Supplementary Information

Chemical Protein Ubiquitylation with Preservation of the Native Cysteine Residues

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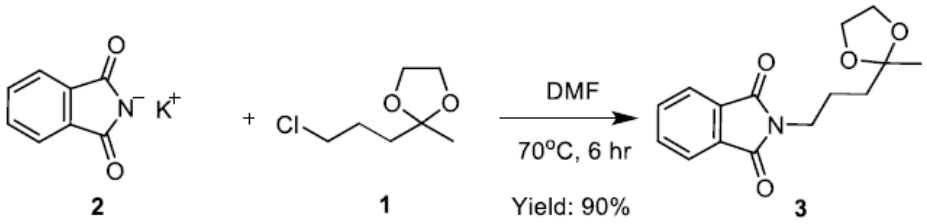
Methods

General Information for compound synthesis

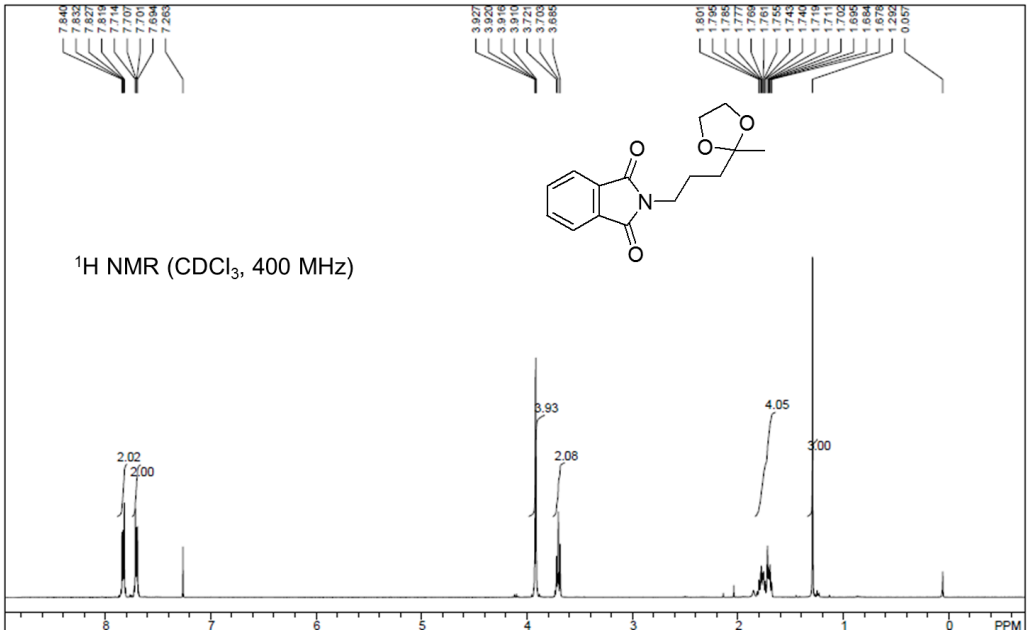
Chemical reagents were obtained from Sigma-Aldrich, Alfa and Acros of the highest available grade and used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AV400 NMR Spectrometer with a CryoProbe. Chemical shifts are reported in δ (ppm) units using ^{13}C and residual ^{1}H signals from deuterated solvents as references. Analytical thin layer chromatography (TLC) was performed on silica gel 60 GF254 (Merck). Column chromatography was conducted on silica gel (230-400 mesh).

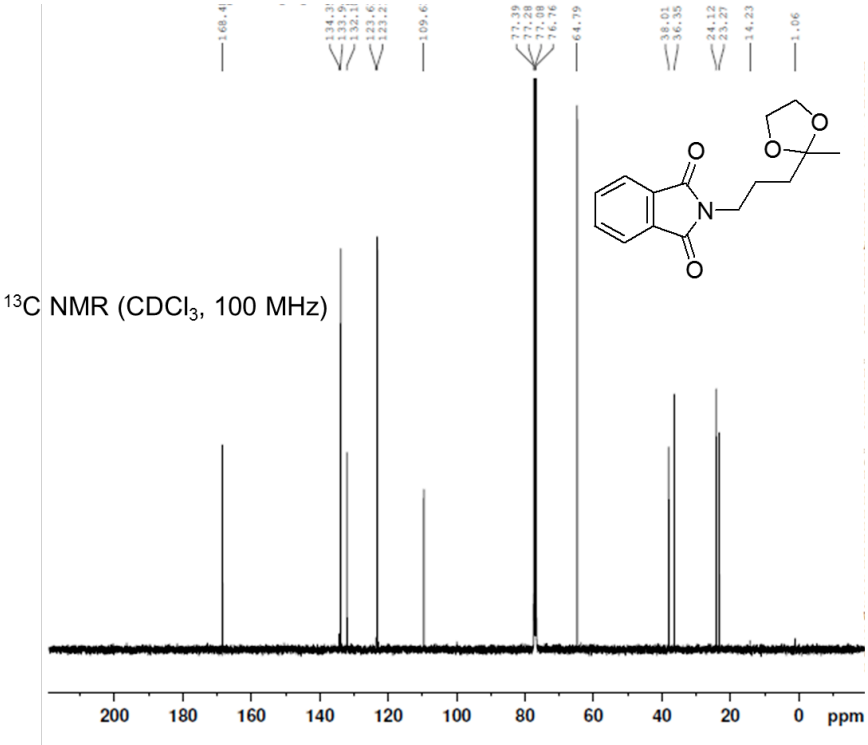
Synthesis of 2-(3-(2-methyl-1,3-dioxolan-2-yl)propyl)isoindoline-1,3-dione (Compound 3)



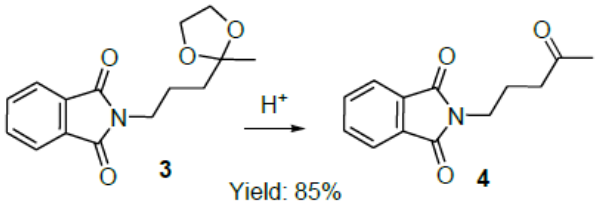
2-(3-chloropropyl)-2-methyl-1,3-dioxolane (13.1 g, 80 mmol) was dissolved in DMF (300 mL). Potassium phthalimide (16.0 g, 86 mmol) was added and the suspension was stirred at 70°C for 6 hr. Then the reaction mixture was cooled down to room temperature, quenched with H_2O and extracted with ethyl acetate. The combined organic layer was washed with H_2O and dried over anhydrous Na_2SO_4. The solvent was evaporated and the residue was purified by silica gel column chromatography with Hexane/EtOAc (3:1) to provide compound 3 (20 g, 73 mmol, 90%) as a white solid. ^{1}H NMR ($CDCl_3$, 400 MHz)

δ 7.84-7.82 (m, 2H), 7.71-7.69 (m, 2H), 3.93-3.91 (m, 4H), 3.72-3.69 (t, *J*=7.2 Hz, 2H), 1.80-1.68 (m, 4H), 1.29 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 168.5, 134.0, 132.1, 123.4, 109.6, 64.8, 38.0, 36.4, 24.1, 23.3; MS (ESI, positive) *m/z* calcd. for $C_{15}H_{18}NO_4$ $[M+H]^+$: 276, found: 276.

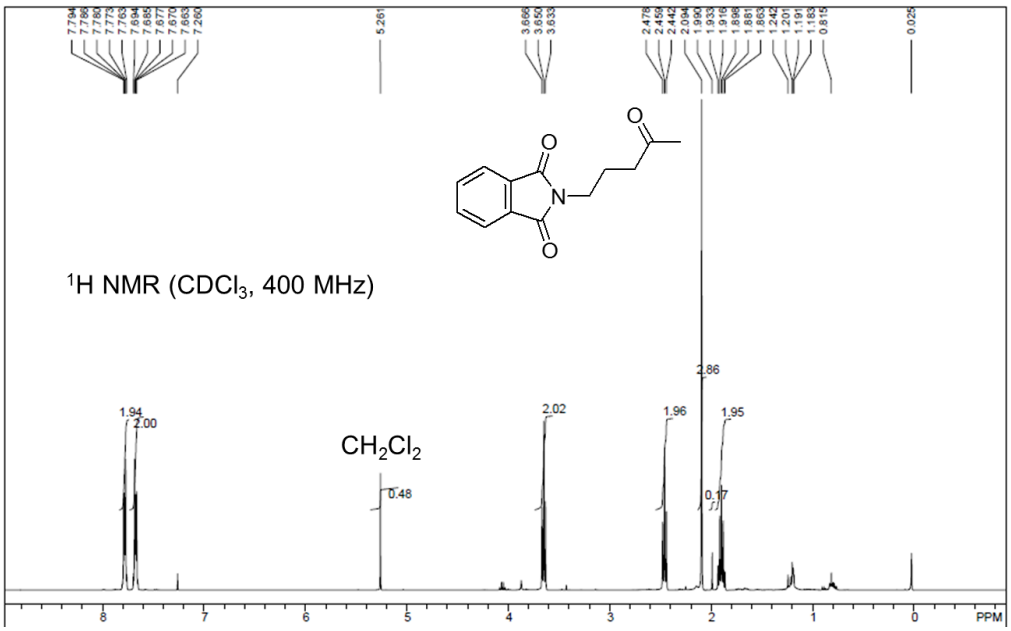




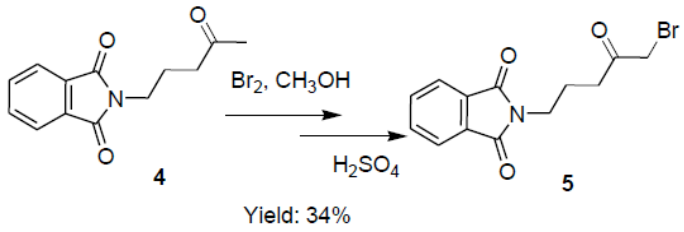
Synthesis of 2-(4-oxopentyl) isoindoline-1,3-dione (Compound 4)



