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Supporting Information

Artificially Linked Ubiquitin Dimers Characterised Structurally and Dynamically by NMR Spectroscopy

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Expression and purification of CxUb (x = 11; 48)

cDNAs encoding human CxUb (replacement of Lys at position x = 11 or 48 by Cys) were cloned into the pET3a vector by *Nde* I/*BamH* I double digest and T4 DNA ligase ligation. *E. coli* BL21 (DE3) transformed with pET3a-CxUb were cultured in LB medium containing 100 mg/L carbenicillin at 37 °C overnight. Overnight cultures were diluted with pre-warmed fresh LB/carbenicillin to OD_{600} = 0.1. At OD_{600} = 0.6, expression of CxUb was induced by addition of 1 mM IPTG and the culture was incubated for another 5 h at 37 °C. Cells were pelleted, resuspended in 20 mM sodium acetate (pH 4.5), and lysed by sonication. The lysate was clarified by centrifugation at 18000 g for 30 min. The supernatant was heated at 65 °C 20 min followed by centrifugation (again g value). The supernatant was cooled down to 4 °C and adjusted to pH 4.5. Then, CxUb was purified by cation exchange chromatography (HiTrap SP HP, 1 mL, GE) on an ÄKTA FPLC system using a linear salt gradient (buffer A = 20 mM NaOAc (pH 4.5), buffer B 20 mM NaOAc (pH 4.5), 1 M NaCl) . The purified CxUb molecules were dialysed against 20 mM Tris-HCl (pH 7.5). Protein concentration was measured by a BCA assay (ThermoFisher).

Modification of CxUb with propargyl acrylate (PA)

Samples containing 100 µM CxUb (x = 11; 48) were treated with 10 eq. TCEP in 20 mM Tris-HCl (pH 7.5) at 37 °C for 0.5 h. Then, CxUb/TCEP was diluted to 20 µM, incubated with 100 eq. propargyl acrylate (PA) in 20 mM Tris-HCl (pH 7.5)/MeCN = 9:1 at 25 °C and incubated by shaking at 180 rpm for 2 h. To determine the efficiency of PA modification of CxUb, 20 µL aliquots were withdrawn and reacted with 25 eq. fluorescein-5-maleimide (F5M) in the dark at 37 °C for 20 min. As control, the same amount of untreated CxUb was incubated with F5M under the same conditions. The samples were analyzed by SDS-PAGE and visualized under UV-light. The same gel was stained by Coomassie blue. To remove potential excess of PA, reaction mixtures were transferred to dialysis tubes (3500 MWCO) and dialyzed sequentially against 20 mM Tris-HCl (pH 7.0)/MeOH = 1:1, 20 mM Tris-HCl (pH 7.0)/MeOH = 4:1, and 20 mM Tris-HCl (pH 7.0). Precipitated proteins were dissolved in 20 mM Tris-HCl (pH 7.0) containing 6 M guanidine hydrochloride, refolded via dialysis against progressively decreasing concentrations of guanidine hydrochloride (i.e. 3 M, then 1.5 M), and finally against 20 mM Tris-HCl (pH 7.0). PA-modified proteins (CxUb-PA) were lyophilized in aliquots and stored at 4 °C. Before use, the required amount of lyophilisate was dissolved in water and quantified by BCA assay (ThermoFisher).

Expression and purification of Aha75Ub

The cDNA encoding human G75M_Ub (replacement of Gly75 by Met; deletion of Met1 and Gly76) was cloned into the pGEX2TK vector by *BamH* I/*EcoR* I double digest and T4 DNA ligase ligation. Methionine auxotrophic *E.coli* B834 (DE3) transformed with pGEX2TK-G75M_Ub were cultured in LB medium containing 100 mg/L carbenicillin at 37 °C overnight. The overnight culture was diluted with NMM medium containing 0.06 mM methionine and 100 mg/L carbenicillin to an OD_{600} = 0.1 and grown at 37 °C to an OD_{600} = 1.3. Cells were harvested and resuspended in fresh NMM supplemented with 0.5 mM azidohomoalanine (Aha). After incubation at 37 °C for 30 min, expression of GST-

Aha75Ub was induced by addition of 1 mM IPTG. Cells were grown at 25 °C overnight to an OD_{600} ~ 2.7. Cells were pelleted, resuspended in 1x PBS buffer containing 1% triton X-100 and lysed by sonication. The lysate was clarified by centrifugation (g value) and he supernatant incubated with glutathione agarose beads (company) at 4 °C for 5 h. The beads were washed three times with 1x PBS and incubated with 10 units of thrombin at room temperature overnight. The released Aha75Ub was collected with 9x500 µL of 1x PBS. The fractions were analyzed by SDS-PAGE and quantified by BCA assay.

Electrospray-ionization mass spectrometry analysis

Proteins (C11Ub-PA, C48Ub-PA, Aha75Ub, $Ub_{2\text{-}PA}^{11}$, or $Ub_{2\text{-}PA}^{48}$) were analysed by direct infusion on a LTQ-Orbitrap Discovery mass spectrometer (Thermo Fisher). Mass spectrometric data were evaluated using the ProMass Deconvolution 2.8 (Thermo Scientific) software. Respective spectrograms are shown in Figure S8.

