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Supporting Information

Development of Diubiquitin-Based FRET Probes To Quantify Ubiquitin Linkage Specificity of Deubiquitinating Enzymes

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Supporting Information

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1. Supporting figures

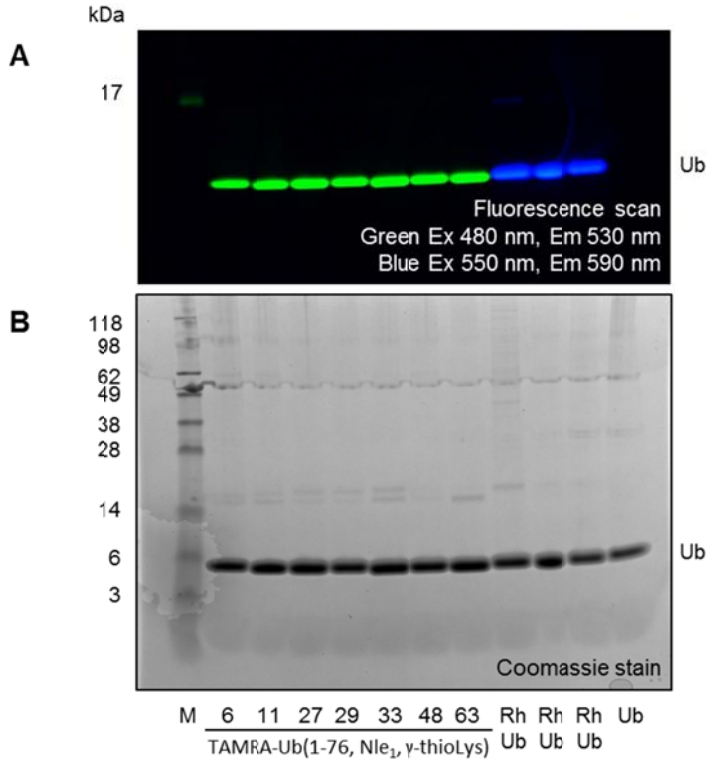


Figure S1. Characterization of TAMRA–Ub(1-76, Nle_1, γ-thioLys) (**16a-g**) and Rh-Ub(1-76, Nle_1)-$S(CH_2)_2CO_2Me$ (**14**) by SDS-PAGE analysis. A) Fluorescence scan. Green: Excitation 480 nm and emission 530 nm. Blue: Excitation 550 nm and emission 590 nm. B) Coomassie Brilliant Blue staining. Loading: 2 μg/lane (~22.1 μM).

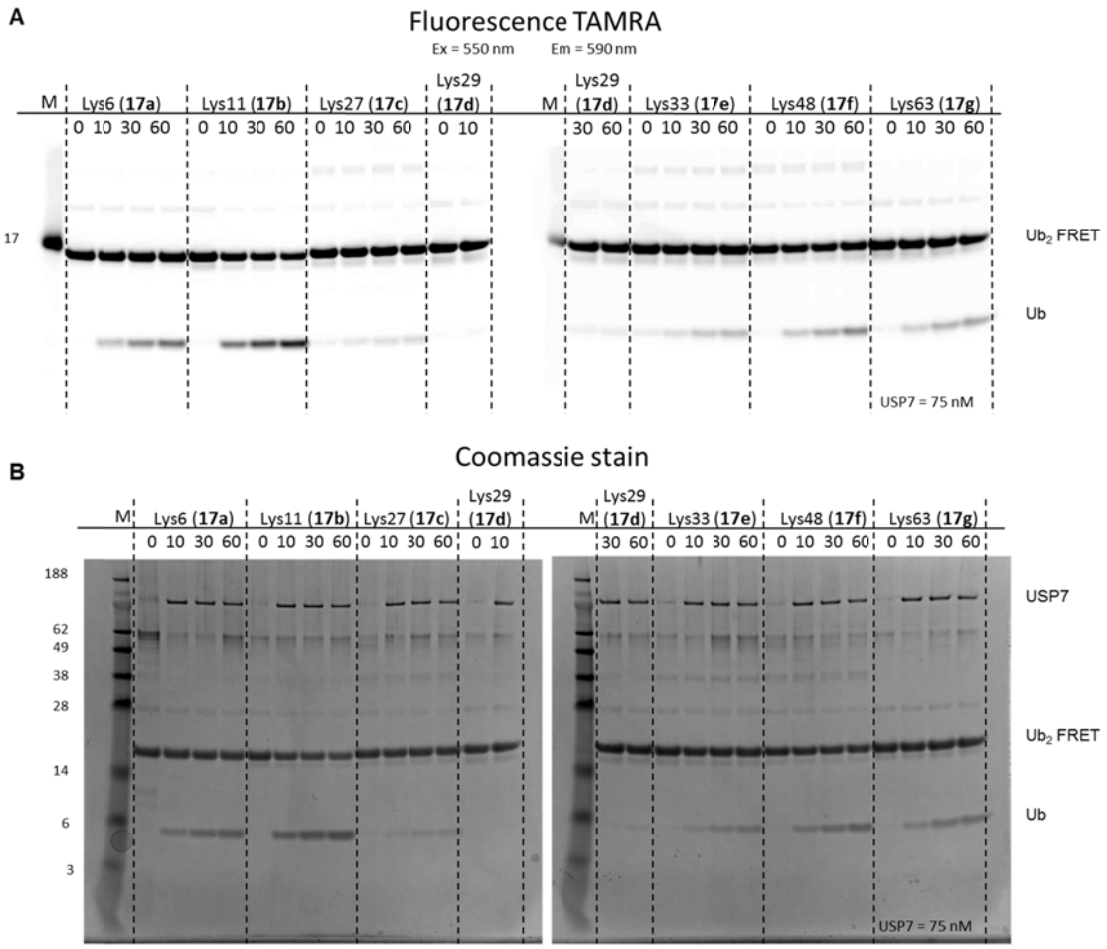


Figure S2. USP7 mediated hydrolysis of all seven diUb FRET pair linkages (**17a-g**), analyzed by SDS-PAGE. USP7 [51-1102] was incubated (75 nM final concentration) with the seven different diUb FRET linkages (**17a-g**) (0.1 mg/mL final concentration) in a buffer containing 50 mM Tris·HCl, 100 mM NaCl, pH 7.6, 5 mM DTT, 1 mg/mL 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS). Samples were taken after 10, 30 and 60 minutes and the reaction was quenched by addition of sample buffer containing β-mercaptoethanol, followed by heating the sample to 95°C for 5 minutes. As a control sample buffer containing β-mercaptoethanol was added to the seven solutions containing the seven different diUb FRET linkages (**17a-g**) (0.1 mg/mL final concentration) in the same buffer without addition of USP7 and this solution was also heated to 95°C for 5 minutes. The samples were resolved by gel electrophoresis (12% bis-tris precast gel, MES buffer), detection by A) fluorescence scanning ($\lambda_{ex/em}$ 550/590 nm) and B) Coomassie Brilliant Blue staining. Loading: 0.89 μg/lane (4.98 μM).

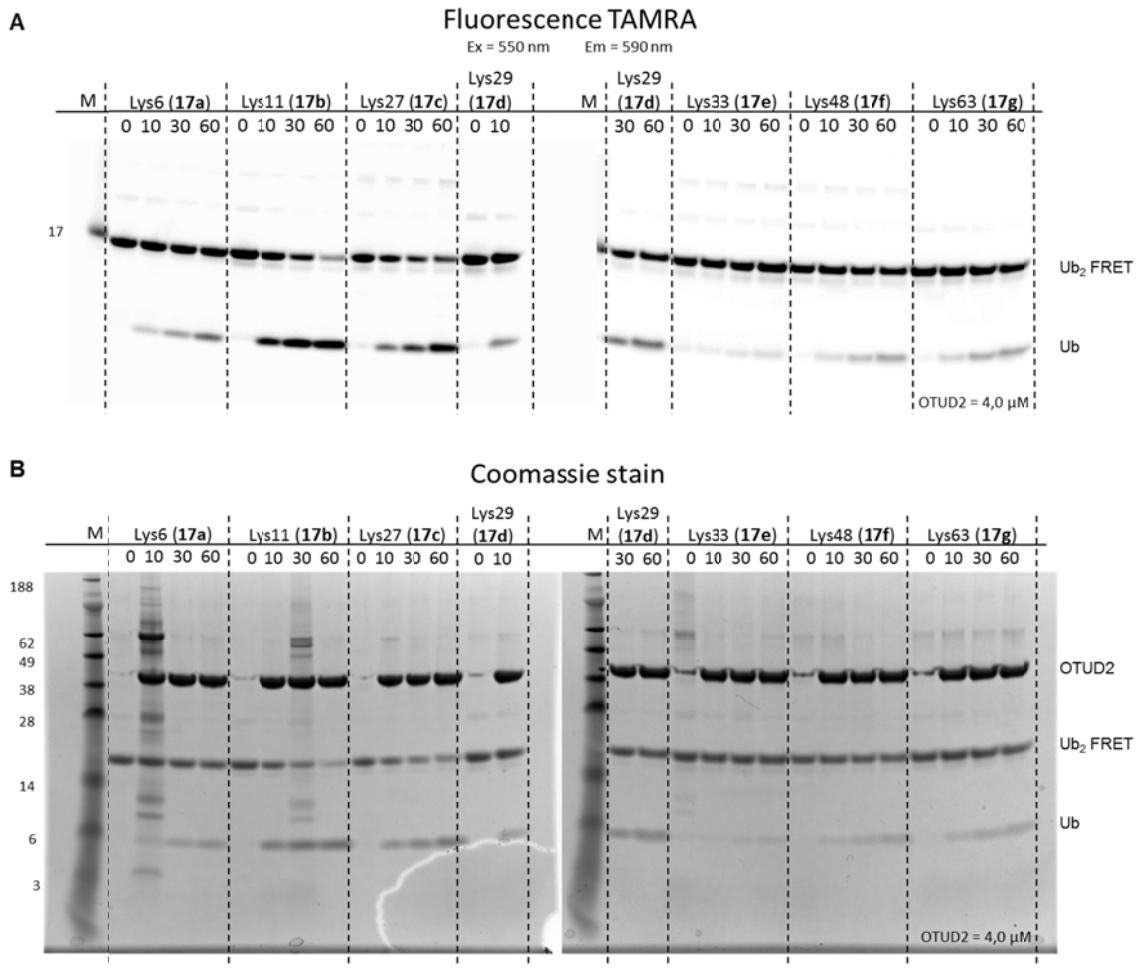


Figure S3. OTUD2 mediated hydrolysis of all seven diUb FRET pair linkages (**17a-g**), analyzed by SDS-PAGE. OTUD2 [1-348] was incubated (4 μM final concentration) with the seven different diUb FRET linkages (**17a-g**) **(**0.1 mg/mL final concentration) in a buffer containing 50 mM Tris·HCl, 100 mM NaCl, pH 7.6, 5 mM DTT, 1 mg/mL 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS). Samples were taken after 10, 30 and 60 minutes and the reaction was quenched by addition of sample buffer containing β-mercaptoethanol, followed by heating the sample to 95°C for 5 minutes. As a control sample buffer containing β-mercaptoethanol was added to the seven solutions containing the seven different diUb FRET linkages (**17a-g**) (0.1 mg/mL final concentration) in the same buffer without addition of OTUD2 and this solution was also heated to 95°C for 5 minutes. The samples were resolved by gel electrophoresis (12% bis-tris precast gel, MES buffer), detection by A) fluorescence scanning ($\lambda_{ex/em}$ 550/590 nm) and B) Coomassie Brilliant Blue staining. Loading: 0.89 μg/lane (4.98 μM).

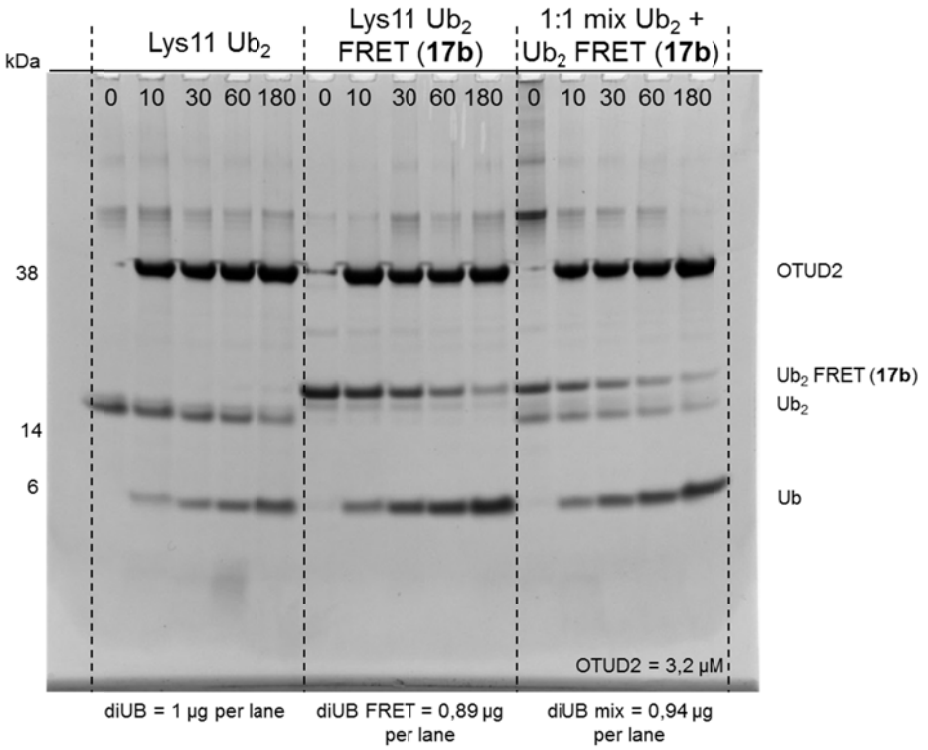


Figure S4. OTUD2 mediated hydrolysis of K11-linked diUb, K11-linked diUb FRET and a 1:1 mixture of both, analyzed by SDS-PAGE. OTUD2 [1-348] (3.2 μM final concentration) was incubated with the K11-linked diUb (0.1 mg/mL final concentration) in a buffer containing 50 mM Tris·HCl, 100 mM NaCl, pH 7.6, 5 mM DTT, 1 mg/mL 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS). Samples were taken after 10, 30, 60 and 180 minutes and the reaction was quenched by addition of sample buffer containing β-mercaptoethanol, followed by heating the sample to 95°C for 5 minutes. As a control sample buffer containing β-mercaptoethanol was added to the K11-linked diUb (0,1 mg/mL final concentration) in the same buffer without addition of OTUD2 and this solution was also heated to 95°C for 5 minutes. The samples were resolved by gel electrophoresis (12% bis-tris precast gel, MES buffer) and stained with Coomassie Brilliant Blue for detection.

