Genetically Encoded Quinone Methides Enabling Rapid, Site-specific, and Photo-controlled Protein Modification with Amine Reagents

Jun Liu[1‡], Rujin Cheng[2‡] Ned Van Eps[3‡], Nanxi Wang[1], Takefumi Morizumi[3], Wei-Lin Ou[3], Paul C. Klauser[1], Sharon Rozovsky[2], Oliver P. Ernst[3,4], and Lei Wang[1*]

1. University of California, San Francisco, Department of Pharmaceutical Chemistry, 555 Mission Bay Blvd. South, San Francisco, California, United States, 94158
2. University of Delaware, Department of Chemistry and Biochemistry, Newark, Delaware, United States, 19716
3. Department of Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada
4. Department of Molecular Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada

* E-mail: Lei.wang2@ucsf.edu
‡ These authors contributed equally to this work.

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Experimental Procedures:

Reagents and molecular biology

Primers (Supplementary Table 1) were synthesized and purified by Integrated DNA Technologies (IDT), and plasmids were sequenced by GENEWIZ. All molecular biology reagents were obtained from New England Biolabs. Cy3 amine was obtained from Lumiprobe (Life science solutions), Biotin-PEG_2-amine, 4-Fluorobenzhydrazide, and 4-Amino-2,2,6,6-tetramethylpiperidinooxy (4-Amino-TEMPO) were obtained from fisher scientific. D-Galactosamine HCl was obtained from CARBOSYNTH LLC. Propargylamine, allylamine, 4-Fluorobenzylamine, and 3-Azido-1-propanamine were obtained from SIGMA-ALDRICH INC. Primers T4L9TAG NdeI for, 131TAG Rev, 131TAG For, T4L HindIII Rev were used to clone T4L (9/131 TAG) to pBAD by Overlap PCR. The template is from the source described previously[1]. Primers Ubiquitin_6TAG_NdeI For and Ubiquitin_WT_HindIII Rev were used to clone ubiquitin(6TAG) from pTAK-Ubiquitin (6TAG)[2] to pBAD plasmid. Primers Ubiquitin_WT_NdeI For and Ubiquitin_WT_HindIII Rev were used for ubiquitin WT cloning. Primers *poQMRS* SpeI For, *poQMRS* SalI Rev, *poQMRS* L125F For, *poQMRS* L125F Rev, *poQMRS* N166A/V168G For, and *poQMRS* N166A/V168G Rev were used to transplant FnbYRS to *Ma*PylRS/*Ma*^{Pyl}tRNA to generate pEVOL-*poQMRS*. Primers eCPX NdeI For and eCPX HindIII Rev were used to clone eCPX (5TAG) from pTAK-eCPX (5TAG)[3] to pBAD plasmid.

poQMRS amino acid sequence

MTVKYTDAQIQRLREYGNGTYEQKVFEDLASRDAAFSKEMSVASTDNEKKIKGMIANPSRHGL
TQLMNDIADALVAEGFIEVRTPIFISKDALARMTITEDKPLFKQVFWIDEKRALRPMLAPNFYSVM
RDLRDHTDGPVKIFEMGSCFRKESHSGMHLEEFTMLALGDMGPRGDATEVLKNYISVVMKAA
GLPDYDLVQEESDVYKETIDVEINGQEVCSAAVGPHYLDAAHDVHEPWSGAGFGLERLLTIRE
KYSTVKKGGASISYLNGAKIN

Red: L125F/N166A/V168G mutations of *poQMRS*

Ubiquitin(6TAG):

MQIFVXTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKEST
LHLVLRLRGGHHHHHH

Black: human ubiquitin amino acid sequence; Red: amber codon TAG at 6th position; Orange: His_6 tag.

Ubiquitin WT:

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKEST
LHLVLRLRGGHHHHHH

Black: human Ubiquitin WT amino acid sequence; Orange: His_6 tag.

T4 lysozyme (9TAG/131TAG)

MNIFEMLRXDEGLRLKIYKDTEGYYTIGIGHLLTKSPSLNAAKSELDKAIGRNTNGVITKDEAEKL
FNQDVDAAVRGILRNAKLKPVYDSLDAVRRAALINMVFQMGETGVAGFTNSLRMLQQKRWDE
AAXNLAKSRWYNQTPNRAKRVITTFRTGTWDAYKNLHHHHHH

Black: T4 lysozyme amino acid sequence; Red: amber codon TAG at 9th and 131th position; Orange: His_6 tag.

MBP-Z (24TAG)

MKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWA
HDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKT
WEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAG
LTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPS

KPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELAKDPRIAAT
MENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNSSSNNNNNNNNNLG
SSGLVPRGSHGTSVDNKFNKEQQNAFYEILHLPNLNXEQRNAFIQSLKDDPSQSANLLAEAKKL
NDAQAPKHHHHHH
Black: MBP-Z amino acid sequence; Red: amber codon TAG at 9th position of Z protein; Orange: His_6 tag.

MBP-Z WT
MKHHHHHHHHGGPCMKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFP
QVAATGDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEA
LSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKY
DIKDVGVDNAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKV
NYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVAL
KSYEEELAKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTN
SSSNNNNNNNNNLGSSGLVPRGSHGTSVDNAFNAEQQNAFYEILHLPNLNEEQRNAFIQSLK
DDPSQSANLLAEAKKLNDAQAPKLEHHHHHH
Black: MBP-Z amino acid sequence; Orange: N-terminal His_8 and C-terminal His_6 tag.

eCPX (TAG)
MKKIACLSALAAVLAFTAGTSVAGQSGXSHHHHHHSSQGGQSGQSGDYNKNQYYGITAGP
AYRINDWASIYGVVGVGYGKFQTTEYPTYKHDTSDYGFSYGAGLQFNPMENVALDFSYEQ
SRIRSVDVGTWILSVGYRFGSKSRRATSTVTGGYAQSDAQGQMNKMGGFNLKYRYEEDNS
PLGVIGSFTYTEKSRTAS
Black: eCPX amino acid sequence; Blue: Signal peptide; Orange: N-terminal His_6 tag; Red: amber codon TAG; Underline: Linker and insertion site.

Incorporation of FnbY and FmnbY into MBP-Z(24TAG), ubiquitin(6TAG)
For the incorporation of FnbY and FmnbY into MBP-Z(24TAG) and ubiquitin(6TAG), the procedure was carried out as previously described with slight modifications[4]. Briefly, plasmid pBAD-MBP-Z(24TAG), pBAD-ubiquitin(6TAG) was co-transformed with pEVOL-FnbYRS into DH10B, and plated on LB agar plate supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. Several colonies were picked from above freshly transformed plate, and inoculated in 25 mL 2x YT (5 g/L NaCl, 16 g/L Tryptone, 10 g/L Yeast extract). The cells were grown at 37 °C, 220 rpm to an OD 1, with good aeration and the relevant antibiotic selection. The medium was then added with either 0.2% L-arabinose only or 0.2% L-arabinose plus 1 mM FnbY, 0.2% L-arabinose plus 1 mM FnbY and the expression were carried out at 18 °C, 220 rpm for 18-22 h. Cells were harvested at 3000 g, 4 °C for 10 min. The cell pellet was washed with cold IMAC buffer (25 mM sodium phosphate, 20 mM imidazole, 500 mM NaCl, pH 7.5), centrifuged again at 3000 g, 4 °C for 10 min, and resuspended in 15 mL IMAC buffer. The tube was then frozen by dry ice and stored in −80 °C. For protein purification, the frozen cells were thawed quickly and resuspended well, and supplemented with EDTA free protease inhibitor cocktail, 0.5 mg/mL lysozyme, 1 μg/mL DNase, and vortex for 2 min. The cells were opened by sonification, after which the cell lysis solution was centrifuged at 25,000 g at 4 °C for 40 min. The supernatant was collected and incubated with 500 μL TALON® metal affinity resin. The resin was washed with equal volume of IMAC buffer for 2 times at 4 °C, and then transferred into a Pierce™ centrifuge column (ThermoFisher Scientific). After 2 times wash with 500 μL IMAC buffer, the protein was eluted five times with 500 μL 25 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl, pH 7.5. The fractions containing the target protein were analyzed by running 12% Tris-tricine SDS−PAGE gel. For FmnbY incorporation, the procedure is the same but using only pEVOL-poQMRS. FnbY incorporation work equally well with pEVOL-poQMRS.

Protein expression and purification of MBP-Z WT and ubiquitin WT
To obtain wild type MBP-Z, pHis8-MBP-Z and pBAD-Ubiquitin WT plasmids were transformed to DH10b cells, and the protein expression and purification were carried out similar as described above.

Incorporation of FnbY into T4L(9/131TAG)
For the incorporation of FnbY into T4L(9/131TAG). Briefly, plasmid pBAD-T4L(9/131TAG) was co-transformed with pEVOL-FnbYRS into C321.ΔA.exp[5], and plated on LB agar plate supplemented with 100 μg/mL ampicillin, 34 μg/mL chloramphenicol, and 5 μg/mL bleomycin. Several colonies were picked from the above freshly transformed plate, and inoculated in 100 mL 2x YT (5 g/L NaCl, 16 g/L Tryptone, 10 g/L Yeast extract). The protein expression and purification were carried out following the procedure as described above, except using Ni-NTA resin for protein purification.

