Supporting Information

Synthesis of Defined Ubiquitin Dimers

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Detailed Experimental Procedures

Materials

All chemicals were of analytical grade and purchased from Sigma-Aldrich, ABCR or Bachem. NMR spectra were acquired on a Bruker AVANCE III 400 MHz spectrometer with proton resonance frequencies of 400 MHz.

LB medium was from Roth (Lennox, LB-broth), anti-Ubiquitin antibody (rabbit polyclonal IgG) was from Upstate, alkaline phosphatase conjugated affinity purified goat anti rabbit IgG was from Millipore.

Chemical Synthesis

Azidohomoalanine (Aha) was prepared as previously described by Link *et al.*[1] 5-(Dimethylamino)-*N*-(2-propynyl)-1-naphthalenesulfonamide (**Y**) was synthesized according to Bolletta *et al.*[2] and the biotin-alkyne **X** according to Gurcel *et al.*[3]. The propargyl-protected lysine derivative Plk was prepared as described by Nguyen *et al.*[4].

Nutrient Media

All expression experiments with Aha were performed in New Minimal Medium (NMM). This was prepared from autoclaved 5x stock solutions of inorganic salts (37.5mM $(NH_4)_2SO_4$, 42.5mM NaCl, 110mM KH_2PO_4, 250mM K_2HPO_4, 5mM $MgSO_4$, 5mg/l $CaCl_2$, 5mg/l $FeCl_2$, 5µg/l $CuCl_2$, 5µg/l $MnCl_2$, 5µg/l $ZnCl_2$). Sterile filtered glucose was added to a final concentration of 20mM, thiamine hydrochloride and d-biotin to a final concentration of 10mg/l. Finally, the medium was supplemented with 50mg/l of all natural amino acids excluding methionine. All bacterial growth was under the selective pressure of 100mg/l carbenicillin.

Expression and Purification of ahaUb

The gene coding for MASUb76M was synthesized by Geneart and cloned into pGDR11 using *EcoRI* and *SacI*. For expression, the plasmid was transformed into met auxotrophic *E. coli B834 (DE3)*. Cells were cultured in NMM with 0.04mM Met until they reached stationary growth (OD_{600} = 0.8). Medium was then changed to fresh NMM supplemented with 0.5mM Aha and induced with 1mM IPTG after 30min. After 4h, cells were harvested by centrifugation and stored at -80°C until further use.

Pellets were resuspended in lysis-buffer (20mM Tris*HCl, pH8.8) and solubilized by sonication. Heating the suspension to 75°C for 20min led to denaturation of most *E. coli* proteins. After centrifugation at 20000g for 20min the supernatant was further purified by anion exchange chromatography (Q-Sepharose).

Expression and Purification of plkUb

The gene coding for (63TAG)Ub was synthesized by Geneart and cloned into pET11a using *XbaI* and *HindIII*. All other (TAG)Ub mutants were created by site directed mutagenesis based on 63(TAG)Ub. The gene for the pyrrolysine tRNA synthetase pylS was obtained from genomic DNA from *Methanosarcina barkeri* and cloned into pRSFduet using *BfuAI* and *SacI*. The gene for the pyrrolysine tRNA was synthesized together with the lipoprotein promoter lpp and the terminator RRN b/c and cloned into the backbone of pET11a already containing the gene for (TAG)Ub in its multiple cloning site. Both vectors were co-transformed into *BL21(DE3)* for expression. Cells were cultured at 37°C. At OD_{600} = 0.3, Plk was added to a final concentration of 1mM. Protein expression was induced with 1mM IPTG at OD_{600} = 0.8. After 12-16h cells were harvested by centrifugation and protein was isolated as described above.