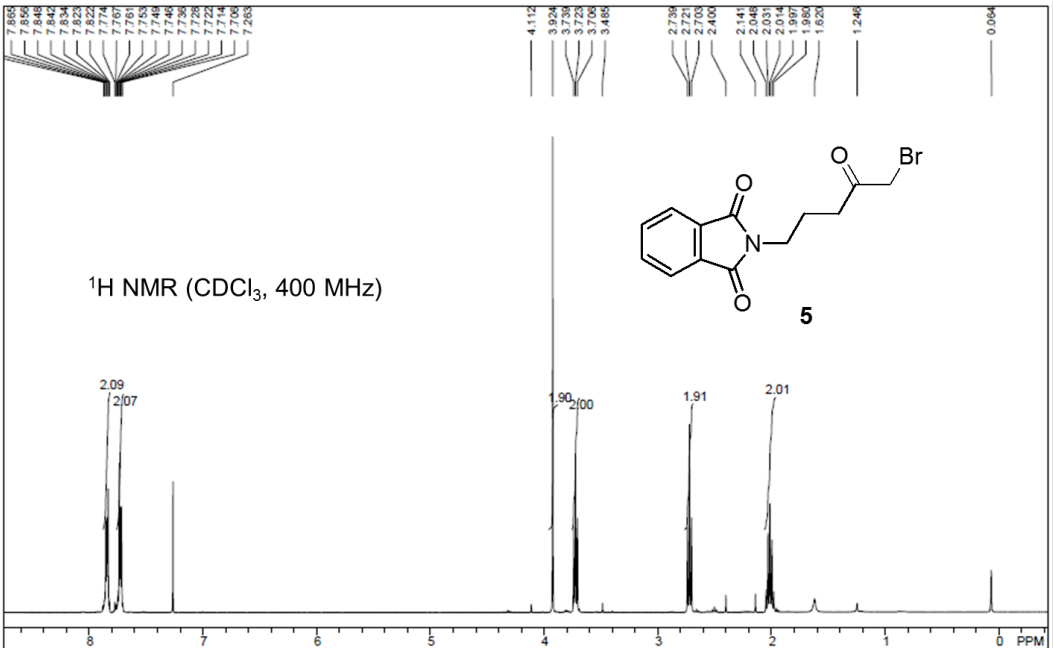
Compound **3** (23.5 g, 85 mmol) was dissolved in 200 mL methanol, and 15 mL 18% HCl was added. The reaction mixture was stirred at r.t. overnight. TLC showed the reaction was complete. The reaction mixture was quenched with saturated $NaHCO_3$, and MeOH was removed *in vacuo*. The residue was then extracted with CH_2Cl_2 and the combined organic layer was washed with saturated $NaHCO_3$, dried over Na_2SO_4. The solvent was evaporated and the residue was purified by silica gel column chromatography with Hexane/EtOAc (3:1) to provide compound **4** (16.7 g, 72 mmol, 85%) as a white solid. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.79-7.76 (m, 2H), 7.69-7.66 (m, 2H), 3.67-3.63 (t, *J*=6.6 Hz, 2H), 2.48-2.44 (t, *J*=7.2 Hz, 2H), 2.69 (s, 3H), 1.93-1.86 (m, 2H); MS (ESI, positive) *m/z* calcd. for $C_{13}H_{14}NO_3$ $[M+H]^+$: 232, found: 232.

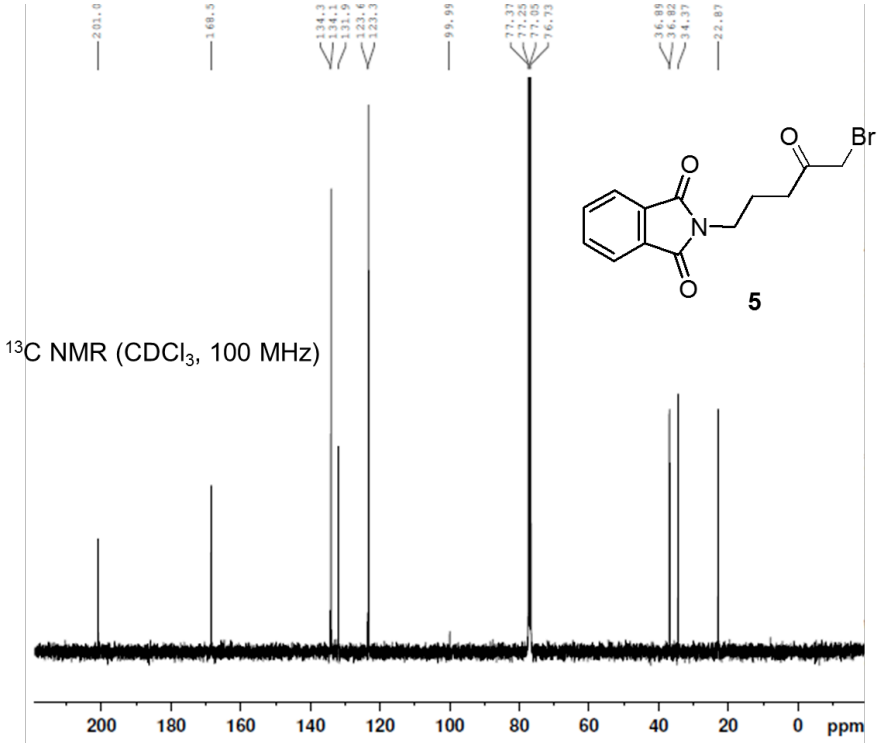


Synthesis of 2-(5-bromo-4-oxopentyl) isoindoline-1,3-dione (Compound 5)

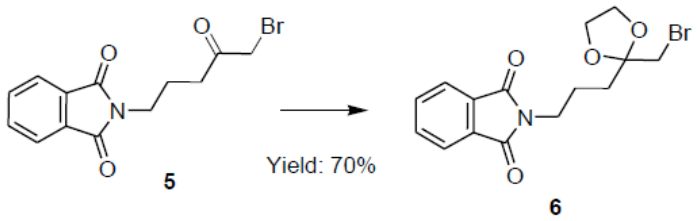


Compound **5** was prepared according to a modified method of Eriks et al[1]. Compound **4** (16.7 g, 72 mmol) was dissolved in 200 mL MeOH. Then the solution was cooled to 0^{o}C and while stirring, Br_2 (3.7 mL, 63.7mmol) was dropwise added. The reaction mixture was warmed to r.t. and reacted at r.t. overnight. To the above solution was added 15 mL of 10 M H_2SO_4, and the reaction mixture was stirred overnight. The white precipitate was collected to get crude product. The crude product was further purified by recrystallization in hot MeOH to provide compound **5** (7.5 g, 24 mmol, 34%) as a white solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.87-7.82 (m, 2H), 7.77-7.71 (m, 2H), 3.92 (s, 2H), 3.74-3.71 (t, *J*=6.6 Hz, 2H), 2.74-2.70 (t, *J*=7.2 Hz, 2H), 2.05-1.98 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 201.0, 168.5, 134.2, 132.0, 123.4, 36.9, 36.8, 344.4, 22.9; MS (ESI, positive) *m/z* calcd. for $C_{13}H_{13}BrNO_3$ $[M+H]^+$: 310, 312, found: 310, 312.

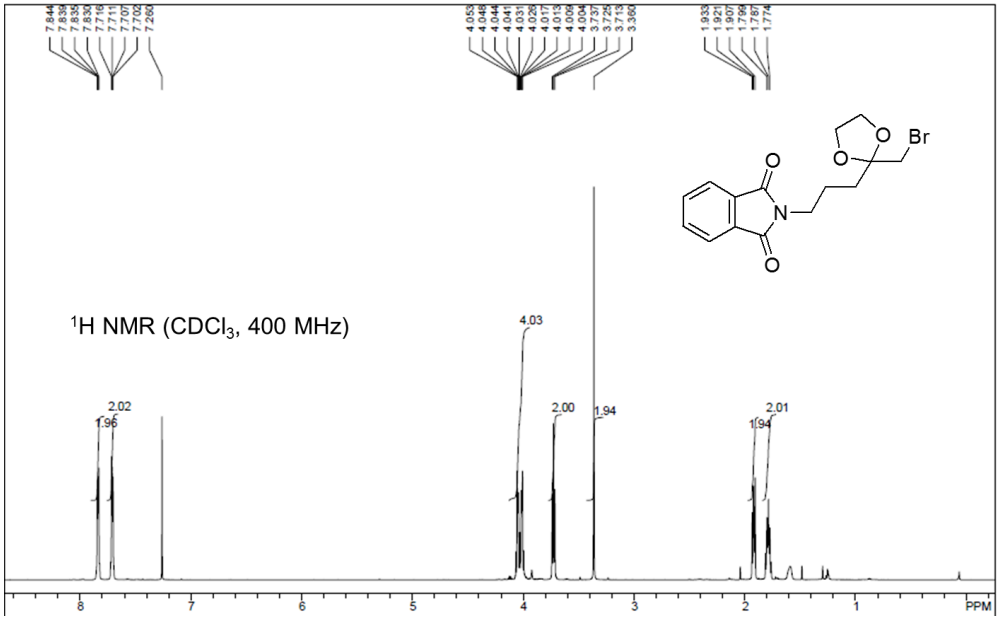

^{1}H NMR ($CDCl_3$, 400 MHz)
5
2.09
2.07
1.90
2.00
1.91
2.01
PPM

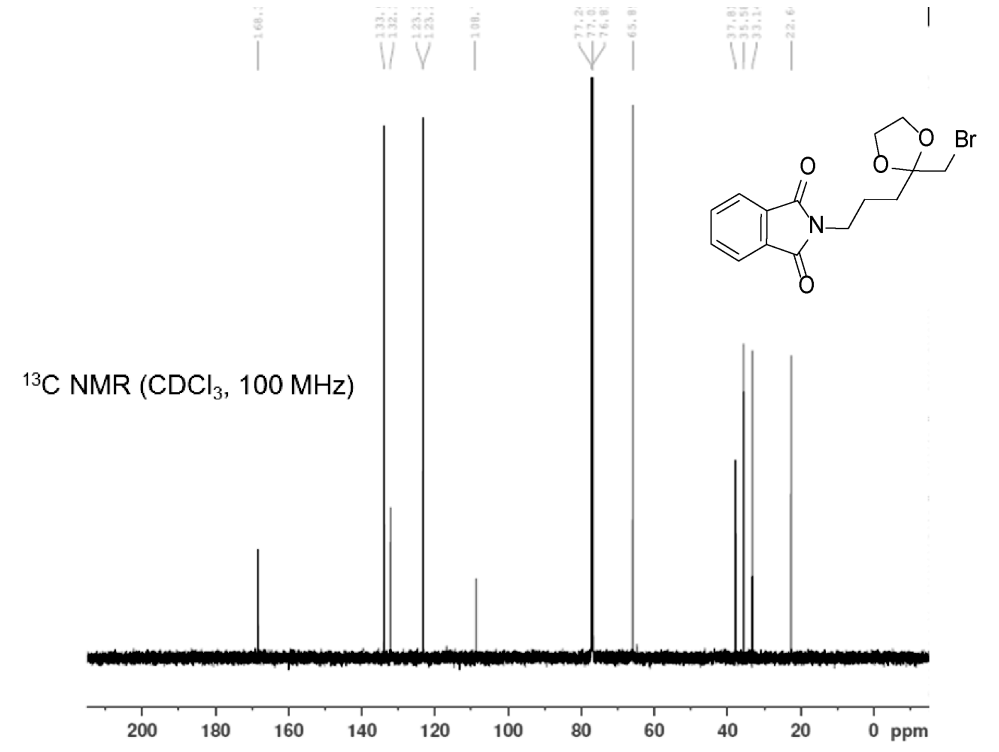

^{13}C NMR ($CDCl_3$, 100 MHz)
5
ppm

Synthesis of 2-(3-(2-(bromomethyl)-1,3-dioxolan-2-yl)propyl)isoindoline-1,3-dione (Compound 6)

