Electronic Supplementary Information

Boronic Acid-Modified Cell-Penetrating Peptides Exhibit Superior Performance Over Natural TAT Peptide

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1. Reagents

TAMRA [5(6)-Carboxytetramethylrhodamine)], 1- Hydroxybenzotriazolemonohydrate (HOBt), {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid (PEG), reagents like O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyl uronium hexafluorophosphate (HATU), 2-(6-Chloro-1H-benzo[d][1,2,3]triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate(V) (HCTU), Triisopropylsilane (TIS), 4-bromomethylphenylboronic acid were purchased from Sigma. Rink amide resin (loading:0.45mmol/g) obtained from Sigma. N,N-Diisopropylethylamine (DIPEA) and Trifluoroacetic acid(TFA) were purchased from Sigma. Solvents (like DMF, MeOH, DCM etc) were of analytical grade. Peptides were prepared by Fmoc-SPPS manually in fritted syringes.

1.1 List of protected amino acids and dipeptide used in peptide synthesis

Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Phe-OH, Fmoc-His(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, FmocLys(Boc)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Asp(OtBu)OH, Fmoc-Glu(OtBu)-OH, Fmoc-Lys(Alloc)-OH, Boc-Cys(Trt)-OH, Fmoc-Nle-OH, Fmoc-LueThr(ψMe,MePro)-OH, Fmoc-Ile-Thr(ψMe,MePro)-OH, Fmoc-Asp(OtBu)-(DMB)Gly-OH and Fmoc-Leu-Ser(ψMe,MePro)-OH were obtained from Sigma.

2. Instruments

Analytical HPLC was performed on Shimadzu LC-20 Series instrument using analytical column C18 (3.5 µm, 4.6 × 150 mm) and C4 (10 µm, 300 Å, 250 × 10 mm) column, at a flow rate of 1 mL/min and preparative HPLC was performed on Shimadzu LC-20 Series instrument, using preparative column (C18, 10 µm, 250 × 19 mm) and C4 (10 µm, 300 Å, 250 × 10 mm) column at a flow rate of 10 mL/min. Products were analyzed using ESI-MS of Quattro Premier XE (Micromass) from Waters. All UV/Vis chromatograms were detected explicitly at 214 nm. To determine the peptide solution concentration, the optical density of the corresponding solutions was measured using the UV-Vis of SHIMADZU-UV-1780 instrument based on TAMRA concentration.

3. Synthesis

Fmoc-SPPS was carried in the presence of 4 eq. of Fmoc-protected amino acid-OH (Fmoc-AAs-OH), 3.85 eq. of activating reagents (HATU/HCTU), 4 eq. HOBt, and 8 eq. of DIPEA (Concentration: 0.2 M) with the resin for 60 min each step. In the case of

PEG, dye, and boronic acid coupling, the timings are different and mentioned in the relevant section. The cleavage cocktail was made with TFA: triisopropyl silane (TIS): water (95:2.5:2.5, v/v/v).

3.1 Synthesis of CPPs:

Rink amide resin for a 10 µmol scale was initially taken in a fritted syringe reactor. After Fmoc cleavage (20% piperidine in DMF, 800 µl, 2x5 min) and washing with DMF (3x, 1mL), the resin was treated with a mixture of 4 eq. of Fmoc-AAs-OH, 3.85 eq. HATU, 4 eq. HOBt and 8 eq. DIPEA in DMF (concentration: 0.2M). After 60 minutes, the resin was washed with DMF (3x, 1mL) followed by Fmoc cleavage (20% piperidine in DMF, 800 µl, 2x5 min) and washing with DMF (3x, 1mL). Thus, the peptide sequence was loaded on the resin and repeated until the desired sequence was reached. After the desired sequence, the Fmoc cleavage (20% piperidine in DMF, 800 µl, 2x5 min) was performed, followed by washing with DMF (3x, 1mL). Then, resin was treated with 4 eq. of PEG, 3.85 eq. HCTU, 4 eq. HOBt and 8 eq. DIPEA in DMF (concentration: 0.2M) for 2h x 2. Afterwards, Fmoc cleavage (20% piperidine in DMF, 800 µl, 2x5 min) was performed and washed with DMF (3x, 1mL). After fmoc removal, the fmoc-cys(trt)-OH was coupled where 4 eq. amino acid, 3.85 eq. HATU and 8 eq. DIPEA for 3h. After this step, the resins were washed with DMF (3x, 1mL), DCM (5x, 1mL) and dried under a vacuum. The global deprotection was performed with the 5 mL cleavage cocktail TFA/triisopropylsilane/H_2O 95:2.5:2.5 (v/v/v) at room temp for 2h. After 2 h the cleavage cocktail was collected by filtration in falcon tube, the resin was washed with the cleavage cocktail (3x 5 mL) and the combined filtrates were concentrated under airflow. Et_2O (10-fold volume) was added to the remaining residue, the suspension was centrifuged (4000 rpm, 20 min). Afterwards, the ether phase was decanted. The remaining peptide pellet was dissolved in ACN: water (1:1, v/v with 0.1% TFA) by vortexing. The solution in the falcon was frozen and lyophilized. Afterwards, it was purified using RP-HPLC.