Click Reaction

Equimolar amounts of AhaUb and PlkUb in 5 to 20mM Tris*HCl (pH8) were mixed with 1mM TCEP, 10µM TBTA and 1mM $CuSO_4$. Reaction vessels were flushed with argon to prevent Cu-induced protein oxidation. After incubation at room temperature for 1h the reaction was stopped by addition of 10mM EDTA. For biotinylation reactions, a 10-fold excess of biotin-alkyne **X** was used and for labelling with fluorophore tags a 10-fold excess of the respective dye **Y**.

SDS-PAGE

Expressed proteins and click reactions were analyzed by glycine SDS-PAGE according to standard procedures including a stacking- and a resolving-gel (15%). Gels were stained using Roti®-Blue Colloidal Coomassie and photographed using ChemiDoc XRS from BioRad.

Mass analysis

ESI-IT mass spectra were recorded on a Bruker Daltonics esquire 3000^{+}. The protein samples were desalted with G25 columns (GE Healthcare) and diluted in 2% acetic acid in water/CH_3CN (1:1). Samples were directly injected and data was analyzed with DataAnalysis from Bruker. For the analysis of the peptide fragments tryptic in-gel digest was conducted. After resolution of the resulting peptide fragments in 0.2% formic acid in water, we performed HPLC-ESI-MS/MS (Thermo Scientific LTQ Orbitrap Discovery). Data was analyzed using DataAnalysis from Bruker and Mascot Search.

References

(1) Link, A. J.; Vink, M. K. S.; Tirrell, D. A.; *Nature Protocols* **2007**, *2*, 1879-1883.
(2) Bolletta, F.; Fabbri, D.; Lombardo, M.; Prodi, L.; Trombini, C.; Zaccheroni, N.; *Organometallics* **1996**, *15*, 2415-2417.
(3) Gurcel, C.; Vercoutter-Edouart, A.-S.; Fonbonne, C.; Mortuaire, M.; Salvador, A.; Michalski, J.-C.; Lemoine, J.; *Anal. Bioanal. Chem.* **2008**, *390*, 2089-2097.
(4) Nguyen, D. P.; Lusic, H.; Neumann, H.; Kapadnis, P. B.; Deiters, A.; Chin, J. W.; *J. Am. Chem. Soc.* **2009**, *131*, 8720-8721.