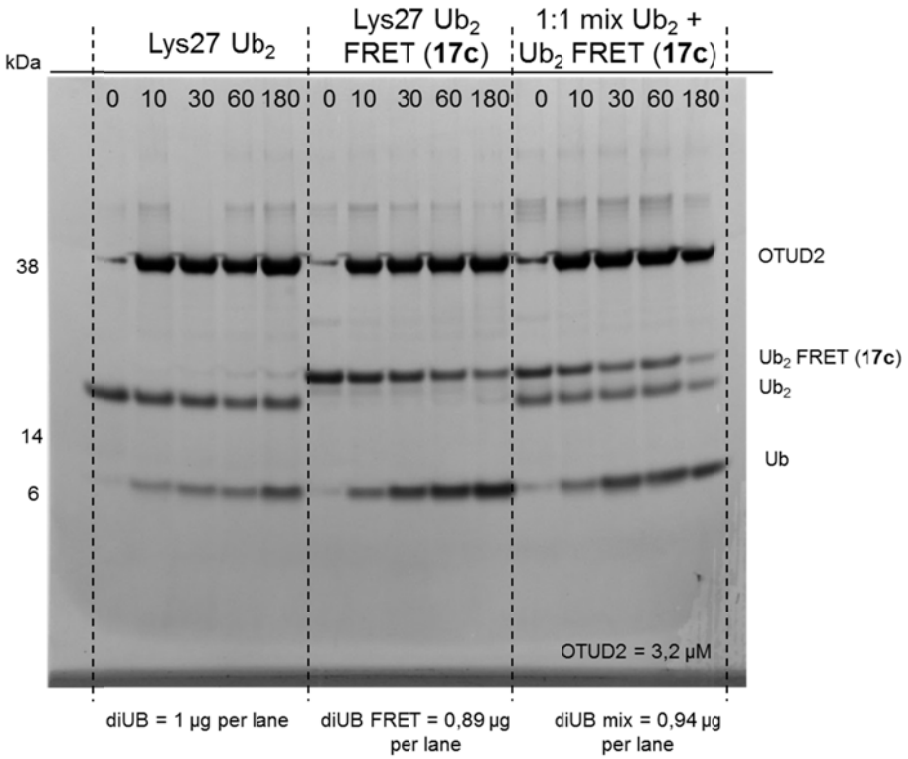


Figure S5. OTUD2 mediated hydrolysis of K27-linked diUb, K27-linked diUb FRET and a 1:1 mixture of labeled and unlabeled K27-linked diUb, analyzed by SDS-PAGE. OTUD2 [1-348] (3.2 μM final concentration) was incubated with the K27-linked diUb (0.1 mg/mL final concentration) in a buffer containing 50 mM Tris·HCl, 100 mM NaCl, pH 7.6, 5 mM DTT, 1 mg/mL 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS). Samples were taken after 10, 30, 60 and 180 minutes and the reaction was quenched by addition of sample buffer containing β-mercaptoethanol, followed by heating the sample to 95°C for 5 minutes. As a control sample buffer containing β-mercaptoethanol was added to the K27-linked diUb (0,1 mg/mL final concentration) in the same buffer without addition of OTUD2 and this solution was also heated to 95°C for 5 minutes. The samples were resolved by gel electrophoresis (12% bis-tris precast gel, MES buffer) and stained with Coomassie Brilliant Blue for detection.

2. Supporting tables

Table S1. Distance between N-terminal amine residues of Ub in diUb constructs calculated from available crystallography data.[a]

Linkage	Distance (Å)	PDB entry	Reference
Lys6	48.8	2XK5	S. Virdee *et al.*[1]
Lys11	51.8	2XEW	A. Bremm *et al.*[2]
Lys27			n.a.[b]
Lys29	32.6	4S22[c]	Y. A. Kristariyanto *et al.*[3]
Lys33	47.6	5AF4	M. A. Michel *et al.*[4]
Lys48	46.7	3NS8	M. Y. Lai *et al.*[5]
Lys63	41.6	2JF5	D. Komander *et al.*[6]

[a] Distances are calculated using UCSF Chimera 1.10.2 software. [b] No crystallography data for Lys27-linked diUb available. [c] Organism: *Bos Taurus*.

Table S2. Overview of all used deubiquitinating enzymes and concentrations used in kinetics assays.

DUB	Origin/Organism	Fragment	Conc. DUB in kinetics assays
USP7	Human	Full length Δ1-50 (51-1102)	Not used for kinetics assays
USP21	Human	Cat. Domain (196-565)	3.5 nM
AMSH	Human	Full length (1-424)	2000 nM
AMSH*	Human – Fused with activator STAM2[4]		2 nM
Cezanne	Human	Cat. Domain (129-438)	1.5 nM
TRABID	Human	Cat. Domain (245-692)	100 nM
vOTU	Crimean-Congo hemorrhagic fever virus (CCHFV)	1-183	1 nM
OTUB1	Human	Full length (1-271)	50 nM
OTUB1*	Human – Fused with activator UBE2D2[4]		3.5 nM
OTUD1	Human	Cat. Domain (287-481)	30 nM
OTUD2/YOD1	Human	Full length (1-348)	456 nM
OTUD3	Human	Cat. Domain (52-209)	250 nM

Table S3. Amplitude-weighted mean fluorescence lifetimes (τ) and calculated FRET efficiencies (*E*), determined by FLIM.

Compound	**Type**	**τ (ns)**	***E***
17a	Lys6	2.21	0.47
17b	Lys11	2.17	0.48
17c	Lys27	2.31	0.45
17d	Lys29	1.69	0.60
17e	Lys33	2.11	0.50
17f	Lys48	2.50	0.41
17g	Lys63	2.00	0.52
Rho-Ub	Donor only	4.20	0.00
TAMRA-Ub	Acceptor only	-	
Rho-Ub + TAMRA-Ub	Mix free donor + acceptor	4.18	0.00

3. Synthetic procedures

General

General reagents were obtained from Sigma Aldrich, Fluka and Acros and used as received. Solvents were purchased from Biosolve or Sigma Aldrich and, where necessary, dried over 4 Å molecular sieves (3 Å for MeOH). Peptide synthesis reagents were purchased from Novabiochem. Analytical thin layer chromatography (TLC) was performed on Merck aluminum sheets (precoated with silica gel 60 F^{254}). Compounds were visualized by UV absorption (254 nm) or by using 20% ninhydrin in ethanol or a solution of $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in water and heating by a heatgun. Column chromatography was carried out on silica gel (0.035-0.070 mm, 90 Å, Acros). Nuclear magnetic resonance spectra (^{1}H-NMR, ^{13}C-NMR) were recorded on a Bruker ARX 400 Spectrometer (^{1}H: 400 MHz, ^{13}C: 100 MHz) at 298 K. Peak shapes in NMR spectra are indicated with the symbols 'd' (doublet), 'dd' (double doublet), 's' (singlet), 't' (triplet), 'q' (quartet) and 'm' (multiplet). Chemical shifts (δ) are given in ppm relative to tetramethylsilane, DMSO-d6 or $CDCl_3$ as internal standard.

LC-MS

LC-MS measurements were performed on a LC-MS system equipped with a Waters 2795 Separation Module (Alliance HT), Waters 2996 Photodiode Array Detector (190-750 nm), Phenomenex Kinetex C18 column (2.1×30 mm, 2.6 μm, 100 Å) or Phenomenex Kinetex C18 column (2.1×100 mm, 2.6 μm, 100 Å) and LCT™ Orthogonal Acceleration Time of Flight Mass Spectrometer. Samples were run using 2 mobile phases: A = 0.1% formic acid in H_2O and B = 0.1% formic acid in CH_3CN. Data processing was performed using Waters MassLynx Mass Spectrometry Software 4.1 (deconvolution with Maxent1 function).

Program 1: Phenomenex Kinetex C18 column (2.1×30 mm, 2.6 μm, 100 Å); flow rate = 0.5 mL/min, runtime = 6 min, column T = 40°C, mass detection = 600 – 1700 Da. Gradient: 0 − 0.5 min: 5% B; 0.5 − 4 min: 5% → 95% B; 4 − 5.5 min: 95% B; 5.5 – 6 min: 95% → 5% B.

Program 2: Phenomenex Kinetex C18 column (2.1×100 mm, 2.6 μm, 100 Å); flow rate = 0.35 mL/min, runtime = 13 min, column T = 40°C, mass detection = 300 – 2500 Da. Gradient: 0 – 1 min: 2% B; 1 - 10 min: 2% → 98% B; 10 – 12 min: 98% B; 12 - 13 min: 98% → 2% B.

RP-HPLC

RP-HPLC purifications were performed on a Waters HPLC equipped with a Waters 2489 UV/Vis detector, Waters fraction collector III and Waters XBridge prep C18 OBD™ (30×150 mm, 5 μm). Flowrate = 37.5 mL/min. Mobile phase: A = H_2O, B = CH_3CN and C = 1% TFA in H_2O.

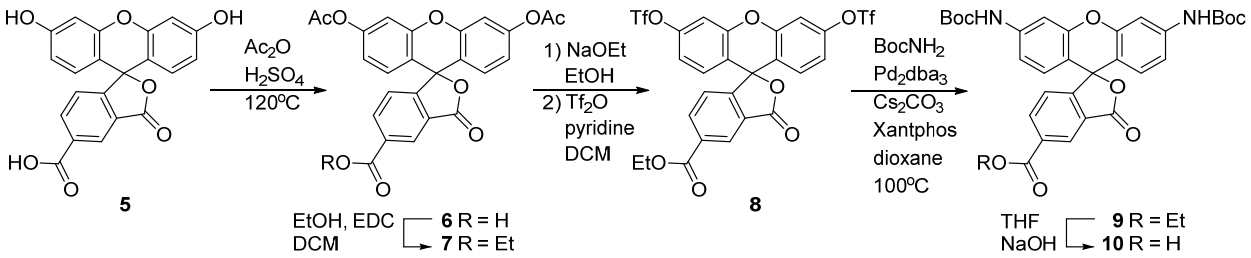
Gradient A: 15% → 50% CH_3CN

Time	A	B	C
0 – 5 min.	90%	5%	5%
5 – 7 min	→ 80%	→ 15%	5%
7 – 18 min.	→ 45%	→ 50%	5%
18 – 18.5 min.	→ 0%	→ 95%	5%
18.5 – 21.6 min.	0%	95%	5%
21.6 – 25 min.	→ 90%	→ 5%	5%

Gradient B: 20% → 45% CH_3CN

Time	A	B	C
0 – 5 min.	90%	5%	5%
5 – 7 min	→ 75%	→ 20%	5%
7 – 18 min.	→ 50%	→ 45%	5%
18 – 18.5 min.	→ 0%	→ 95%	5%
18.5 – 21.6 min.	0%	95%	5%
21.6 – 25 min.	→ 90%	→ 5%	5%

Synthesis of *N*,*N*'-diBoc-5-carboxyrhodamine



3',6'-Diacetoxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylic acid (5-carboxyfluorescein diacetate) (6)

5-Carboxyfluorescein[7] (**5**, 28.8 g, 76.6 mmol) was suspended in acetic acid anhydride (400 mL) and three drops of concentrated sulfuric acid (96%) were added. The reaction mixture was stirred at 120°C for 2 hours. The solvent was removed under reduced pressure and the residual oil stirred with ethanol (300 mL). The precipitated solid was filtered and dried to obtain the product as a colorless solid (yield: 35.1 g, 76.3 mmol, 99.6%). ^{1}H NMR (300 MHz, *DMSO-d6*) δ ppm 8.45 (dd, *J* = 1.46, 0.70 Hz, 1H), 8.32 (dd, *J* = 8.04, 1.51 Hz, 1H), 7.55 (dd, *J* = 8.05, 0.66 Hz, 1H), 7.30 (dd, *J* = 1.79, 0.80 Hz, 2H), 7.00-6.92 (m, 4H), 2.29 (s, 6H). The spectral data corresponded to that reported in literature.[8]

5-(Ethoxycarbonyl)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl diacetate (7)

3',6'-diacetoxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylic acid (**6**, 4.0 g, 8.7 mmol, 1.0 eq.) was dissolved in DCM. 4-(dimethylamino)pyridine (0.14 g, 0.87 mmol, 0.1 eq.), ethanol (2.0 mL, 34.8 mmol, 4.0 eq.) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (2.0 g, 10.4 mmol, 1.2 eq.) were added subsequently. The reaction mixture was stirred overnight, washed twice with 1 M HCl (aq) and once with brine. The organic layer was dried with $MgSO_4$, filtered and evaporated to obtain the product as a yellow fluffy solid (yield: 4.0 g, 8.2 mmol, 94.1%). ^{1}H NMR (300 MHz, *CDCl$_3$*) δ ppm 8.69 (dd, *J* = 1.45, 0.71 Hz, 1H), 8.35 (dd, *J* = 8.05, 1.50 Hz, 1H), 7.27 (dd, *J* = 8.09, 0.71 Hz, 1H), 7.11 (dd, *J* = 1.75, 0.86 Hz, 2H), 6.86-

6.78 (m, 4H), 4.44 (q, *J* = 7.13 Hz, 2H), 2.30 (s, 6H), 1.43 (t, *J* = 7.13 Hz, 3H). ^{13}C NMR (75 MHz, *CDCl$_3$*) δ ppm 168.6, 167.9, 164.7, 156.2, 152.1, 151.3, 136.2, 132.8, 128.7, 126.6, 126.4, 124.2, 117.8, 115.6, 110.4, 81.7, 61.7, 53.4, 20.9, 14.1.

