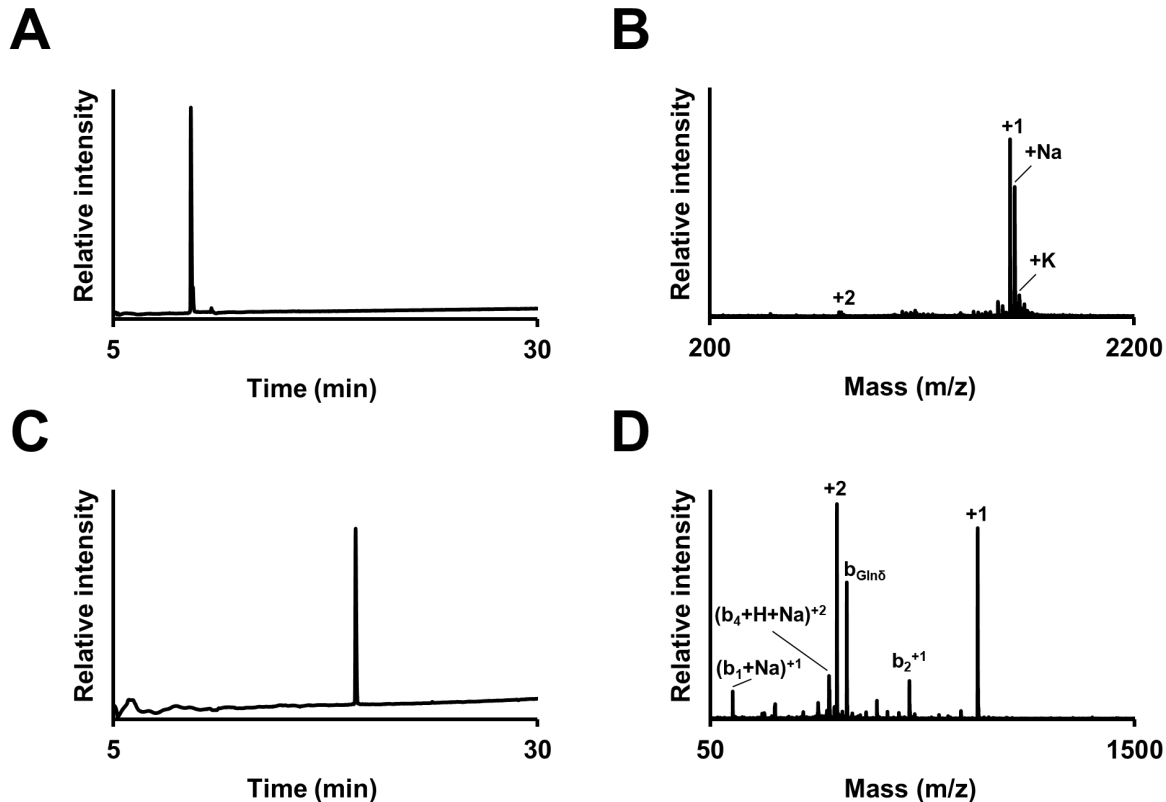
### **N-O bond reduction**

Zinc dust was activated with 5% aqueous HCl with manual agitation until bubbling ceased. The activated Zn was successively washed with water, ethanol, and diethyl ether, before drying *in vacuo*. The protein to be reduced was dissolved in degassed 6 M Gn-HCl, pH 3.0 (1 mL buffer per 1 mg protein) and 200 mg activated zinc added per 1 mg protein. For peptide substrates **2**, **3** and the H3(1-28) peptide, only 20 mg activated zinc was required per 1 mg peptide. The slurry was briefly sonicated then stored under an atmosphere of argon for up to a maximum of 48 h at 37 ° time-point. The solid Zn was pelleted by centrifugation and the supernatant aspirated. The pelleted Zinc was washed 5 times with 6 M Gn-HCl using 1/3<sup>rd</sup> of the initial reduction reaction volume per wash. The supernatant and washes were combined and dialyzed against 1 L buffer containing 100 mM Tris, 150 mM NaCl, 5 mM EDTA, and 1 mM TCEP at pH 7. This was followed by dialysis against 1 L of water. The reduced product in water was concentrated *in vacuo* and purified by C18 analytical RP-HPLC using a gradient of 0-73% B over 30 min. This yielded 0.7 mg (56% yield) of unalkylated **8** from unalkylated **7**, and 0.4 mg (56% yield) of alkylated **8** from alkylated **7**.