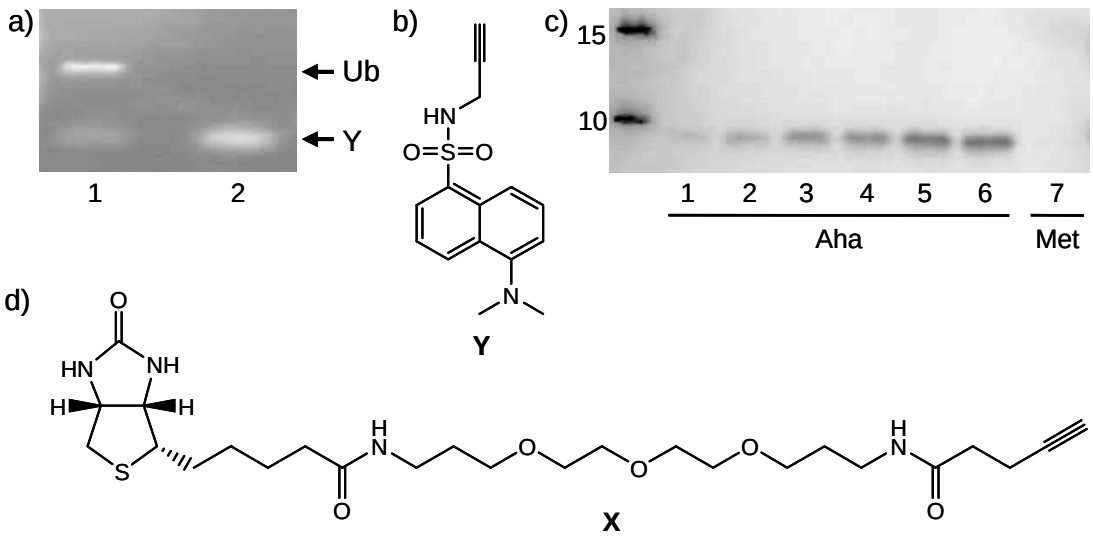

Figure S1. Click reactions with 76AhaUb. a) Fluorescence image (SDS-polyacrylamide gel) of click reaction between **Y** and 76AhaUb (lane 1) and 76MetUb (lane 2) as negative control. b) Alkyne functionalized dansyl **Y**. c) Click reaction between 76AhaUb and **X**; western blot with streptavidin-AP conjugates; lane 1 to 6: click reaction at RT with increasing reaction times: 15min (1), 30min (2), 1h (3), 2h (4), 4h (5) and 8h (6); lane 7: click reaction between 76MetUb and **X** at RT for 8h as negative control. d) Alkyne functionalized biotin **X**.

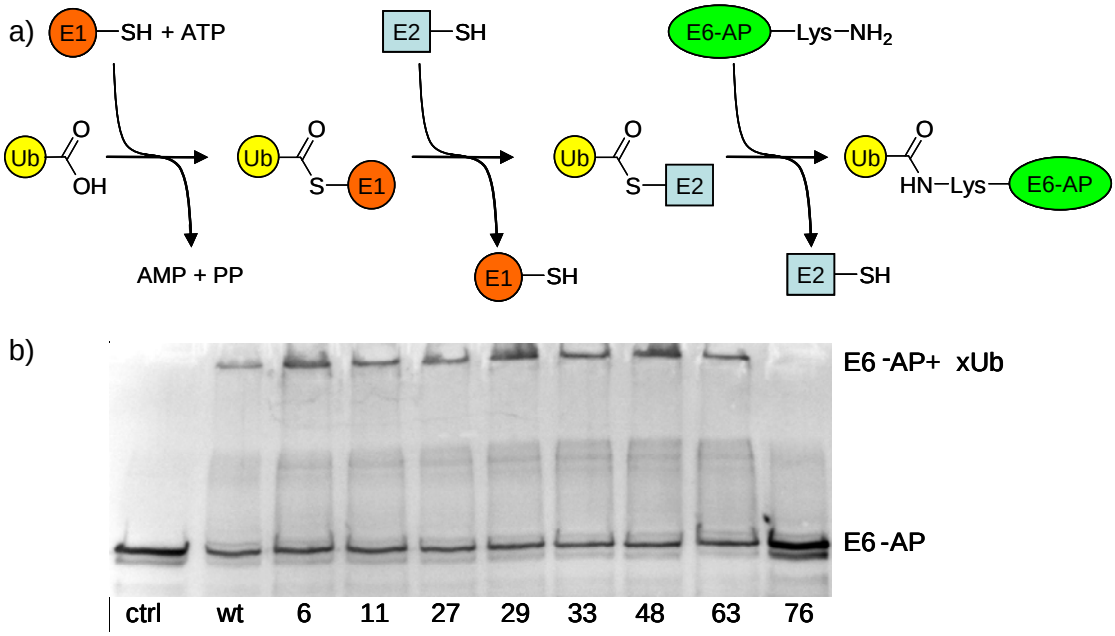

Figure S2. E6-AP auto-ubiquitination assay. a) Schematic overview of ubiquitination. Ub is activated by the Ub-activating enzyme E1 at the expense of ATP by forming a thioester complex between the carboxyl group of the C-terminal Gly of Ub and the thiol group of the active site Cys of E1. Activated Ub is then transferred to the active site Cys of an Ub-conjugating enzyme E2 (here UbcH7). Finally, an isopeptide bond between the C-terminal Gly of Ub and a lysine residue of E3-ligase (here E6-AP) or a lysine residue of a second Ub is formed. b) Western blot with anti-Ub antibodies of E6-AP auto-ubiquitination assay with modified Ub monomers. ctrl: reaction without Ub; wt: wtUb; 6: 6PlkUb; 11: 11PlkUb; 27: 27PlkUb; 29: 29PlkUb; 33: 33PlkUb; 48: 48PlkUb; 63: 63PlkUb; 76: 76AhaUb. E6-AP, non-modified E6-AP; E6-AP+xUB, polyubiquitinated E6-AP.