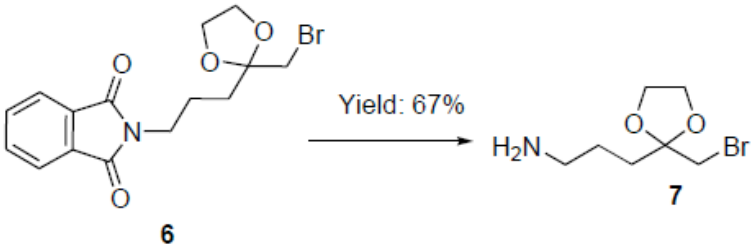


Under N_2 atmosphere, to the mixture of compound **5** (2 g, 6.4 mmol) in 150 mL of benzene was added ethylene glycol (4 g, 64.5 mmol) and p-toluenesulfonic acid (123 mg, 0.64 mmol). The reaction mixture was refluxed overnight, during which H_2O was removed using Dean-Stark trap. After cooling to room temperature, the reaction was quenched with 100 mL of saturated $NaHCO_3$. The mixture was extracted with ether. The combined organic layer was washed with H_2O, dried with Na_2SO_4. The solvent was evaporated and the residue was purified by silica gel column chromatography with Hexane/EtOAc(4:1) to provide compound **6** (2.2 g, 6.2 mmol, 97 %) as a white solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ δ 7.84-7.83 (m, 2H), 7.72-7.70 (m, 2H), 4.05-4.00 (m, 4H), 3.74-3.71 (t, *J*=4.8 Hz, 2H), 3.36 (s, 2H), 1.93-1.91 (t, *J*=5.2 Hz, 2H), 1.80-1.77 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 168.4, 133.9, 132.2, 123.2, 108.9, 65.7, 37.8, 35.6, 33.1, 22.6; MS (ESI, positive) *m/z* calcd. for $C_{15}H_{17}BrNO_4$ $[M+H]^+$: 354, 356, found: 354, 356.


^{1}H NMR ($CDCl_3$, 400 MHz)
4.03
2.02
1.96
2.00
1.94
2.01
1.94
Br
O
N
8
7
6
5
4
3
2
1
PPM

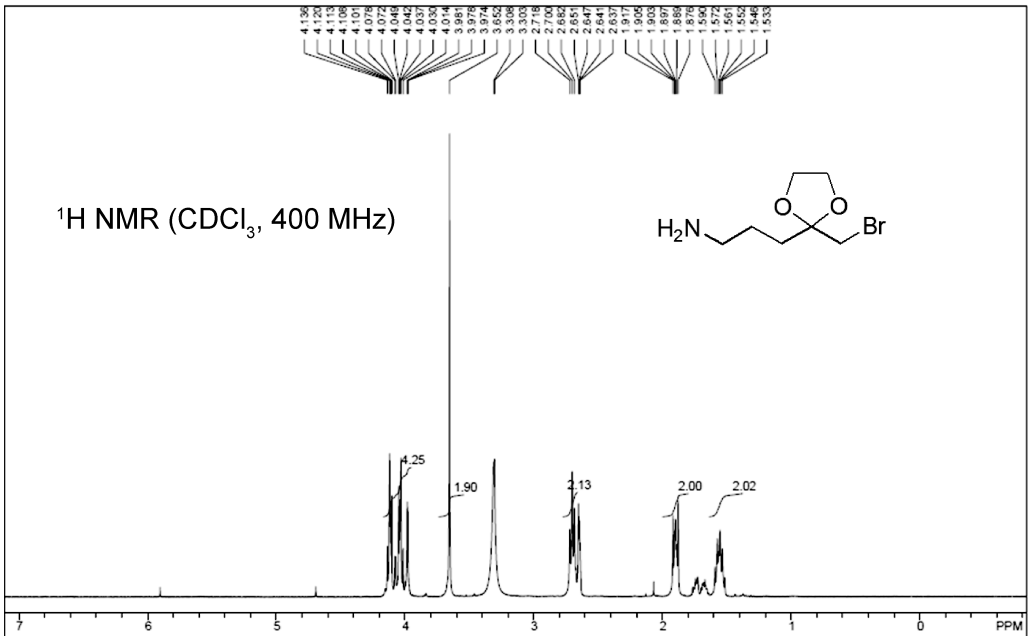

^{13}C NMR ($CDCl_3$, 100 MHz)
Br
O
N
200
180
160
140
120
100
80
60
40
20
0 ppm

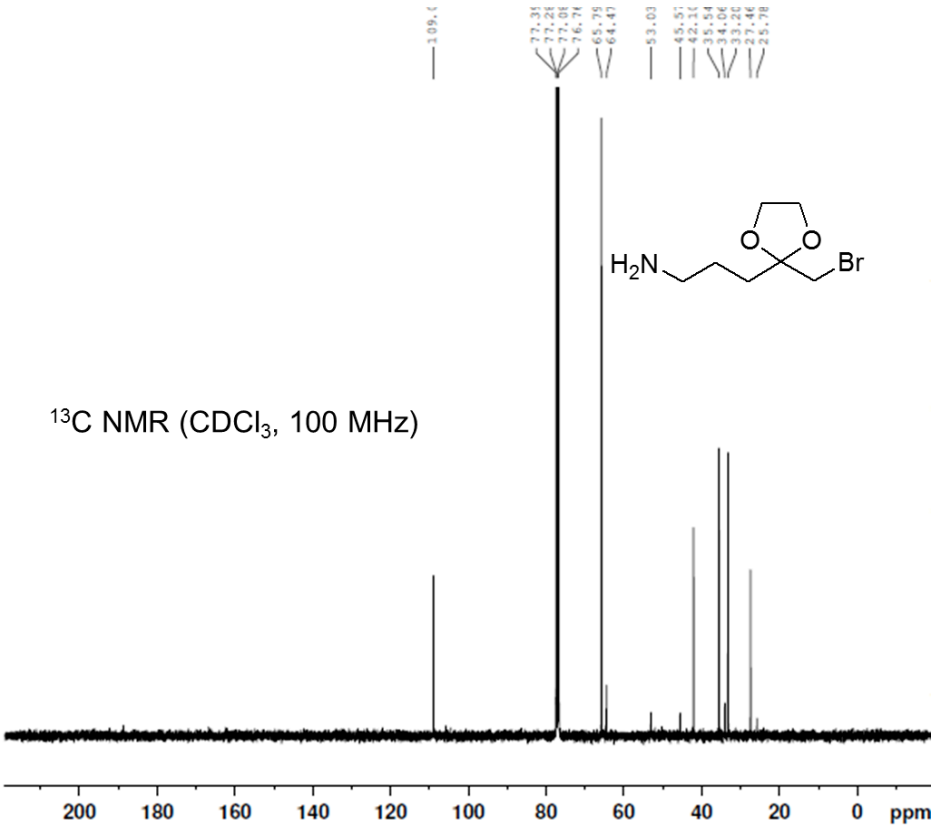
Synthesis of 3-(2-(bromomethyl)-1,3-dioxolan-2-yl)propan-1-amine (Compound 7)



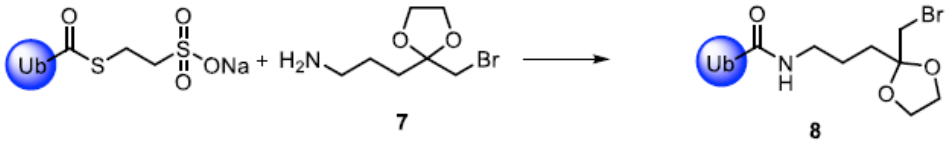
6 **7**

Under N_2 atmosphere, to a mixture of compound **6** (5 g, 14 mmol) and 300 mL of methanol was added 10 mL of hydrazine hydrate. The reaction mixture was stirred at r.t. for 48 h. The solvent was evaporated and the residue was purified by silica gel column chromatography with NH_3 saturated CH_2Cl_2/CH_3OH (10:1) to provide compound **7** (2.1 g, 9.3 mmol, 67 %) as a colorless oil. ^{1}H NMR (d_6-DMSO, 400 MHz) δ 4.14-3.97 (m, 4H), 3.65 (s, 2H), 2.72-2.68 (m, 2H), 1.92-1.88 (m, 2H), 1.59-1.53 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 109.0, 65.8, 64.5, 42.1, 35.5, 33.2, 27.5; MS (ESI, positive) *m/z* calcd. for $C_7H_{15}BrNO_2$ $[M+H]^+$: 224, 226, found: 224, 226.