Ethyl 3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylate

5-(ethoxycarbonyl)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl diacetate (**7**, 29.9 g, 61.3 mmol, 1.0 eq.) was suspended in dry ethanol (300 mL). The reaction mixture was cooled to 0°C and sodium ethoxide (8.3 g, 122 mmol, 2.0 eq.) was added. The reaction mixture was stirred overnight at room temperature. The reaction mixture was cooled to 0°C and a second batch of sodium ethoxide (2.1 g, 0.5 eq.) was added. The reaction mixture was stirred overnight at room temperature again. The reaction mixture was cooled to 0°C and a third batch of sodium ethoxide (2.1 g, 0.5 eq.) was added. The reaction mixture was stirred for 6 hours at room temperature. Concentrated HCl (aq) was added until pH 1. The solvent was evaporated and the residual solid co-evaporated twice with ethanol. The solid was dried and used in the next step without further purification (crude yield: 37.4 g (151%)).

Ethyl 3-oxo-3',6'-bis(((trifluoromethyl)sulfonyl)oxy)-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylate (8)

Pyridine (29.7 mL, 368 mmol, 15 eq.) was added to a suspension of ethyl 3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylate (purity ~66%) (15.0 g, 24.5 mmol, 1.0 eq.) in dry DCM (225 mL). The reaction mixture was cooled to 0°C and triflic anhydride (16.5 mL, 98.0 mmol, 4.0 eq.) was added dropwise. The reaction was stirred overnight at room temperature. The reaction mixture was washed with 1 M HCl (aq) three times and once with water. The organic layer was dried over $MgSO_4$, filtered and evaporated to leave a solid which was stirred for two hours with diethyl ether. The solid was filtered and dried to obtain the product as an orange solid (yield: 9.3 g, 13.8 mmol, 56.5%). ^{1}H NMR (300 MHz, *DMSO-d6*) δ ppm 8.48 (dd, *J* = 1.47, 0.68 Hz, 1H), 8.35 (dd, *J* = 8.08, 1.53 Hz, 1H), 7.80 (d, *J* = 2.48 Hz, 2H), 7.68 (dd, *J* = 8.09, 0.64 Hz, 1H), 7.31 (dd, *J* = 8.85, 2.52 Hz, 2H), 7.21 (d, *J* = 8.88 Hz, 2H), 4.41 (q, *J* = 7.10 Hz, 2H), 1.37 (t, *J* = 7.10 Hz, 3H). ^{13}C NMR (75 MHz, *DMSO-d6*) δ ppm

167.0, 164.2, 155.2, 150.6, 149.9, 136.4, 132.4, 130.8, 125.9, 125.9, 125.0, 118.5, 118.1 (q, *J* = 320.98 Hz), 117.9, 110.8, 79.9, 61.5, 14.0. LC-MS (50→98% ACN/H_2O): R_t (min): 9.46 (MS (*m/z*): 669.0 ($M+H^+$)).

Ethyl 3',6'-bis((*tert*-butoxycarbonyl)amino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylate (9)

This compound was synthesized following the procedure reported by J. B. Grimm *et. al.*[9]

Ethyl 3-oxo-3',6'-bis(((trifluoromethyl)sulfonyl)oxy)-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylate (**8**, 9.3 g, 13.8 mmol, 1.0 eq.), *tert*-butyl carbamate (4.1 g, 34.6 mmol, 2.4 eq.), 4,5-bis(diphenylphosphino)-9,9-dimethylxanthene (Xantphos) (2.4 g, 4.2 mmol, 0.3 eq.), tris(dibenzylideneacetone)dipalladium(0) (Pd_2dba_3) (1.3 g, 1.4 mmol, 0.1 eq.) and cesium carbonate (12.6 g, 38.9 mmol, 2.8 eq.) were weighed in a 250 mL round bottom flask. The flask was evacuated and refilled with argon three times. Dioxane (70 mL) was added and the flask was again evacuated and refilled with argon three times. The reaction mixture was stirred at 100°C overnight. The reaction mixture was cooled and DCM (70 mL) was added. The suspension was filtered through hyflo and the hyflo bed was washed with DCM. The filtrate was evaporated under reduced pressure and the resulting dark brown oil was purified by flash chromatography (eluents: *n*-heptane/EtOAc, (3/1) → (2/1)). The product was obtained as a colorless foam (yield: 6.8 g, 11.3 mmol, 81.8%). ^{1}H NMR (300 MHz, *DMSO-d6*) δ ppm 9.70 (s, 2H), 8.43 (d, *J* = 0.76 Hz, 1H), 8.30 (dd, *J* = 8.07, 1.52 Hz, 1H), 7.56 (d, *J* = 2.01 Hz, 2H), 7.41 (dd, *J* = 8.07, 0.46 Hz, 1H), 7.15 (dd, *J* = 8.75, 2.10 Hz, 2H), 6.72 (dd, *J* = 8.72, 3.39 Hz, 2H), 4.40 (q, *J* = 7.10 Hz, 2H), 1.48 (s, 19H), 1.37 (t, *J* = 7.10 Hz, 3H). ^{13}C NMR (75 MHz, *DMSO-d6*) δ ppm 167.6, 164.4, 156.2, 152.5, 150.8, 142.0, 135.9, 131.8, 128.4, 126.5, 125.4, 124.6, 114.3, 111.1, 104.8, 82.4, 79.6, 62.4, 27.9, 14.0. LC-MS (50→98% ACN/H_2O): R_t (min): 8.04 (MS (*m/z*): 603.2 ($M+H^+$)).

3',6'-Bis((*tert*-butoxycarbonyl)amino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylic acid (10)

Ethyl 3',6'-bis((*tert*-butoxycarbonyl)amino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylate (**9**, 4.0 g, 6.6 mmol, 1.0 eq.) was dissolved in THF (50 mL). Sodium hydroxide (0.66 g, 16.6 mmol, 2.5 eq.) dissolved in water (6 mL) was added and the reaction mixture was stirred overnight. The reaction mixture was cooled to 0°C and acidified to pH 1 using 1 M HCl (aq). The reaction mixture was extracted three times with EtOAc. The organic layer was washed with brine once, dried with $MgSO_4$, filtered and concentrated under reduced pressure. The oily residue was stirred with diethyl ether/*n*-heptane (1/1) (40 mL) overnight. The precipitated solid was filtered and dried to obtain the product as an off-white solid (3.6 g, 6.3 mmol, 95.0%). ^{1}H NMR (300 MHz, *DMSO-d6*) δ ppm 9.71 (s, 2H), 13.74-13.38 (m, 1H), 8.42 (d, *J* = 0.65 Hz, 1H), 8.30 (dd, *J* = 8.05, 1.47 Hz, 1H), 7.57 (d, *J* = 1.98 Hz, 2H), 7.43-7.33 (m, 1H), 7.16 (dd, *J* = 8.76, 2.09 Hz, 2H), 6.74 (d, *J* = 8.71 Hz, 2H), 5.76 (s, 1H), 1.49 (s, 17H). ^{13}C NMR (75 MHz, *DMSO-d6*) δ ppm 168.3, 166.5, 156.6, 153.1, 151.4, 142.5, 136.7, 133.4, 129.0, 126.9, 126.1, 125.0, 114.9, 111.9, 105.4, 82.9, 80.2, 28.5. LC-MS (50$\rightarrow$98% ACN/H_2O): R_t (min): 6.88 (MS (*m/z*): 575.2 ($M+H^+$)).

Synthesis of diUb FRET pairs (17a-g)

General method for the N-terminal modification of Ub

Ub(1-75, Nle_1) (**11**) and seven Ub(1-76, Nle_1) (**15 a-g**) polypeptides with a single γ-thiolysine in positions 6, 11, 27, 29, 33, 48 or 63 with a free N-terminus were synthesized on a trityl resin; TentaGel® R TRT-Gly Fmoc (Rapp Polymere GmbH; RA1213), following the previously reported procedure[10] on 40 μmol scale. The resin was washed with NMP and DCM, prior to further modifications. A solution of PyBOP (4 eq., 83.3 mg, 160 μmol) in NMP (0.6 mL) was made. A second solution of 5-carboxytetramethylrhodamine (5-carboxy-TAMRA) (4 eq., 68.8 mg, 160 μmol) in case of Ub(1-76, Nle_1) (**15 a-g**) or *N*,*N*'-Boc-protected 5-carboxyrhodamine (**10**) (4 eq., 91.8 mg, 160 μmol) in case of Ub(1-75, Nle_1) (**11**) and DiPEA (15 eq., 104 μL, 77.5 mg, 600 μmol) in NMP (0.6 mL) was made. These two solution were mixed and after a pre-activation of 5 minutes the solution was added to the resin-bound peptides (1 eq., 40 μmol). The mixture was shaken overnight. The resin was filtered and washed three times with NMP and three times with DCM. To follow the reaction progress a mini-cleavage was done. A small amount of resin was taken and the protein was cleaved from the resin under general cleavage conditions. The reaction was checked by LC-MS analysis (Program 1). When the reaction was complete, the resin was washed alternately with DCM and MeOH and followed by alternately washing with DCM and Et_2O. Finally the resin was washed with Et_2O and dried under high vacuum.

General procedure for mini-cleavage

The progress of the reaction was analyzed by a mini cleavage. A small amount of resin in DCM was parted from the reaction mixture and washed with DCM and Et_2O. The resin was air-dried, dissolved in TFA/H_2O/Phenol/iPr_3SiH (90.5/5/2.5/2; v/v/v/v; 100 μL) and shaken for 2.5 h. The reaction mixture was added to cold Et_2O/*n*-pentane (3/1; v/v; 1.5 mL) and the protein was precipitated and centrifuged. The precipitated protein was washed three times with Et_2O (1 mL) and centrifuged. The pellet was dissolved in

DMSO (50 μL). The DMSO solution (2 μL) was diluted in 0.1% aqueous formic acid (80 μL) and analyzed by LC-MS (Program 1).

Global deprotection and cleavage of TAMRA-Ub from the resin

The polypeptide was deprotected and detached from the resin by treatment with TFA/H_2O/Phenol/iPr_3SiH (90.5/5/2.5/2; v/v/v/v; 7.5 mL) for 2.5 h. The reaction mixture was filtered directly into cold Et_2O/*n*-pentane (3/1; v/v; 35 mL) and the resin was washed with TFA (2×4mL). The precipitated protein was centrifuged, the Et_2O/*n*-pentane was removed and the protein was washed three times with Et_2O (30 mL) and centrifuged. The pellet was dissolved in H_2O/CH_3CN/formic acid (65/35/10; v/v/v; 15 mL) and lyophilized.

Synthesis of Rh-Ub(1-76, Nle_1)-S$(CH_2)_2CO_2Me$ (14)

Resin-bound Rh-Ub(1-75, Nle_1) (**12**) was washed with DCM. A solution of DCM/HFIP (4/1; v/v; 5 mL) was added to the resin, shaken for 20 minutes and filtered. The resin was rinsed two times with DCM (8 mL) and the combined filtrates were concentrated under reduced pressure. The residual resin was treated a second time following the same procedure. The protected protein was co-evaporated three times with DCE to remove traces of HFIP and lyophilized overnight. The protected protein was dissolved in DCM (5 mL) and EDC (3 eq., 23 mg, 120 μmol), HOBt (3 eq., 18.3 mg, 120 μmol) and HCl·H-Gly-S$(CH_2)_2CO_2$Me (3 eq., 25.7 mg, 120 μmol) were added to the solution. The reaction mixture was stirred overnight. To follow the reaction progress a mini-deprotection was done. A small amount of the reaction mixture was taken and the protection groups were removed under general cleavage conditions. The reaction was checked by LC-MS analysis (Program 1). When the reaction was complete, the reaction mixture was extracted with 1 M $KHSO_4$ (2×30 mL). The organic layer was dried with $MgSO_4$, filtered and concentrated under reduced pressure. The residue was treated with TFA/H_2O/Phenol/iPr_3SiH (90.5/5/2.5/2; v/v/v/v; 7.5 mL) for 2.5 h. The reaction mixture was directly added to cold Et_2O/*n*-pentane (3/1; v/v; 35 mL) and the protein precipitated. The precipitated protein was centrifuged, the Et_2O/*n*-pentane was removed

and the protein was washed three times with Et_2O (30 mL) and centrifuged. The pellet was dissolved in H_2O/CH_3CN/formic acid (65/35/10; v/v/v; 15 mL) and lyophilized.