Preparation of ^{15}N-C48Ub and ^{15}N-$Ub_{2\text{-}PA}^{48}$

E.coli BL21 (DE3) transformed with pET3a-K48C_Ub were cultured in 50 mL of LB/carbenicillin and incubated overnight at 37 °C and 150 rpm. An aliquot of the overnight culture was diluted with 50 mL ^{15}N-M9 Medium (22 mM KH_2PO_4, 8.55 mM NaCl, 1 mM $MgSO_4$, 1.62 µM H_3BO_3, 134 µM EDTA, 31 µM $FeCl_3$, 0.42 µM $CoCl_2$, 0.76 µm $CuCl_2$, 6.2 µM $ZnCl_2$, 0.3 Mm $CaCl_2$, 0.081 µM $MnCl_2$, 33.7 mM Na_2HPO_4, 9.35 mM $^{15}NH_4Cl$, 22.2 mM glucose, 1 mg/L biotin, 1 mg/L thiamine, 100 mg/L carbenicillin) to an OD_{600} = 0.1 and the pre-culture was incubated overnight at 37 °C and 150 rpm. One liter prewarmed ^{15}N-M9 Medium was inoculated with the pre-culture to an OD_{600} = 0.1 and incubated at 37 °C and 150 rpm until stationary growth was reached (OD_{600} = 0.5 – 0.7). Protein expression was induced with 1 mM IPTG followed by incubation overnight at 25 °C and 150 rpm. Cells were harvest by centrifugation (provide g value, rpms are not telling if the rotor used is not indicated), resuspended in lysis buffer (20 mM NaOAc, pH 4) and lysed by sonification. After centrifugation at 18000 g for 30 min, the supernatant was heated to 65 °C for 20 min. Upon centrifugation at 18000 g for 30 min, K48C_Ub was purified via cation exchange chromatography (ÄKTA FPLC) with a linear salt gradient (buffer A: 25 mM NaOAc, pH 4.5; buffer B: 25 mM NaOAc, pH 4.5, 1 M NaCl,). Fractions containing ^{15}N-C48Ub were pooled and dialysed against 20 mM Tris-HCl, pH 7.5 at 4 °C overnight. Protein concentration was determined by BCA assay. For NMR analysis, an aliquot of ^{15}N-C48Ub was dialysed against 20 mM Na_3PO_4, pH 6.8 at 4 °C overnight.

PA-modification of ^{15}N-C48Ub and the synthesis of ^{15}N-$Ub_{2\text{-}PA}^{48}$ were performed as described above. After click reaction, samples were dialysed against 10 mM Tris-HCl, pH 7.5 at 4 °C, and the dimers were isolated via gel filtration (buffer: 25 mM Tris-HCl, pH 7.5, 1 M NaCl). Fractions containing pure ^{15}N-$Ub_{2\text{-}PA}^{48}$ were pooled and concentrated via Amicon Ultra 4 mL centrifugal devices (molecular mass cutoff 3000 Da). The concentrated samples were prepared for NMR analysis by dialysis against 20 mM Na_3PO_4, pH 6.8 at 4 °C overnight. The concentration of the protein was determined by BCA assay.

Western blot analysis

Proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked by 1x Roti-Block solution (Roth) at room temperature for 1 h. Then, the proteins were probed with a K48-linkage specific antibody (concentration of 1 µg/mL) (Millipore), which was subsequently decorated with an HRP conjugated secondary antibody (Jackson ImmunoResearch). Finally, antibody-bound proteins were detected by chemiluminescence (ECL reagent, Thermal Scientific).

E6AP auto-ubiquitylation assay

Auto-ubiquitylation assays were performed in 20 µl reaction volumes containing 100 ng UBA1, 125 ng UBCH5b, 800 ng E6AP, 2 mM ATP, 2 mM $MgCl_2$, 1 mM DTT, and 1 µg of wild-type Ub monomer, $Ub_{2\text{-}PA}^{11}$ or $Ub_{2\text{-}PA}^{48}$ in 25 mM Tris-HCl (pH 7.5), 50 mM NaCl. Reactions mixtures were incubated at 30 °C for 90 min and subsequently analyzed by SDS-PAGE followed by Commassie blue staining.

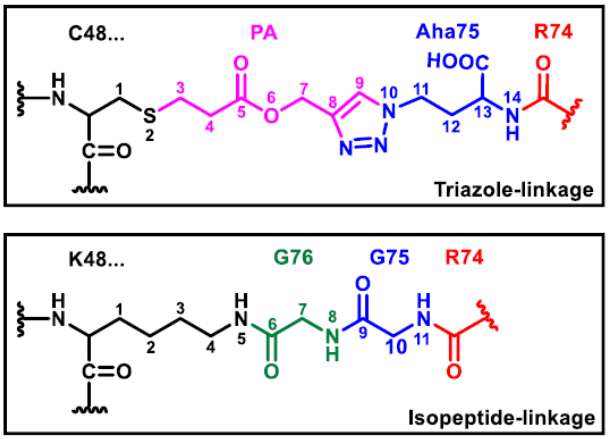


Figure S1. Chemical structures of the triazole linkage (C48-PA---Aha75) and the isopeptide linkage present in the artificial and the native Ub dimer, respectively. Note that any Lys of Ub can be replaced by Cys for Ub dimer formation.