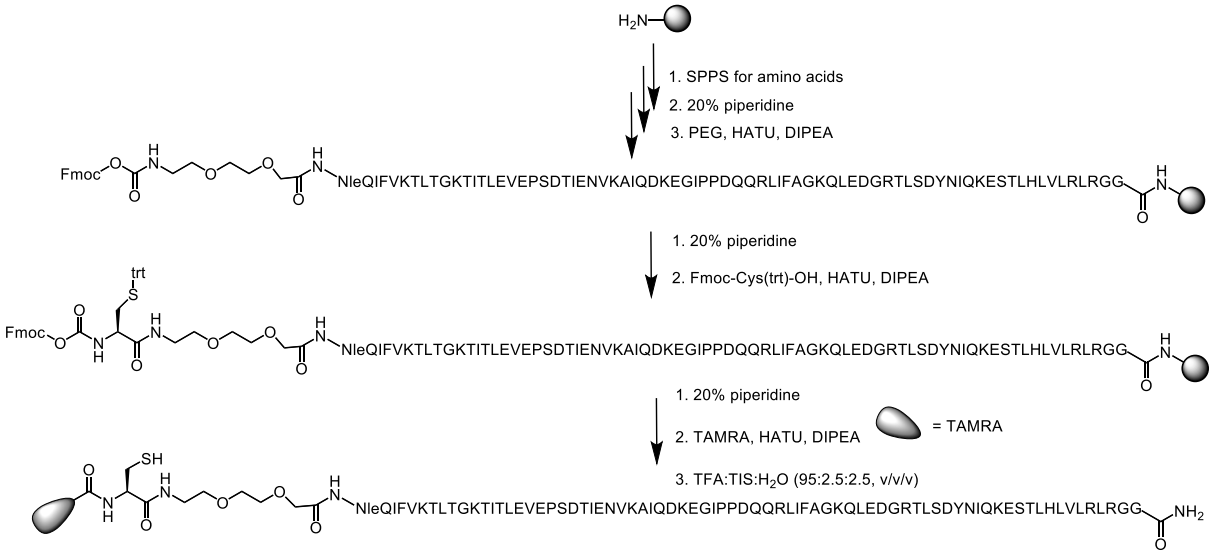
3.2 Synthesis of TAMRA-Cys-PEG-Ub(1-76)-$CONH_2$

[Ub sequence:

NIeQIFVKT*LT*GKT*IT*LEVEPSDTIENVKAIQDKEGIPPDQQRLIFAGKQLE*DG*RT*LS*DYNIQK ESTLHLVLRLRGG-$CONH_2$]

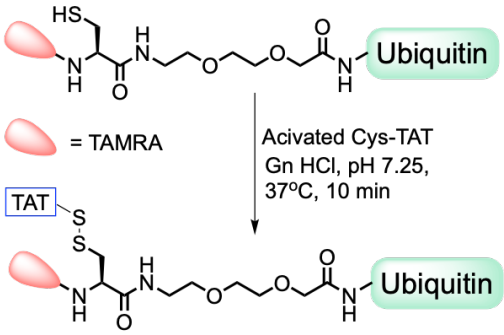
TAMRA-Cys-PEG-Ub (1-76)-$CONH_2$ was prepared on pre-swollen Rink amide resin (0.025 mmol) (scheme S4). Fmoc amino acids were coupled manually using 4 eq. amino acids, 3.85 eq. HCTU, 4eq. HOBt and 8 eq. of DIPEA. Different dipeptides were