Purification

A minimal amount of DMSO (max. 1.75 mL) was added to the crude protein and the solution was warmed until the protein was properly dissolved. The DMSO was carefully diluted into H_2O (32.9 mL) and 5 M NaOAc/HOAc pH 4.8 (0.35 mL) was added dropwise to the solution to create a 50 mM NaOAc/HOAc buffer solution (35 mL) with dissolved protein – the final DMSO amount was kept as low as possible (max. 5% of the final volume). The solution was loaded onto a MonoS cation exchange column (Workbeads 40S) and the protein was purified on a Büchi automatic chromatography system using a 0 → 1 M NaCl gradient in 50 mM NaOAc/HOAc buffer at pH 4.8. The fractions were analyzed by LC-MS (Program 1) and the fractions containing the product were combined and purified on RP-HPLC following the general procedure using gradient A. The fractions were analyzed by LC-MS analysis and pure fractions were combined, lyophilized, dissolved in H_2O/CH_3CN/formic acid (65/35/10; v/v/v; 15 mL) and lyophilized again.

TAMRA–Ub(1-76, Nle_1, γ-thioLys)

The products were obtained as a pink-purple solids. LC-MS analysis using Program 2. Yields:

TAMRA-Ub(1-76, Nle_1, γ-thioLys$_6$) (**16a**) = 27.9 mg, 3.08 μmol, 7.7%. LC-MS: R_t 5.21 min: MS ES+ (amu) calculated: 9078.3 [M]; found 9078.5.

TAMRA-Ub(1-76, Nle_1, γ-thioLys$_{11}$) (**16b**) = 31.3 mg, 3.45 μmol, 8.6%. LC-MS: R_t 5.25 min: MS ES+ (amu) calculated: 9078.3 [M]; found 9077.5.

TAMRA-Ub(1-76, Nle_1, γ-thioLys$_{27}$) (**16c**) = 15.6 mg, 1.71 μmol, 4.3%. LC-MS: R_t 5.15 min: MS ES+ (amu) calculated: 9078.3 [M]; found 9077.0.

TAMRA-Ub(1-76, Nle_1, γ-thioLys$_{29}$) (**16d**) = 32.0 mg, 3.52 μmol, 8.8%. LC-MS: R_t 5.14 min: MS ES+ (amu) calculated: 9078.3 [M]; found 9077.0.

TAMRA-Ub(1-76, Nle_1, γ-thioLys$_{33}$) (**16e**) = 22.5 mg, 2.48 μmol, 6.2%. LC-MS: R_t 5.10 min: MS ES+ (amu) calculated: 9078.3 [M]; found 9077.0.

TAMRA-Ub(1-76, Nle_1, γ-thioLys_{48}) (**16f**) = 47.5 mg, 5.23 μmol, 13.1%. LC-MS: R_t 5.02 min: MS ES+ (amu) calculated: 9078.3 [M]; found 9077.0.

TAMRA-Ub(1-76, Nle_1, γ-thioLys_{63}) (**16g**) = 33.6 mg, 3.70 μmol, 9.3%. LC-MS: R_t 5.08 min: MS ES+ (amu) calculated: 9078.3 [M]; found 9077.0.

Rh-Ub(1-76, Nle_1)-S$(CH_2)_2CO_2$Me (14)

The product was obtained as a yellow-orange solid. LC-MS analysis using Program 2. Yield:

Rh-Ub(1-76, Nle_1)-S$(CH_2)_2CO_2$Me (**14**) = 66.9 mg, 7.43 μmol, 18.6%. LC-MS: R_t 4.90 min: MS ES+ (amu) calculated: 9005.3 [M]; found 9003.0.

Native Chemical Ligation of TAMRA-Ub(1-76, Nle_1, γ-thioLys)(16a-g) and Rh-Ub(1-76, Nle_1)-S$(CH_2)_2CO_2$Me (14)

TAMRA-Ub(1-76, Nle_1, γ-thioLys) (**16a-g**, 1 eq., 10.0 mg, 1.10 μmol) and Rh-Ub(1-76, Nle_1)-S$(CH_2)_2CO_2$Me (**14**, 1.5 eq., 15.0 mg, 1.67 μmol) were dissolved in 500 μL of aqueous buffer containing 6.0 M Gn·HCl, 0.15 M Na_2HPO_4 and 0.25 M MPAA at pH 7.2 and shaken overnight at 37°C. The progress of the reaction was checked by LC-MS analysis (Program 1). The formed diUbs were purified by RP-HPLC by first adding 1 M aqueous TCEP solution pH 7.0 (250 μL), followed by dilution with H_2O to a final volume of 20 mL prior to injection. For RP-HPLC purification gradient B was used. The fractions were analyzed by LC-MS analysis and the fractions containing only product were combined. The fractions which contained product and MPAA were also combined. The combined fractions were lyophilized, dissolved in H_2O/CH_3CN/formic acid (65/35/10; v/v/v; 15 mL) and lyophilized again.

The mixture of product and MPAA was dissolved in H_2O/CH_3CN/AcOH (72/18/10; v/v/v; 15 mL) and the MPAA was removed using a 3 kDa cutoff spin-column (Amicon®Ultra)-15. The removal of MPAA was checked by means of LC-MS analysis (Program 1). The residue was lyophilized, dissolved in H_2O/CH_3CN/formic acid (65/35/10; v/v/v; 15 mL) and lyophilized again.

Desulfurization

Thiol-containing diUbFRET pair was dissolved in aqueous buffer containing 6.0 M Gn·HCl, 0.15 M Na_2HPO_4 and 0.25 M TCEP at pH 7.0 to a concentration of 1 mg/mL protein. Reduced glutathione (GSH) was added to the solution to a concentration of 100 mM. The pH of the solution was adjusted to pH 7.0 by addition of 1 M NaOH. VA-044 was added to the solution to a final concentration of 75 mM. The reaction mixture was flushed with argon and shaken overnight at 37°C. The progress of the reaction was checked by LC-MS analysis (Program 1). When the reaction did not go to completion half of the initial GSH amount was added to the solution, the pH was re-adjusted to pH 7.0 and half of the initial amount of VA-044 was added to the reaction mixture. The reaction was flushed with argon again and shaken at 37°C overnight. The desulfurized diUbs were purified by RP-HPLC using gradient B by first diluting the desulfurized diUbs reaction mixtures with H_2O (the same amount as reaction volume) and 5 M NaOAc/HOAc buffer (5% of the initial volume) prior to HPLC purification. The fractions were analyzed by LC-MS analysis and the fractions containing only product were combined, lyophilized, dissolved in H_2O/CH_3CN/formic acid (65/35/10; v/v/v; 15 mL) and lyophilized again. The desulfurized diUb FRET pairs were then purified by gel filtration using a Biorad NGC Chromatography system on a size exclusion S75 16/600 superdex PG-GE healthcare column with a volume bed of 120 mL and a 3-70 kDa separation range using a filtered aqueous buffer containing 50 mM TRIS·HCl and 100 mM NaCl at pH 7.6 at a flowrate of 1 mL/min.

DiUbFRET pairs 17a-g

The products were obtained as a pink solution in 50 mM TRIS·HCl and 100 mM NaCl buffer at pH 7.6. LC-MS analysis using Program 2. Yield:

DiUbFRET pair Lys6 linked (**17a**) = 3.9 mg, 0.22 μmol, 19.4%. LC-MS: R_t 7.14 min: MS ES+ (amu) calculated: 17843.8 [M]; found 17846.0.

DiUbFRET pair Lys11 linked (**17b**)= 5.5 mg, 0.31 μmol, 26.6%. LC-MS: R_t 7.33 min: MS ES+ (amu) calculated: 17843.8 [M]; found 17846.0.

DiUbFRET pair Lys27 linked (**17c**) = 1.4 mg, 0.08 μmol, 7.4%. LC-MS: R_t 7.18 min: MS ES+ (amu) calculated: 17843.8 [M]; found 17846.0.

DiUbFRET pair Lys29 linked (**17d**) = 2.6 mg, 0.15 μmol, 13.3%. LC-MS: R_t 7.14 min: MS ES+ (amu) calculated: 17843.8 [M]; found 17846.0.

DiUbFRET pair Lys33 linked (**17e**) = 2.2 mg, 0.12 μmol, 10.9%. LC-MS: R_t 7.14 min: MS ES+ (amu) calculated: 17843.8 [M]; found 17846.5.

DiUbFRET pair Lys48 linked (**17f**) = 2.6 mg, 0.14 μmol, 12.1%. LC-MS: R_t 7.10 min: MS ES+ (amu) calculated: 17843.8 [M]; found 17846.0.

DiUbFRET pair Lys63 linked (**17g**) = 6.6 mg, 0.37 μmol, 32.1%. LC-MS: R_t 7.14 min: MS ES+ (amu) calculated: 17843.8 [M]; found 17846.0.

4. Biochemical procedures

SDS-PAGE analysis

A sample of the reaction mixture was diluted in sample buffer (3×), containing NuPAGE® LDS sample buffer (4×, Invitrogen) (450 μL), β-mercaptoethanol (45 μL) and milli-Q (105 μL), heated at 95°C for 5 minutes and loaded on 12% NuPAGE® Novex® Bis-Tris Mini Gels (Invitrogen) using MES-SDS running buffer. SeeBlue Plus2 Pre-stained Standard (Invitrogen, cat# LC5925) was used as marker. Fluorescence gel scans were performed on a Perkin Elmer ProXpress 2D proteomic Imaging System and the Coomassie Brilliant Blue stains were scanned using a Biorad ChemiDoc XRS+ with Image Lab Software.

Emission spectra

Emission spectra were recorded on a BMG Labtech Clariostar using "non binding surface flat bottom low flange" black 384-well plates (Corning). Solutions of diUb FRET pairs (**17a-g**) in a buffer containing 50 mM Tris·HCl, 100 mM NaCl, pH 7.6 at a concentration of ~200-400 nM were added to the wells. The emission was recorded between 491-618 nm at an excitation of 466 nm. Fluorescence intensities were normalized to the highest intensity value for each diUb FRET pair and plotted.

FRET efficiency determination by Fluorescence Lifetime Imaging Microscopy (FLIM)

FLIM was measured by time-correlated single-photon counting (TCSPC) using a Leica/PicoQuant SP8-SMD system equipped with 80 MHz pulsed white-light laser set at 488 nm. Solutions (1.0 μM) of all diUb FRET pairs (**17a-g**), Rho-Ub-S$(CH_2)_2CO_2$Me (**14**) and TAMRA-Ub (**16f**) in a buffer containing 50 mM Tris·HCl, 100 mM NaCl, pH 7.6, 1 mg/mL CHAPS and 0.5 mg/mL bovine gamma globulins (BGG) were made. 500 μL of each solution was transferred to a glass-bottom dish and placed on the 60x water immersion objective. Detection of fluorescence emission was restricted to a narrow passband between 494-520 nm to reject acceptor fluorescence. Fluorescence decay

curves were collected for 10 seconds each and analyzed with PicoQuant software. Mean FRET efficiency values, *E*, were calculated from

$$E = 1 - \tau_{DA}/\tau_D$$

Where τ_{DA} is the amplitude-weighted mean fluorescence lifetime of donor (Rho) in the diUb FRET pair. τ_D is the mean fluorescence lifetime of donor (Rho) in Rho-Ub, calculated from the fit to the fluorescence lifetime decays.

Determination of ideal DUB concentrations for kinetics assays

The assays were performed in “non binding surface flat bottom low flange” black 384-well plates (Corning) at room temperature in a buffer containing 50 mM Tris·HCl, 100 mM NaCl, pH 7.6, 2.0 mM DTT, 1 mg/mL 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS) and 0.5 mg/mL γ-globulins from bovine blood (BGG). Each well had a volume of 20 µL.

For all nine DUBs, one linkage was chosen to determine the ideal DUB concentration for kinetics assays, based on reported specificities. A concentration series (final concentrations 2000, 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 2.0 and 0 nM) of DUB in buffer were predispensed and the appropriate diUb FRET pair (final concentration 0.45 µM) was added by which the reaction was started. Fluorescence intensities of donor emission were measured on a BMG Labtech Clariostar in intervals of 2 minutes by excitation of the donor at 487 nm and emission at 535 nm. The fluorescence intensity was plotted against time. Based on this plot, the ideal kinetics assay concentration was chosen as such that the reaction rate was constant during the first 30 minutes with a good signal-to-background ratio (see Table S2).

Michaelis-Menten kinetics assay

The assays were performed in “non binding surface flat bottom low flange” black 384-well plates (Corning) at room temperature in a buffer containing 50 mM Tris·HCl, 100 mM NaCl, pH 7.6, 2.0 mM DTT, 1 mg/mL 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS) and 0.5 mg/mL γ-globulins from bovine blood (BGG).

Each well had a volume of 15 μL. The substrates were prepared in a 96-well plate by making a mixtures of diUbFRET pairs (1.0 μM) and a concentration series of the corresponding unlabeled diUbs (56.9, 28.5, 14.2, 7.1, 3.6, 1.8, 0.9, 0.4, 0.2, 0.1, 0.06 μM) in buffer. These substrate mixtures (2× final concentration, 7.5 μL) were predispensed and the reaction was started by addition of the enzyme solution (2× final concentration, 7.5 μL).

Kinetics assays were performed on a BMG Labtech Clariostar. Fluorescence intensities were measured in intervals of 2 or 4 minutes at a certain gain and focal height by excitation of the donor at 487 nm emission at 535 nm. Fluorescence intensities values were converted to “amounts of converted substrate” using a calibration curve, measured at the same gain and focal height.

The calibration curve was constructed by making mixtures of diUbFRET, TMR-Ub and Rh-Ub of the appropriate ratios at an ‘original concentration’ of 0.5 μM diUbFRET, thereby mimicking conversions of 0%, 5%, 10%, 20%, 35%, 50%, 75% and 100%.