FnbY mediated T4L double conjugation with 4-Amino-TEMPO
After purification, 21 μM T4L(9/131FnbY) were buffer exchanged to 50 mM sodium phosphate, 200 mM NaCl, pH 7.5. The protein mixture was added with 30 mM 4-amino-TEMPO, and illuminated with UV at 365 nm with a low-power UVP lamp (3UV-38 UV lamp, irradiance output of 1.5 mW/cm^2) for 10 min at RT. The protein reaction mixture was then diluted 10 times with 50 mM sodium phosphate, 200 mM NaCl, pH 7.5. Excess 4-amino-TEMPO was removed by concentration and dilution followed by a second IMAC column rebinding and constant wash with excess 50 mM sodium phosphate, 200 mM NaCl, pH 7.5 buffer. The labelled protein was eluted from the column and buffer exchanged to 50 mM sodium phosphate, 200 mM NaCl, pH 7.5.

Photo-controlled site specific protein labeling of ubiquitin(6FnbY) and ubiquitin(6FmnbY) with amine derivatives
For rapid functionalization of protein via photo-controlled amine-QM conjugation, 10 μM ubiquitin(6FnbY) or ubiquitin(6FmnbY) was treated with 1 mM biotin hydrazide, 50 mM allyl amine, 50 mM propargylamine, 10 mM or 50 mM biotin-PEG$_2$-amine, 1.5 or 50 mM 4-amino-TEMPO used for FmnbY labeling, 30 mM 4-amino-TEMPO used for FnbY labeling, 50 mM 3-azido-1-propanamine, 25 mM 4-aminobenzene-1-sulfonyl fluoride, or 100 mM D-galactosamine, in 50 mM sodium phosphate, 200 mM NaCl, pH 7.5. Typically, the reaction mixture was illuminated with UV at 365 nm wavelength, at RT for 10 min with a low-power UVP lamp (3UV-38 UV lamp, irradiance output of 1.5 mW/cm^2) or 30 s with a high-power UV lamp (Omnicure S1000, irradiance output of 5.3 W/cm^2). The protein conjugation was detected by ESI-MS. The percent of labeling (conversion efficiency) was evaluated by mass spectrometry [Intensity of labeled peak / Intensity of (labeled peak + hydrolysis peak + original FnbY or FmnbY Ub peak)].

Photo-controlled site specific protein labeling of ubiquitin(6FmnbY) with thiol derivatives
For photo-controlled thiol-QM conjugation, 10 μM ubiquitin(6FmnbY) was treated with 1 mM or 10 mM 1-thio-β-D-glucose, in 50 mM sodium phosphate, 200 mM NaCl, pH 7.5. For conjugation of ubiquitin with mertansine, 0.75 mM mertansine was slowly added to the ubiquitin reaction mixture containing 10% DMSO and the pH was adjusted to 9. The reaction mixture was illuminated for 30 s at wavelength 365 nm with a high-power UV lamp (Omnicure S1000, irradiance output of 5.3 W/cm^2). The protein conjugation was detected by ESI-MS.

Photo-controlled site specific protein labeling of MBP-Z with Cy3-amine
For site-specific fluorescent labelling through FnbY mediated amine-QM conjugation, 3 μM MBP-Z WT or MBP-Z (24FnbY) was treated with 1 mM, 1.5 mM, or 3 mM Cy3-amine. The reaction mixture was illuminated with or without UV at 365 nm wavelength (low-power 3UV-38 UV lamp), at 0 °C, 4 °C, or RT for 10 min. The sample were treated with SDS loading dye with 100 mM DTT,

and samples were boiled at 95 °C for 5 mins and the protein were imaged for Cy3 fluorescence as well as for Coomassie blue staining.

Photo-controlled site specific protein labeling of MBP-Z with biotin-PEG_2-amine

To site-specific label a target protein with a biotin tag, 3 μM MBP-Z WT or MBP-Z (24FnbY) was treated with 10 mM biotin-PEG_2-amine. The reaction mixture was illuminated with or without UV at 365 nm wavelength (low-power 3UV-38 UV lamp), at RT for 10 min. The sample were treated with SDS loading dye with 100 mM DTT, and samples were boiled at 95 °C for 5 mins and the protein were subjected to SDS-PAGE for both Western blot with NeutrAvidin™ HRP conjugate antibody and coomassie blue staining.

Photo-controlled site specific *E. coli* live cell labeling

To utilize QM for rapid *E. coli* live cell labeling with fluorophore, 15 mL 2XYT of DH10B with freshly transformed pEVOL-poQMRS and pBAD-eCPX(5TAG) was grown at 37 °C, 220 rpm supplemented with relevant antibiotics. The cells were induced as described above for protein expression and incorporation of FmnbY with 1 mM FmnbY and 0.2% arabinose. The cells were harvested and washed with PBS, pH 7.4 for 2 times. After resuspension in a 1.5 mL microcentrifuge tube with a volume of final 200 μL, the cells were treated with 0.1 mM Alexa Fluor 488 hydrazide, and mixed well. Then the cells were quickly illuminated with or without UV at wavelength 365 nm (high-power UV lamp Omnicure S1000, irradiance output of 5.3 W/cm^2) on ice for 1 min. The excess dye was removed by thoroughly wash with 1 mL PBS buffer for 5 times with a benchtop centrifuge. The cells were resuspended in 1 mL PBS buffer, 20 μL was dropped on the coverslip and fixed with a thin-layer of 1.5 % LB agar. Images were captured with a NIKON Ti microscope with TIRF and N-STORM using NIS-Elements software.

CW EPR measurements

X-band continuous wave (CW) EPR measurements were acquired using a Bruker ELEXSYS E500 spectrometer equipped with a Bruker EP 041 MR microwave bridge. Samples were loaded into 0.6 mm ID and 0.84 mm OD capillaries and inserted into an ER 4123D dielectric resonator for data acquisition. Measurements were acquired under ambient atmospheric conditions. The field sweep for data collection was 100-G, sweep time 20.97 s, modulation amplitude 2-G, modulation frequency 100 kHz, and incident microwave power was 0.3165 mW. Each spectrum was acquired at room temperature.

DEER measurements

For DEER measurements, deuterated glycerol was added to the samples as a cyro-protectant (final concentration 20%). Labeled T4 lysozyme samples were loaded into quartz capillaries (1.5 mm ID and 1.8 mm OD) and snap frozen using a dry ice / ethanol bath. After freezing, the capillaries were loaded into an ER 5107D2 Q-band flexline resonator and Q-band measurements were performed at 80 Kelvin on a Bruker Elexsys 580 spectrometer with a Super Q-FTu Bridge. A 32-ns π-pump pulse was applied to the low field peak of the nitroxide field swept spectrum, and the observer π/2 (16 ns) and π (32 ns) pulses were positioned 20 G upfield at the nitroxide center line. Distance distributions were extracted from the raw data using the LabView (National Instruments) program "LongDistances" [developed by Christian Altenbach and Wayne L. Hubbell, University of California, Los Angeles (UCLA)] that can be downloaded from http://www.biochemistry.ucla.edu/Faculty/Hubbell/.

Mass spectrometry

Mass spectrometric measurements were performed as previously described.[6] Briefly for electrospray ionization mass spectrometry, mass spectra of intact proteins were obtained using a QTOF Ultima (Waters) mass spectrometer, operating under positive electrospray ionization

(+ESI) mode, connected to an LC-20AD (Shimadzu) liquid chromatography unit. Protein samples were separated from small molecules by reverse phase chromatography on a Waters Xbridge BEH C4 column (300 Å, 3.5 µm, 2.1 mm x 50 mm), using an acetonitrile gradient from 30-71.4%, with 0.1% formic acid. Each analysis was 25 min under constant flow rate of 0.2 mL/min at RT. Data were acquired from m/z 350 to 2500, at a rate of 1 sec/scan. Alternatively, spectra were acquired by Xevo G2-S QTOF on a Waters ACQUITY UPLC Protein BEH C4 reverse-phase column (300 Å, 1.7 µm, 2.1 mm x 150 mm). An acetonitrile gradient from 5%-95% was used with 0.1% formic acid, over a run time of 5 min and constant flow rate of 0.5 mL/min at RT. Spectrum were acquired from m/z 350 to 2000, at a rate of 1 sec/scan. The spectra were deconvoluted using maximum entropy in MassLynx.