used which were attached to the sequence manually Fmoc-LueThr(ψMe,MePro)-OH, Fmoc-Ile-Thr(ψMe,MePro)-OH, Fmoc-Asp(OtBu)-(DMB)Gly-OH and Fmoc-Leu-Ser(ψMe,MePro)-OH, the positions were coupled manually at positions 8-9 for LT, 13–14 for IT, 52-53 for DG and 56 -57 for LS using 3 eq. of the dipeptide, 2.85 eq. of HATU and 6 eq. of DIPEA for 2 h. After the desired sequence, Fmoc cleavage (20% piperidine in DMF, 800 µl, 2x5 min) was performed and washed with DMF (3x, 1mL). Then, the linker [2-[2-(Fmoc-amino)ethoxy]ethoxy]acetic acid (PEG) was coupled manually, using 4eq. PEG, 3.85 eq. HATU and 8 eq. DIPEA for 4h. After fmoc removal, the fmoc-cys(trt)-OH was coupled where 4 eq. amino acid, 3.85 eq. HATU and 8 eq. DIPEA for 3h. After completion of the sequence, the peptide resin was washed with DMF (5x, 1mL), followed by fmoc deprotection, and TAMRA was coupled where 1.5 eq. TAMRA, 1.35 eq. HATU, and 3 eq. DIPEA were used in DMF (Concentration: ~33 mM) for 3h. After this step the resins were washed with DMF (3x, 1mL), DCM (5x, 1mL) and dried under a vacuum. The global deprotection was performed with the 10 mL cleavage cocktail TFA/triisopropylsilane/H_2O 95:2.5:2.5 (v/v/v) at room temp for 2h. After 2 h the cleavage cocktail was collected by filtration in 50 mL falcon, the resin was washed with the cleavage cocktail (3x 5 mL) and the combined filtrates were concentrated under airflow. Et_2O (10-fold volume) was added to the remaining residue, the suspension was centrifuged (4000 rpm, 20 min). Afterwards, the ether phase was decanted. The remaining peptide pellet was dissolved in ACN: water (1:1, v/v with 0.1% TFA) by vortexing. The solution in the falcon was frozen and lyophilized. Afterwards, it was purified using RP-HPLC.



Scheme S1. Schematic representation of Synthesis of TAMRA-Cys-PEG-Ub (Ub_{TAMRA}).

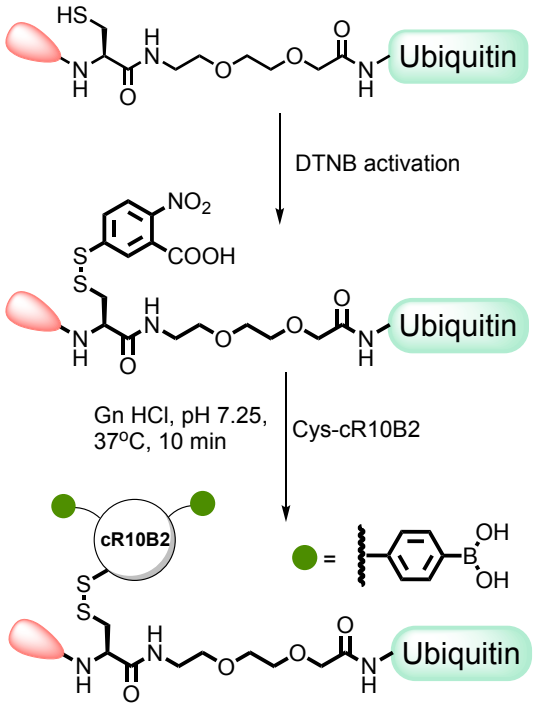
3.3. Synthesis of Ub-TAT



Scheme S2. Schematic representation of the synthesis of TAT-Ub_{TAMRA}.

The Cys residue of the TAT peptide was activated using 20 eq. 2,2'-Dithiobis(5-nitropyridine) (DTNP) in 95% TFA/H2O for 1h, producing the activated Cys-TAT after purification. The activated TAT was dissolved in degassed 6M guanidium phosphate buffer at pH 7.3, added to the TAMRA-Cys-PEG-Ub in 2mM final concentration, and incubated at 37oC for 7 min to generate the Ub-TAT conjugate.

