Electronic Supplementary Information (ESI) for

N-Methylcysteine-Mediated Total Chemical Synthesis of Ubiquitin Thioester

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Solid Phase Peptide Synthesis:

Solid-phase chemistry was carried out manually in syringes, equipped with teflon filters, purchased from Torviq or by using an automated peptide synthesizer (CS336X, CSBIO). If not differently described, all reactions were carried out at room temperature. Analytical HPLC was performed on a Thermo instrument (Spectra System p4000) using an analytical column (Jupiter 5 micron, C18, 300A 150 x 4.6 mm) and a flow rate of 1.2 mL/min. Preparative HPLC was performed on an ECOM instrument using a preparative Column (Jupiter 5 micron, C18, 300A, 250 x 10 mm) and a flow rate of 25 mL/min. DMF was purchased in biotech grade. Commercial reagents were used without further purification. Resins, protected amino acids and HBTU were purchased from Novabiochem.

HPLC and MS analysis of peptide 1.

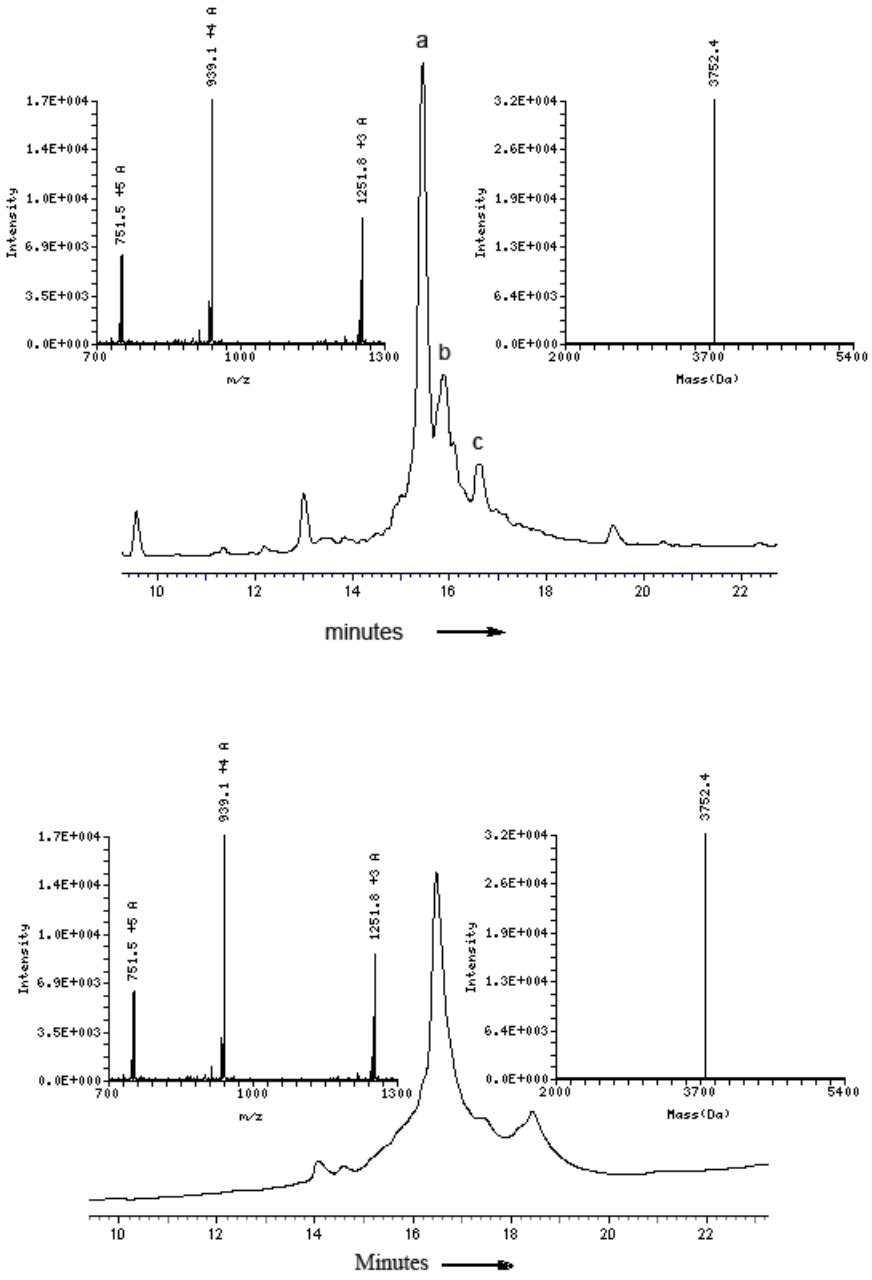
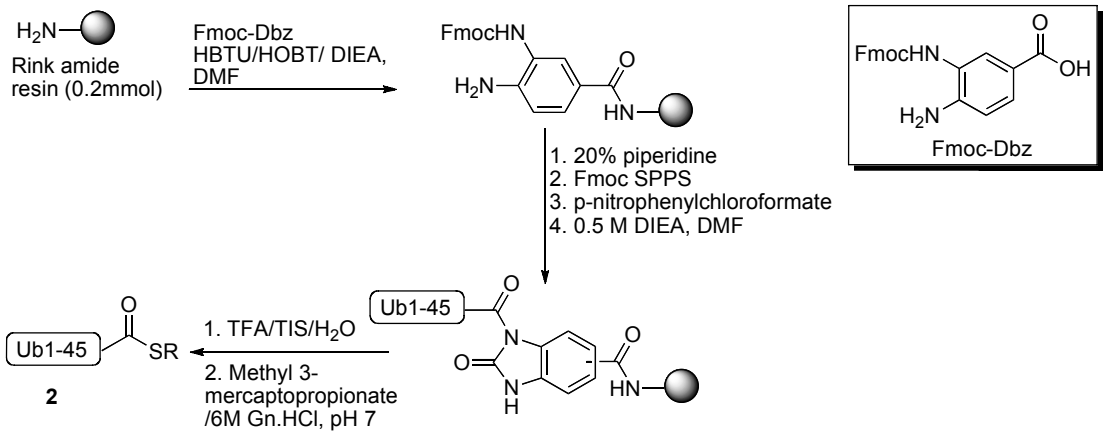
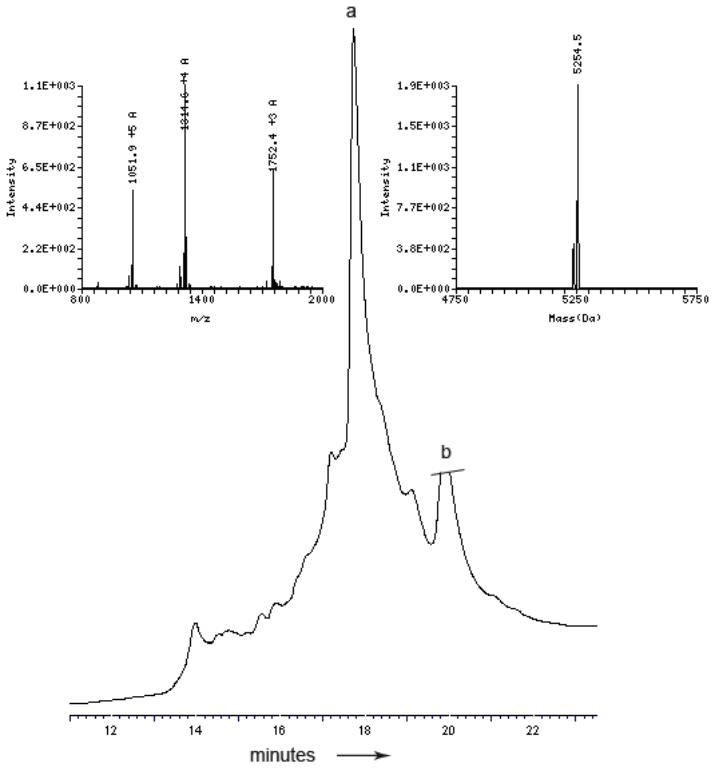


Figure 1: Analytical HPLC of the crude (top) and pure (bottom) peptide 1. Peak a corresponds to the desired peptide with the observed mass 3752.4 Da (calcd m/z 3753 Da). Peak b and peak c correspond to unidentified by-products.