Crystal structure determination

Crystals of T4L double conjugated with 4-amino TEMPO at sites 9 and 131 were grown using the hanging-drop vapor diffusion method over a 500 µL well solution containing 2.4 M NaH_2PO_4/K_2HPO_4 (pH 7.4), 150 mM NaCl, 100 mM 1,6-hexanediol, and 3% 2-propanol. Crystals grew in 1 month in a 5 µL drop composed of 2.5 µL of a solution containing 5 mg/mL spin labeled T4L, 50 mM MOPS (pH 6.8), and 25 mM NaCl and 2.5 µL of the well solution suspended over the well. Diffraction data for the ~100–200 µm single crystals of spin labeled T4L (PDB access code 6V51) were collected with a Pilatus 6M detector, using a wavelength of 1.0332 Å, at beamline 23-ID-D at the Advanced Photon Source (Argonne National Laboratory, Lemont, Illinois, USA). Diffraction data were processed using the software package available at the beamline and the indexed and integrated reflections were scaled and merged using Aimless (CCP4). Molecular replacement was conducted with Phaser (Phenix) using a T4L model (PDB access code 1P2L). Initial model was built in *Coot*.[7] Structure and density maps were refined and validated using Phenix.refine[8] with the TEMPO structure factor file.[9] Data collection and refinement statistics are given in Table S3.

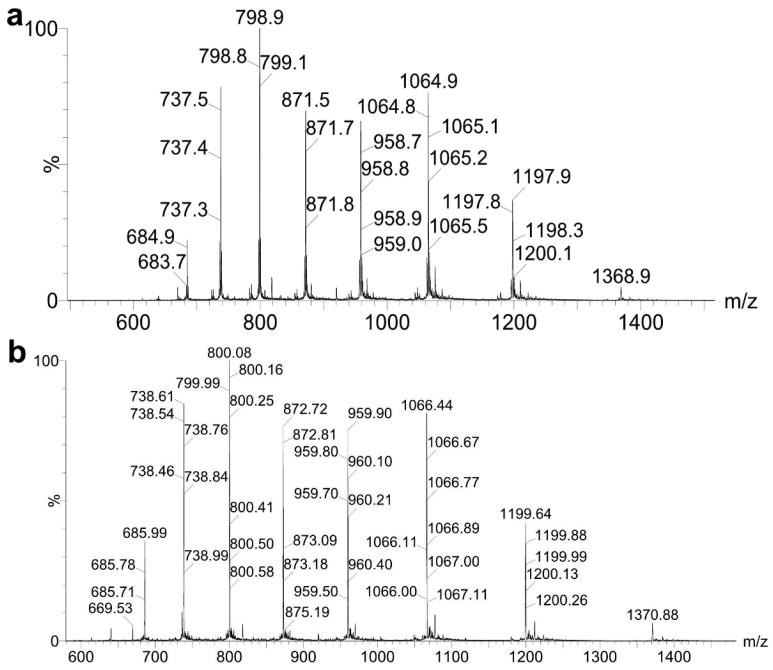

Figure S1. Detection of ubiquitin(6FnbY) and ubiquitin(6FmnbY) by mass spectrometry. a) M/Z spectrum of ubiquitin (6FnbY) before mass deconvolution. b) M/Z spectrum of ubiquitin (6FmnbY) before mass deconvolution.

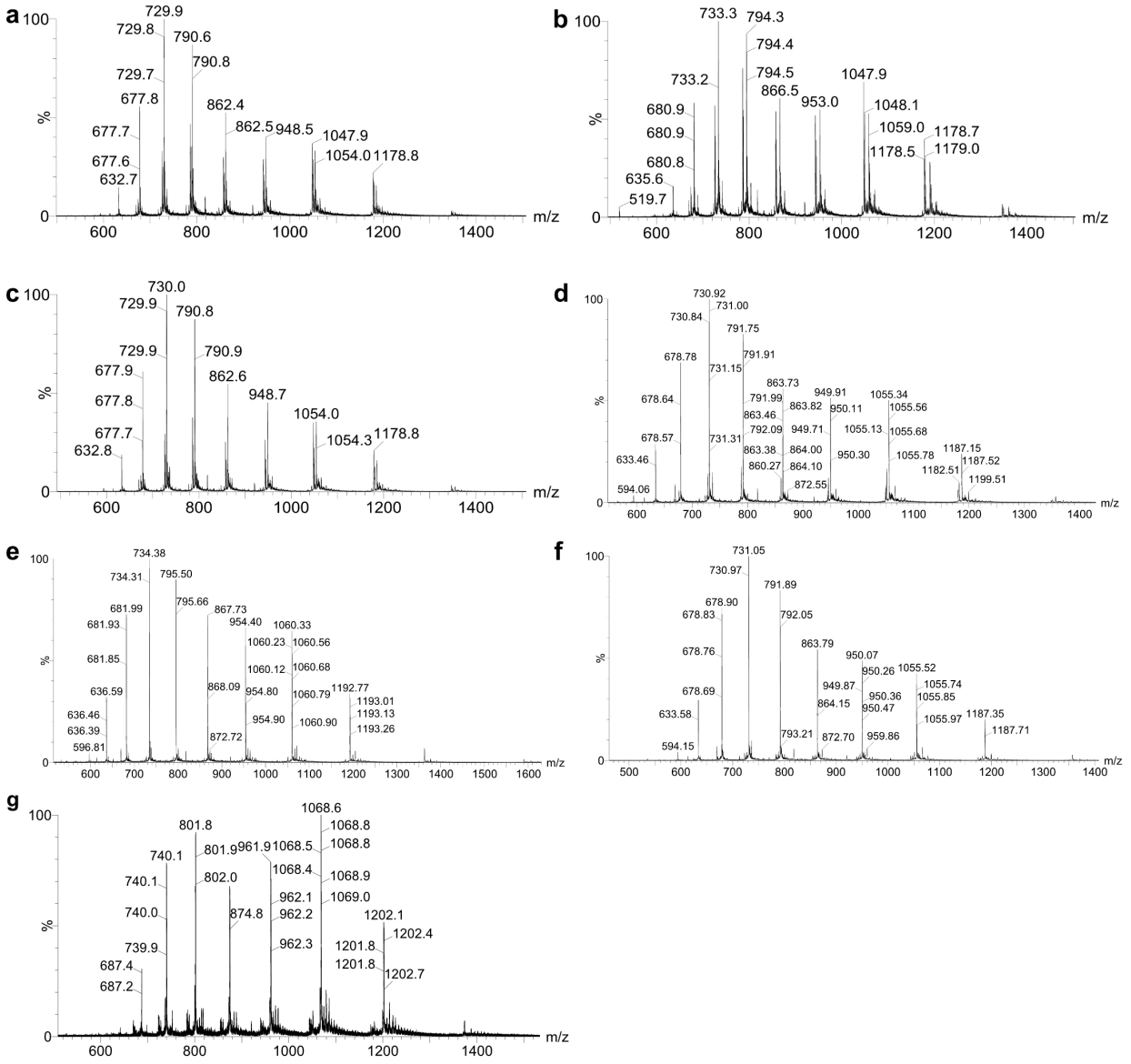

Figure S2. M/Z spectrum of ubiquitin(6FnbY) and ubiquitin(6FmnbY) after rapid functionalization. a) M/Z spectrum of ubiquitin(6FnbY) conjugated with propargylamine. b) M/Z spectrum of ubiquitin(6FnbY) conjugated with 3-Azido-1-propanamine. c) M/Z spectrum of ubiquitin(6FnbY) conjugated with allylamine. d) M/Z spectrum of ubiquitin(6FmnbY) conjugated with propargylamine. e) M/Z spectrum of ubiquitin(6FmnbY) conjugated with 3-azido-1-propanamine. f) M/Z spectrum of ubiquitin(6FmnbY) conjugated with allylamine. g) M/Z spectrum of ubiquitin(6FmnbY) conjugated with 4-aminobenzene-1-sulfonyl fluoride.