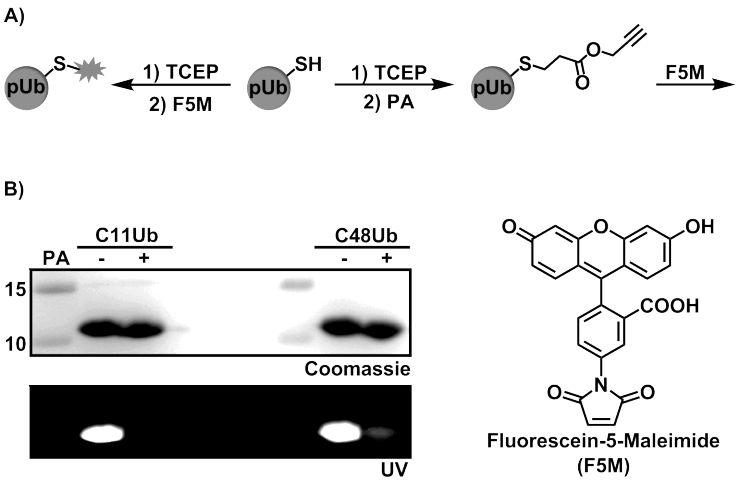


Figure S2. Generation of the alkyne-functionalized Ub unit (CxUb-PA). A) Schematic of the modification of CxUb (x = 11; 48) by propargyl acrylate (PA) and fluorescein-5-maleimide (F5M). B) SDS-PAGE analysis of the F5M labelling of PA-treated (+) and untreated (-) CxUb. The gel was visualised under UV light (λ = 302 nm, lower panel) followed by Coomassie blue staining (upper panel).

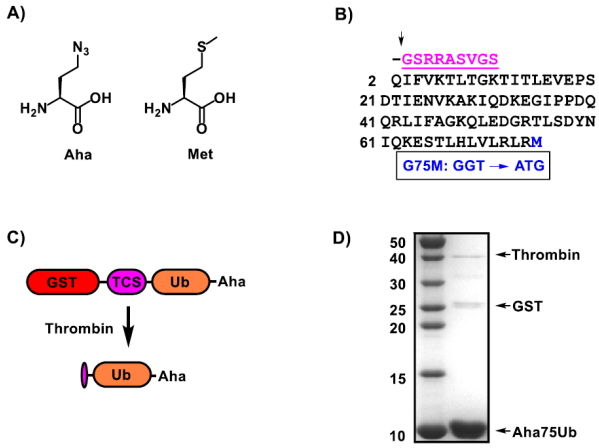


Figure S3. Generation of the azide-functionalized Ub unit (Aha75Ub). A) Chemical structures of azidohomoalanine (Aha) and methionine (Met). B) Sequence of Ub G75Aha as used in the present study. The arrow indicates the thrombin cleavage site. The remaining N-terminal extension is underlined. C) Scheme of the fusion protein GST-TCS-Aha75Ub and thrombin cleavage. D) SDS-PAGE analysis of Aha75Ub followed by Coomassie blue staining. GST, glutathione S-transferase. TCS, thrombin cleavage site.

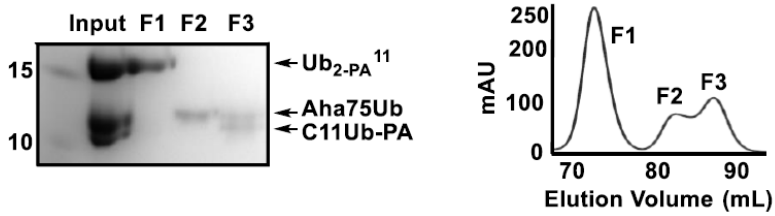


Figure S4. SDS-PAGE analysis of the preparation (Input) of Ub dimer $Ub_{2\text{-}PA}^{11}$ (left; Coomassie blue staining) and the corresponding fractions (F) obtained by size exclusion chromatography (right). The chromatogram (determined at a wavelength of 214 nm) indicates the separation of Ub dimer (F1) and unreacted monomers (F2 and F3).