The amount of converted substrate was plotted against time from which the initial velocities (v_i) of the reaction were determined. The initial velocities (v_i) were used to determine the Michaelis-Menten constants (K_M, V_{max}, and k_{cat}) by fitting the data according to the Michaelis-Menten equation below (where $k_{cat}=V_{max}/[E]$). All experimental data was processed using Ms Excel and Prism 6 (GraphPad Software, Inc.).

$$v_i = (V_{max} \times S_0) / (K_M + S_0)$$

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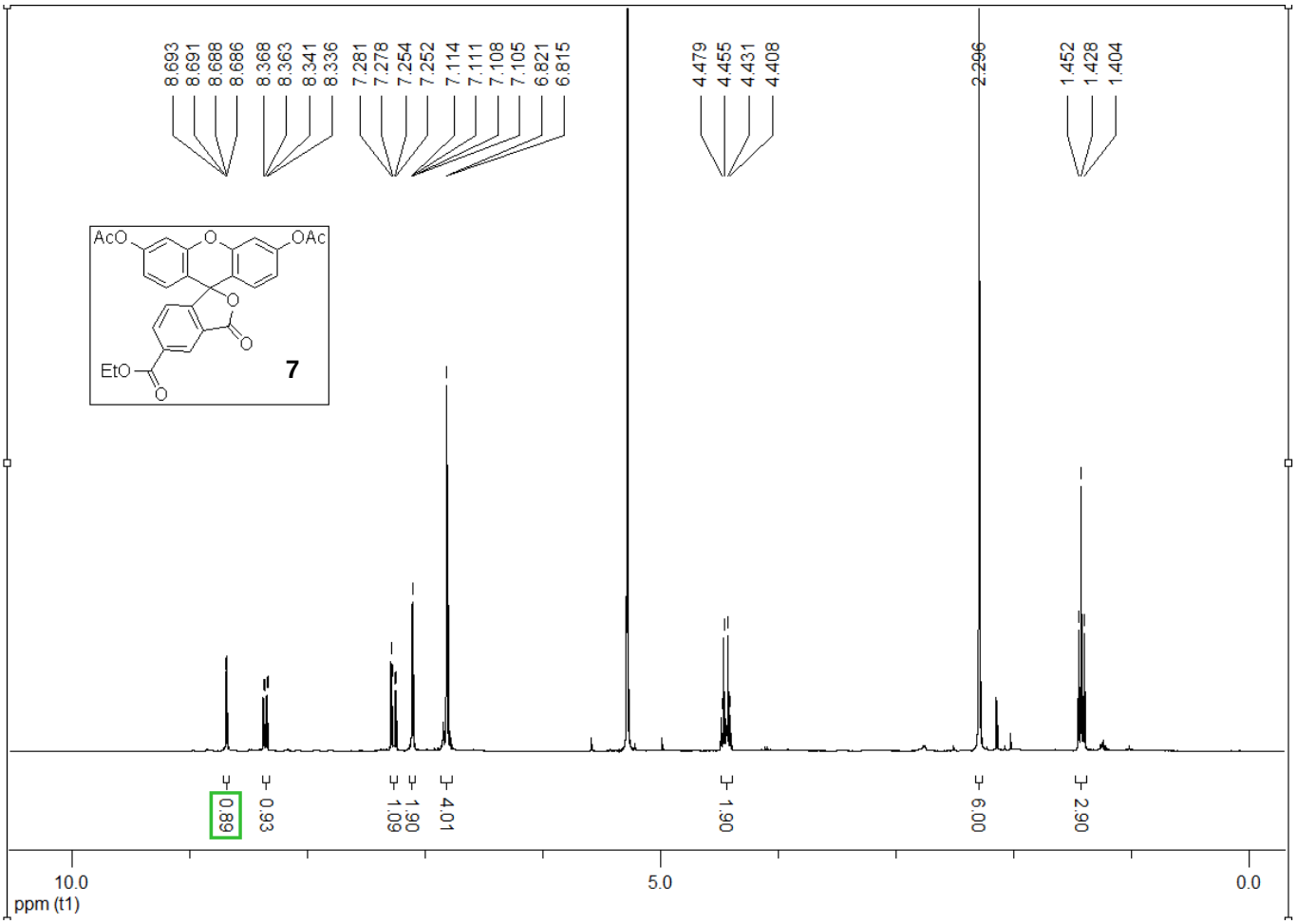