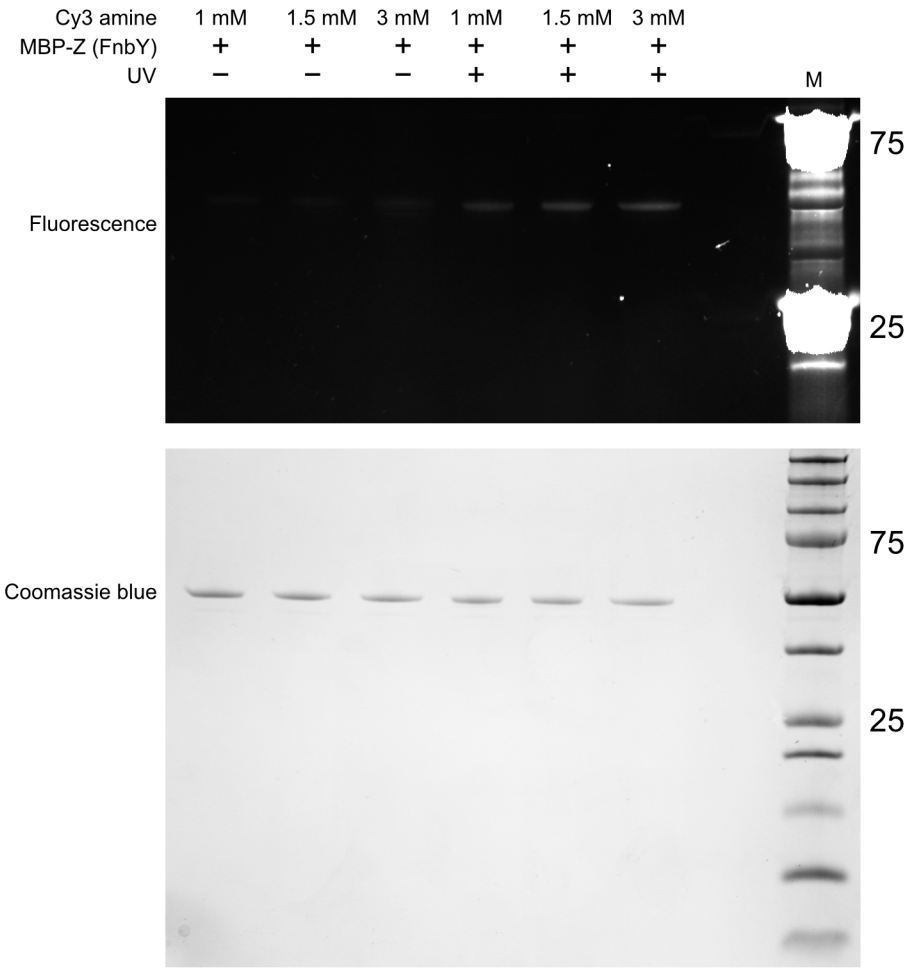

Figure S3. MBP-Z(24FnbY) conjugated with various concentration of Cy3-amine. MBP-Z(24FnbY) were added with 1 mM, 1.5, or 3 mM Cy3-amine, illuminated with UV at 365 nm for 10 min. The Cy3 fluorescence signal can be detected under each concentration only in the presence of UV illumination (upper panel: fluorescent gel; lower panel: SDS-PAGE stained with coomassie blue).

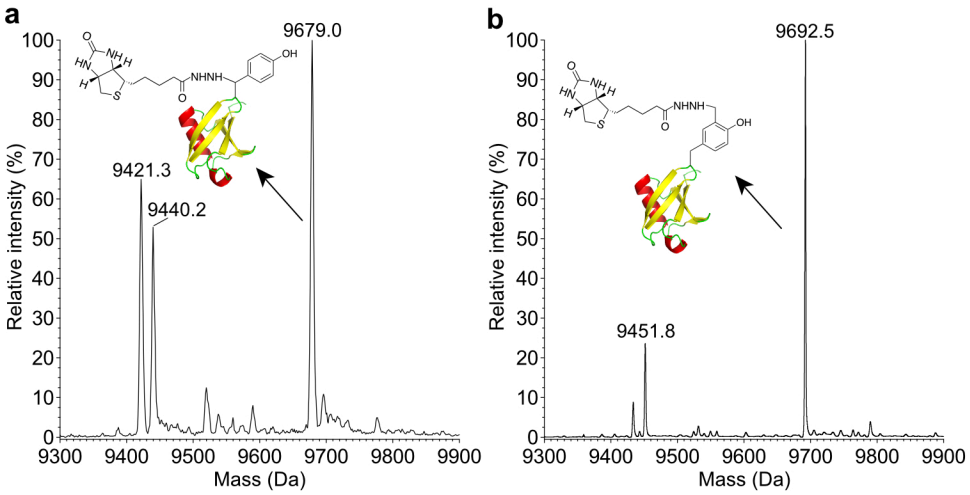

Figure S4. Ubiquitin(6FnbY) and ubiquitin(6FmnbY) conjugated with 1 mM biotin hydrazide. a) Mass spectrum of ubiquitin(6FnbY) conjugated with 1 mM biotin hydrazide. Expected, 9679.0 Da; measured 9679.0 Da. The peak at 9440.2 Da corresponds to conjugation with H_2O, and the peak at 9421.3 Da corresponds to loss of F, suggesting intramolecular Michael addition of nucleophilic residue. b) Mass spectrum of ubiquitin(6FmnbY) conjugated with 1 mM biotin hydrazide. Expected, 9693.0 Da; measured 9692.5 Da. The peak at 9451.8 Da corresponds to conjugation with H_2O.

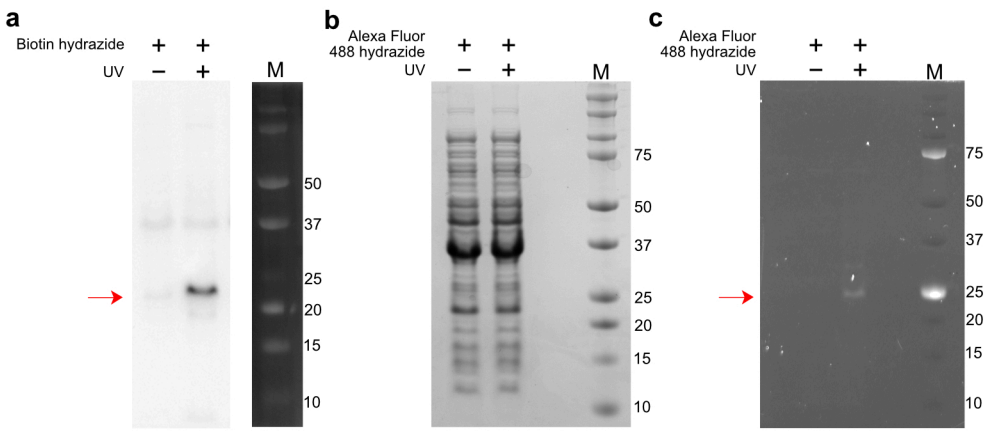

Figure S5. Detection of *E. coli* live cell labeling with biotin hydrazide and Alexa Fluor 488 hydrazide by Western blot and fluorescent imaging. a) Western blot detection of eCPX(5FmnbY) labelled with biotin hydrazide using an anti-biotin antibody. b) Coomassie blue staining of cell lysate of *E. coli* cells expressing eCPX(5FmnbY) and labeled with Alexa Fluor 488 hydrazide. c) b was imaged for Alexa Fluor 488 fluorescence before Coomassie blue staining.

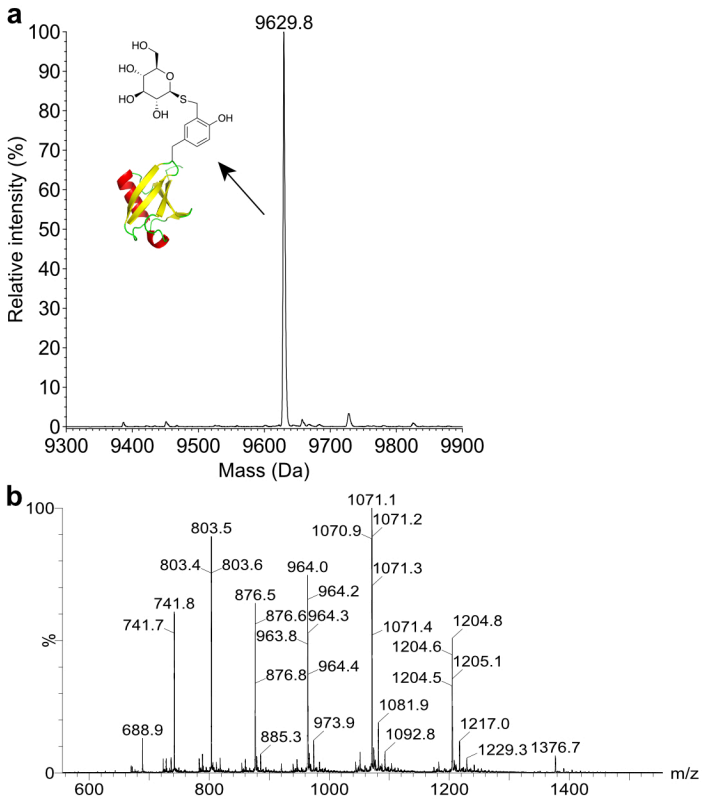

Figure S6. Ubiquitin(6FmnbY) conjugated with 10 mM 1-thio-β-D-glucose. a) Mass spectrum of ubiquitin(6FmnbY) conjugated with 10 mM 1-thio-β-D-glucose. b) M/Z spectrum of ubiquitin(6FmnbY) conjugated with 1-thio-β-D-glucose.