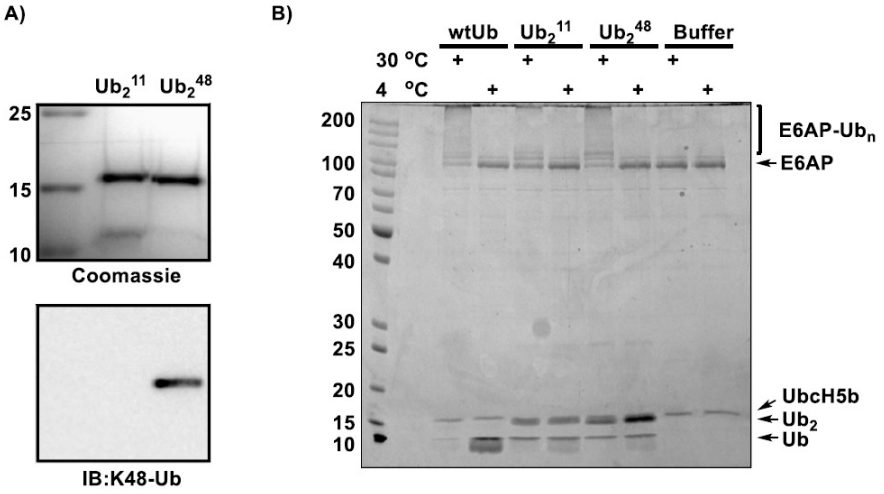
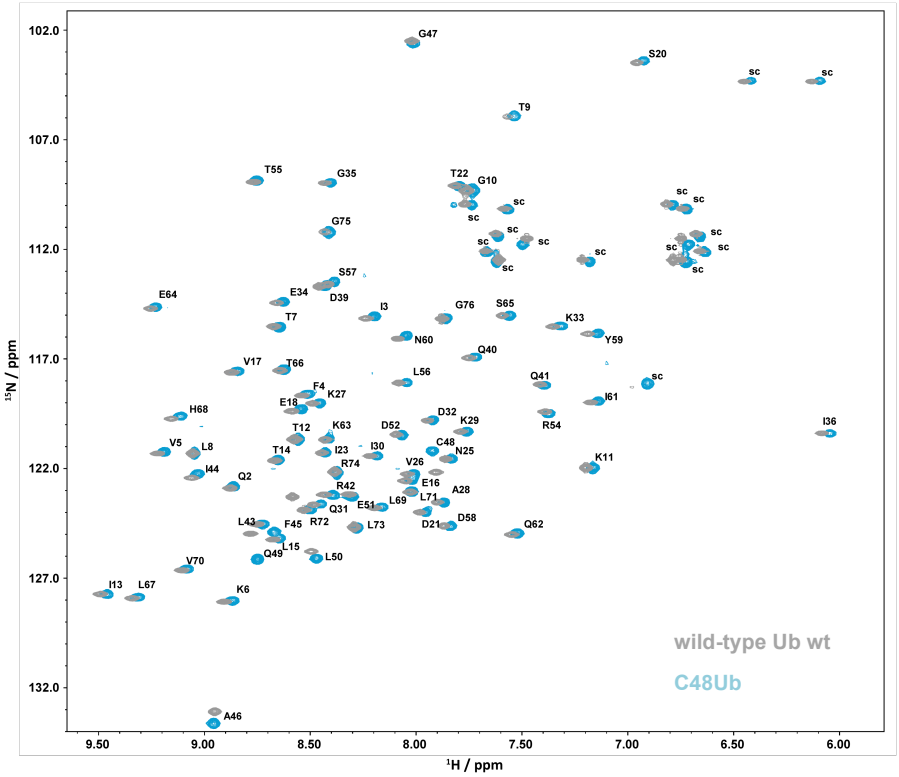
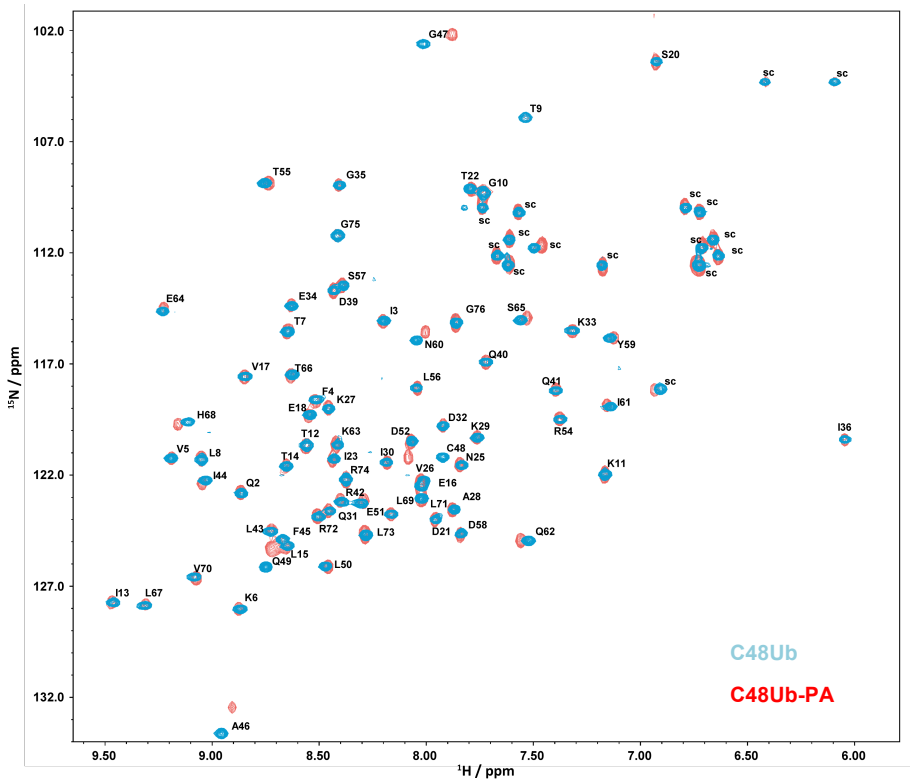


Figure S5. A) Western blot analysis (bottom) of synthesized Ub dimers ($Ub_{2\text{-}PA}^{11}$ and $Ub_{2\text{-}PA}^{48}$) using a K48 Ub chain-specific antibody (IB:K48-Ub). The same samples were resolved by SDS-PAGE analysis followed by Coomassie blue staining (top). B) SDS-PAGE analysis of an E6AP-auto-ubiquitylation assay performed for 90 minutes at two temperatures 4 °C and 30 °C, respectively. w/o Ub, reaction in the absence of ubiquitin. As shown, Ub dimers were used by E6AP for auto-ubiquitylation, albeit $Ub_{2\text{-}PA}^{48}$ somewhat more efficiently than $Ub_{2\text{-}PA}^{11}$. This result shows that the chemistry used for dimer formation does not affect Ub conformation with respect to its ability to be activated and conjugated by the Ub conjugation machinery.