3.3. Synthesis of Ub-cR10B2



Scheme S3. Schematic representation of the synthesis of cR10B2-Ub_{TAMRA}.

The Cys residue of the Ub_{TAMRA} was activated using 20 eq. 5,5′-dithiol-bis-[2-nitrobenzoicacid], DTNB in 95% TFA/H2O for 1h, producing the activated Ub(DTNB) after purification. The activated Ub(DTNB) was dissolved in degassed 6M guanidium

phosphate buffer at pH 7.3, added to the Cys-cR10B2 in 2mM final concentration, and incubated at 37°C for 2h to generate the desired Ub-cR10B2 conjugate.

4. Cell Culture and Biological Experiments

4.1 Cell culture procedure:

U2OS cells (obtained from ATCC) were cultured in high glucose DMEM, 10% FBS, 1% L-Gln, and 1% antibiotics (penicillin-streptomycin) in a humidified 37 °C incubator at 5% CO_2. To detach cells from culture flasks, the media was aspirated, and the flask was washed with sterile calcium and magnesium-free PBS, then cells were treated with 0.25% Trypsin containing 0.02% EDTA solution and returned to the incubation chamber for 5 min. Trypsin was quenched by adding the supplemented full medium. The cell suspension was collected, and the cells were pelleted (5 min at 500-RPM), washed with PBS once, pelleting, followed by homogeneous suspension in full medium. For microscopy, U2OS cells were seeded on poly-L-lysine eight well µ-slides in 5 × 10^4 $cells/well$ in full medium and were allowed to reach ~90% confluency.

4.2 Cell delivery experiments:

Cells in ~90% confluency were washed three times with warm PBS, followed by incubation with peptides dissolved in serum-free medium for 60 minutes at 37°C. After 60 minutes, cells were washed with PBS x 2 and PBS containing heparin sulfate once (with 5 min of incubation) and rewashed with PBS x 2. Afterward, the cells were stained using Hoechst (2 µg/ml) for nuclear staining, and imaging was done by microscopy. All experiments were repeated three times. The distribution of peptides in live cells was analyzed using a confocal laser scanning microscope (Leica TCS SP8). During microscopy, the samples were kept at room temperature. Image analysis was performed using Fiji.

Flow cytometry was performed using Cytoplex Flow cytometry, and data was analysed using Cytexpert software. Suspended cells were used for the experiment in PBS buffer at 37°C. After treatment with probes, washing was carried out using PBS buffer x 2, followed by centrifugation, then washed again with PBS containing Heparin Sulphate once (with 5 min of incubation), followed by washing with PBS once. Centrifugation was done at 1500 RPM for 5 minutes, and a minimum 10,000 cell count was used.

5. UPLC and MS Traces

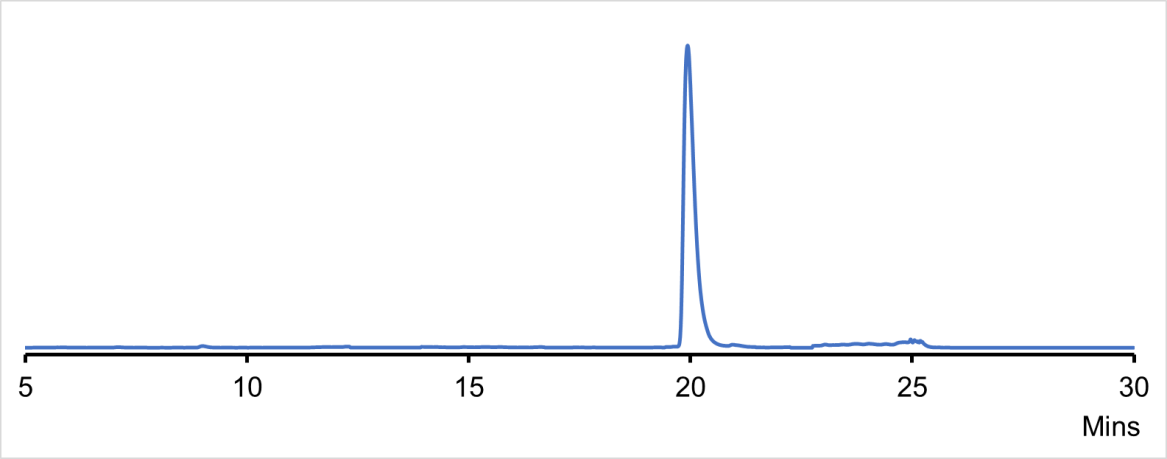


Fig. S1 Analytical HPLC traces of pure Ub-cR10B2.