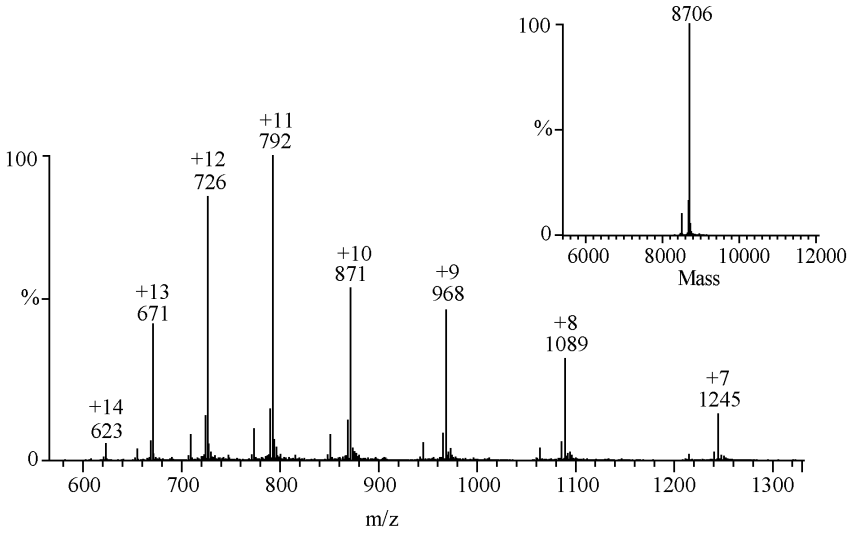

^{1}H NMR ($CDCl_3$, 400 MHz)
H_2N
O
O
Br
4.25
1.90
2.13
2.00
2.02
7
6
5
4
3
2
1
0
PPM


^{13}C NMR ($CDCl_3$, 100 MHz)
H_2N
O
O
Br
200
180
160
140
120
100
80
60
40
20
0
ppm

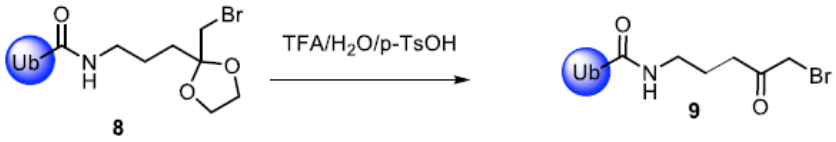
Generation of Ubiquitin species 8



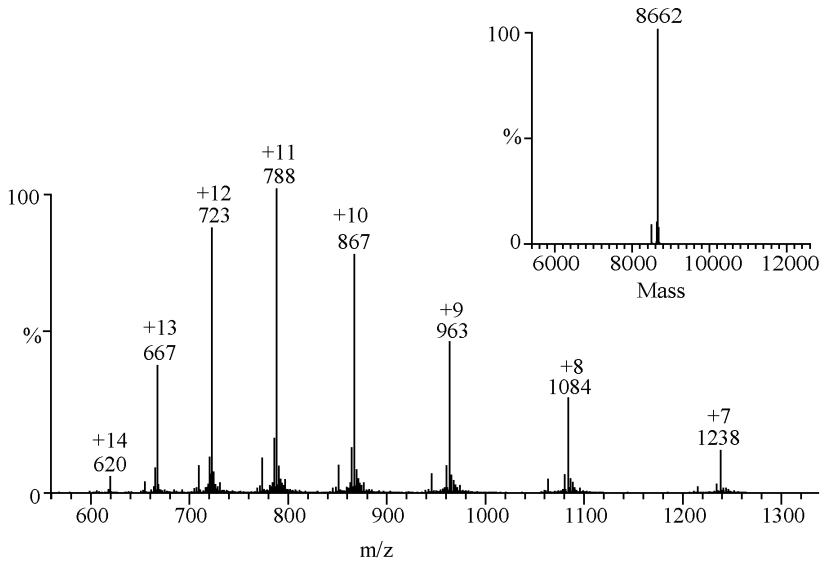
y-Ub_{1-75}-MESNa was purified following a reported protocol[2]. Before the ligation of y-Ub_{1-75}-MESNa with compound **7**, y-Ub_{1-75}–MESNa was buffer exchanged with HEPES buffer (pH 6.7) to remove MESNa. To a solution of y-Ub_{1-75}-MESNa (1.2 mg/mL) was added 0.18 M of compound **7** (dissolved in the HEPES buffer). The mixture was immediately vortexed and reacted at r.t. overnight. Then the reaction mixture was spinned down to remove the precipitate. The supernatant was buffer exchanged to remove unreacted compound **7**. The molecular weight of the ubiquitin species **8** was determined by ESI-MS to 8,706 Da (calculated molecular weight is 8,706 Da).



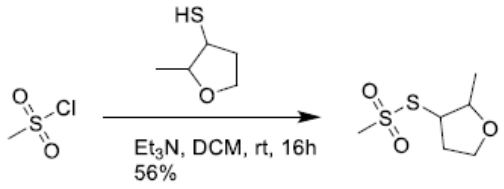
Generation of Ubiquitin species 9 (Ub-Br)



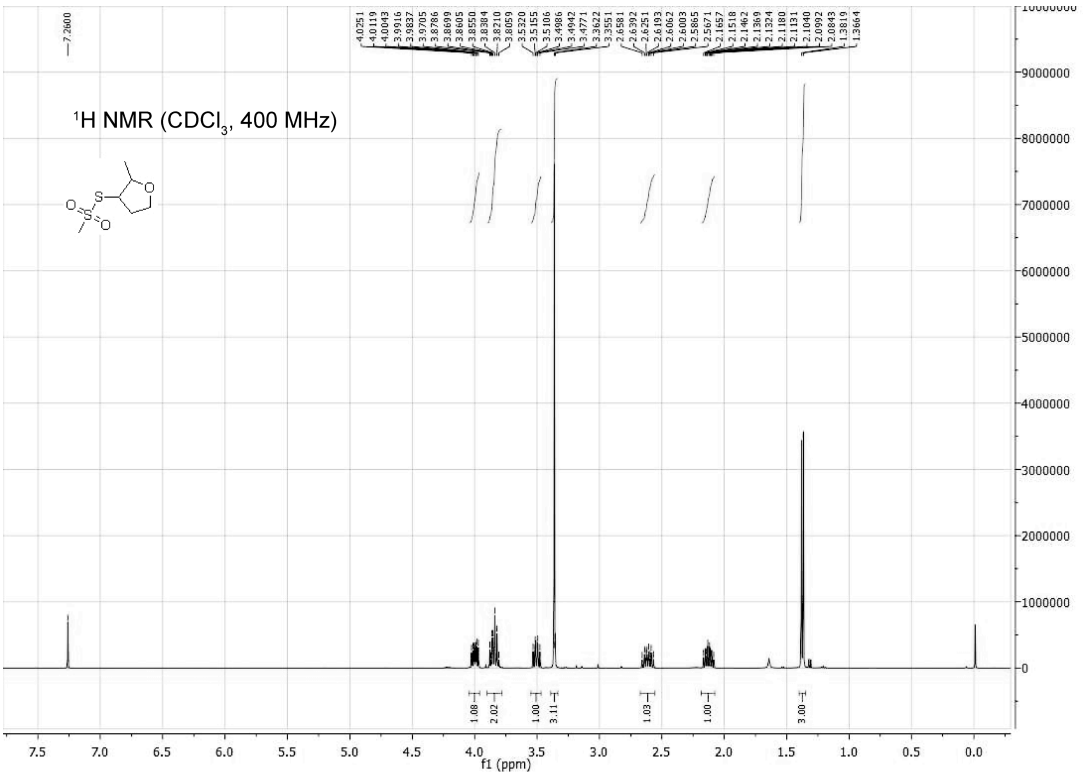
Ubiquitin species **8** was added to a solution of p-TsOH dissolved in H_2O and TFA to obtain a solution containing 0.04 M p-TsOH, 54% (v/v) TFA and 0.5 mg/mL ubiquitin species **8**. The reaction was allowed at r.t. overnight. The crude mixture was precipitated with ten times volume of cold ether, washed with cold ether and air dried. Then the crude product was dissolved in buffer (100 mM Na_2HPO_4, 8 M urea; 500 mM NaCl, pH 6.0) at a protein concentration of approximately 0.5 mg/ml and buffer exchanged into buffer (20 mM Na_2HPO_4, 100 mM NaCl, pH 6.0). The molecular weight of the ubiquitin species **9** was determined by ESI-MS to 8,662 Da (theoretical MW is 8,662 Da).