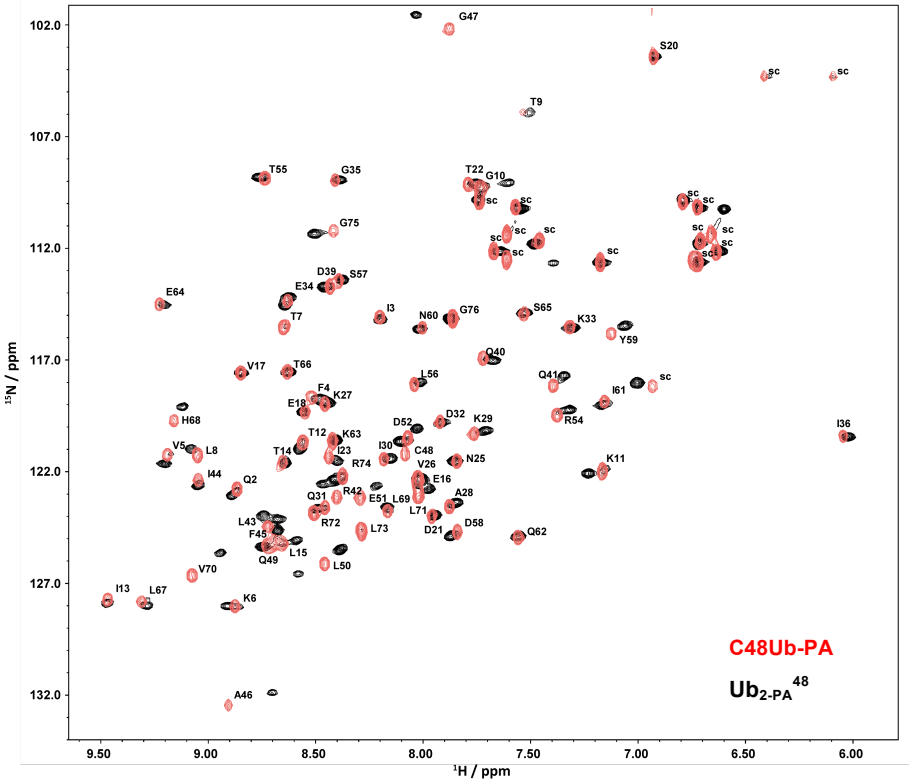
A)


wild-type Ub wt
C48Ub
^{15}N / ppm
^{1}H / ppm

B)


C48Ub
C48Ub-PA
^{15}N / ppm
^{1}H / ppm

C)



D)

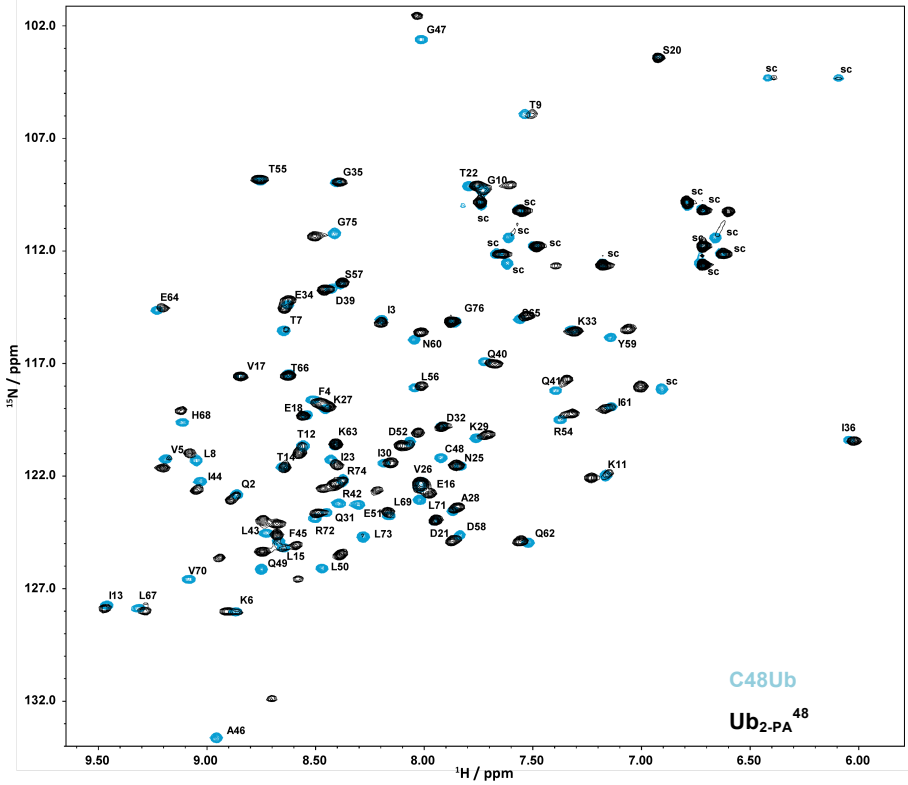


Figure S6. Two-dimensional ^{1}H-^{15}N HSQC spectra acquired for different variants of monomeric and dimeric ubiquitin (see labelling in respective panel). Signals originating from the side chains of arginine, asparagine

and glutamine residues are labelled with sc. All spectra have been measured in 20 mM Na_3PO_4, pH 6.8, 5 % (v/v) D_2O at T = 298 K.