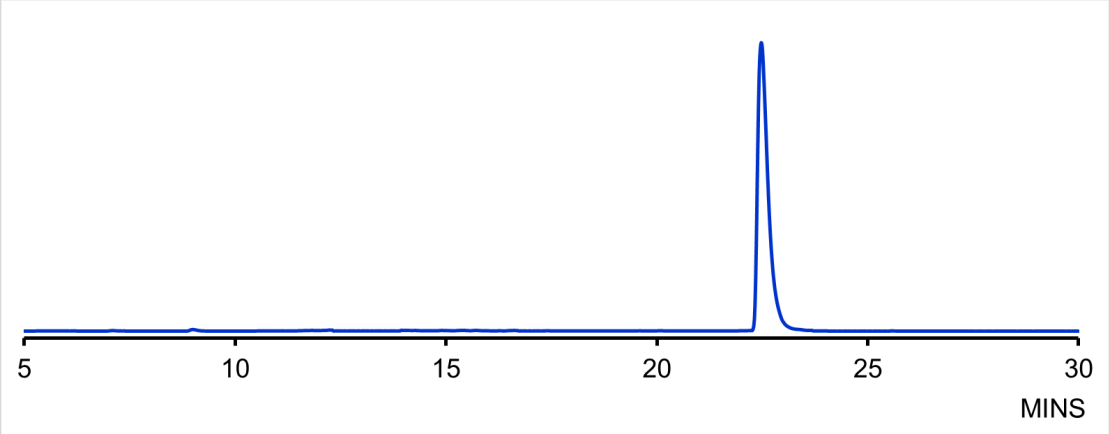


Fig. S2 Analytical HPLC traces of pure Ub-TAT.