7. NMR and LC-MS data and Michaelis-Menten kinetics graphs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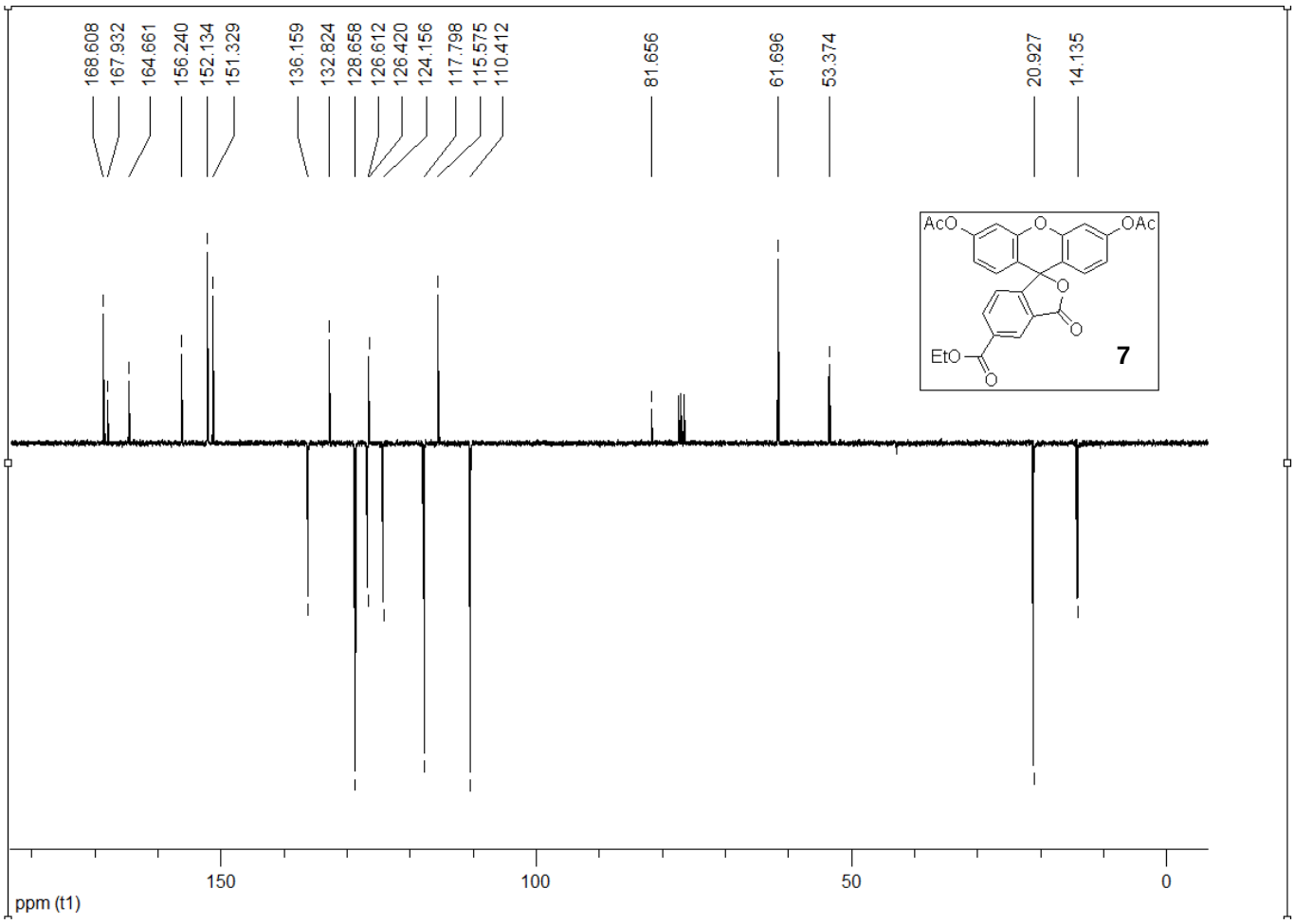
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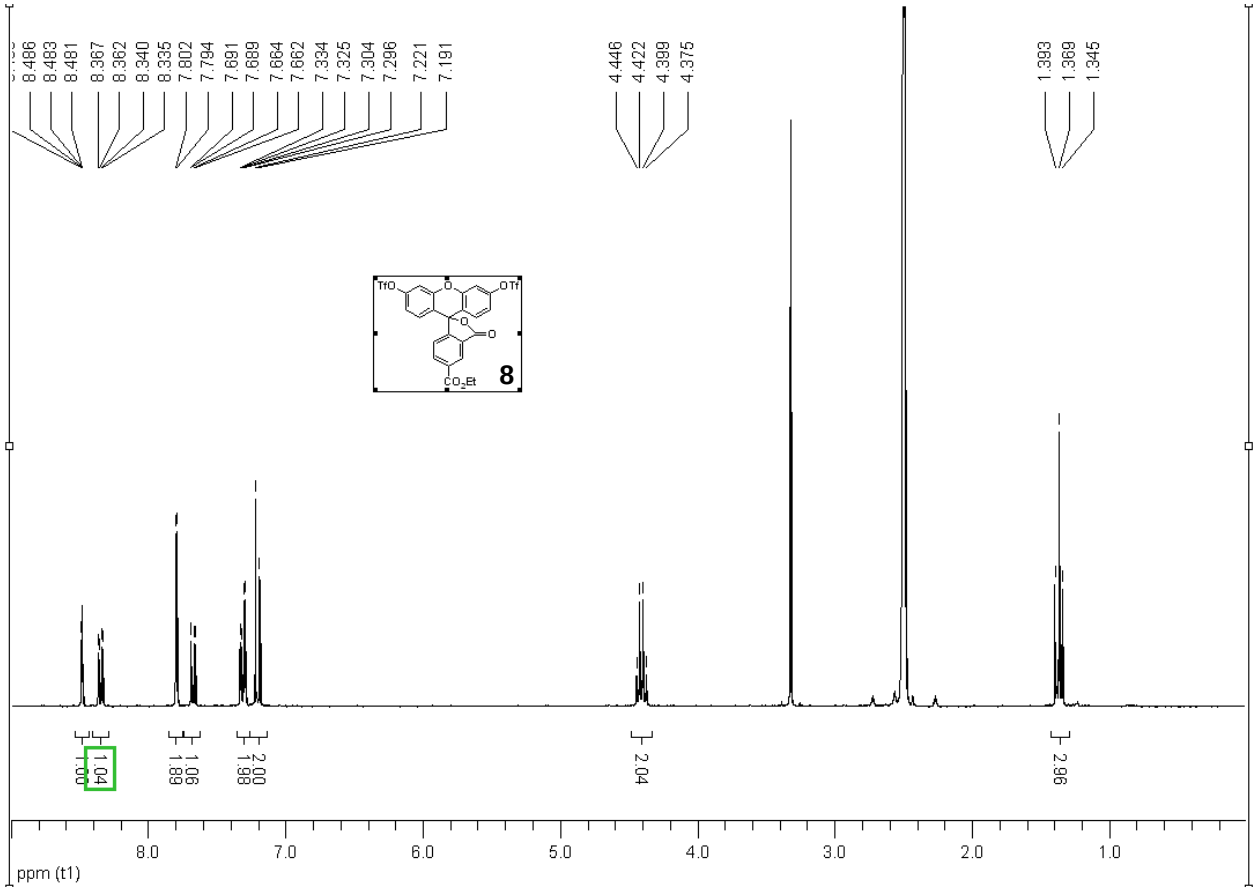
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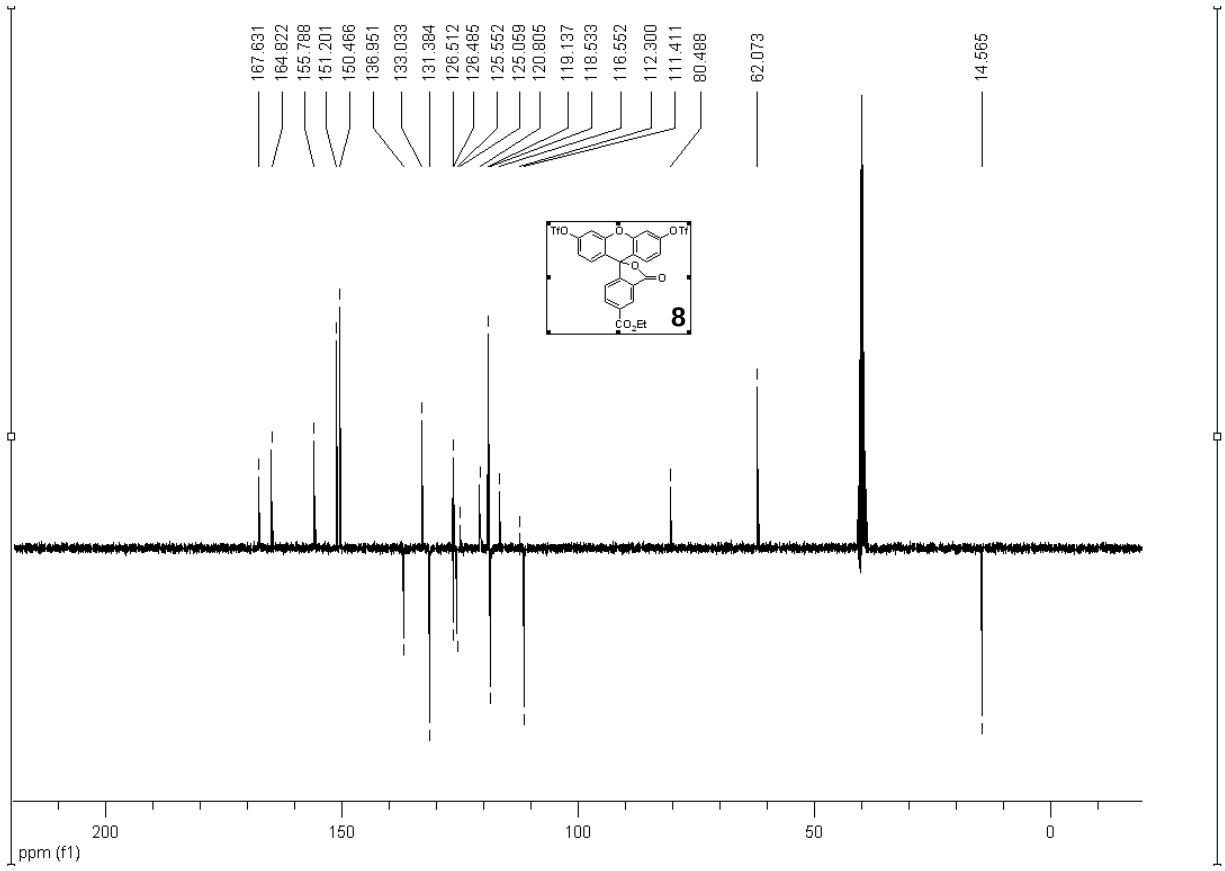
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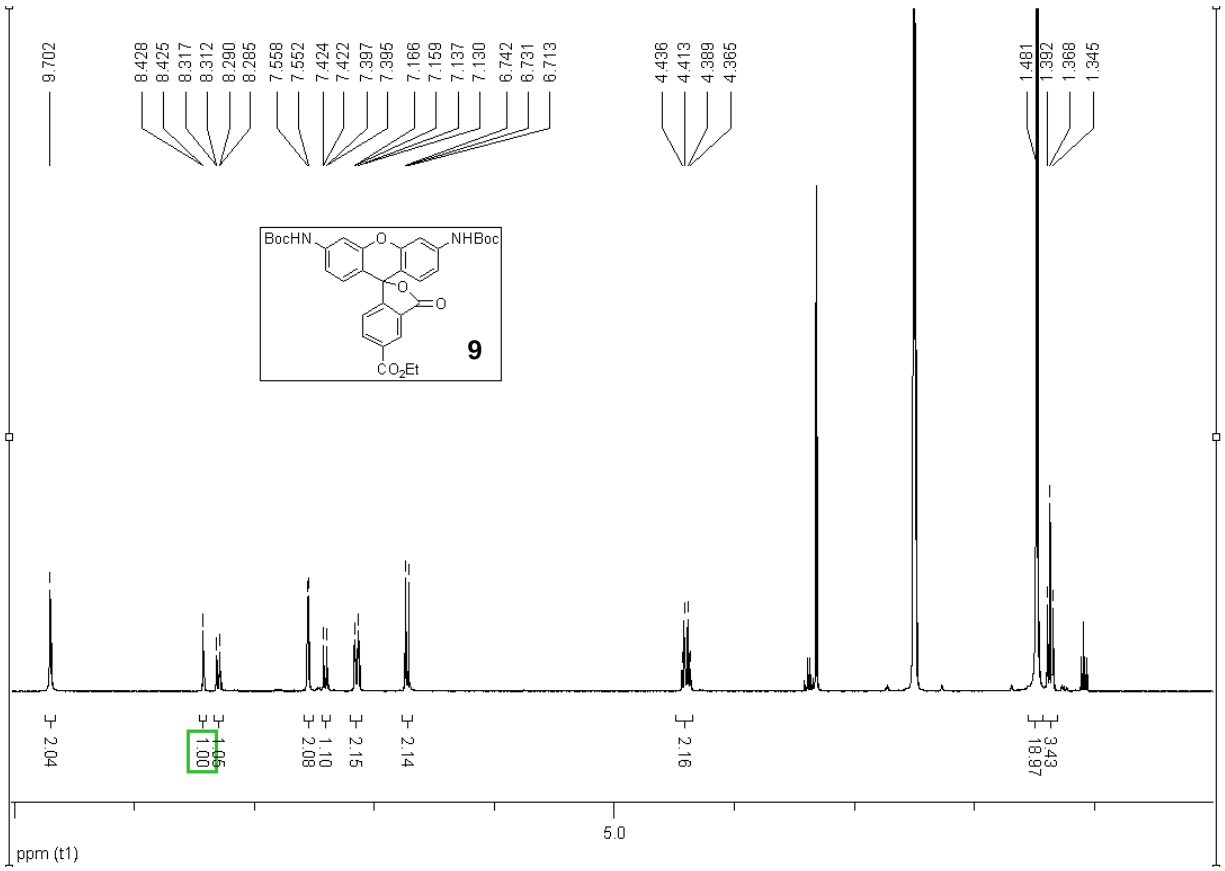
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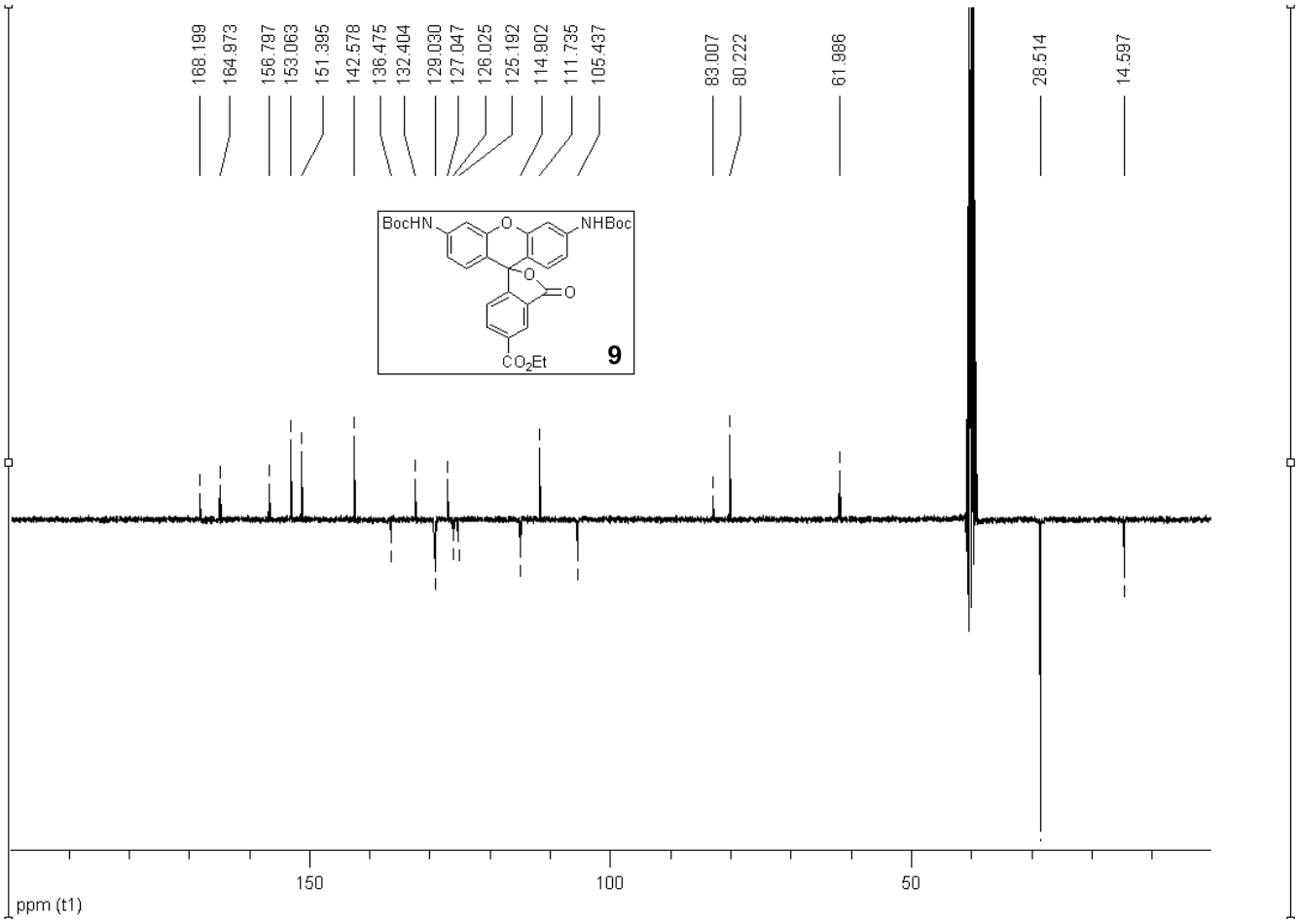