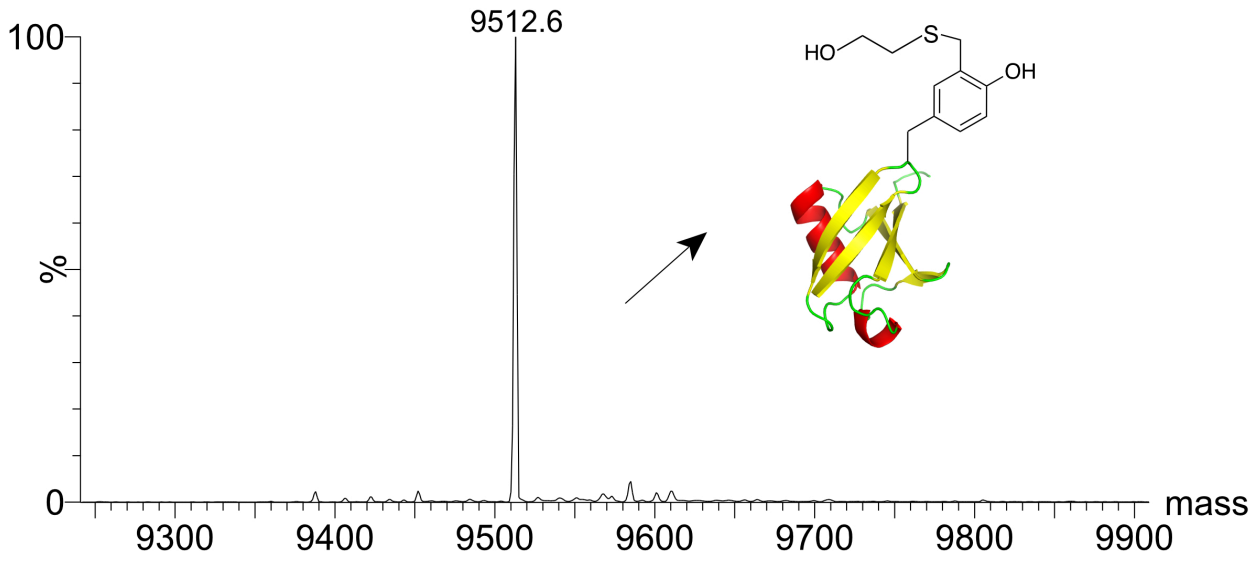

Figure S7. Ubiquitin(6FmnbY) conjugated with 0.1 mM 2-mercaptoethanol. Mass spectrum of 10 μM ubiquitin(6FmnbY) conjugated with 0.1 mM 2-Mercaptoethanol. The calculated MW for conjugated-ubiquitin is 9512.8 Da (observed 9512.6 Da)

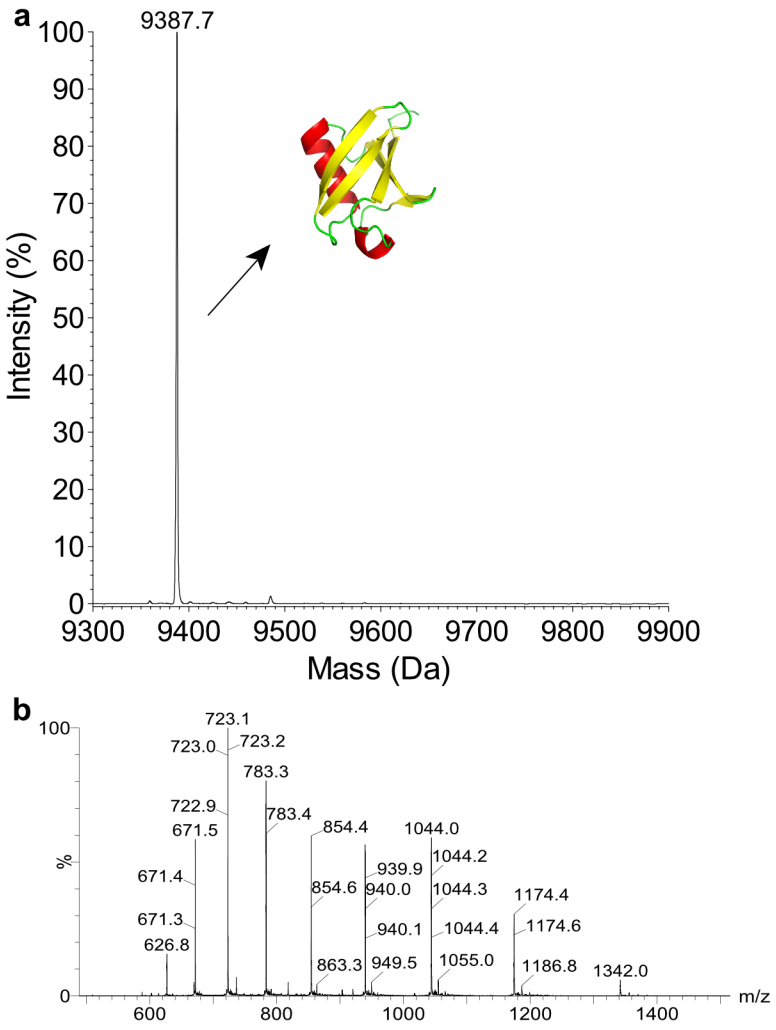

Figure S8. Ubiquitin WT did not conjugate with hydrazide-biotin. a) Mass spectrum of ubiquitin WT conjugated with 5 mM biotin-hydrazide and activated by the high-power UV lamp for 1 min. No labelling was found. The calculated MW for ubiquitin WT is 9387.7 Da (observed 9387.7 Da). b) M/Z spectrum of ubiquitin WT conjugated with biotin-hydrazide.

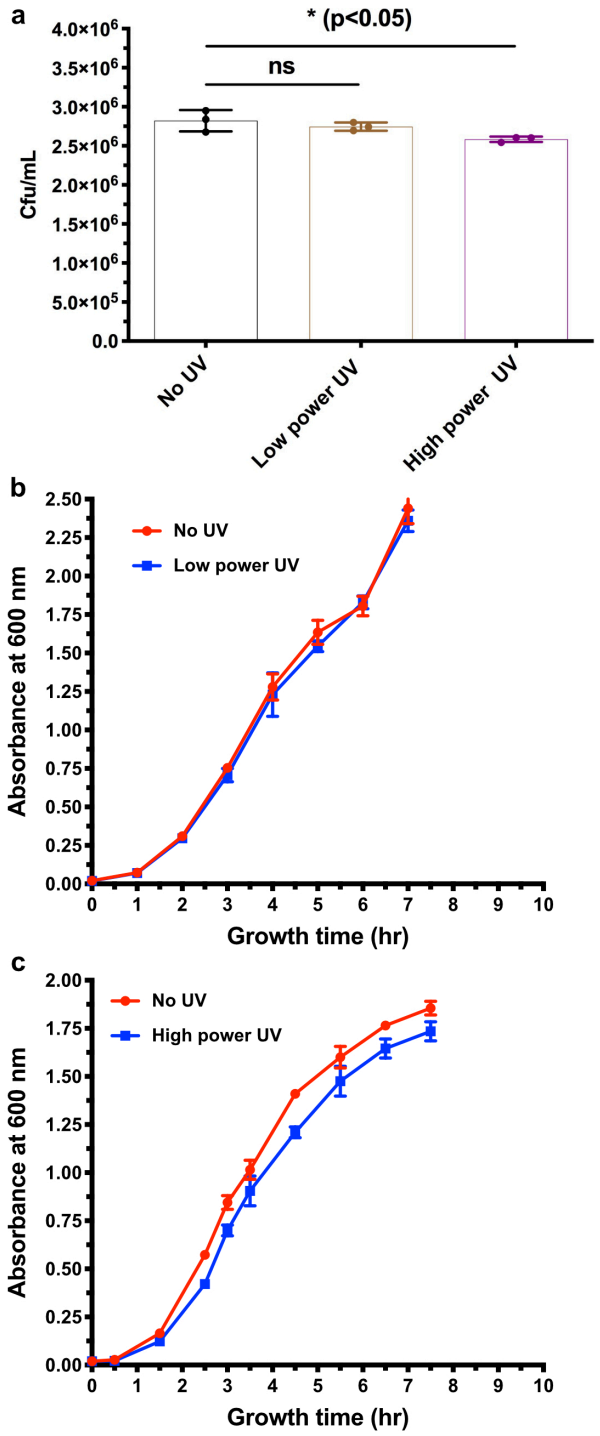

Figure S9. Effect of UV labeling conditions on *E. coli* survival and growth. a) *E. coli* survival on agar plate after exposure to low power UV (365 nm) for 6 min or high power UV (365 nm) for 10 s. Colony forming unit (cfu) was compared with that of *E. coli* cells without UV exposure. b-c) Growth curve of *E. coli* in 2xYT liquid media after low power UV treatment for 6 min, high power UV treatment for 10 s, or no UV treatment. The UV labeling conditions were selected based on results in Figure 5. Error bars represent s.d.; n = 3 biological replicates; ns, not significant; * $P < 0.05$ one-way ANOVA.