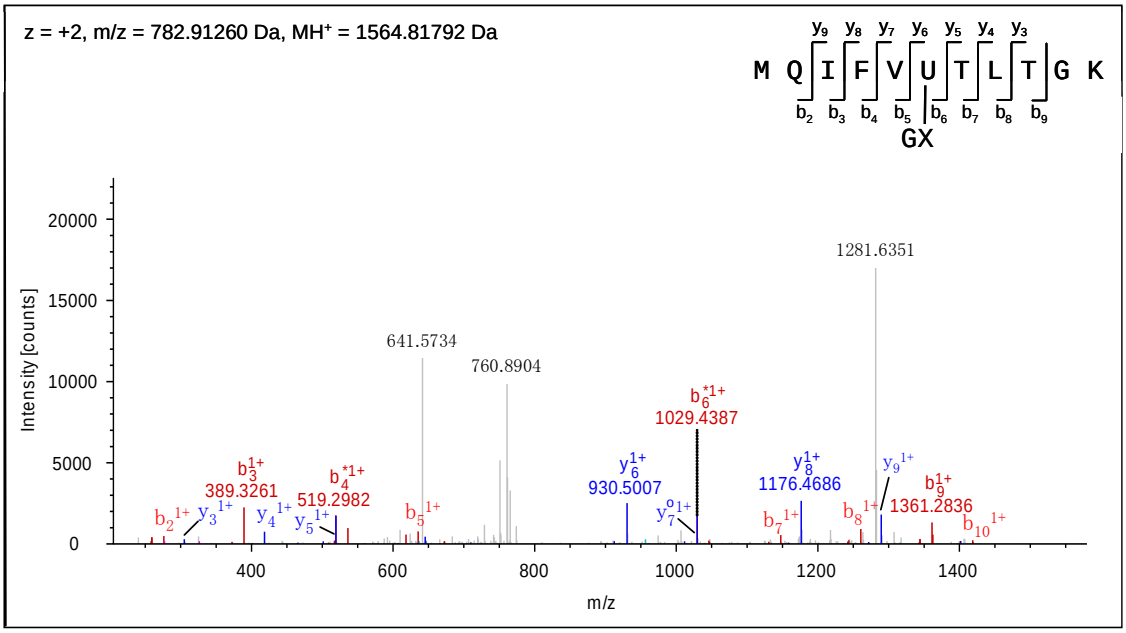

Figure S3. MS/MS spectrum of triazole linked fragment of 6PlkUb-76AhaUb after tryptic digest. All b- and y-ions marked in the peptide sequence were found in the corresponding MS/MS spectrum. b/y°: b/y minus H_2O; b/y*: b/y minus NH_3. First methionine is oxidized during sample preparation.