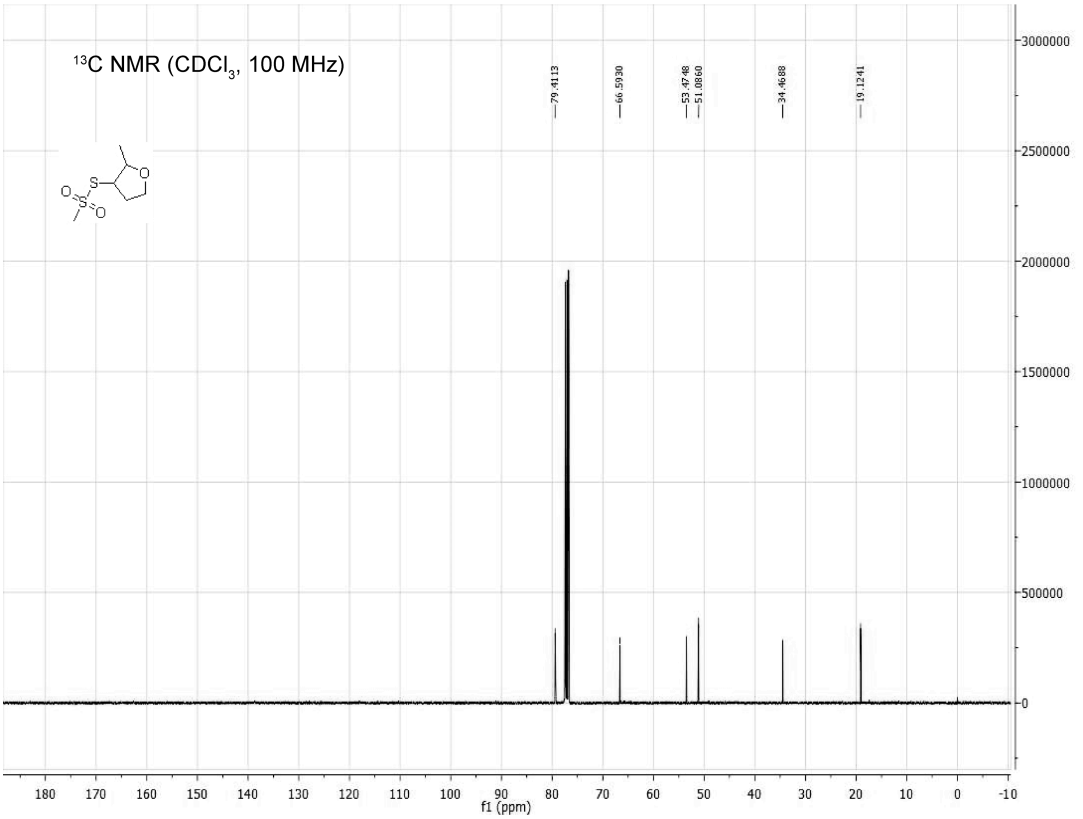


Synthesis of 2-methyl tetra-hydrofuran-methanethiosulfonate



To a solution of 2-methyl tetra-hydrofuran-3-thiol (0.37 mL, 3.26 mmol) in dry DCM was added triethylamine (1.10 eqv., 0.49 mL, 3.59 mmol); then the reaction mixture was cooled to 0 °C, and a solution of methane sulfonyl chloride, (1.10 equiv, 0.28 mL, 3.59 mmol) in 3 mL of DCM was added over 20 min. After stirring for 16 h, the solution was successively washed with water, saturated aqueous $NaHCO_3$ solution, then brine, and dried over $MgSO_4$. After evaporating the solvents, the product was column purified with Hexane:EA=10:1 as a white oil (358 mg, 56% yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.97-4.02 (m, 1H), 3.81-3.88 (m, 2H), 3.53(dt, *J*=6.6, 8.6 Hz, 1H), 3.36 (s, 3H), 2.65 (ddd, *J*=8.6, 13.2, 21.2 Hz, 1H), 2.08-2.16 (m, 1H), 1.40 (d, *J*=8.6Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 79.4, 66.6, 53.5, 51.1, 34.5, 19.1.


^{1}H NMR ($CDCl_3$, 400 MHz)
f1 (ppm)


^{13}C NMR ($CDCl_3$, 100 MHz)
79.4113
66.5930
53.4748
51.0860
34.4688
19.1241
f1 (ppm)

Purification of caged Cys-PCNA

GFP150TAG-His6 from the vector psfGFP150TAGPylT-His6[3] was replaced by His6-PCNA164TAG to form psfHis6-PCNA164TAGPylT. psfHis6-PCNA164TAGPylT was then transformed into TOP10 cells together with pBK-PCC2RS[3]. A single colony was picked from the LB-agar plate containing 50 μg/ml kanamycin and 12 μg/ml tetracycline and then grown in 100 ml 2xYT media. When OD_{600} reached 0.8, cells were harvested and resuspended in 100 ml 2xYT media containing 2 mM *S*-[(*R*,*S*)-1-{4',5'-(methylenedioxy)-2'-nitrophenyl}ethyl]-L-cysteine[3], 50 μg/ml kanamycin , 12 μg/ml tetracycline and 0.2% L-arabinose and then kept growing at 30 °C for 20 hours. Cells were harvested and lysed in a buffer containing 50 mM NaH_2PO_4 (pH 8.0), 500 mM NaCl, 5% glycerol and 10 mM imidazole by sonication. Cell lysates were spun down and the supernatant was collected and incubated with 1 ml cobalt affinity resin at 4 °C for 1 hour. The cobalt affinity resin was then washed extensively with the lysis buffer and the protein was eluted with a buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5% glycerol and 50 mM imidazole. Pure fractions were collected and the protein was then buffer exchange into a buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5% glycerol. The incorporation of the caged cysteine to PCNA was confirmed by Waters Q-TOF mass spectrometry.

Purification of wt PCNA

Yeast PCNA gene was amplified and inserted into pET15b- vector using NdeI and XhoI restriction sites. PCNA-pET15b was then transformed into Rosetta(DE3) cells. A single colony was picked from the LB-agar plate containing 100 μg/ml ampicillin and 34 μg/ml

chloramphenicol and then grown in 1 L LB media. When OD_{600} reached 0.8, 0.5 mM IPTG was added to induce the protein expression for overnight at 16℃. The cells were harvested and the protein was purified following the protocol used for the purification of caged Cys-PCNA.

Protection of the native cysteines on PCNA

The small molecules (MMTS, MTHFMTS or TBMTS) were dissolved in DMSO to prepare 1 M stock and then diluted into a buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5% glycerol to the concentration of 100 mM. 3 mg caged Cys-PCNA was added to the buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5% glycerol together with 100 eqv. small molecule. The mixture was incubated at room temperature for 1 hour. The modification was checked by mass spectrometry and the protein was buffer exchanged extensively to the buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5% glycerol.

UV irradiation of caged Cys-PCNA$(SMe)_4$ and ligation to Ub-Br

1 mg caged Cys-PCNA$(SMe)_4$ was first diluted to 1 mg/ml in a buffer containing 25 mM MES (pH 6.0), 50 mM NaCl, 5% glycerol and 4 mM L-ascorbic acid. 1 ml protein (1 mg/ml) was then split into 5 tubes and treated with 365 nm UV at 4 ℃ for 1 minute. Following UV irradiation, 3 eqv. Ub-Br was immediately added and incubated at room temperature for 2 hours. After ligation, the products were injected to Q column and eluted with 50-1000 mM NaCl gradient buffer 50 mM Tris-HCl (pH 8.0), 5% glycerol. The fractions were loaded onto a 20% SDS-PAGE gel and the pure fractions were collected and concentrated to 2 mg/ml.

Tryptic digestion of PCNA or Ub-PCNA

A solution containing 50 µg PCNA or Ub-PCNA was incubated at 100 °C for 5 min. The protein precipitate was pelleted by centrifugation and resuspended in 50 mM ammonium bicarbonate (pH 8.0) buffer containing 1 µg trypsin. The mixture was incubated at 37 ºC overnight. The peptide solution after digestion was analyzed on a Thermo Q-Exactive Orbitrap LC-MS/MS.

Stability of Ub-PCNA in the presence of DTT

A reaction mixture containing 4 µg Ub-S-S-PCNA or Ub-PCNA with non-cleavable linker in 20 mM Tris (pH 7.0) with 50 mM NaCl and DTT (5 mM, 10 mM or 100 mM) was incubated at room temperature for 20 min. The sample was analyzed using a 20% denaturing SDS-PAGE gel without any reducing agent.

Stability of chemically ubiquitylated PCNA in yeast cell lysates

S. cerevisiae cell lysate was prepared as previously described[4]. 4 µg non-cleavable Ub-PCNA was incubated with 20 µg yeast cell lysate at room temperature for different time periods (2 or 4 hours). The samples were analyzed using a 15% denaturing SDS-PAGE gel.

Stimulation of ATPase activity of RFC by Ub-PCNA

Yeast RFC was purified following the previously reported procedure[5]. Native Ub-PCNA was prepared following the protocol described previously[6]. The ATPase activity of RFC was determined using a phosphoenolpyruvate kinase/lactate dehydrogenase-coupled assay at 25 °C in an assay solution containing 200 nM RFC, 200 nM native Ub-PCNA or

non-cleavable Ub-PCNA and 200 nM primer/template DNA, 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 25 mM NaCl, 1 mM ATP, 4 mM phosphoenolpyruvate (PEP), 400 μM NADH and 3 units of phosphoenolpyruvate kinase-lactate dehydrogenase mix. The rates of ATP hydrolysis were determined by measuring the UV absorbance change at 340 nm.

DNA synthesis of Polη on an abasic site-containing DNA

The DNA synthesis assay was carried following a reported procedure[7]. Briefly, A 23 nt primer was labeled with [γ-^{32}P]-ATP by T4 polynucleotide kinase (PNK) and then annealed to a 100 nt abasic site-containing DNA oligo with biotin at both 5' and 3' ends. The annealed DNA substrate was incubated with excess amount of streptavidin for 30 min at 30 ºC to block the two ends of the DNA. A typical 25 μl reaction mixture contained 10 nM DNA substrate, 50 nM PCNA species, 50 nM RFC, 1 mM ATP and 100 μM dNTPs in a reaction buffer containing 40 mM Tri-HCl (pH 7.5), 5 mM $MgCl_2$, 150 mM NaCl, and 0.1 mg/ml BSA. Proteins were mixed on ice and then incubated at 30 ºC for 5 minutes, DNA synthesis was initiated by the addition of 1 nM Polη. The reaction was allowed for 5 minutes before being quenched by 500 mM EDTA. DNA products were isolated and analyzed on a 15% denaturing urea polyacrylamide gel. The DNA product bands were imaged using PhosphorImager (Storm, GE Healthcare Bioscience) and quantified using Imagequant 5.2 software (Molecular Dynamics). The incorporation efficiency was determined by quantifying the DNA synthesis band opposite the abasic site as the percentage of total primer extension product plus the unextended primer.