### **In vitro sumoylation assays**

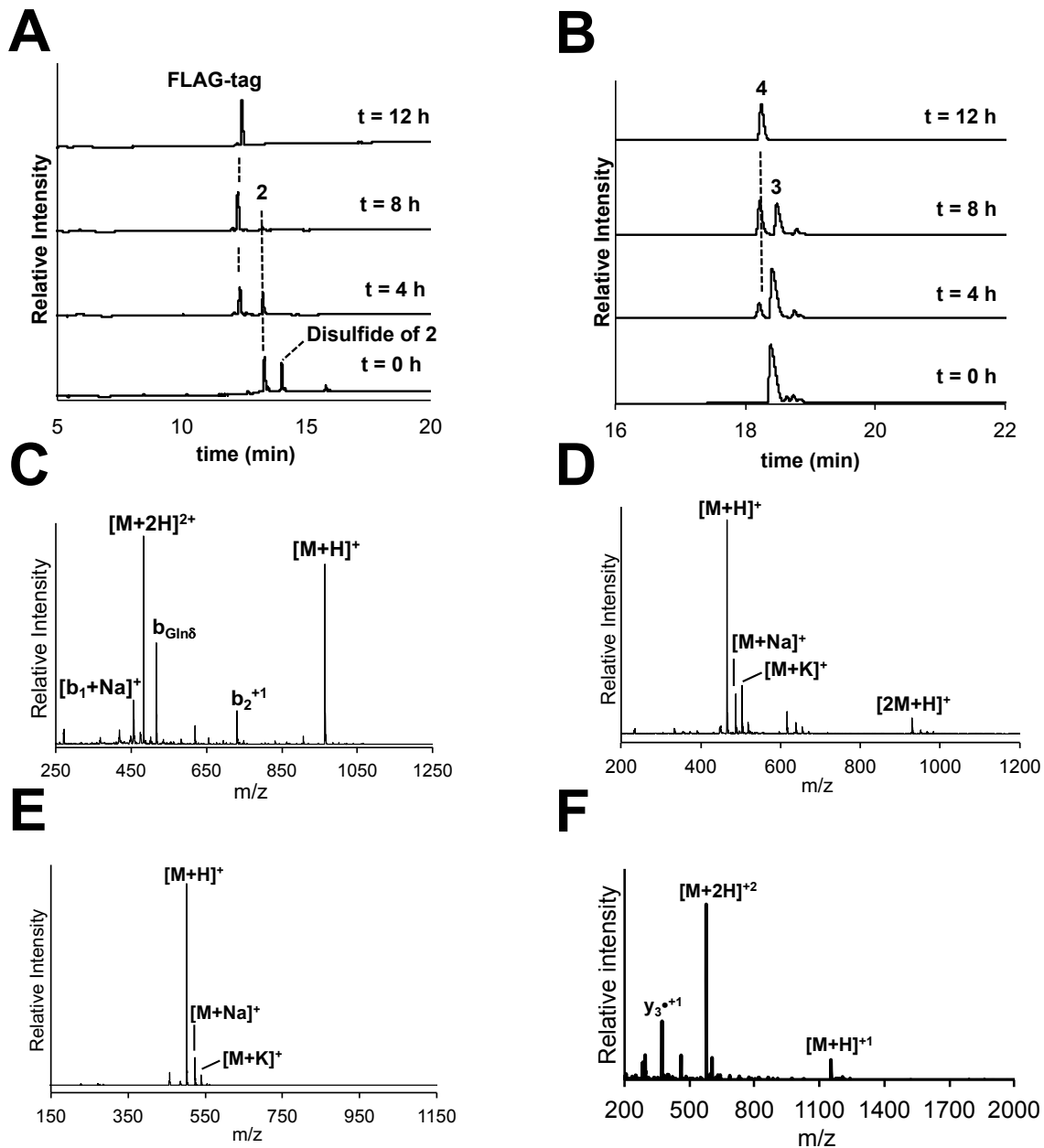
In vitro sumoylation was performed following a reported procedure.<sup>5</sup> Briefly, either 3 μM cysteineless SUMO-3(2-92) or 3 μM of the N-O bond cleaved product **8** were mixed with 0.45 μM Uba2, 1.5 μM Ubc9, and 6 μM SP-100 in sumoylation buffer consisting of 20 mM HEPES-KOH, pH 7.3, 110 mM KOAc, 2 mM Mg(OAc)<sub>2</sub>, 1 mM EGTA, 1 mM DTT, 0.05 % (v/v) Tween-20, and 1x cOmplete protease inhibitor cocktail (Roche). Sumoylation assays were conducted for 12 h at 30 °C prior to quenching with 6xLaemmli buffer. Assay products were visualized by 15% SDS-PAGE stained with coomassie brilliant blue.

## Supplemental Figures



**Figure S1. Characterization of peptides 2 and 3.** **A.** C18 Analytical RP-HPLC chromatogram of purified **2**, 30 min, 0-73% B gradient. **B.** ESI-MS of purified **2**. Obsd.  $[M+H]^{+1}$  1,614.65  $\pm$  0.60 Da. Calcd. 1,614.57 Da. **C.** C18 Analytical RP-HPLC chromatogram of purified **3**, 30 min, 0-73% B gradient. **D.** ESI-MS of purified **3**. Obsd.  $[M+H]^{+1}$  963.18  $\pm$  0.56 Da. Calcd. 963.09 Da. For  $[b_1+Na]^{+1}$  fragment, Obsd. 126.695 Da. Calcd. 127.137 Da. For  $[b_4+H+Na]^{+2}$  fragment, Obsd. 455.861 Da. Calcd. 456.016 Da. For  $[b_{Gln\delta}+H]^{+1}$  fragment, Obsd. 516.524 Da. Calcd. 515.630 Da. For  $[b_2+H]^{+1}$  fragment, Obsd. 730.551 Da. Calcd. 731.885 Da. All reported masses are for the isotopic average ( $M_{av}$ ). Fragments arise from the parent ion of **3** only during MS analysis.