Synthesis of peptide 2, Ub(1-45)



A



B

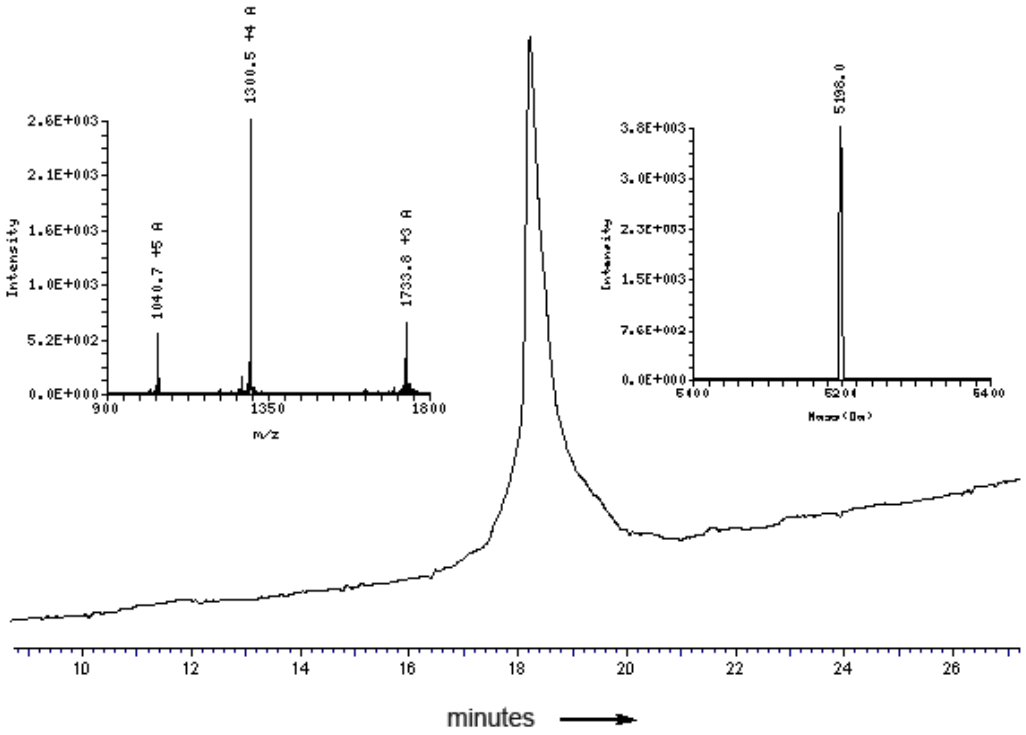


Figure 2: Analytical HPLC of the crude (A) and pure (B) peptide **2** with the observed mass 5198 Da (calcd m/z 5198.9 Da).