Supplementary Figures

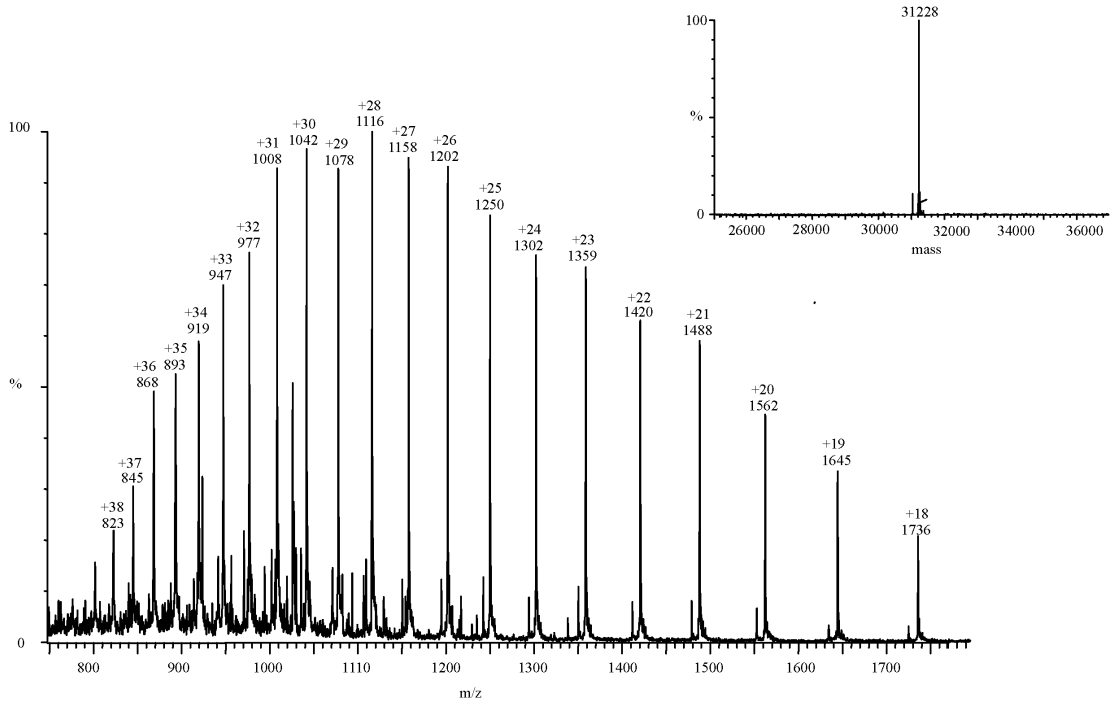


Fig. S1 Mass spectrometry of caged Cys-PCNA. The detected MW of 31,228 Da agreed well with the calculated MW of 31,231 Da.

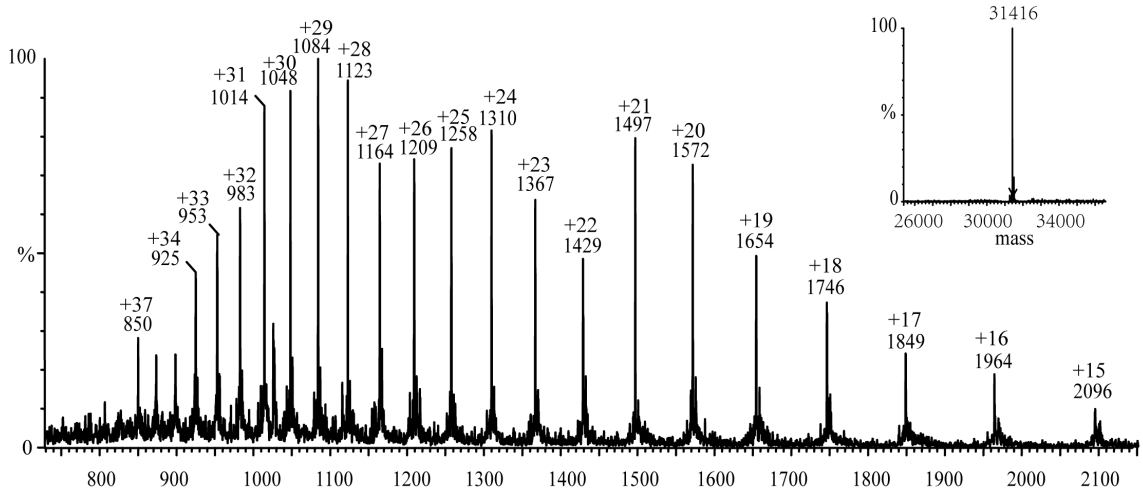


Fig. S2 (A) Mass spectrometry of caged Cys-PCNA(SMe)$_4$. The detected MW of 31,416 Da agreed well with the calculated MW of 31,415 Da.

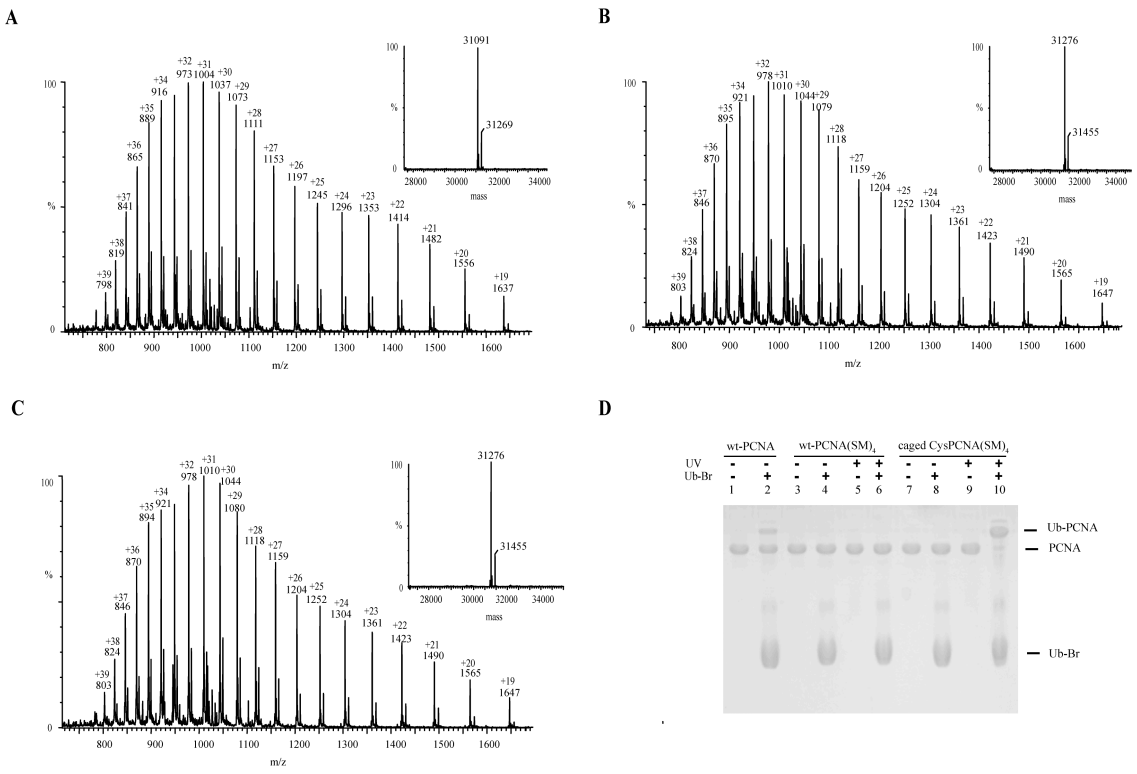


Fig. S3 (A) Mass spectrometry of wt-PCNA. The detected MW of 31,091 Da agreed well with the calculated MW of 31,090 Da. The peak with the MW of 31269 Da is possibly due to the α-N-6 gluconoylation of the N-terminus His tag on PCNA[8]. (B) Mass spectrometry of wt-PCNA$(SMe)_4$. The detected MW of 31,276 Da agreed well with the calculated MW of 31,274 Da. (C) Mass spectrometry of wt-PCNA$(SMe)_4$ after 1 minute UV irradiation at the wavelength of 365 nm. (D). wt-PCNA, wt-PCNA$(SMe)_4$, wt-PCNA$(SMe)_4$ following 1 minute UV irradiation, caged Cys-PCNA$(SMe)_4$ or caged Cys-PCNA$(SMe)_4$ following 1 min UV irradiation was incubated with Ub-Br respectively at room temperature for 2 hours. The products were resolved on a 20% SDS-PAGE gel and stained with Coomassie blue.

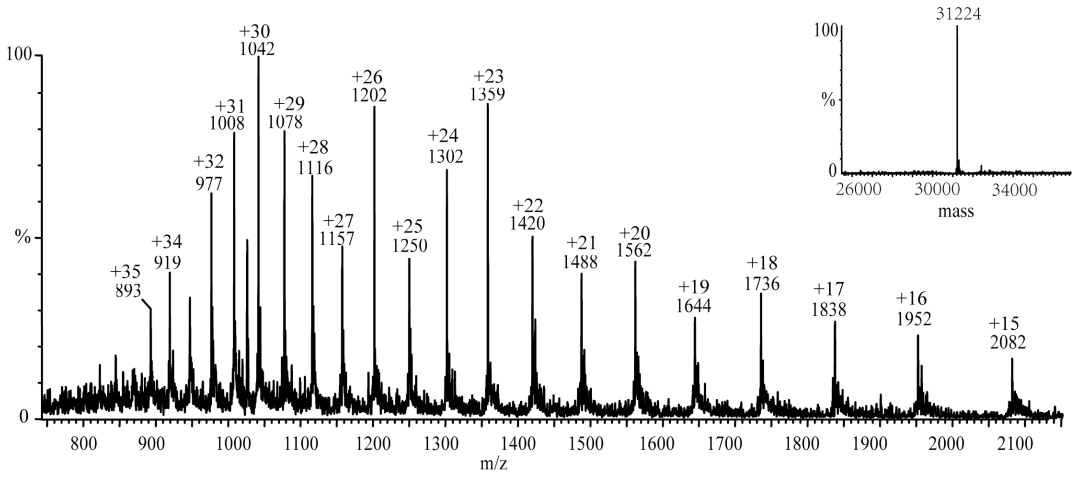


Fig. S4 Mass spectrometry of Cys-PCNA$(SMe)_4$. The detected MW of 31,224 Da agreed well with the calculated MW of 31,223 Da.