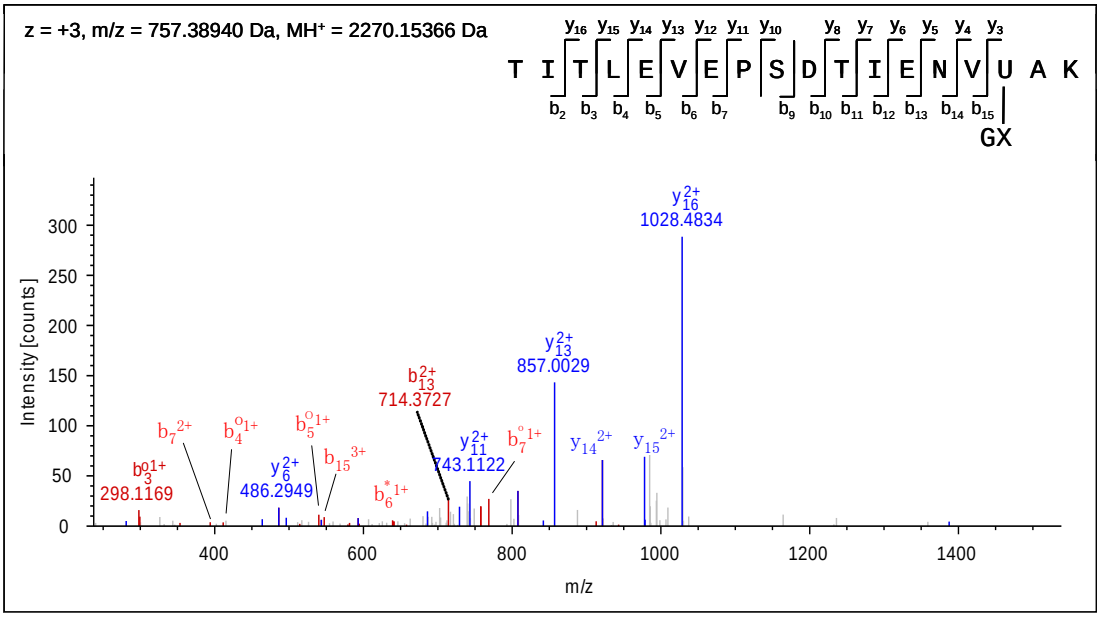

Figure S4. MS/MS spectrum of triazole linked fragment of 27PlkUb-76AhaUb after tryptic digest. All b- and y-ions marked in the peptide sequence were found in the corresponding MS/MS spectrum. b/y°: b/y minus H_2O; b/y*: b/y minus NH_3.

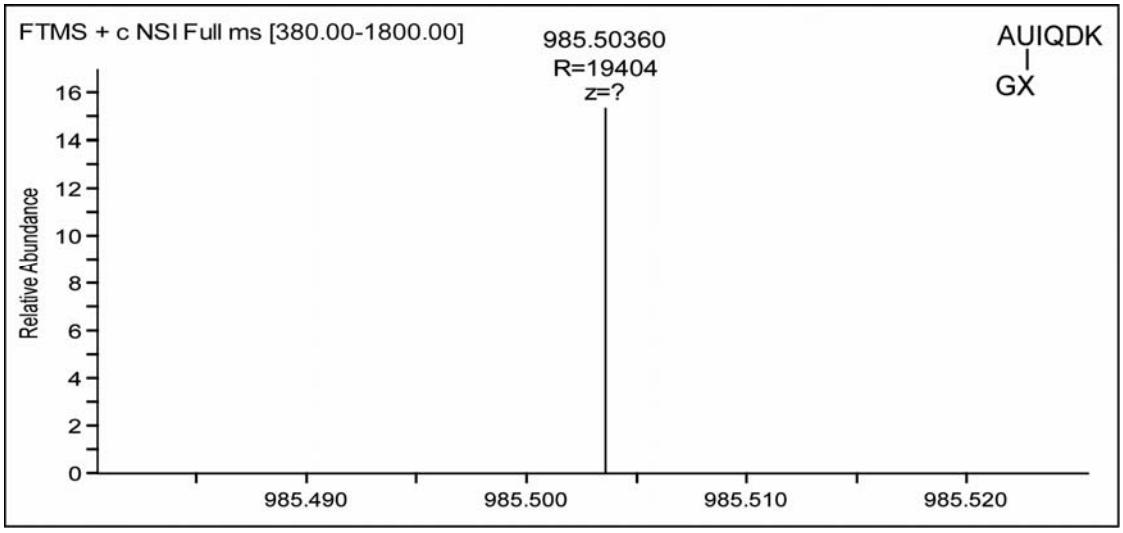

Figure S5. MS spectrum of triazole linked fragment of 29PlkUb-76AhaUb after tryptic digest. Due to small fragment size, intensity too low for MS/MS fragmentation.