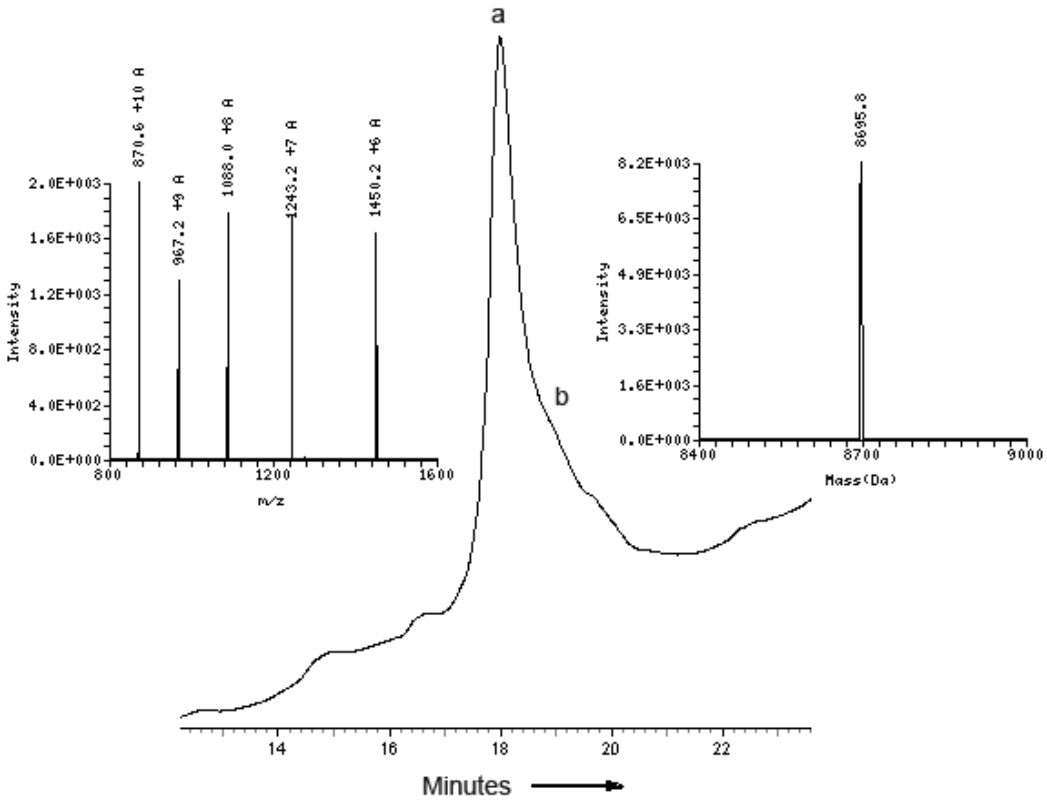


Figure 3: Analytical HPLC and mass spectrometry analysis of the ubiquitin after photolysis showing the product with the observed mass of 8695.8 Da (calcd m/z 8695.9 Da). Peak b corresponds to the protein forming disulfide bond with the unprotected Cys and MPA in the photolysis buffer with the observed mass of 8800 Da.