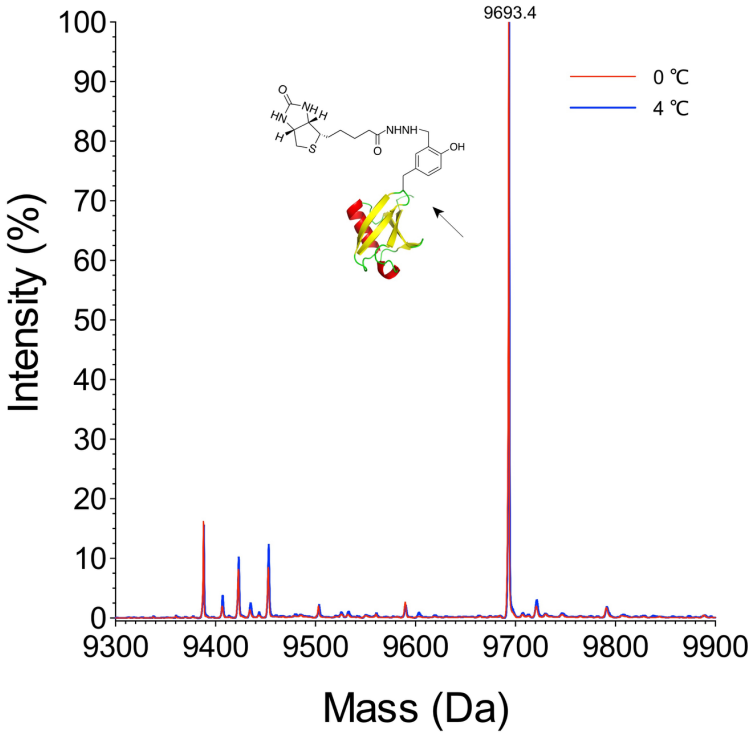

Figure S10. Labeling of Ub(6FmnbY) with biotin-hydrazide at 0 or 4 °C was completed when activated by the high power UV lamp for 10 s. Ub(6FmnbY) was incubated with 5 mM biotin-hydrazide at 0 or 4 °C and activated by the high power UV lamp for 10 s. Samples were analyzed with MS. The observed 9693.4 peak corresponds to the labeled biotin (expected 9693.0 Da).

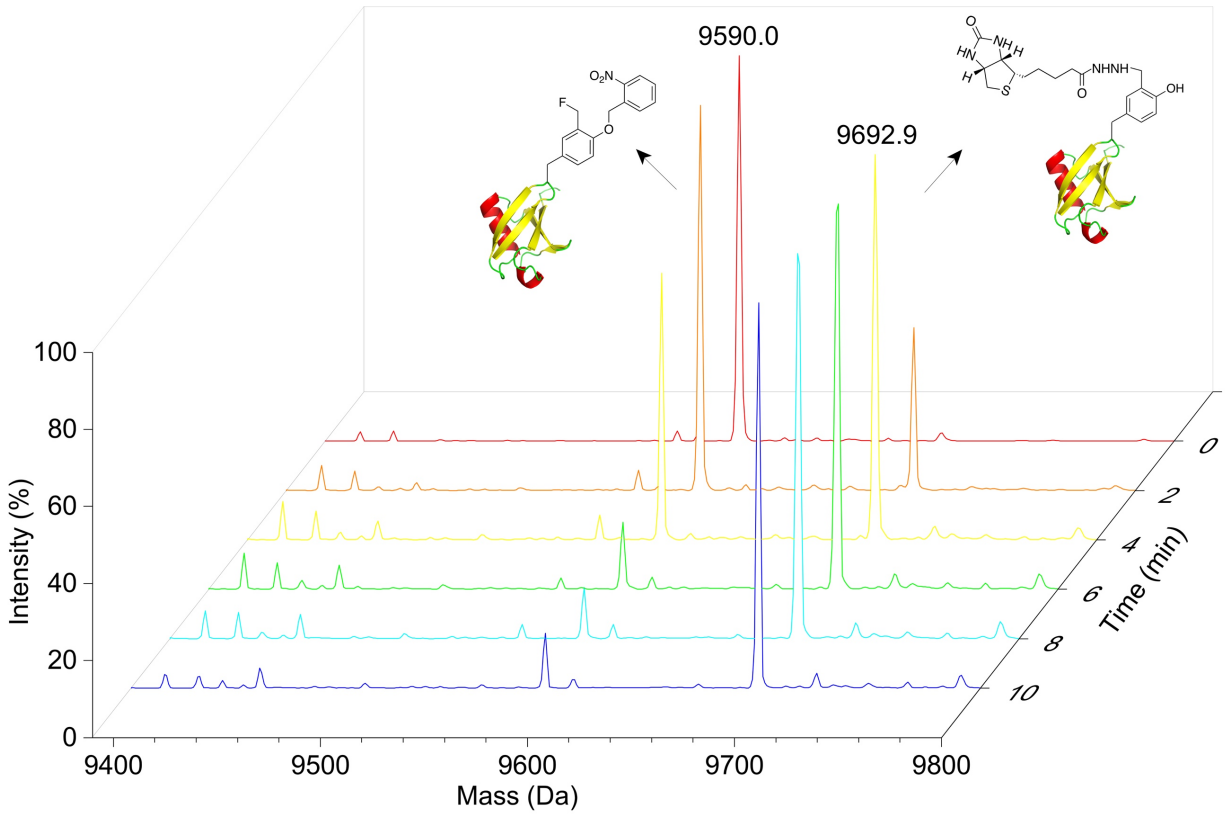

Figure S11. Mass spectra of Ub(6FmnbY) conjugation with biotin-hydrazide (5 mM) at 4 °C with the reaction activated by low power UV lamp for indicated time duration. The peak observed at 9590.0 Da corresponds to Ub(6FmnbY) (expected 9589.8 Da), and the peak observed at 9692.9 Da corresponds to the biotin labeled Ub (expected 9693.0 Da).

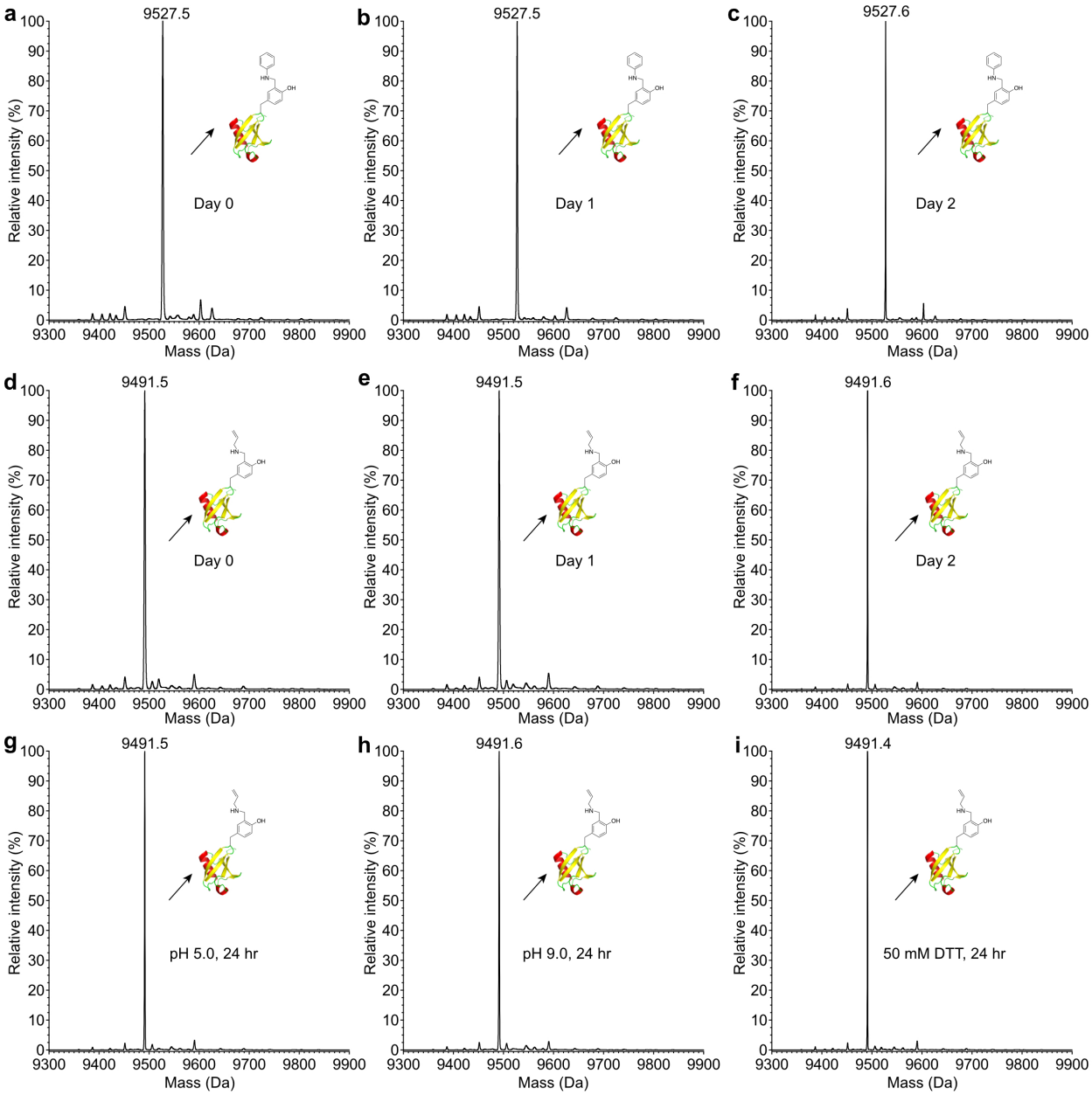

Figure S12. Stability study of ubiquitin(6FmnbY) conjugated with aniline and allylamine. a-f) Ubiquitin(6FmnbY) was conjugated with 50 mM aniline (a-c) or 50 mM allylamine (d-f) in 50 mM sodium phosphate, 200 mM NaCl, pH 7.5. The conjugation product was left on bench at RT for 1 day or 2 days. The stability was analyzed by mass spectrometry. g-h) The pH of the buffer of the conjugated product was also adjusted to 5.0 or 9.0, and the mass spectrometry was run to check the remaining labelling. i) In a separate experiment, the conjugated product was also incubated with 50 mM DTT for 24 h, followed by mass spectrometric analysis.