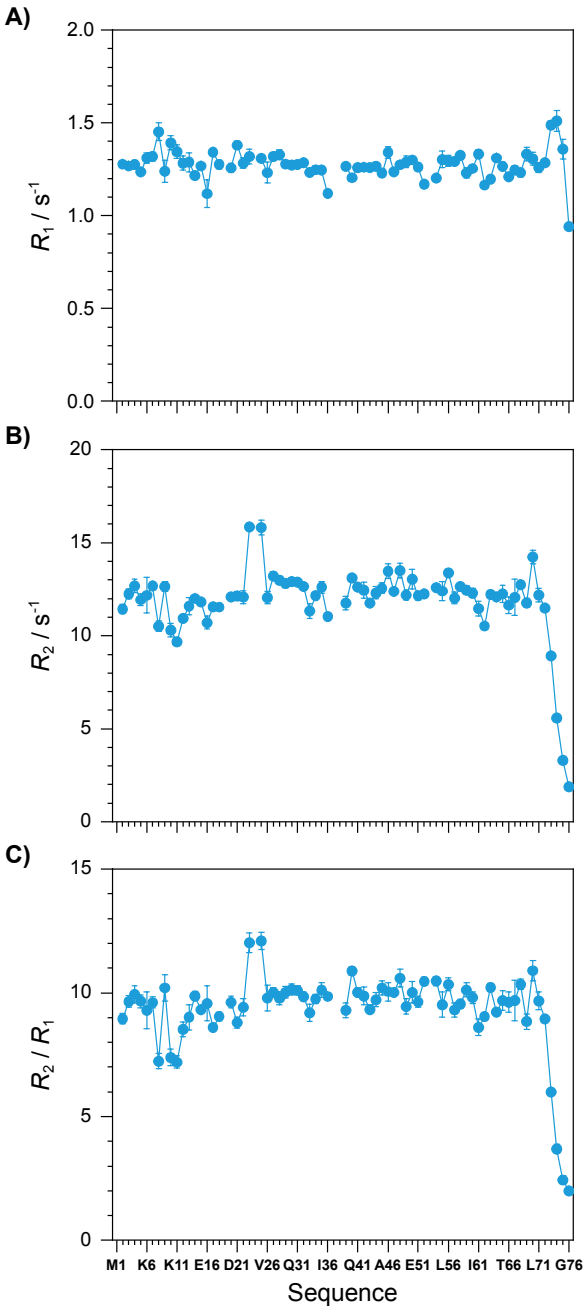
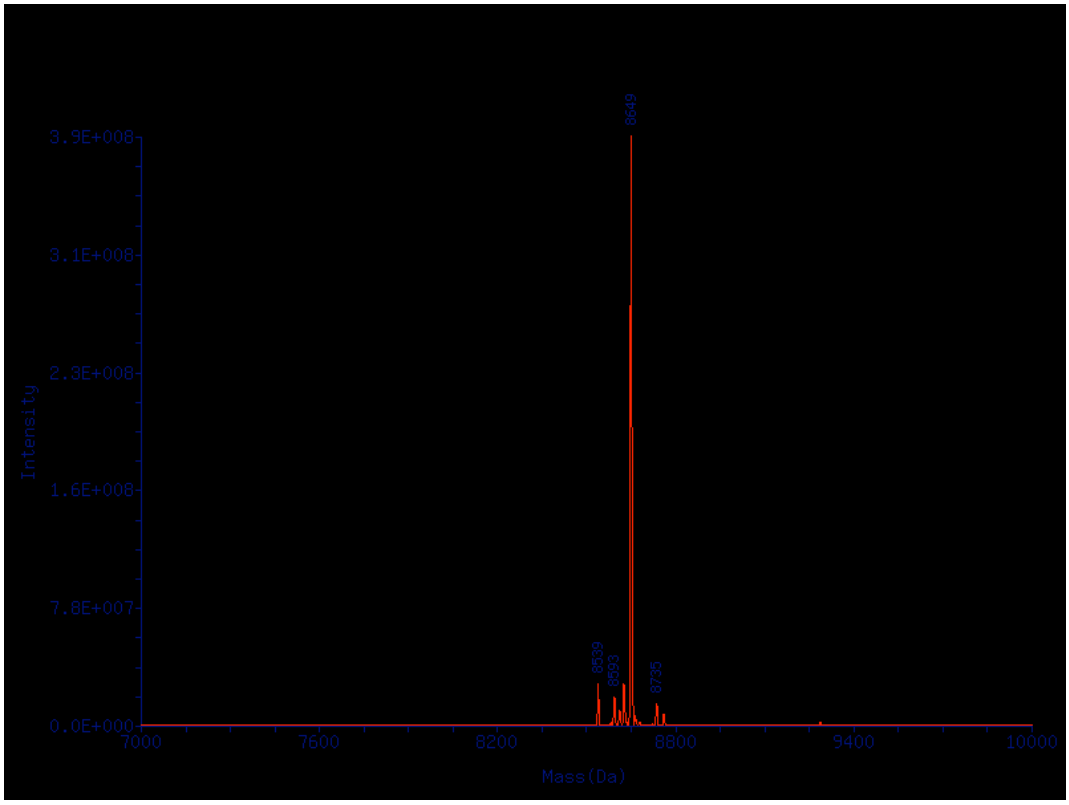


Figure S7. A) ^{15}N longitudinal (R_1) and B) transverse relaxation rate constants (R_2) for each residue of the proximal unit of $Ub_{2\text{-}PA}$[48] determined at T = 298 K. C) Ratio of relaxation rate constants R_2 and R_1.