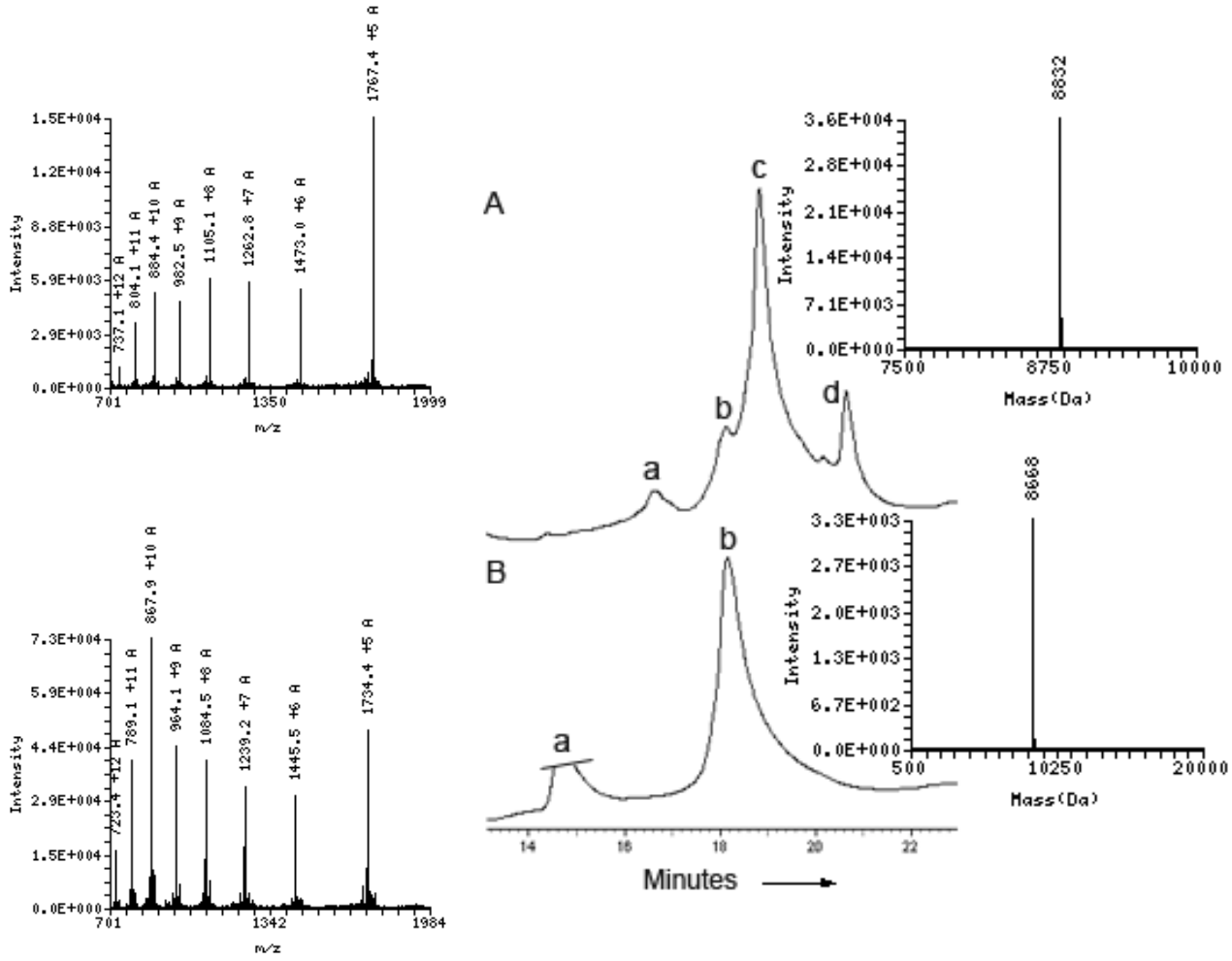


Figure 4. Representative analytical HPLC traces / (ESMS) of the ligation reaction between 1 and 2 (A) followed by thioester formation (B). Reported mass is for total protein. A) Ligation after 8 h: Peak a, unreacted peptide 1 with the observed mass of 3752.1 Da. Peak b, thioester hydrolysis by product (Ub1-45-COOH) with the observed mass 5096.1 Da. Peak c, ligation product with the observed mass of 8832 Da (calcd m/z 8830.9 Da). Peak d, unreacting benzyl thioester of peptide 2 with the observed mass of 5201.2 Da (1.1 eq of peptide thioester was used in the ligation reaction). B) Photolysis (2 h) of the ligation product (365 nm) followed by treatment with 20% 3-mercaptopropionic acid (12 h): Peak a, photolysis mixture. Peak b, ubiquitin thioester with the observed mass of 8668 Da (calcd m/z 8668.8 Da).