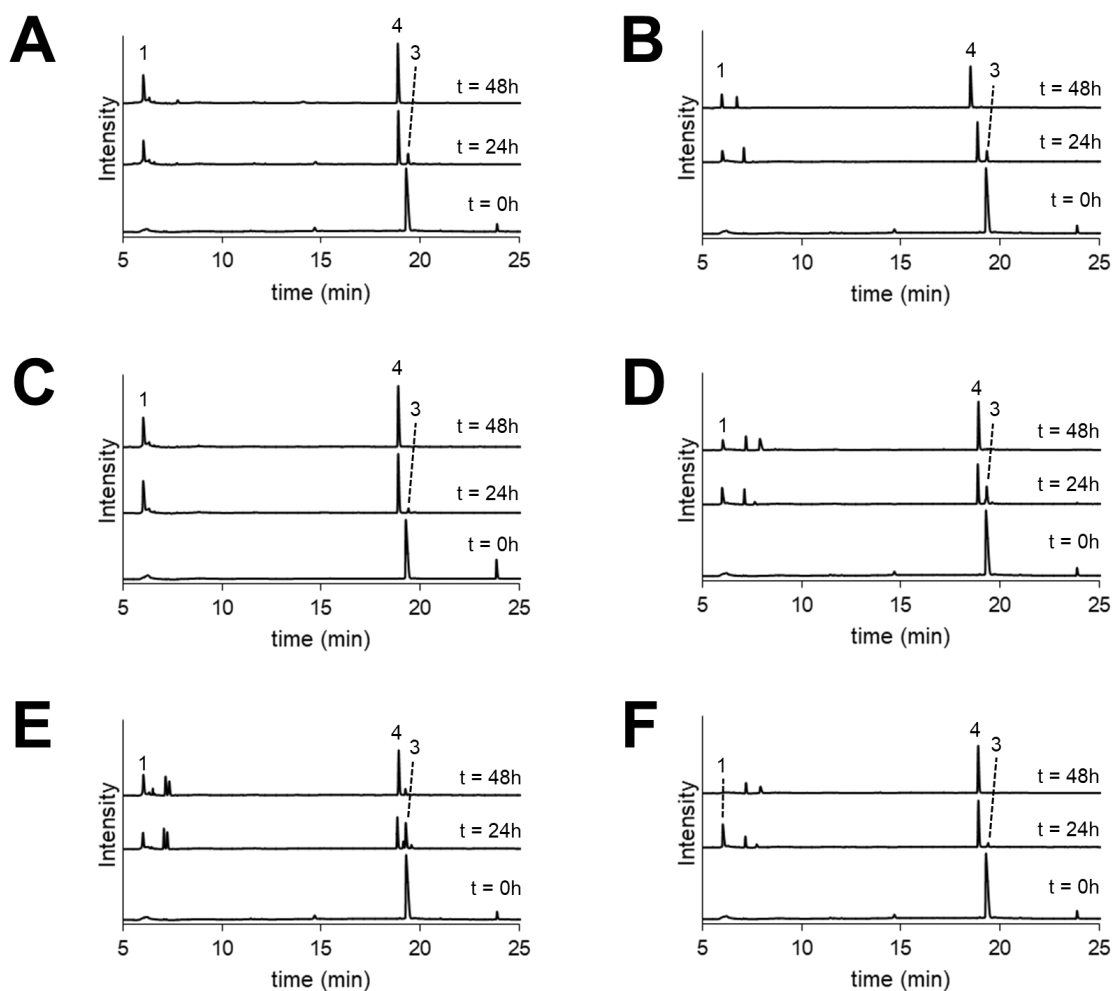


**Figure S2. Characterization of N-O bond cleavage in peptides 2 and 3.** **A.** Time-course of N-O bond reduction for peptide **2** in 6 M Gn-HCl, pH 3 followed by C18 analytical RP-HPLC, 30 min, 0-35% B gradient. **B.** Time-course of N-O bond reduction for peptide **3** in 6 M Gn-HCl, pH 3 followed by C18 analytical RP-HPLC, 30 min, 0-35% B gradient. **C.** ESI-MS of **3**. Calcd.  $[M+H]^+$  964.1 Da, found  $963.2 \pm 0.6$  Da. Fragment b-ions arising from the parent ion during MS analysis are indicated. **D.** ESI-MS of peptide **1**. Calcd.  $[M+H]^+$  464.5 Da, found 464.5 Da. **E.** ESI-MS of biotin derivative **4**. Calcd. 500.6 Da, found 500.48 Da. **F.** ESI-MS of cleaved FLAG-tag.  $[M+H]^{+1}$  Calcd. 1,152.44 Da, Obsd. 1,152.45 Da.  $[M+2H]^{+2}$  Calcd. 576.73, Obsd. 576.37 Da.  $y_3^{+1}$  Calcd. 375.15, Obsd. 373.30. All reported masses are for the isotopic average ( $M_{av}$ ). Fragments arise from the parent ion of **3** and FLAG-tag only during MS analysis.

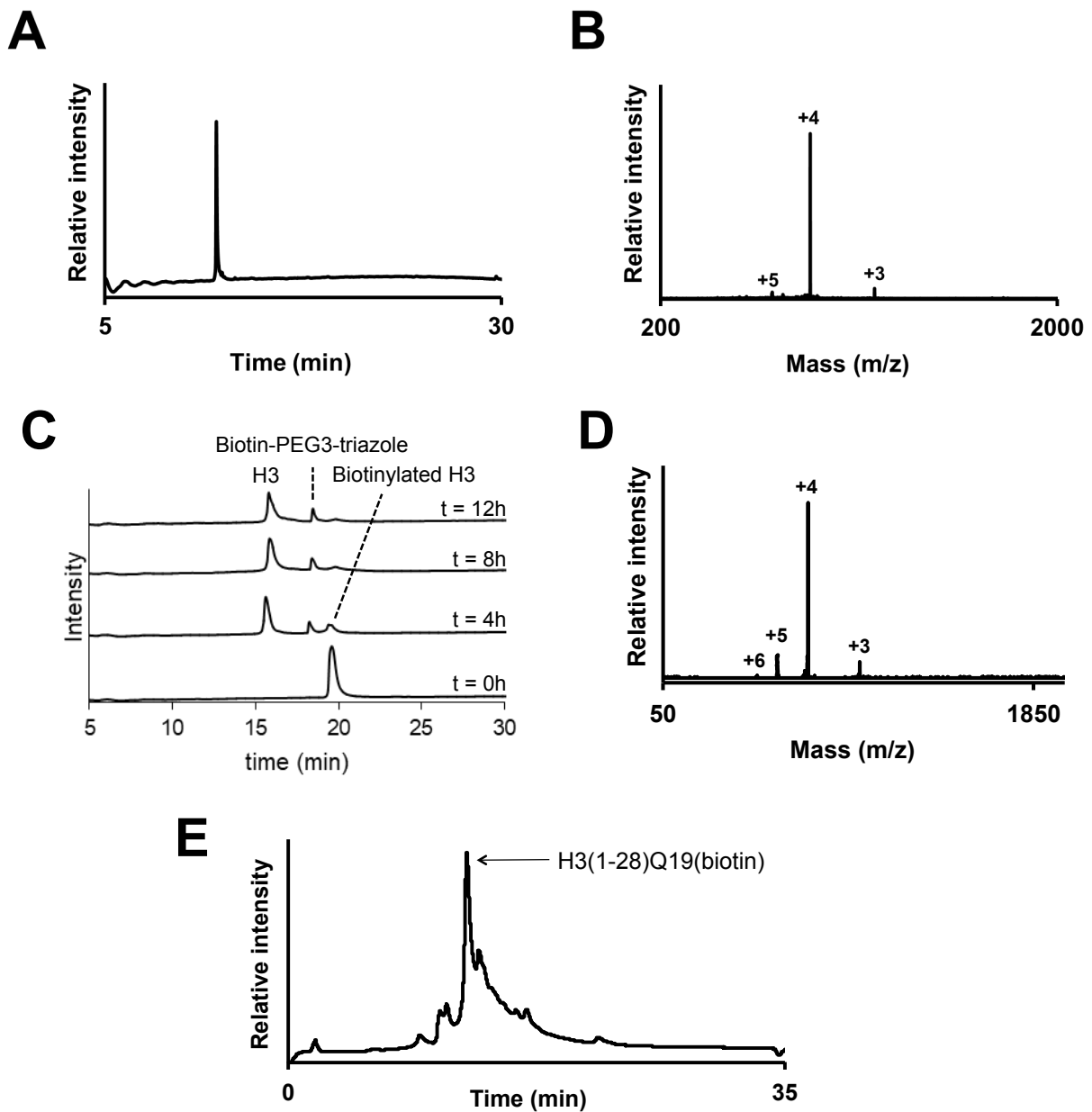
**Table S1. Reduction of the N-O bond in 3.** Effect of pH and denaturant on the extent of Zn-mediated N-O bond reduction of **3**.