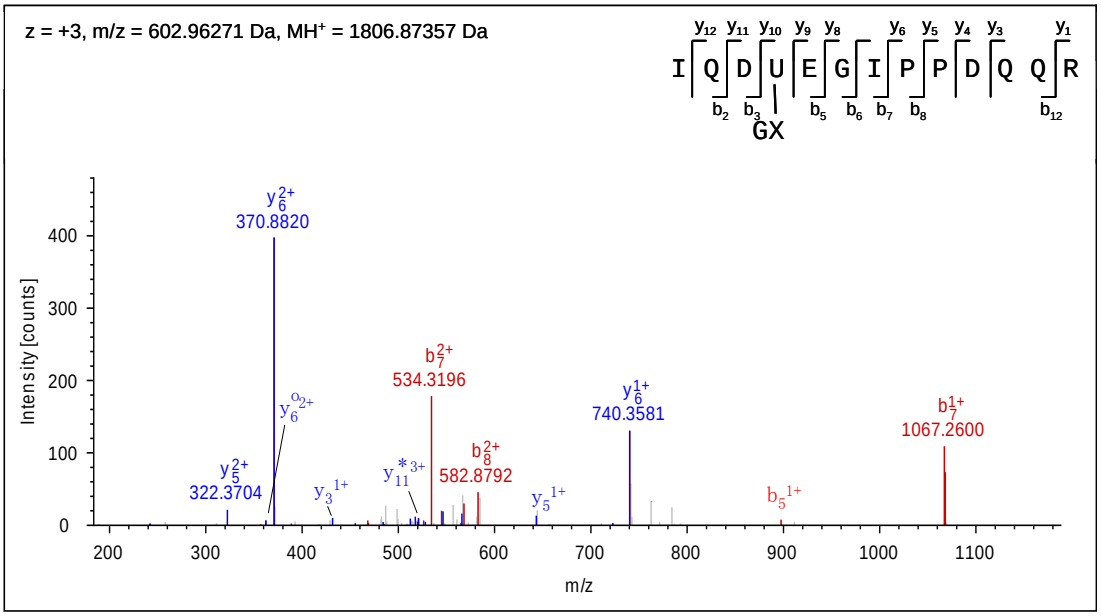

Figure S6. MS/MS spectrum of triazole linked fragment of 33PlkUb-76AhaUb after tryptic digest. All b- and y-ions marked in the peptide sequence were found in the corresponding MS/MS spectrum. b/y°: b/y minus H_2O; b/y*: b/y minus NH_3.