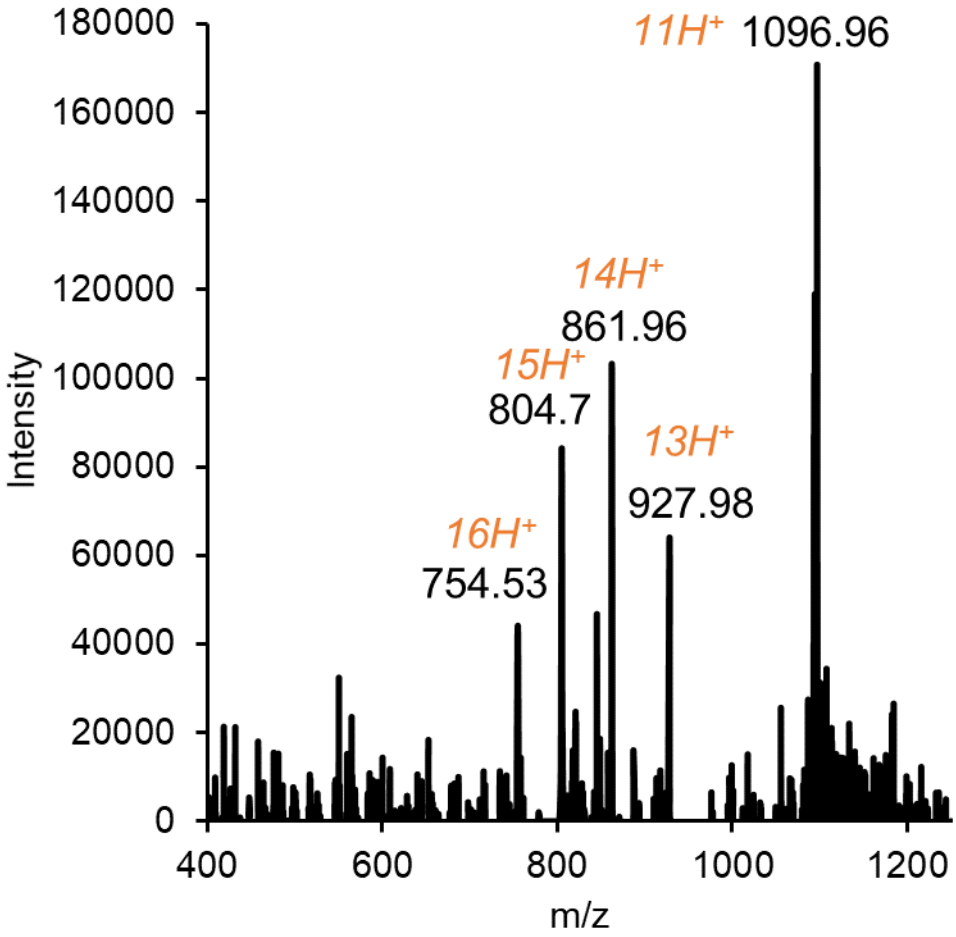


Fig. S3. ESI-MS of UPLC traces of Ub-cR10B2, full mass calcd (Da): 12053 (+1), 1096.72 (+11), 928.15 (+13), 861.92 (+14), 804.53 (+15), 754.31 (+16); found (Da): 1096.96 (+11), 928.98 (+13), 861.96 (+14), 804.7 (+15), 754.53 (+16).

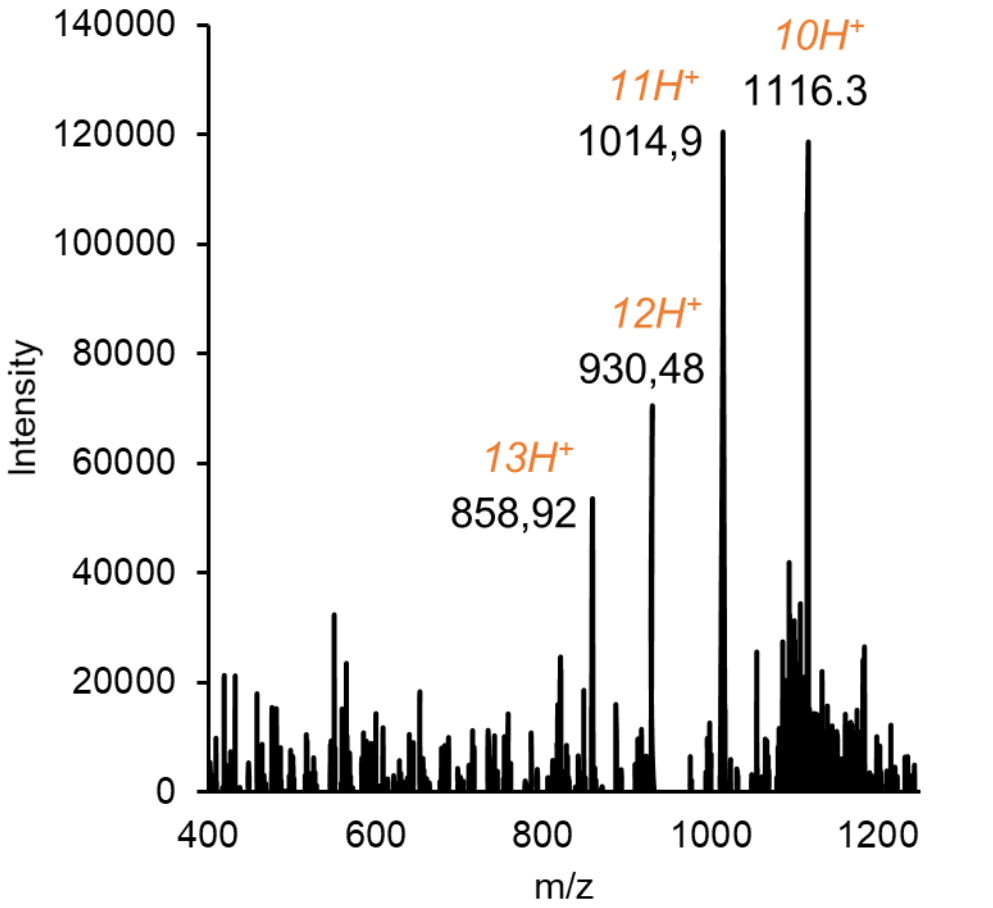


Fig. S4. ESI-MS of UPLC traces of Ub-TAT, full mass calcd (Da): 11155 (+1), 1116.5 (+10), 1015.0 (+11), 930.5 (+12), 859.0 (+13); found (Da): 1116.3 (+10), 1014.9 (+11), 930.48 (+12), 858.92 (+13).