<b>Buffer</b>	<b>Time (h)</b>	<b>% Reduced*</b>
39.7 mM citric acid, 20.6 mM sodium phosphate, pH 3	24	90%
	48	99%
18.4 mM citric acid, 63.1 mM sodium phosphate, pH 6	24	84%
	48	99%
6.5 mM citric acid, 87.0 mM sodium phosphate, pH 7.2	24	97%
	48	100%
6 M guanidine hydrochloride, pH 3	24	91%
	48	100%
6 M guanidine hydrochloride, 100 mM sodium phosphate, pH 6	24	93%
	48	99%
6 M guanidine hydrochloride, 100 mM sodium phosphate, pH 7.2	24	74%
	48	95%

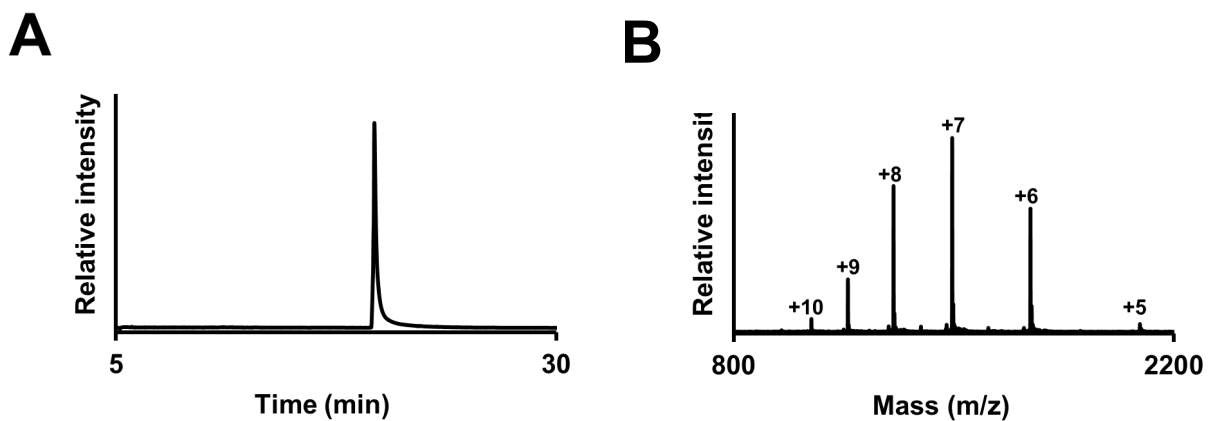
\*Percentage reduced was quantified by area under the analytical RP-HPLC peak for **3**.



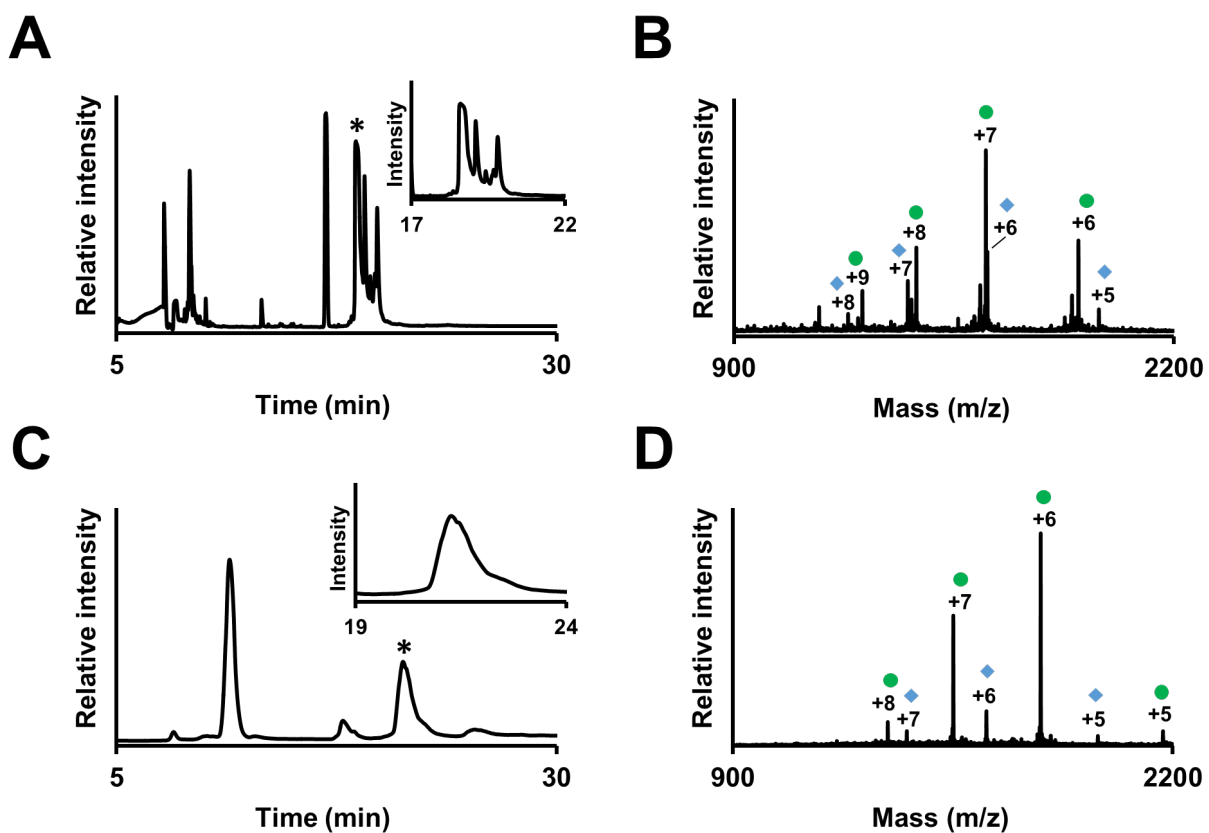
**Figure S3. Comparison of N-O bond reduction efficiency with varying pH and buffer conditions.** **A.** Time-course of **3** N-O bond reduction in 6 M Gn-HCl, pH 3 followed by C18 analytical RP-HPLC, 30 min, 0-35% B gradient. **B.** Time-course of **3** N-O bond reduction in 39.7 mM citric acid, 20.6 mM sodium phosphate, pH 3 followed by C18 analytical RP-HPLC, 30 min, 0-35% B gradient. **C.** Time-course of **3** N-O bond reduction in 6 M Gn-HCl, 100 mM sodium phosphate, pH 6 followed by C18 analytical RP-HPLC, 30 min, 0-35% B gradient. **D.** Time-course of **3** N-O bond reduction in 18.4 mM citric acid, 63.1 mM sodium phosphate, pH 6 followed by C18 analytical RP-HPLC, 30 min, 0-35% B gradient. **E.** Time-course of **3** N-O bond reduction in 6 M Gn-HCl, 100 mM sodium phosphate, pH 7.2 followed by C18 analytical RP-HPLC, 30 min, 0-35% B gradient. **F.** Time-course of **3** N-O bond reduction in 6.5 mM citric acid, 87.0 mM sodium phosphate, pH 7.2 followed by C18 analytical RP-HPLC, 30 min, 0-35% B gradient.