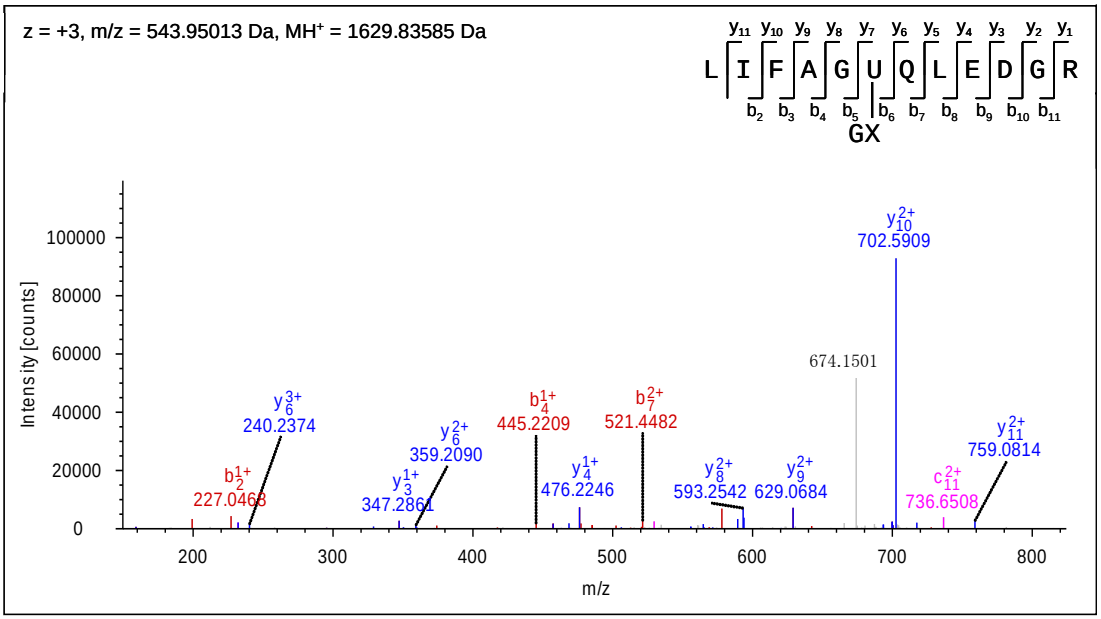

Figure S7. MS/MS spectrum of triazole linked fragment of 48PlkUb-76AhaUb after tryptic digest. All b- and y-ions marked in the peptide sequence were found in the corresponding MS/MS spectrum. b/y°: b/y minus H_2O; b/y*: b/y minus NH_3.

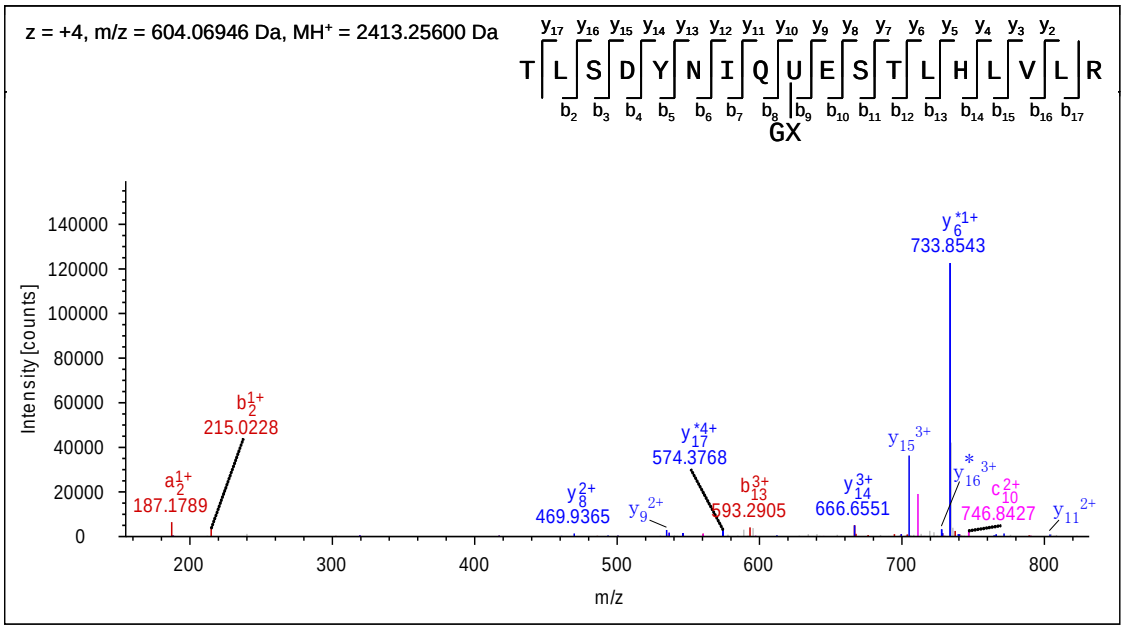

Figure S8. MS/MS spectrum of triazole linked fragment of 63PlkUb-76AhaUb after tryptic digest. All b- and y-ions marked in the peptide sequence were found in the corresponding MS/MS spectrum. b/y°: b/y minus H_2O; b/y*: b/y minus NH_3.