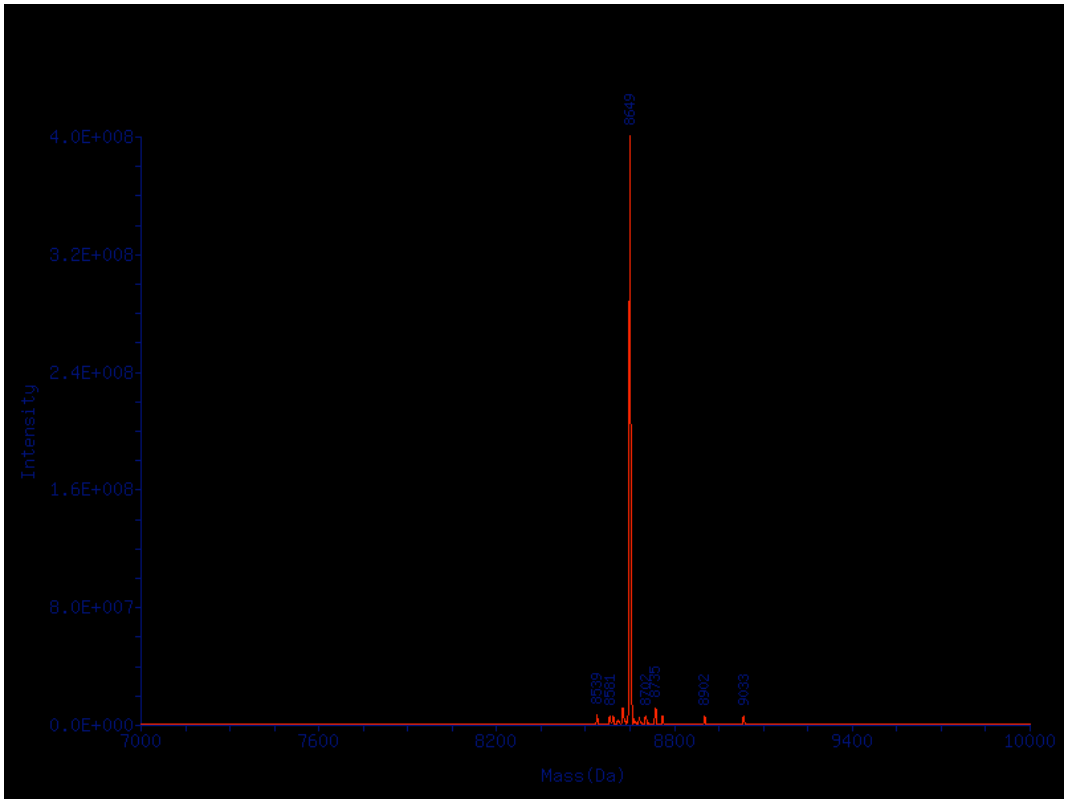
A)

C11Ub-PA



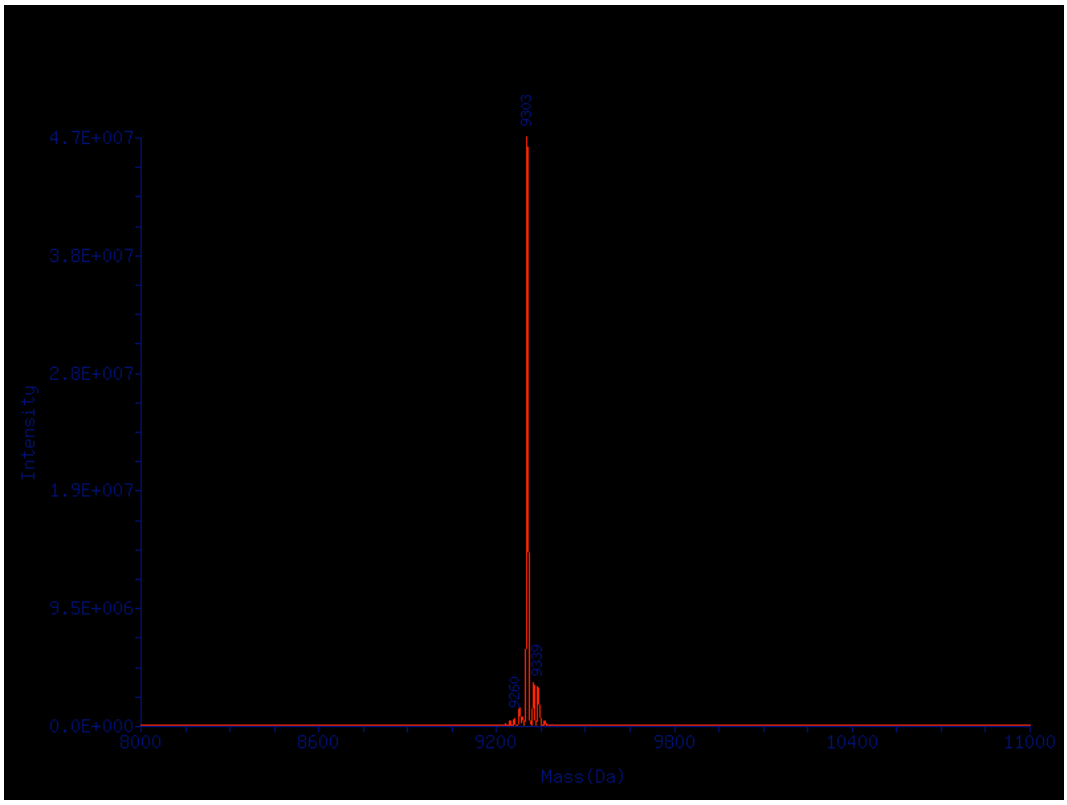
B)