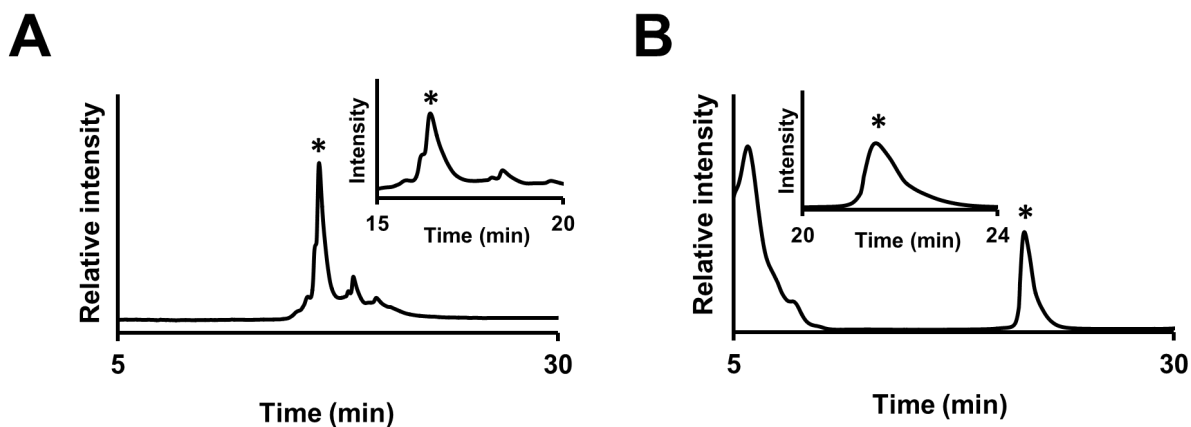
**Figure S4. Characterization of H3(1-28) biotinylated peptide and time-course of N-O bond cleavage.** **A.** C18 Analytical RP-HPLC chromatogram of purified H3(1-28)Q19(biotin) peptide, 30 min, 0-73% B gradient. **B.** ESI-MS of purified H3(1-28)Q19(biotin) peptide. Obsd.  $[M+H]^+$  3,512.32  $\pm$  3.87 Da. Calcd. 3,510.15 Da. **C.** Time-course of H3(1-28)Q19(biotin) N-O bond reduction in 6 M Gn-HCl, pH 6 followed by C18 analytical RP-HPLC, 30 min, 0-35% B gradient. **D.** ESI-MS of purified N-O bond cleaved H3(1-28) peptide. Obsd.  $[M+H]^+$  3,015.21  $\pm$  4.19 Da. Calcd. 3,011.55 Da. **E.** C18 Analytical RP-HPLC chromatogram of crude H3(1-28)Q19(biotin) peptide after SPPS and cleavage, 30 min, 0-73% B gradient.



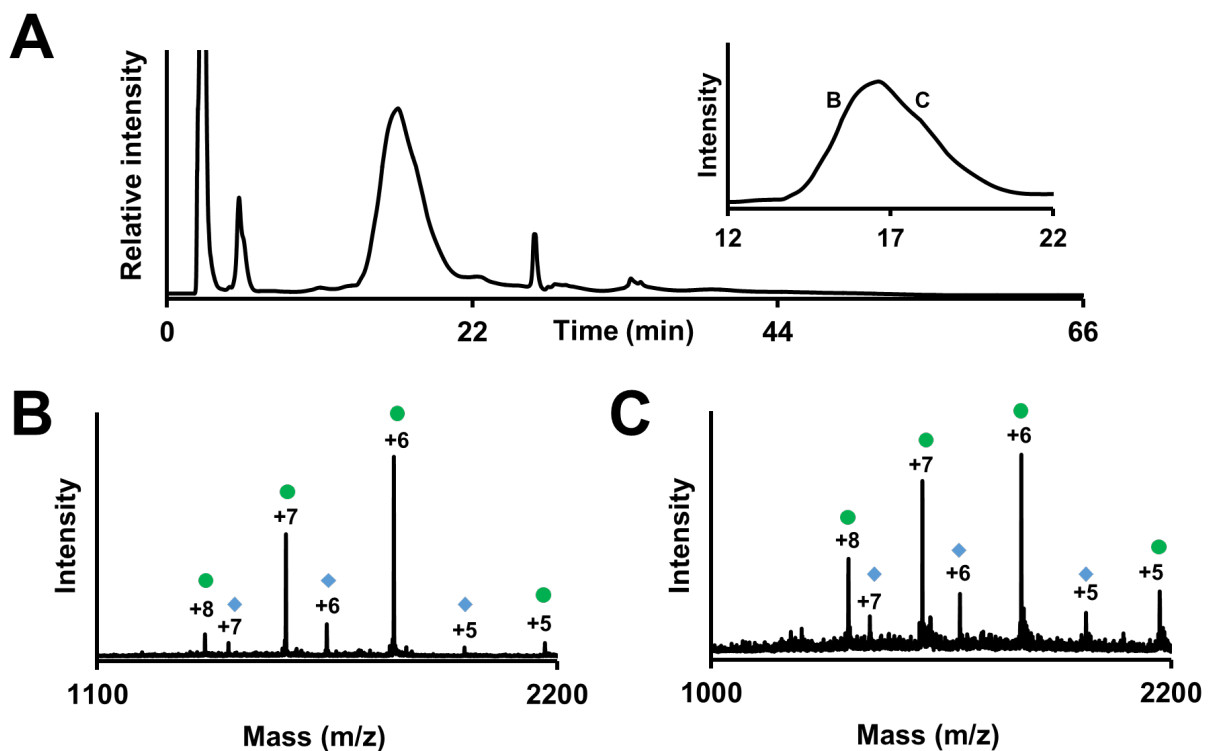
**Figure S5. Characterization of 5.** **A.** C18 Analytical RP-HPLC chromatogram of purified **5**, 30 min, 0-73% B gradient. **B.** ESI-MS of purified **5**. Obsd.  $[M+H]^+$   $10,462.46 \pm 2.22$  Da. Calcd. 10,461.7 Da.



**Figure S6. Expressed protein ligation of 5 with either 2 or 3.** **A.** C18 analytical RP-HPLC chromatogram of crude expressed protein ligation products of **2** with **5**, 30 min, 0-73% B gradient. **B.** ESI-MS of the indicated peak marked with an asterisk in **A**. Green circles indicate the desired product **6**. Obsd.  $[M+H]^+ 11,503.60.24 \pm 1.98$  Da. Calcd. 11,503.68 Da. Blue diamonds indicate the by-product SUMO-3(2-87)-CO<sub>2</sub>H. Obsd. for  $[M-18+H]^+ 9,888.44 \pm 1.17$  Da. Calcd. 9,887.95 Da. **C.** C18 analytical RP-HPLC chromatogram of expressed protein ligation products of **3** with **5**, 30 min, 0-73% B gradient. **D.** ESI-MS of the indicated peak marked with an asterisk in **C**. Green circles indicate the desired product **7**. Obsd.  $[M+H]^+ 10,852.42 \pm 1.47$  Da. Calcd. 10,851.04 Da. Blue diamonds indicate the by-product SUMO-3(2-87)-CO<sub>2</sub>H. Obsd. for  $[M-18+H]^+ 9,888.47 \pm 1.31$  Da. Calcd. 9,887.95 Da.