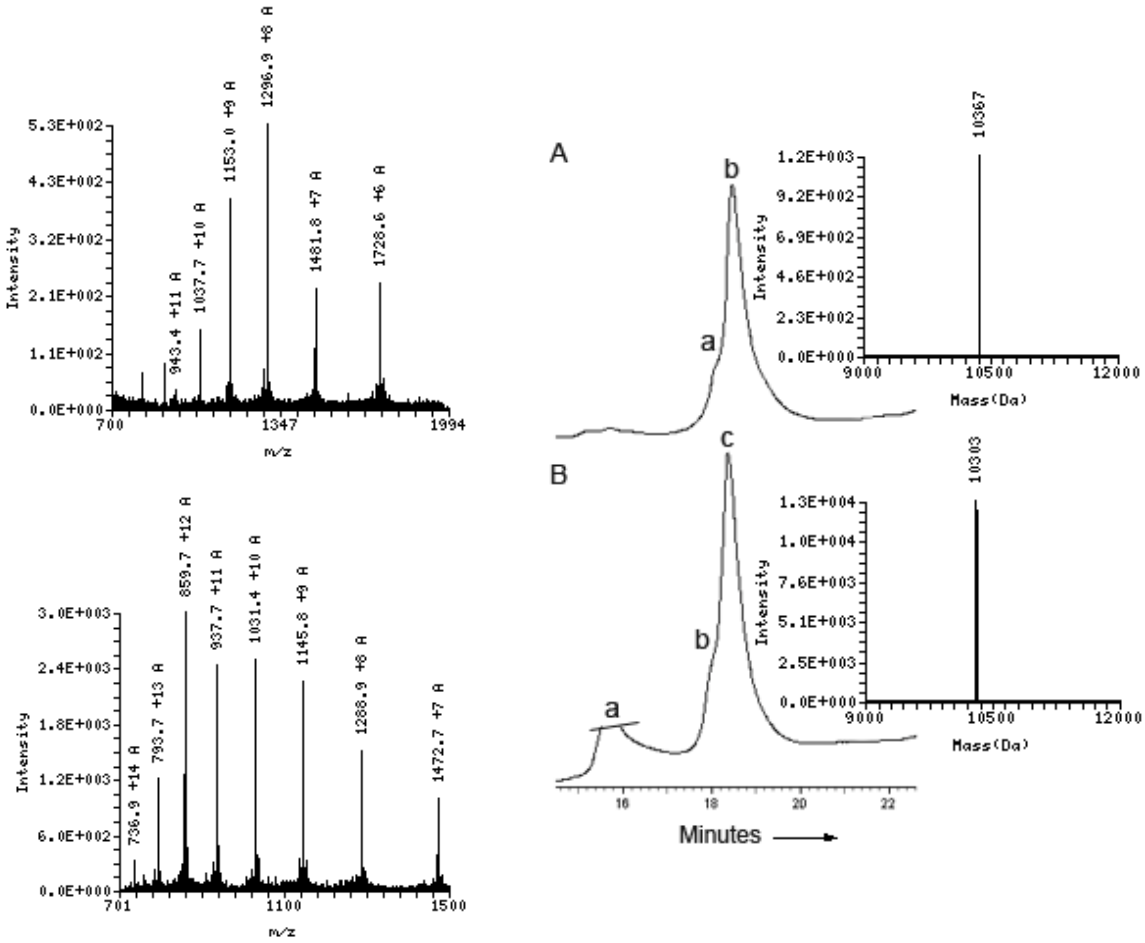


Figure 5. Representative analytical HPLC traces / (ESMS) of ubiquitylation reaction between **5** and **7** (A) followed by desulfurization (B). Reported mass is for total protein. A) Ubiquitylation after 4 h: Peak a, hydrolyzed thioester that was not fully seperated from the previous step with the observed mass of 5096.1 Da. Peak b, the desired ubiquitylation product with the observed mass 10367 Da (calcd m/z 10367.9 Da). B) Desulfurization after 3 h: Peak a, desulfurization mixture. Peak b, by-product carried from previous step. Peak c, the desired desulfurized product with the observed mass 10303 Da (calcd m/z 10303.8 Da).

Enzymatic cleavage of isopeptide: Purified peptide **8** was dissolved in 482 µL of assay buffer (50 mM Tris, 150 mM NaCl, 1 mM DTT, pH 7.5) to a final concentration of ~100 µM and reacted with recombinant human ubiquitin C-terminal hydrolase L3 (UCH-L3, Aldrich). 10 µg of UCH-L3 in 15.5 µL of assay buffer containing 50 mM Tris, 150 mM NaCl, 12 mM DTT, pH 8.0 was incubated for 20 min at 25 ºC. To the reduced UCH-L3 was then added **8** in 187 µL. The mixture was incubated for 12 h at 37 ºC, at which a complete hydrolysis was achieved. The reaction was analyzed using C-4 analytical RP-HPLC employing a gradient of 10-60% B for 30 min, in order to identify the hydrolysis products.

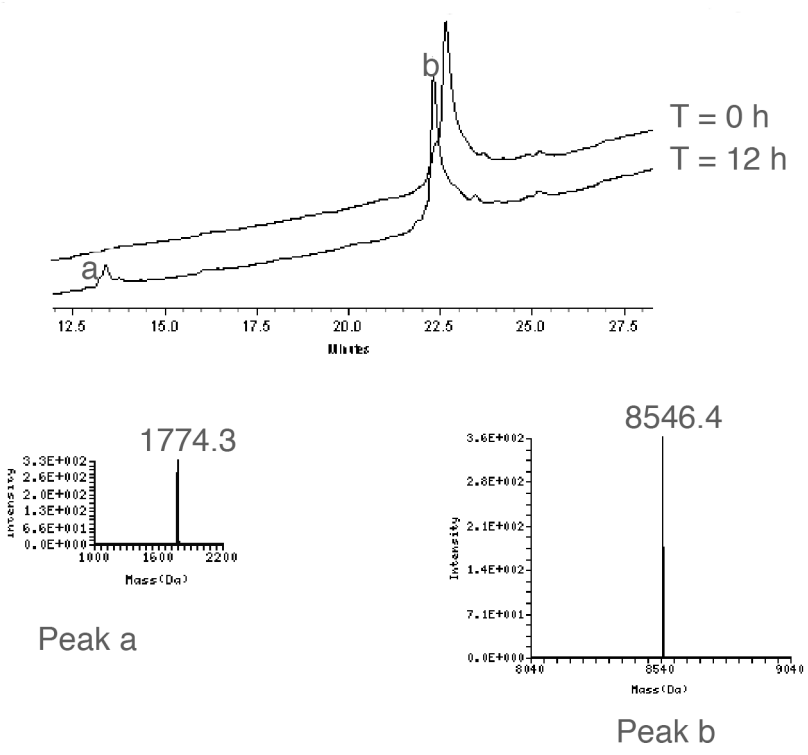


Figure 6. HPLC and mass spectrometry analysis of the enzymatic cleavage of a-Syn(1-17)-Ub at 0 h and after 12 h. Peak a is the a-Syn(1-17) peptide with an observed mass of 1774.3 (calcd. is 1774.8 Da. Peak b is the hydrolyzed ubiquitin with the observed mass of 8546.4 Da calcd. m/z 8547.8 Da.