C48Ub-PA



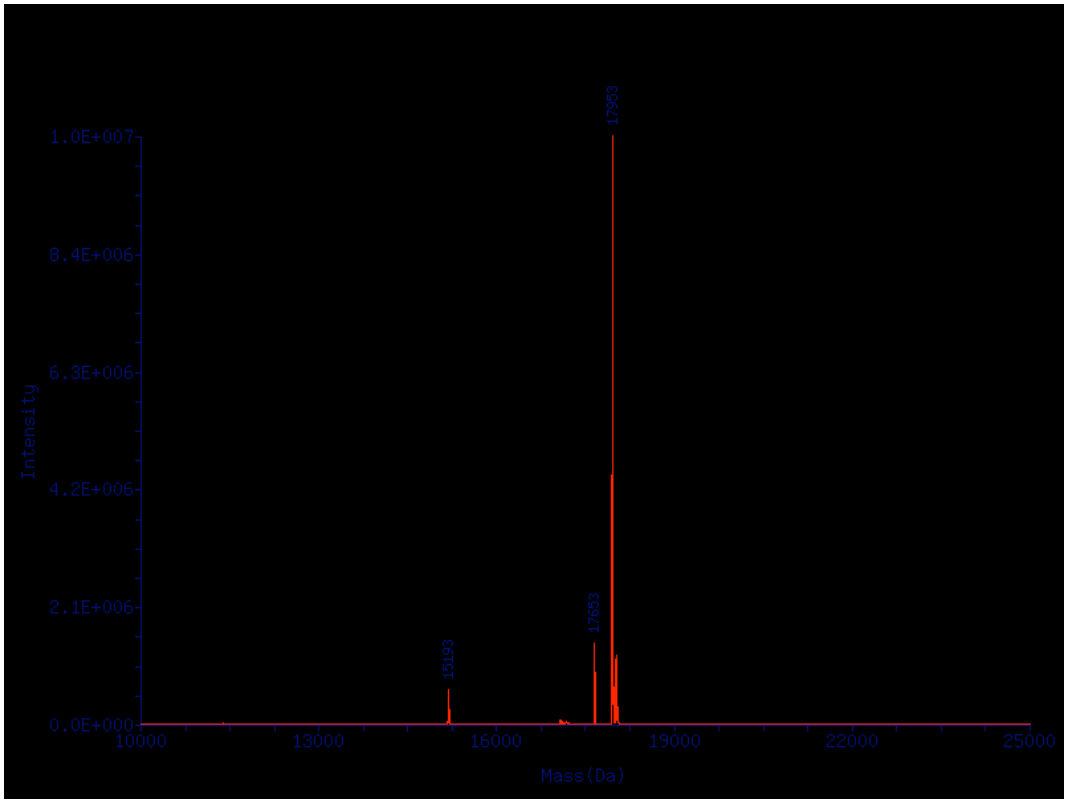
C)

Aha75Ub



D)

$Ub_{2\text{-}PA}$[11]



E)

$Ub_{2\text{-}PA}^{48}$

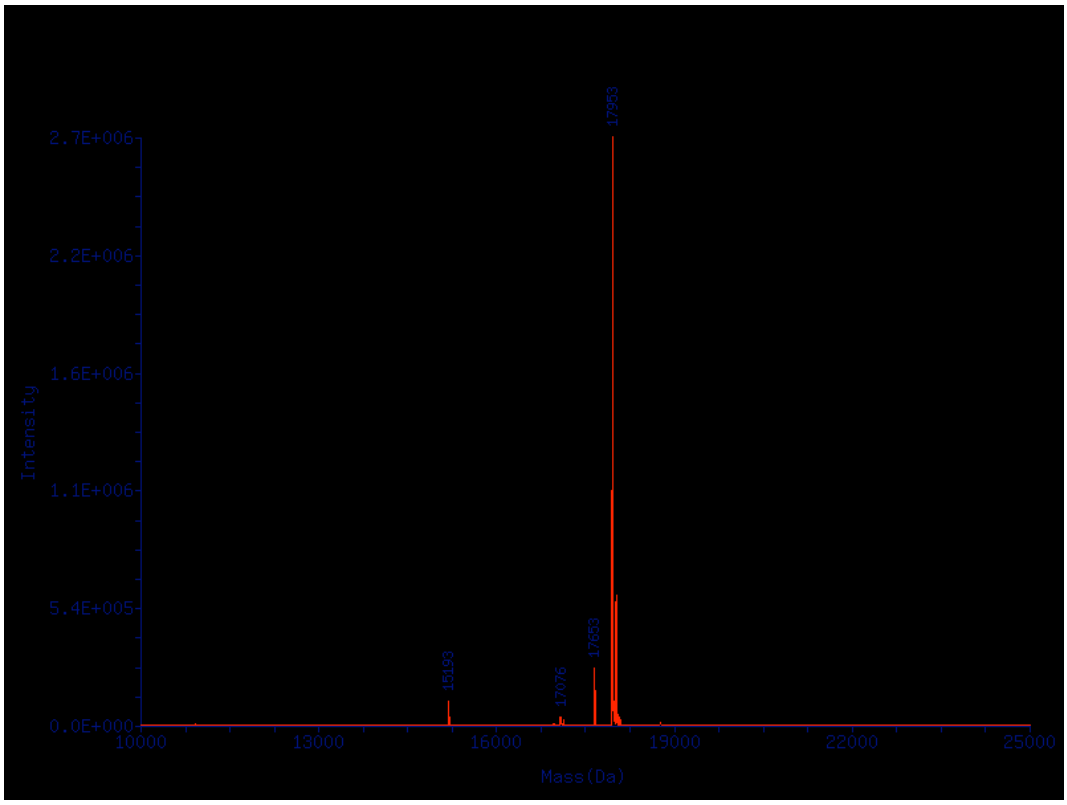


Figure S8. Mass spectrometry analysis of ubiquitin variants investigated in the present study.