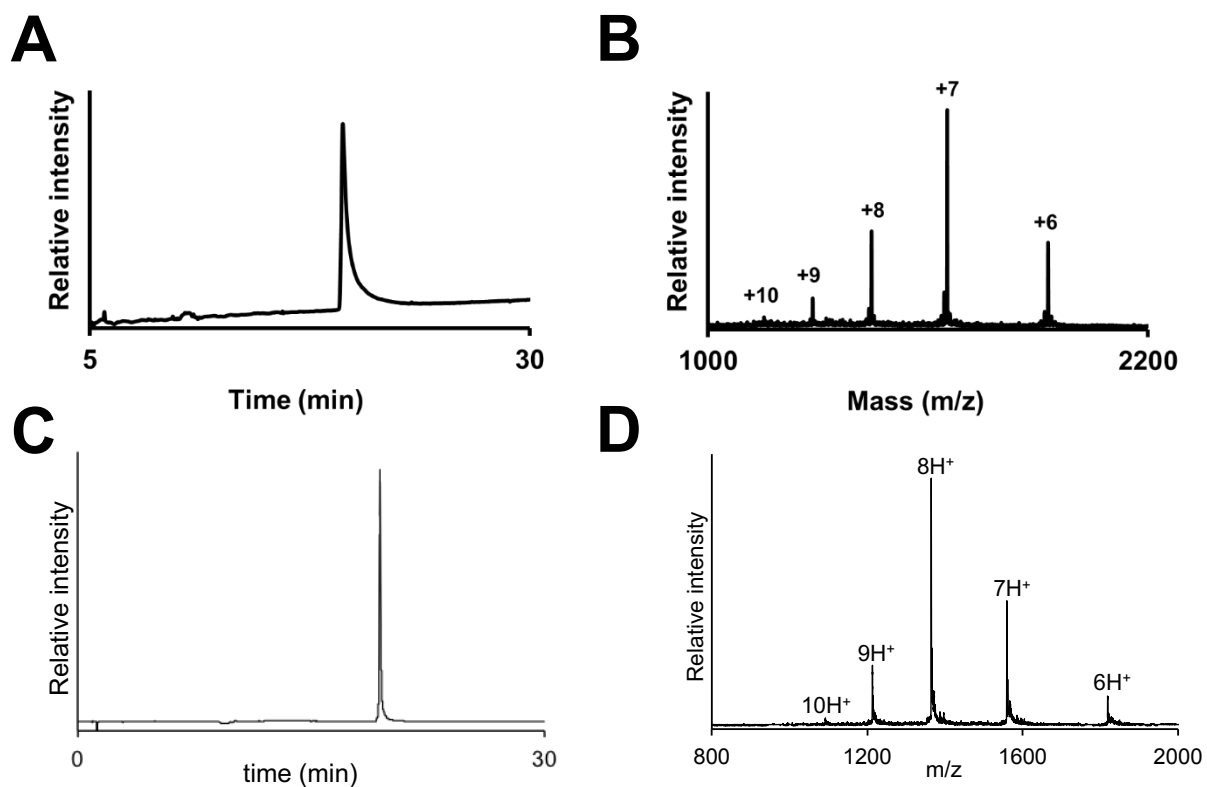


**Figure S7. Expressed protein ligation of 5 with either 2 or 3 followed by S-alkylation. A.** C18 analytical RP-HPLC chromatogram of expressed protein ligation products of **2** with **5**, followed by reduction and alkylation with 2-iodoacetamide. An asterisk indicates the peak containing ligation product and hydrolyzed SUMO-3(2-87)-CO<sub>2</sub>H. Gradient of 28-45% B over 30 min. **B.** C18 analytical RP-HPLC chromatogram of expressed protein ligation products of **3** with **5**, followed by reduction and alkylation with 2-iodoacetamide. An asterisk indicates the peak containing ligation product and hydrolyzed SUMO-3(2-87)-CO<sub>2</sub>H. Gradient of 0-73% B over 30 min.