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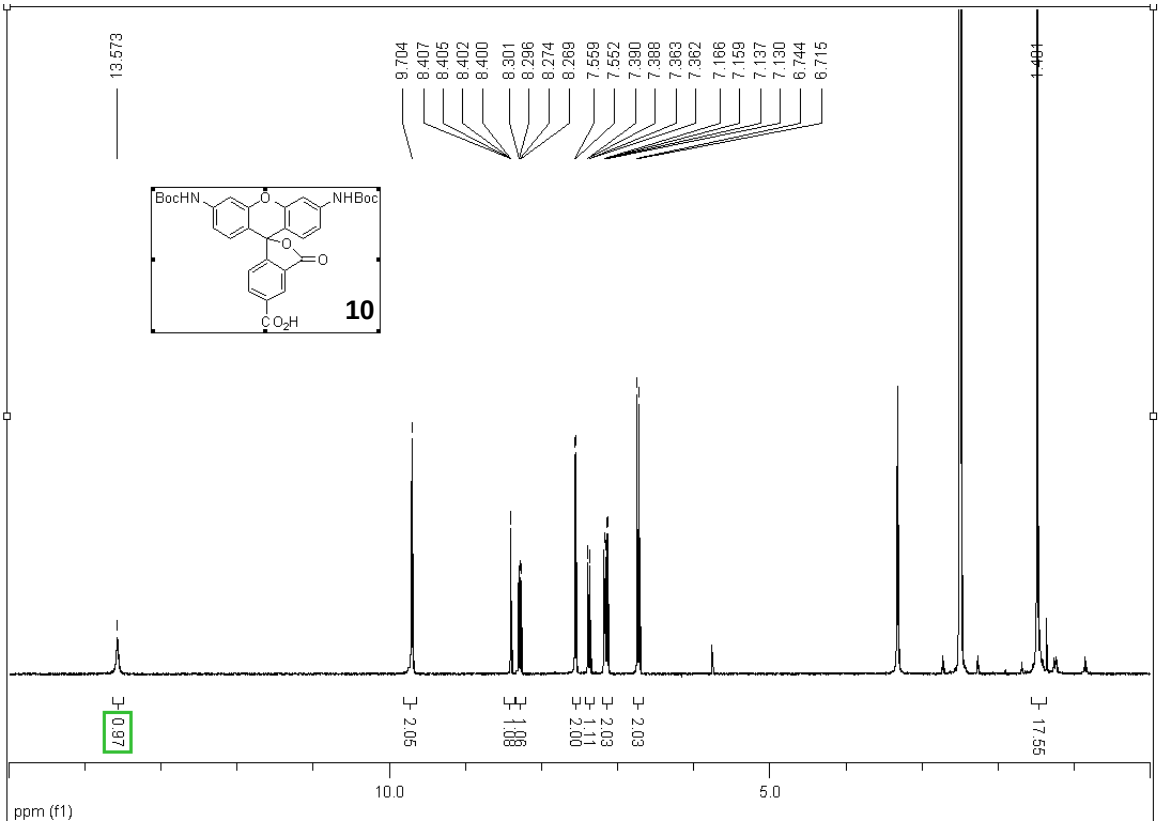
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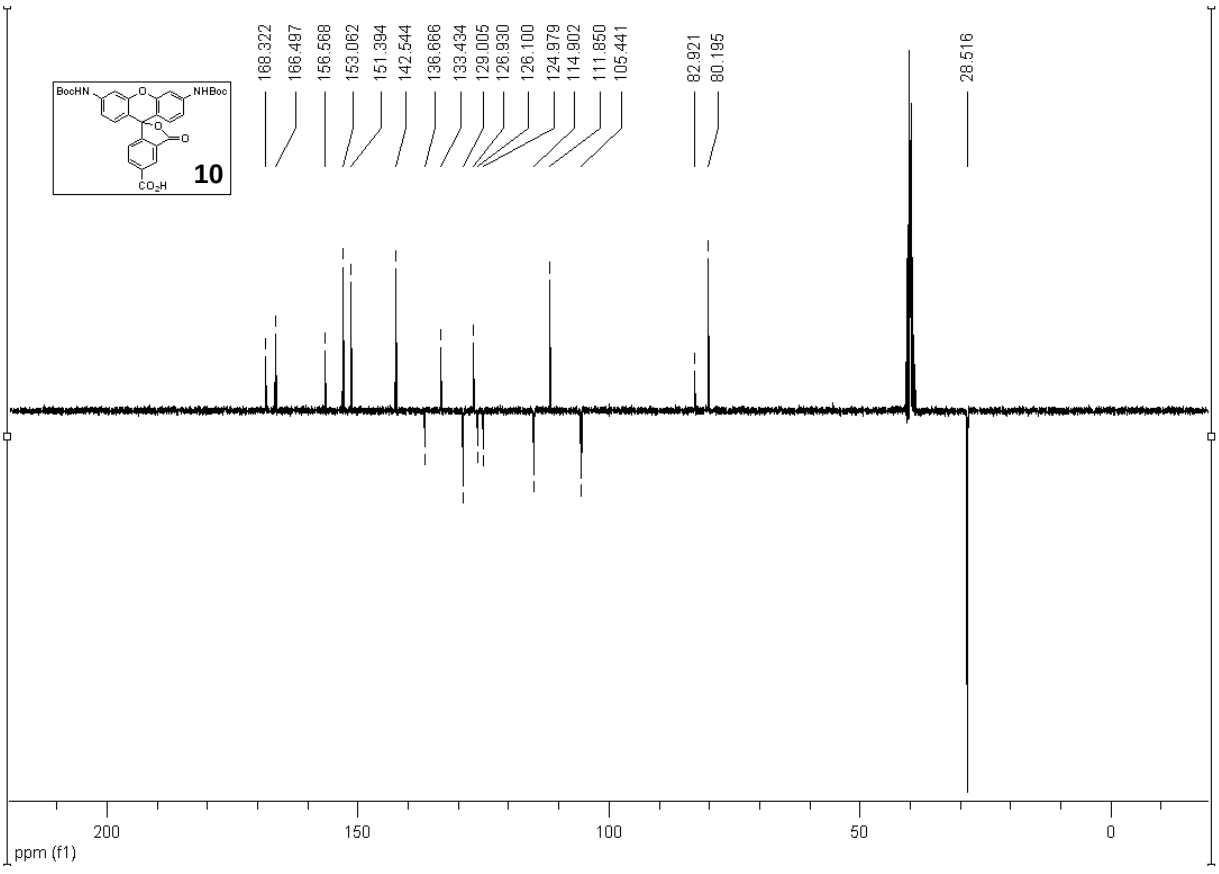
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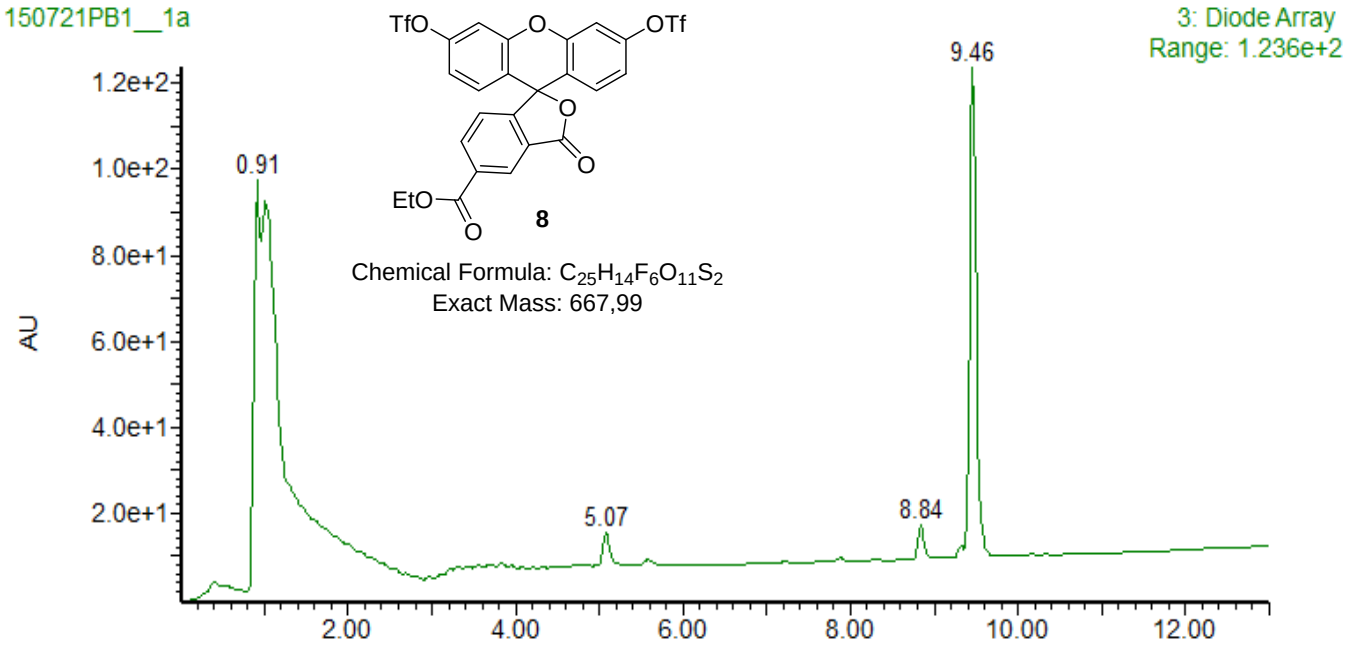
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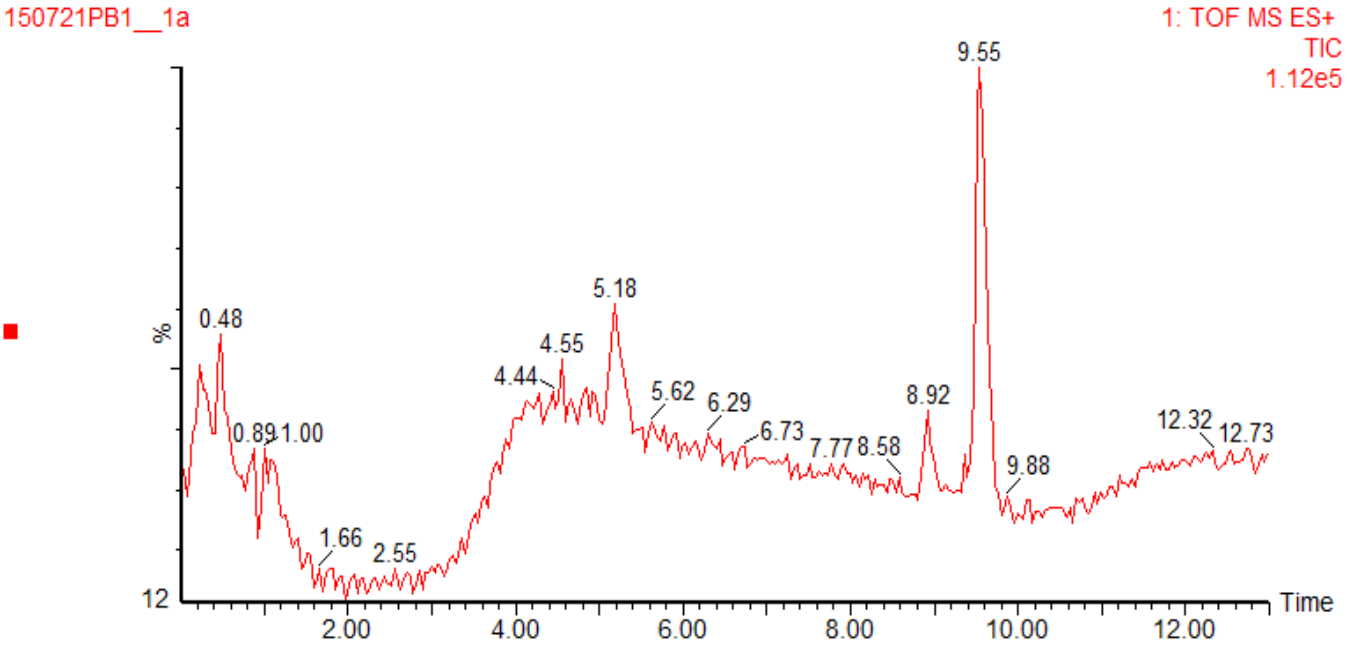
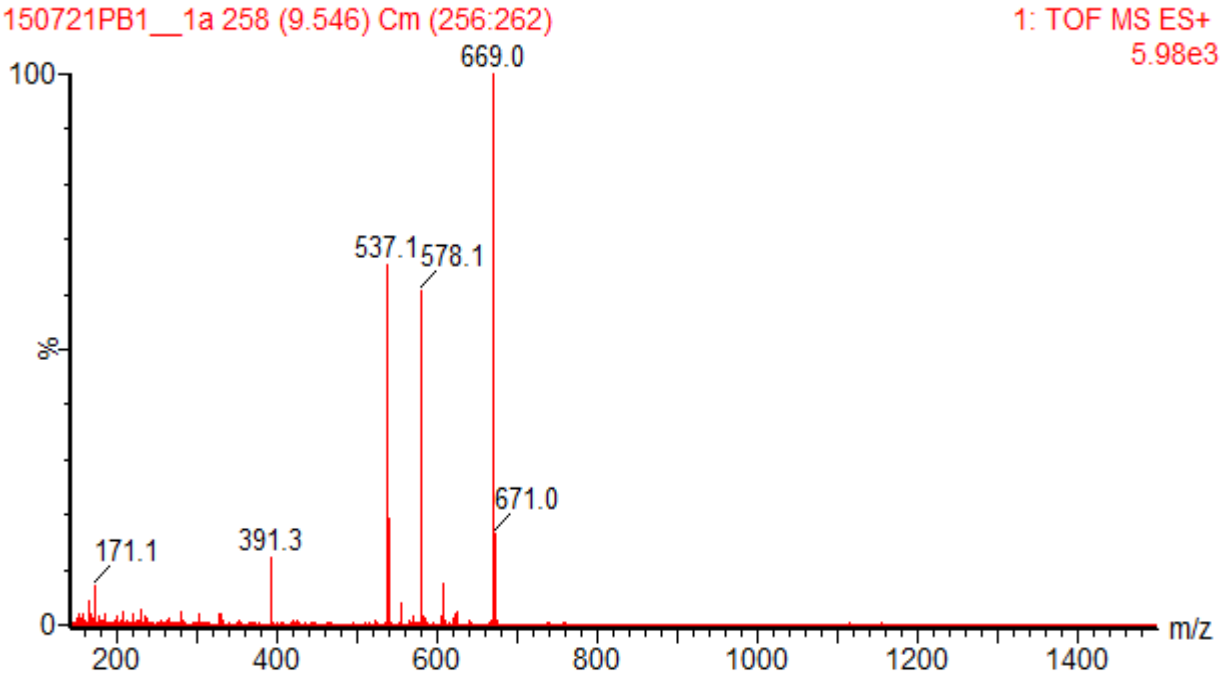
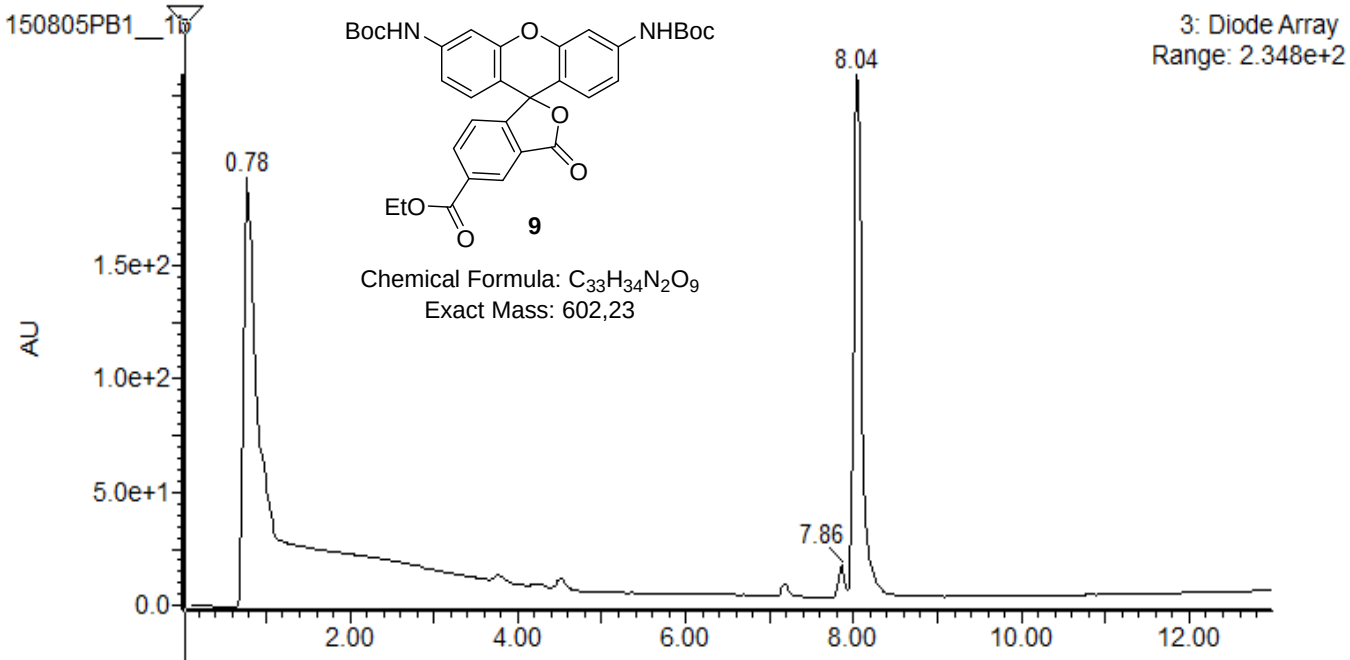
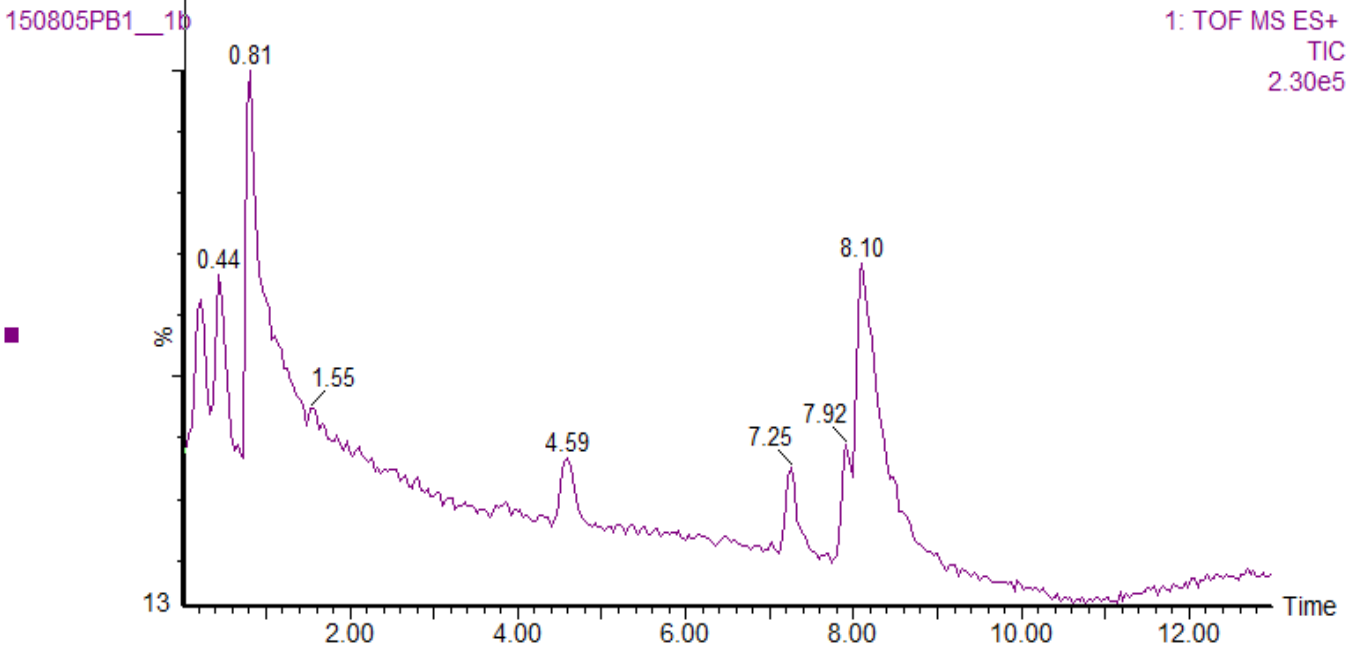
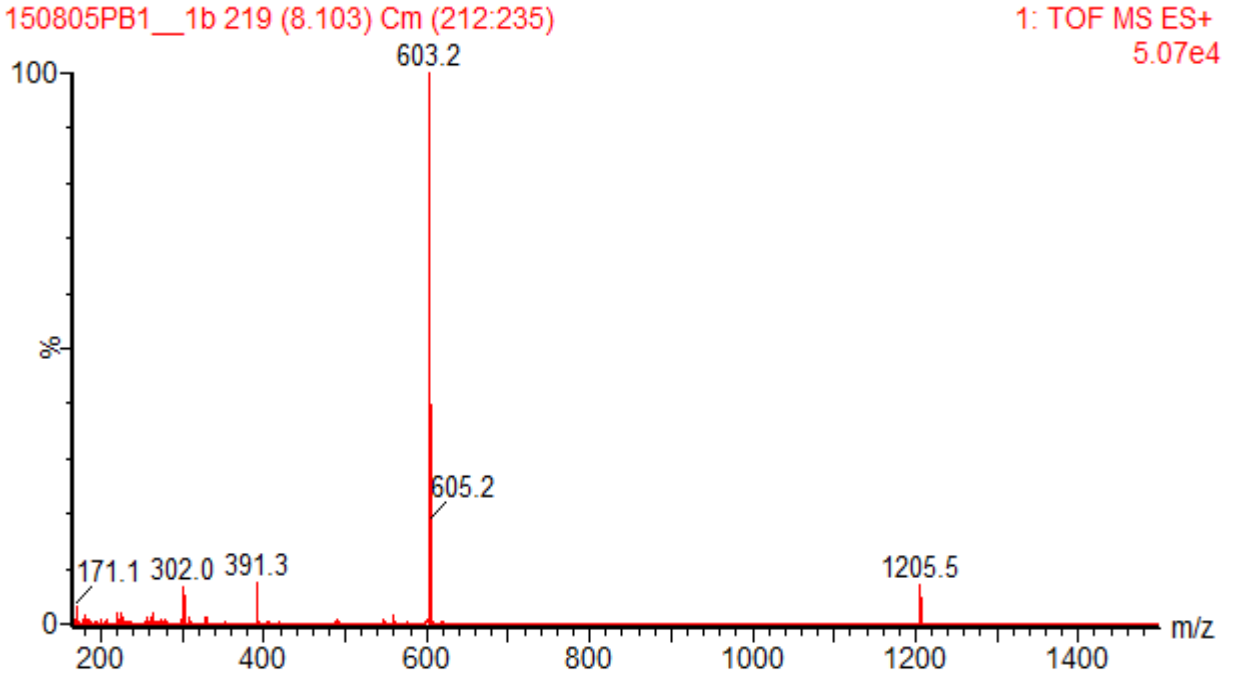
8.84
9.46