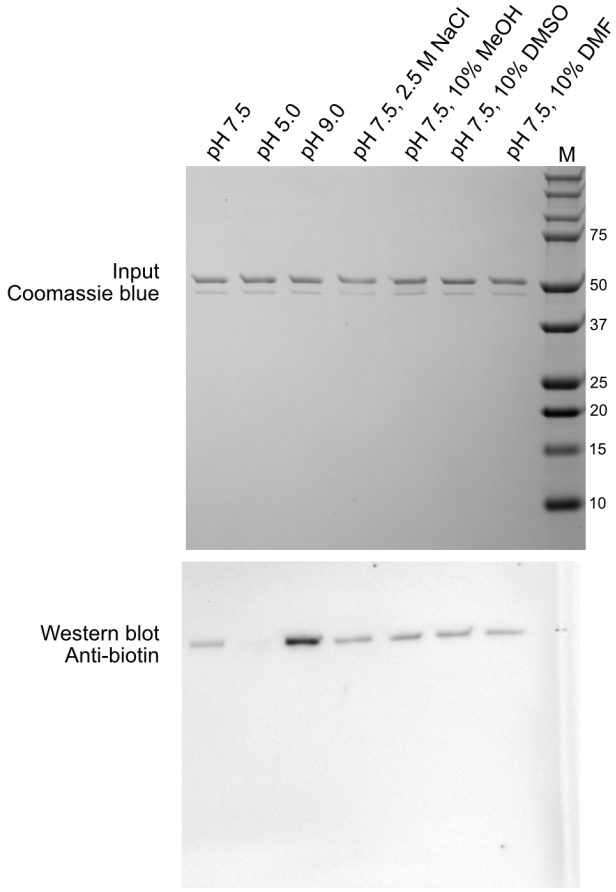

Figure S13. Effect of pH and solvent on amine-QM conjugation. MBP-Z(24FmnbY) was conjugated with 5 mM biotin-(PEG)2-amine in 50 mM sodium phosphate buffer with various pH (7.5, 5.0, or 9.0), high salt (2.5 M NaCl), 10% MeOH, 10% DMSO, or 10% DMF. The reaction was activated for 30 s using the high-power UV lamp, and the labeling was monitored by running Western-blot using an anti-biotin antibody.

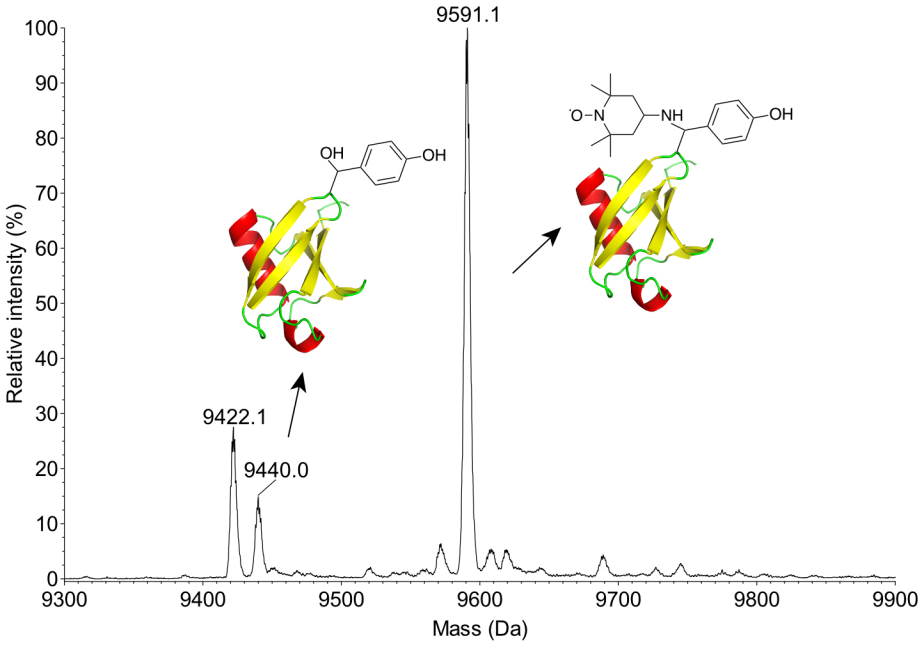

Figure S14. Ubiquitin(6FnbY) conjugated with 4-amino-TEMPO. Mass spectrometric analysis of ubiquitin(6FnbY) conjugation with 30 mM 4-amino-TEMPO upon UV illumination. Expected, 9591.9 Da; measured, 9591.1 Da. The peak of 9440.0 Da corresponds to conjugation with H_2O, and the peak at 9422.1 Da corresponds to loss of F.

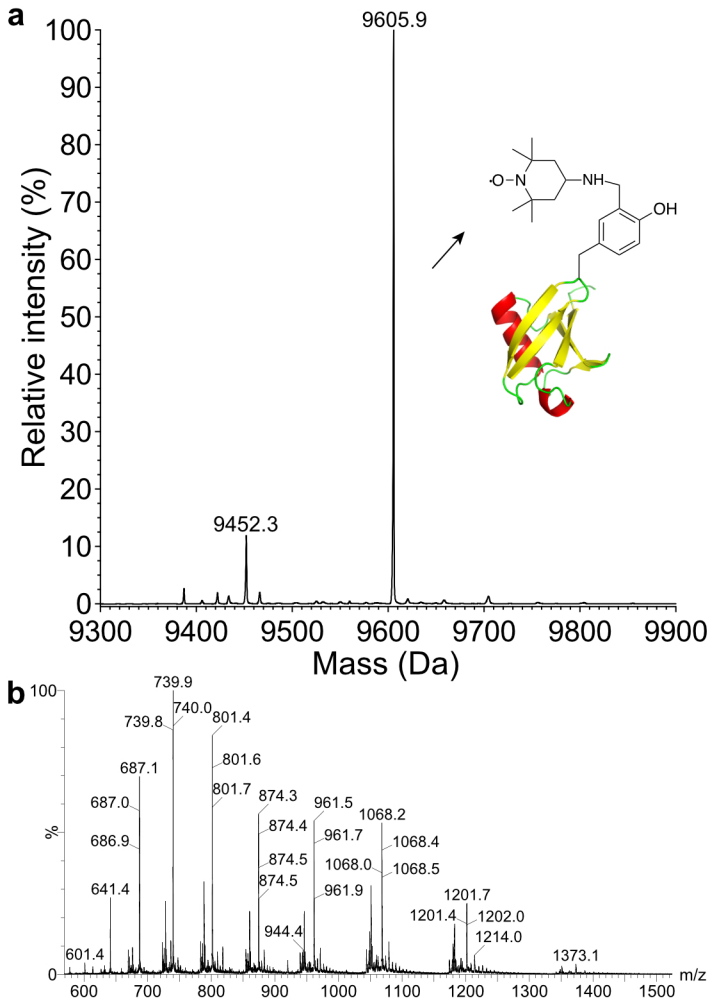

Figure S15. Ubiquitin(6FmnbY) conjugated with 1.5 mM 4-amino-TEMPO. a) Mass spectrometric analysis of ubiquitin(6FmnbY) conjugation with 4-amino-TEMPO (1.5 mM) upon UV illumination. Expected, 9606.0 Da; measured, 9605.9 Da. The peak of 9452.3 Da corresponds to conjugation with H_2O. b) M/Z spectrum of ubiquitin(6FmnbY) conjugated with 1.5 mM 4-amino-TEMPO.