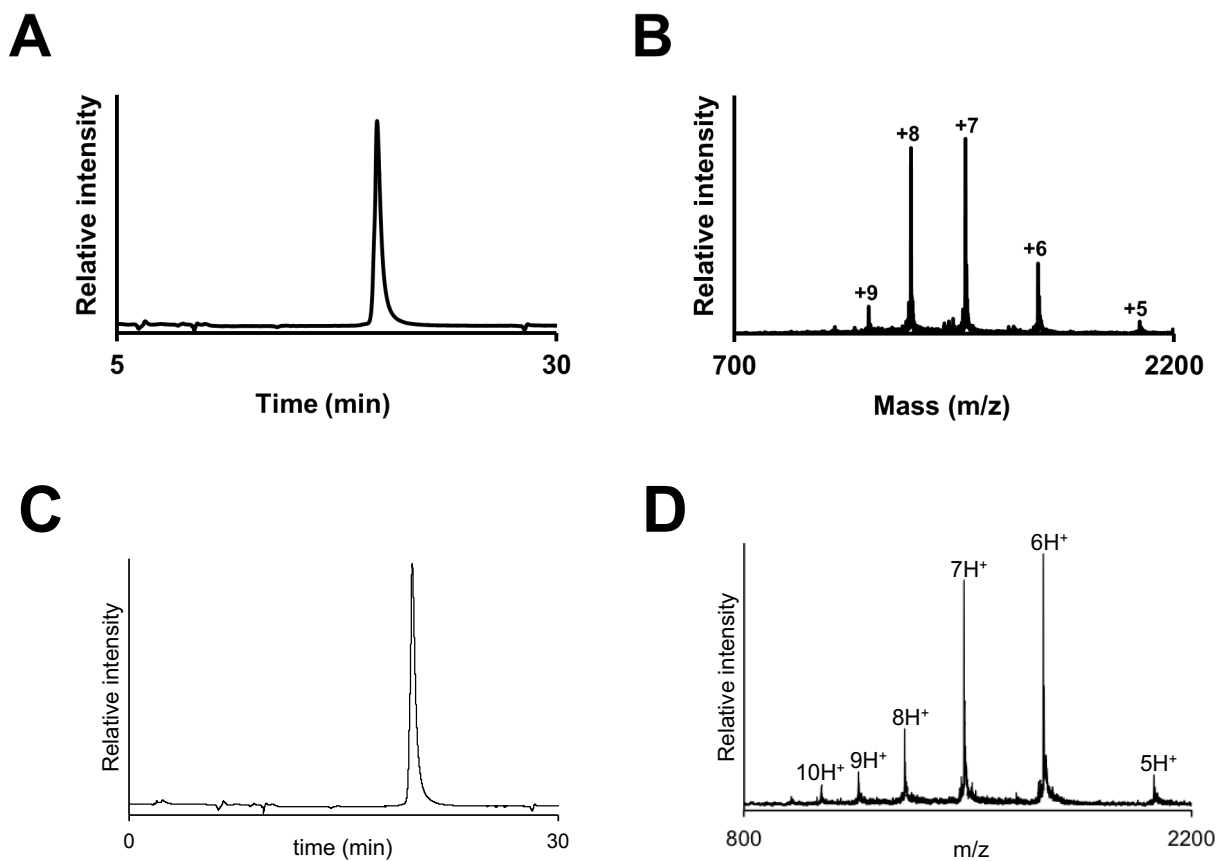


**Figure S8. Inseparable expressed protein ligation products of 5 and 3.** **A.** C18 analytical RP-HPLC chromatogram of expressed protein ligation of 5 with 3, 60 min, 30-50% B gradient. **B.** ESI-MS of fraction B from the leading edge of the 17 min peak in (A). Green circles indicate desired product 7. Obsd.  $[M+H]^{+1}$   $10,851.24 \pm 1.14$  Da. Calcd. 10,851.04 Da. Blue diamonds indicate the by-product SUMO-3(2-87)-CO<sub>2</sub>H. Obsd. for  $[M-18+H]^{+1}$   $9,888.44 \pm 1.17$  Da. Calcd. 9,887.95 Da. **C.** ESI-MS of fraction C from the trailing edge of the 17 minute peak in (A). Green circles indicate desired product 7. Obsd.  $[M+H]^{+1}$   $10,851.91 \pm 4.18$  Da. Calcd. 10,851.04 Da. Blue diamonds indicate the by-product SUMO-3(2-87)-CO<sub>2</sub>H. Obsd. for  $[M-18+H]^{+1}$   $9,888.88 \pm 2.77$  Da. Calcd. 9,887.95 Da.