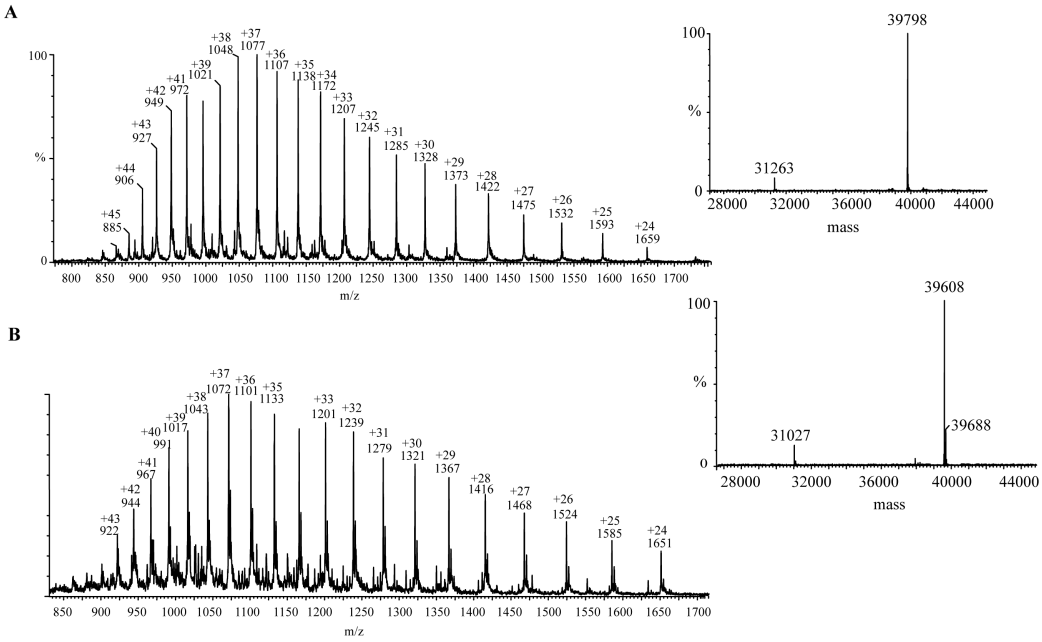


Fig. S5 (A) Mass spectrometry of Ub-PCNA(SMe)$_4$. The detected MW of 39,798 Da agreed well with the calculated MW of 39,795 Da. (B) Mass spectrometry of Ub-PCNA. The detected MW of 39,608 Da agreed well with the calculated MW of 39,611 Da. Peak with MW of 39,688 is possibly $2K^+$ adduct.

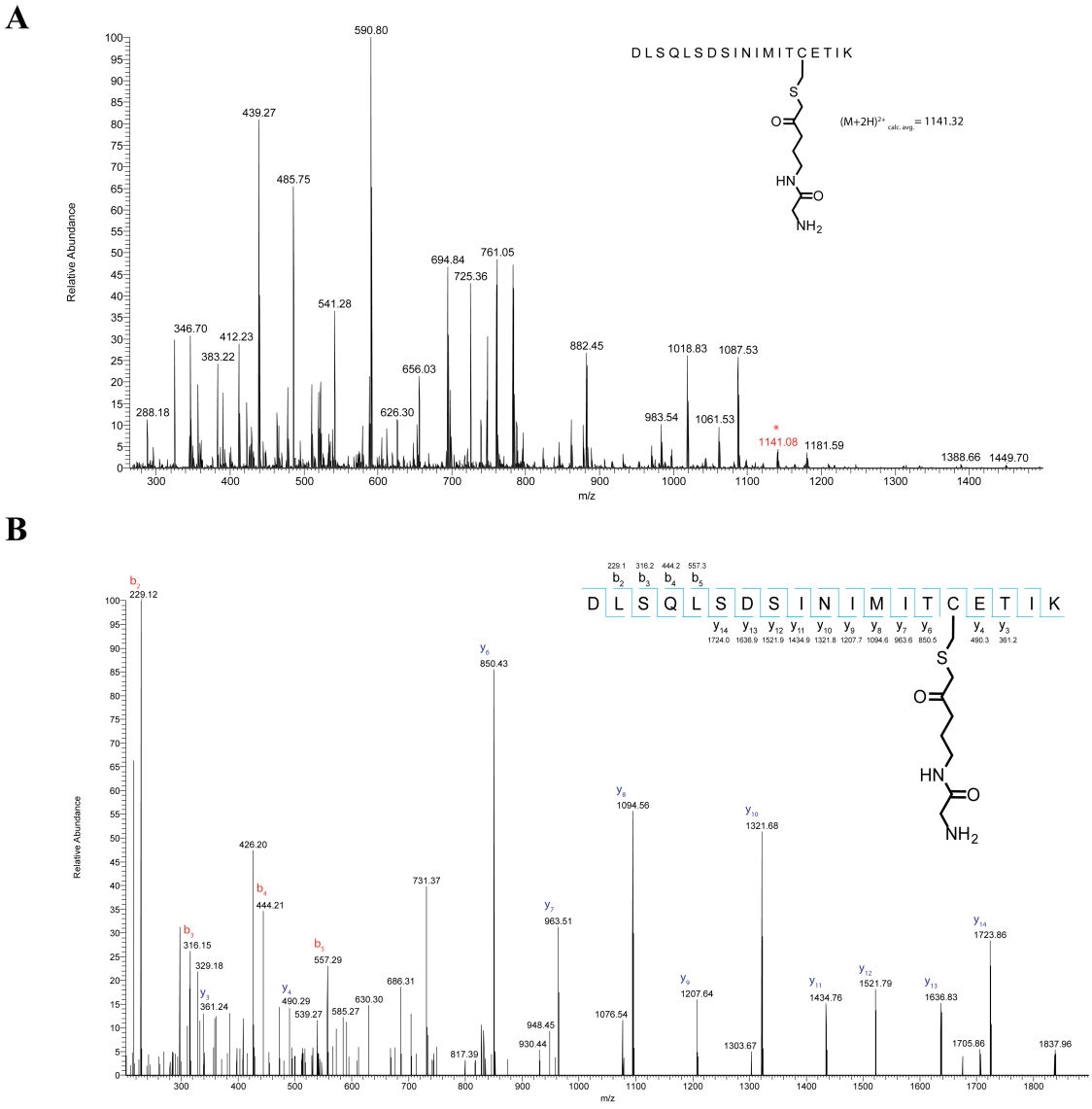


Fig. S6 (A) ESI spectrum of the tryptic digested peptides of Ub-PCNA. The isotopic peak cluster of the PCNA peptide flanking Cys164 (DLSQLSDSINIMITCETIK) with a glycine-non-cleavable linker on ubiquitin is designated by the highest intensity monoisotopic peak (m/z of 1141.08). The inset is the calculated average m/z of the peptide. B) MS/MS spectrum of the monoisotopic peak with m/z of 1141.08 that corresponds to the PCNA peptide flanking Cys164 (DLSQLSDSINIMITCETIK) with a non-cleavable linker conjugated to Gly75 of ubiquitin.

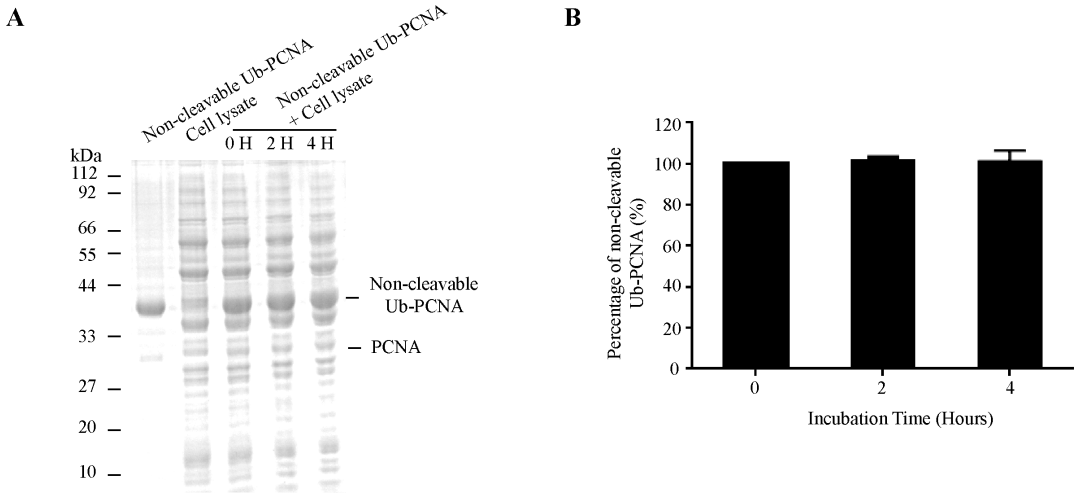


Fig. S7 Stability of non-cleavable Ub-PCNA in cell lysates. (A) 4 μg non-cleavable Ub-PCNA was incubated with 20 μg yeast cell lysate at room temperature for 2 or 4 hours. The proteins were resolved on 20% SDS-PAGE gel and stained with Coomassie blue. (B) Quantification of non-cleavable Ub-PCNA after incubation with cell lysate. The normalized percentage of intact Ub-PCNA after the treatment is showed as bar graph.

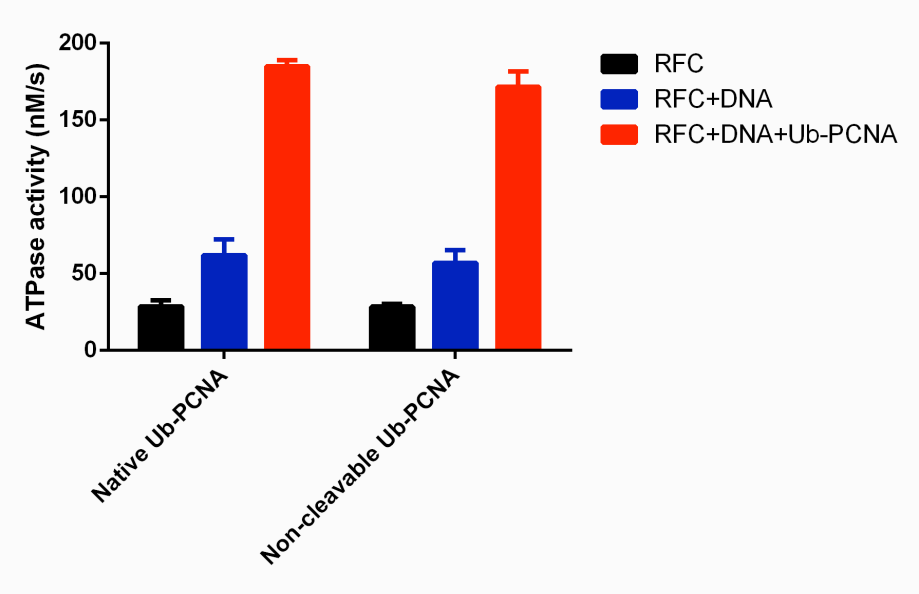


Fig. S8 Stimulation of ATPase activity of RFC by non-cleavable Ub-PCNA. For each condition, the initial rate of ATP hydrolysis is reported.