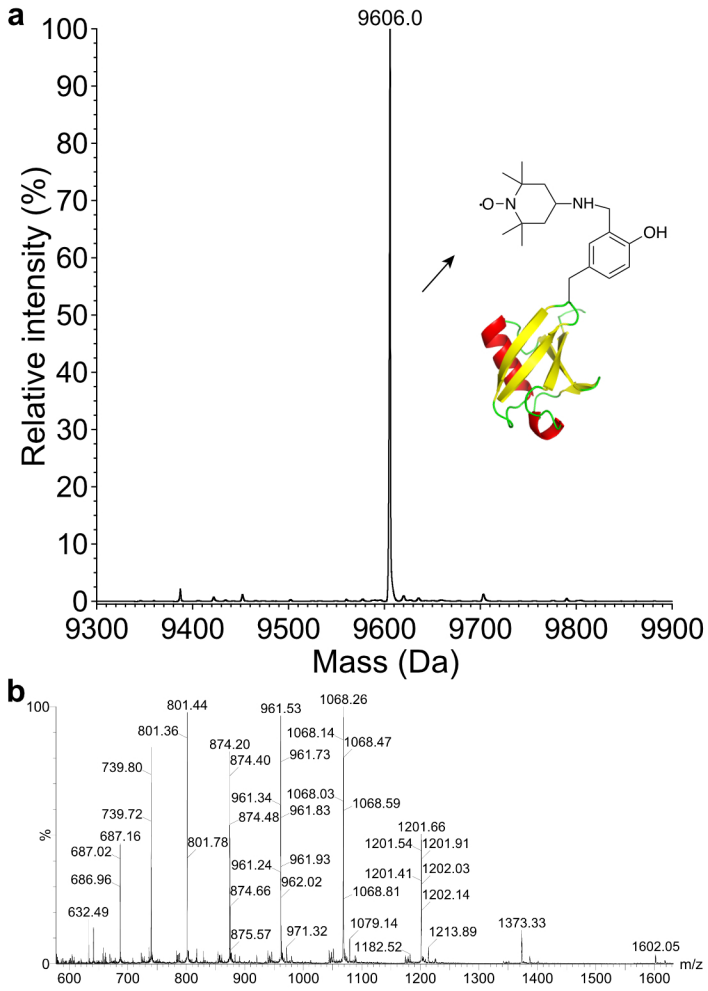

Figure S16. Ubiquitin(6FmnbY) conjugated with 50 mM 4-amino-TEMPO. a) Mass spectrometric analysis of ubiquitin(6FmnbY) conjugation with 4-amino-TEMPO (50 mM) upon UV illumination. Expected, 9606.0 Da; measured, 9606.0 Da. No peak corresponding to water hydrolysis of the photoactivated FmnbY was detected, indicating that higher concentration of 4-amino-TEMPO effectively competes with water and thus preventing water hydrolysis. b) M/Z spectrum of ubiquitin(6FmnbY) conjugated with 50 mM 4-amino-TEMPO.

Supplementary Table 1: Primers used for cloning

Primer	Oligonucleotide sequence (5'→3')
T4L9TAG NdeI For	GTTGTTCATATGAATATATTTGAAATGTTACGTTAGGATGAAGGTCTTAGACTTAAAATC
131TAG Rev	CTACTTTTAGCTAAGTTCTATGCTGCTTCATCCCAG
131TAG For	CTGGGATGAAGCAGCATAGAACTTAGCTAAAAGTAG
T4L HindIII Rev	GTTGTTAAGCTTTTAGTGATGGTGATGGTGATGTAGATTTTTATACGCGTCCCAAG
Ubiquitin_6TAG_NdeI For	GGTGGTCATATGCAGATCTTCGTCTAGACGTTAACCGGTAAAAC
Ubiquitin_WT_NdeI For	GGTGGTCATATGCAGATCTTCGTCAAAACGTTAACCGGTAAAAC
Ubiquitin_WT_HindIII Rev	GTTGTTAAGCTTTTAGTGATGGTGATGGTGATGACCACCTCTTAGTCTTAAGAC
poQMRS SpeI For	GGTGGTACTAGTATGACCGTGAAGTACACCGAC
poQMRS SalI Rev	GTTGTTGTCGACTTAATTGATTTTGGCACCATTC
poQMRS L125F For	CGATGCTGGCGCCAAACTTTTACAGCGTGATGCGTGACCTG
poQMRS L125F Rev	CAGGTCACGCATCACGCTGTAAAAGTTTGGCGCCAGCATCG
poQMRS N166A/V168G For	GAGGAGTTCACCATGCTGGCGCTGGGCGATATGGGTCCGCGCGGCGAC
poQMRS N166A/V168G Rev	GTCGCCGCGCGGACCCATATCGCCCAGCGCCAGCATGGTGAACTCCTC
eCPX NdeI For	GGTGGTCATATGAAAAAAATTGCATGTCTTTCAG
eCPX HindIII Rev	GTTGTTAAGCTTTTAGCTTGCAGTACGGCTTTTC

Supplementary Table 2: Protein yields for FnbY and FmnbY incorporation in comparison to AzF and OnbY.

Uaa	Plasmid-tRNA/aaRS	Target protein (Uaa site)	Yield (mg/L)
FnbY	pEvol-tRNA^{Pyl}/FnbYRS	sfGFP(151TAG)	7.5
FmnbY	pEvol-tRNA^{Pyl}/poQMRS	sfGFP(151TAG)	4.0
FnbY	pEvol-tRNA^{Pyl}/FnbYRS	T4L(9/131TAG)*	4 mg/L
FnbY	pEvol-tRNA^{Pyl}/poQMRS	T4L(9/131TAG)*	2 mg/L
FmnbY	pEvol-tRNA^{Pyl}/poQMRS	MBP-Z(24TAG)	5.7
AzF	pEvol-tRNA^{Tyr}/MjAziRS	sfGFP(151TAG)	8.5
OnbY	pEvol-tRNA^{Pyl}/ONBYRS	sfGFP(151TAG)	3.2

Note: sfGFP, super fold green fluorescent protein; AzF, *p*-azido-phenylalanine; OnbY, *o*-nitrobenzyl-*O*-tyrosine; * C321.ΔA.exp was used for expression and FnbY was incorporated at two sites.

Supplementary Table 3: Summary of calculated and observed molecular masses of the proteins characterized in this study.

Protein	Calculated masses (Da)	Observed masses (Da)
Ubiquitin(6FnbY)	9575.8	9575.1
Ubiquitin(6FnbY)-(Biotin-PEG_2-amine)	9795.2	9794.1
Ubiquitin(6FnbY)-(4-Amino-TEMPO)	9591.9	9591.1
Ubiquitin(6FnbY)-(Propargylamine)	9475.8	9475.0
Ubiquitin(6FnbY)-(3-Azido-1-propanamine)	9520.8	9520.2
Ubiquitin(6FnbY)-(Biotin hydrazide)	9679.0	9679.0
Ubiquitin(6FnbY)-(Allylamine)	9477.8	9477.1
Ubiquitin(6FmnbY)	9589.8	9590.1
Ubiquitin(6FmnbY)-(Biotin-PEG_2-amine)	9809.2	9809.3
Ubiquitin(6FmnbY)-(4-Amino-TEMPO)	9606.0	9606.0
Ubiquitin(6FmnbY)-(Propargylamine)	9489.8	9489.6
Ubiquitin(6FmnbY)-(3-Azido-1-propanamine)	9534.8	9534.8
Ubiquitin(6FmnbY)-(Biotin hydrazide)	9693.0	9692.5
Ubiquitin(6FmnbY)-(Hydrazine)	9466.8	9466.1
Ubiquitin(6FmnbY)-(Allylamine)	9491.8	9491.2
Ubiquitin(6FmnbY)-(D-Galactosamine)	9613.9	9613.7
Ubiquitin(6FmnbY)-(1-Thio-β-D-glucose)	9630.9	9630.8
Ubiquitin(6FmnbY)-(2-Mercaptoethanol)	9512.8	9512.6
Ubiquitin(6FmnbY)-(Mertansine)	10173.0	10171.9
Ubiquitin(6FmnbY)-(Aniline)	9527.8	9527.5
Ubiquitin(6FmnbY)-(4-aminobenzene-1-sulfonyl fluoride)	9609.9	9609.4
Ubiquitin WT	9387.7	9387.7

Supplementary Table 4: Data collection and refinement statistics.

	T4L (9/131FnbY)-(4-Amino-TEMPO [a]
Data collection	
Space group	P 3_2 2 1
Cell dimensions	
a, *b*, *c* (Å)	59.407, 59.407, 95.489
α, β, γ (°)	90, 90, 120
Resolution (Å)	31.83 – 1.5 (1.554 – 1.5) [b]
R_{merge}	0.0093 (0.0434)
$I / \sigma I$	40.17 (14.34)
Completeness (%)	99.97 (100.00)
Redundancy	2.0 (2.0)
Refinement	
Resolution (Å)	31.83 – 1.5
No. reflections	31894 (3148)
R_{work} / R_{free}	0.1684/0.1839
No. atoms	
Protein	1319
Ligand/ion	36
Water	201
B-factors	
Protein	13.28
Ligand/ion	31.37
Water	25.77
R.m.s. deviations	
Bond lengths (Å)	0.006
Bond angles (°)	0.87

[a] One crystal was used.
[b] Values in parentheses are for highest-resolution shell.

Supplementary references

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