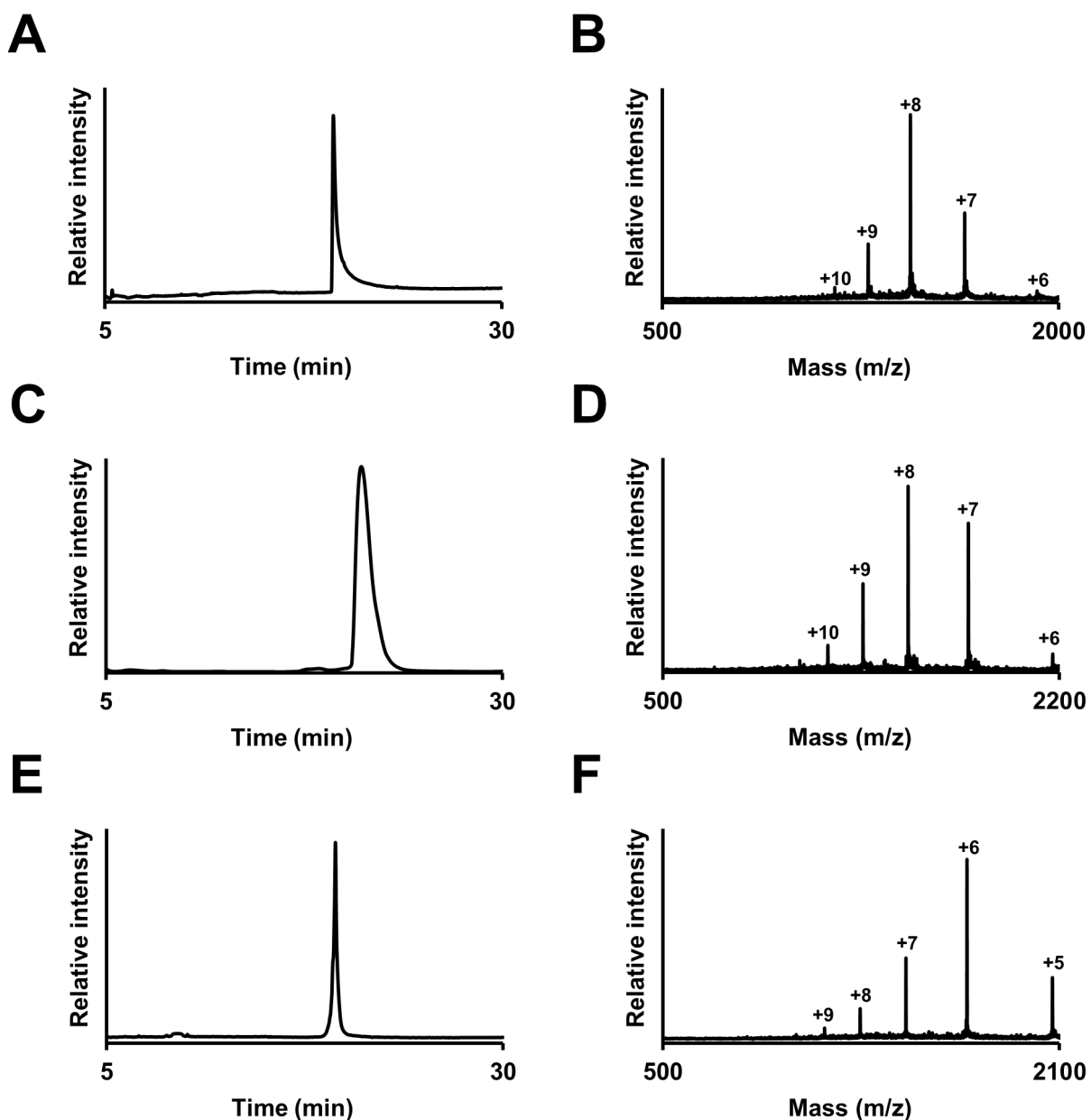




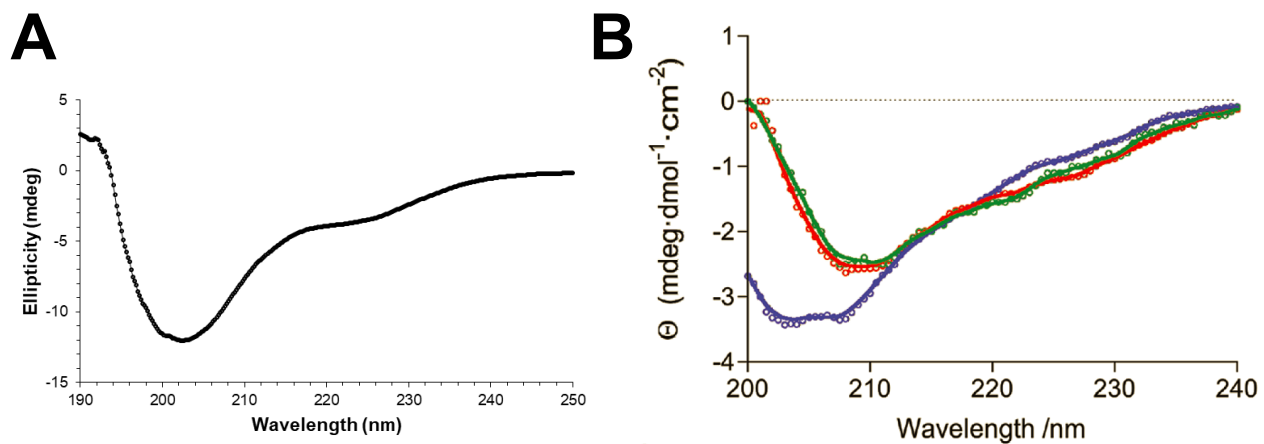
**Figure S9. Characterization of affinity-purified EPL products 6 and 7.** **A.** RP-HPLC chromatogram of purified **6**, 30 min, 0-73% B gradient. **B.** ESI-MS of **6**. Obsd.  $[M+H]^+$   $11,560.7 \pm 1.9$  Da. Calcd. 11,560.7 Da. **C.** RP-HPLC chromatogram of purified **7**, 30 min, 0-73% B gradient. **D.** ESI-MS of **7**. Calcd.  $[M+H]^+$  10,908.1 Da, found  $10,909.1 \pm 3.5$  Da.



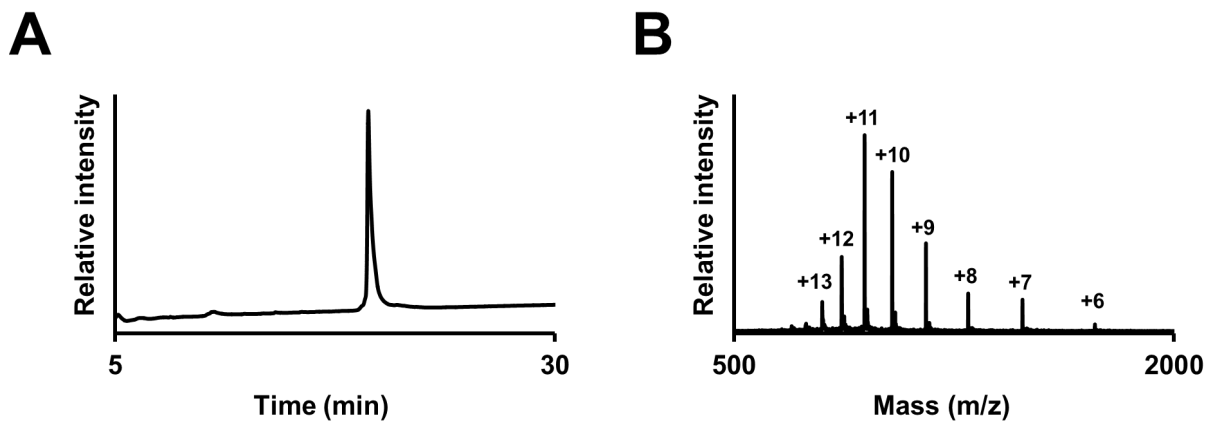
**Figure S10. Characterization of 8 after Zn-mediated reduction of 6 or 7.** **A.** RP-HPLC chromatogram of N-O bond cleaved product **8** obtained from **6**, 30 min, 0-73% B gradient. **B.** ESI-MS of N-O bond cleaved product **8** obtained from **6**. Obsd.  $[M+H]^+$   $10,410.0 \pm 2.1$  Da. Calcd. 10,409.5 Da. **C.** RP-HPLC chromatogram of N-O bond cleaved product **8** obtained from **7**, 30 min, 0-73% B gradient. **D.** ESI-MS of N-O bond cleaved product **8** obtained from **7**. Obsd.  $[M+H]^+$   $10,410.0 \pm 2.1$  Da. Calcd. 10,409.5 Da.



**Figure S11. N-O bond reduction of purified unalkylated **6** and **7**.** **A.** C18 analytical RP-HPLC chromatogram of purified unalkylated **6** (1.4 mg, 61% yield), 30 min, 0-73% B gradient. **B.** ESI-MS of purified unalkylated **6**. Obsd.  $[M+H]^+$  11,504.79  $\pm$  2.96 Da. Calcd. 11,503.68 Da. **C.** C18 analytical RP-HPLC chromatogram of purified unalkylated **7** (2.6 mg, 85% yield), 30 min, 0-73% B gradient. **D.** ESI-MS of purified unalkylated **7**. Obsd.  $[M+H]^+$  10,851.11  $\pm$  2.13 Da. Calcd. 10,851.04 Da. **E.** C18 analytical RP-HPLC chromatogram of purified N-O bond reduced SUMO-3(2-92)Q88C, 30 min, 0-73% B gradient. **F.** purified N-O bond reduced SUMO-3(2-92)Q88C. Obsd.  $[M+H]^+$  10,353.71  $\pm$  2.44 Da. Calcd. 10,352.59 Da. Unalkylated **6** yielded 71% (0.9 mg) and unalkylated **7** yielded 56% (0.7 mg) of SUMO-3(2-92)Q88C from 0.12  $\mu$ mol scale reactions.



**Figure S12. Circular Dichroism of N-O bond reduced product 8. A.** 84  $\mu\text{M}$  of **8** dissolved in 18 mM  $\text{NaH}_2\text{PO}_4$ , 92 mM Sodium citrate, pH 7.4 at 25 °C. **B.** CD spectrum of recombinant full-length SUMO-3 (green), SUMO-2 (red) and SUMO-1 (blue) in phosphate buffer at 25 °C reproduced from Sabate et al.<sup>6</sup>



**Figure S13. Characterization of SUMO-3(2-92)-CO<sub>2</sub>H substrate for in vitro sumoylation. A.** RP-HPLC chromatogram of pure SUMO-3(2-92)-CO<sub>2</sub>H, 30 min, 0-73% B gradient. **B.** ESI-MS of SUMO-3(2-92)-CO<sub>2</sub>H. Obsd. [M+H]<sup>+</sup> 10,380.6 ± 4.6 Da. Calcd. 10,378.6 Da.

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