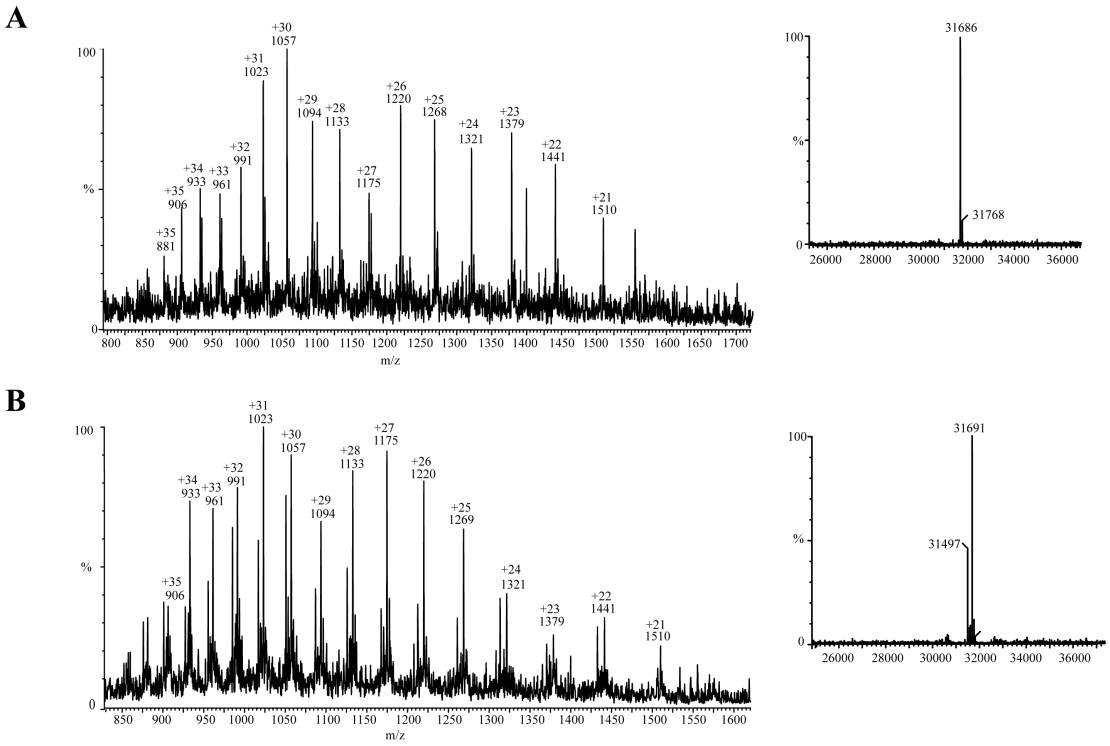


Fig. S9 (A) Mass spectrometry of caged Cys-PCNA(SMeTHF)$_4$. The detected MW of 31,686 Da agreed well with the calculated MW of 31,690 Da. The peak with MW of 31,768 is possibly $2K^+$ adduct. (B) Mass spectrometry of UV irradiated (1 minute) caged Cys-PCNA(SMeTHF)$_4$. The detected MW of 31,691 Da agreed well with the calculated MW of 31,690 Da. The peak with MW of 31,497 Da is Cys-PCNA(SMeTHF)$_4$ with the caging group removed.

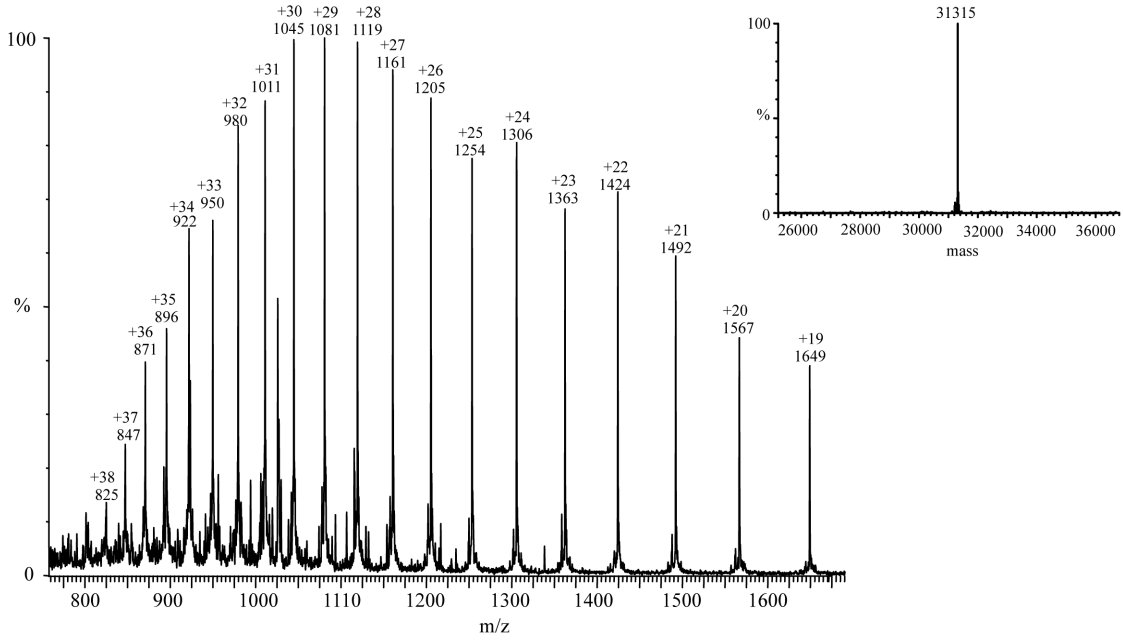


Fig. S10 Mass spectrometry of caged Cys-PCNA(S*t*Bu). The detected MW of 31,315 Da agreed well with the calculated MW of 31,318 Da.

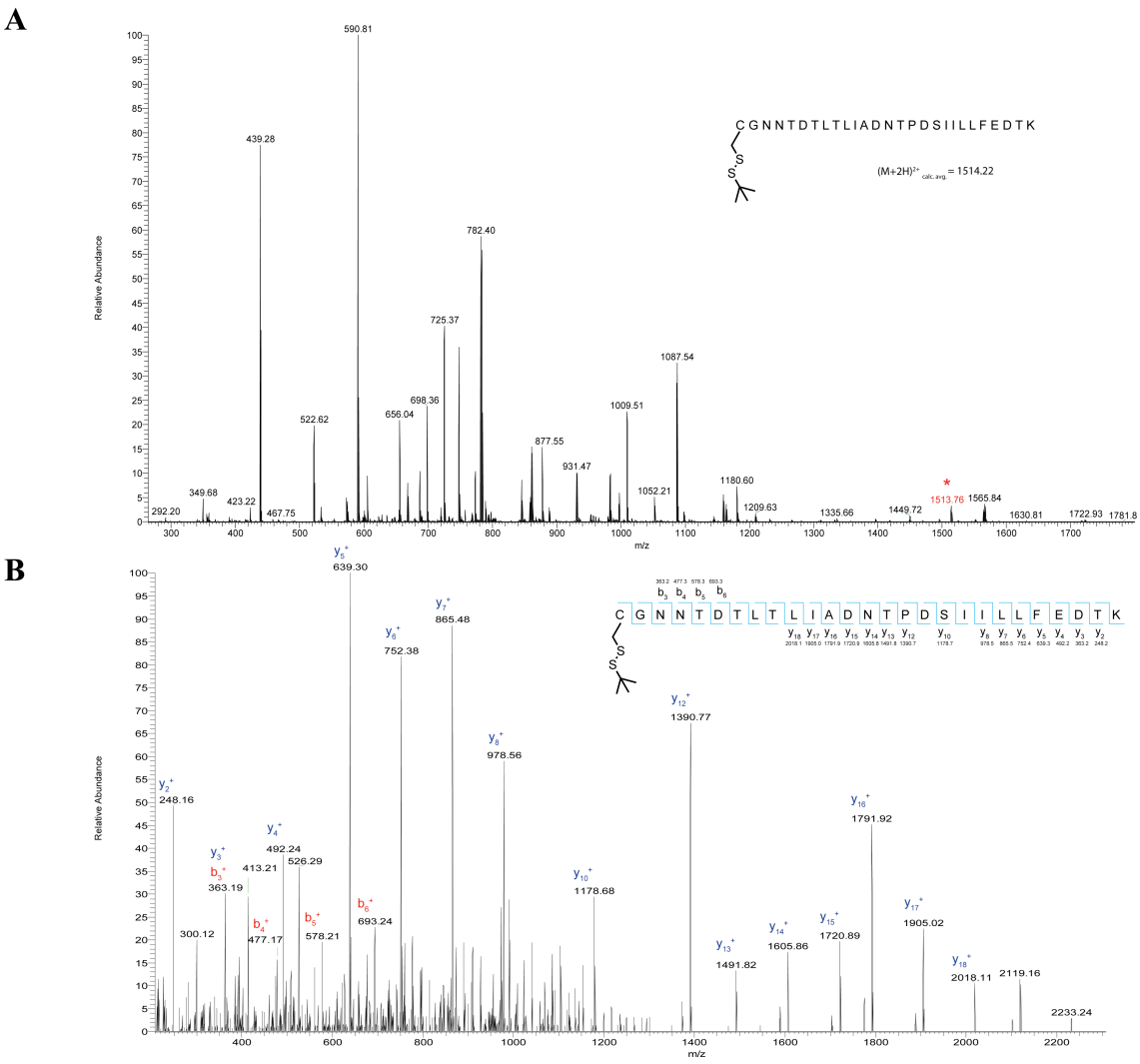


Fig. S11 A) ESI spectrum of the tryptic digested peptides of caged-Cys PCNA(S*t*Bu). The isotopic peak cluster of the PCNA peptide flanking Cys81 (CGNNTDTLTLIADNTPDSIILLFEDTK) with a S*t*Bu conjugate is designated by the highest intensity monoisotopic peak (m/z of 1513.76). The inset is the calculated average m/z of the peptide. B) MS/MS spectrum of one of the monoisotopic peaks with the m/z of 1513.51 that corresponds to the PCNA peptide flanking Cys81 (CGNNTDTLTLIADNTPDSIILLFEDTK) with a S*t*Bu conjugate.

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