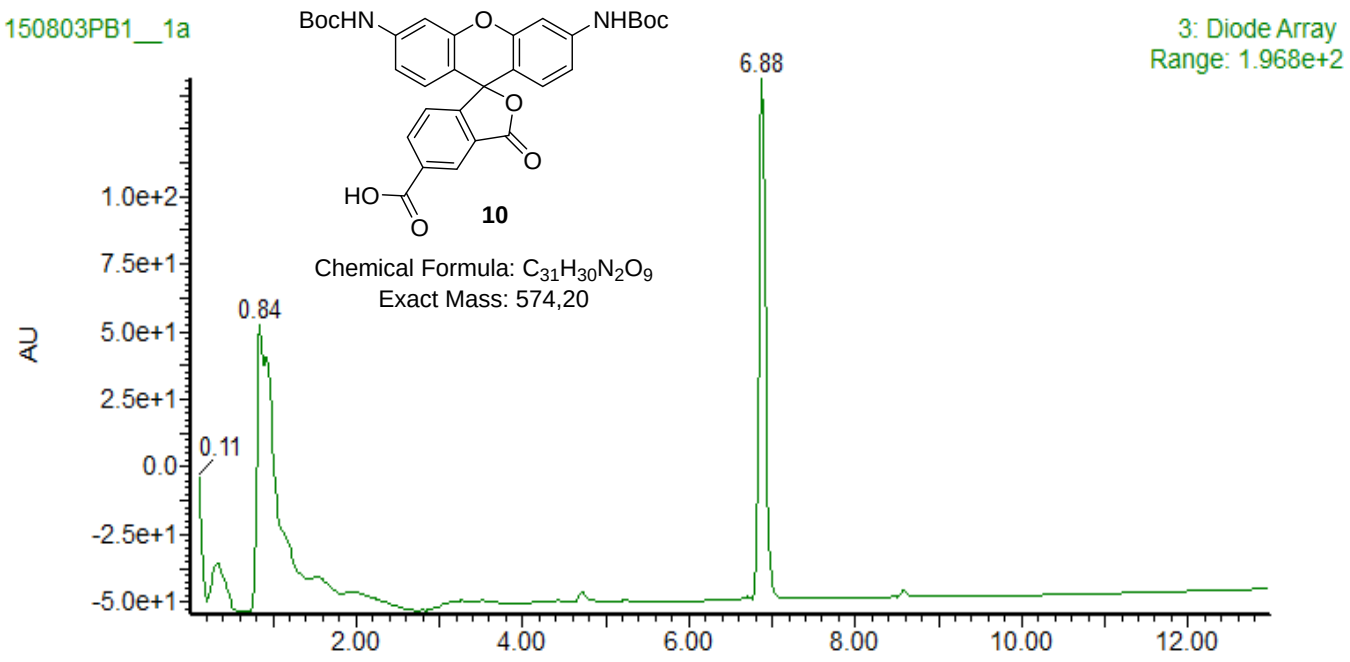

150721PB1__1a
1: TOF MS ES+
TIC
1.12e5
%
0.48
0.89
1.00
1.66
2.55
4.44
4.55
5.18
5.62
6.29
6.73
7.77
8.58
8.92
9.55
9.88
12.32
12.73
Time

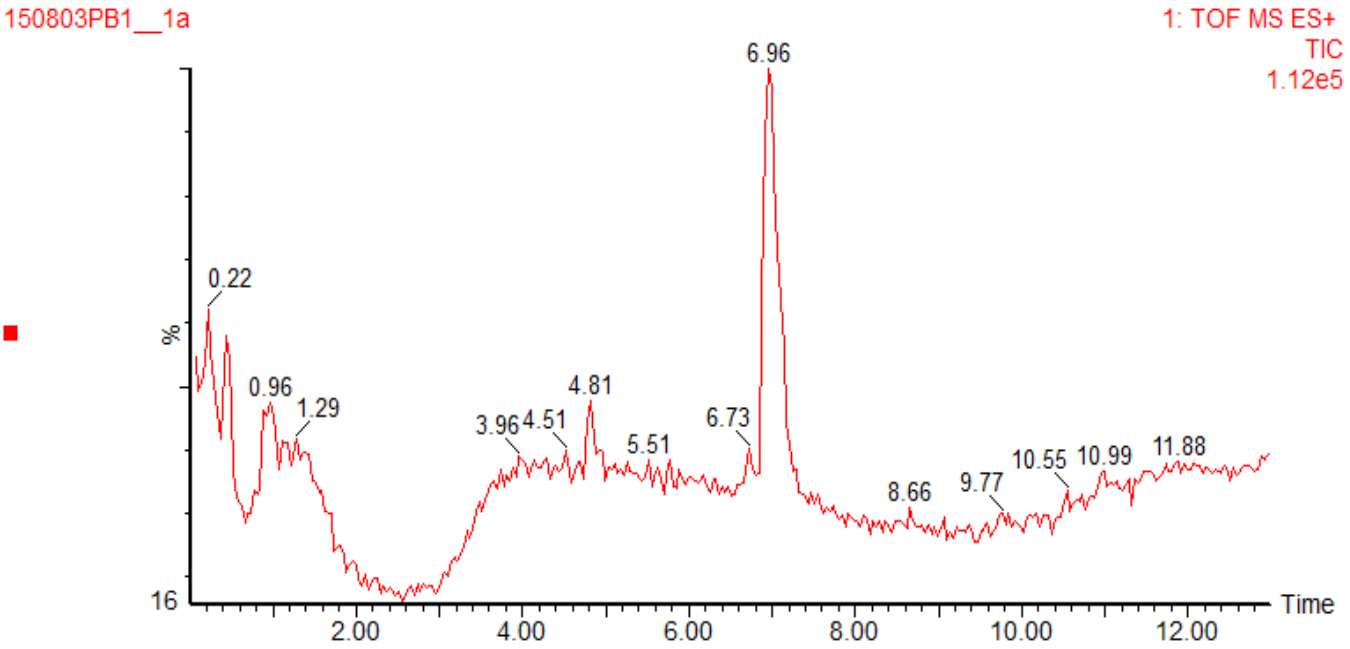

150721PB1__1a 258 (9.546) Cm (256:262)
1: TOF MS ES+
5.98e3
%
669.0
537.1
578.1
671.0
171.1
391.3
m/z

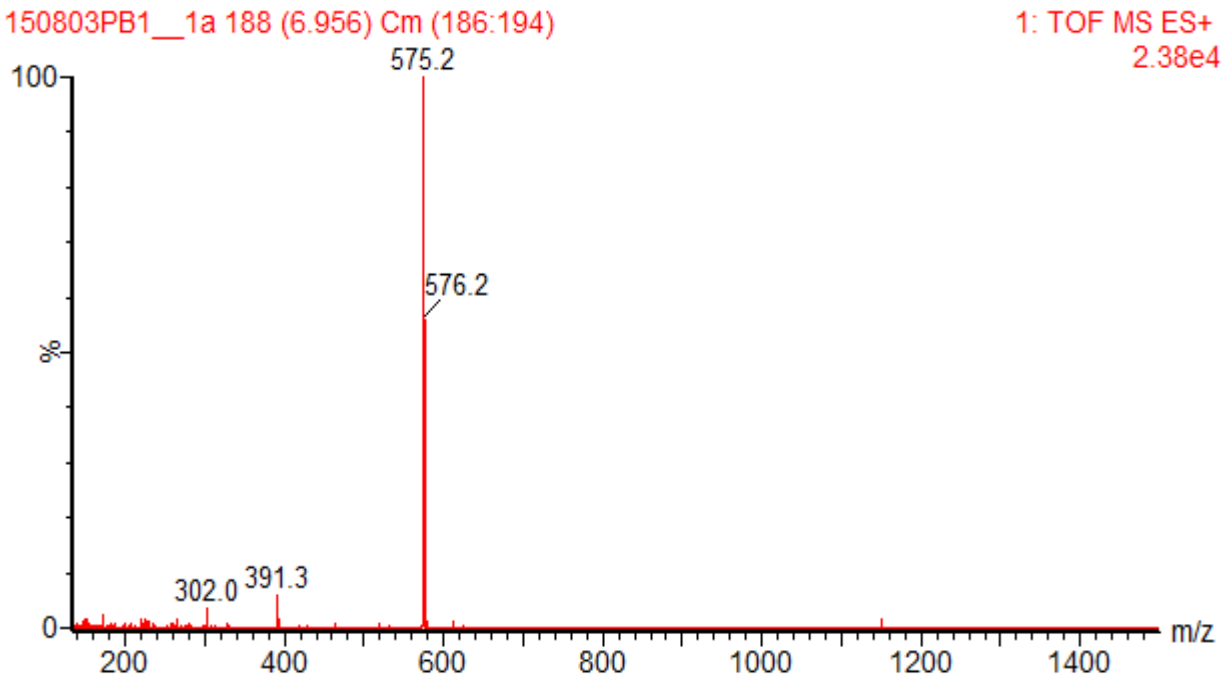

150805PB1__1b
3: Diode Array
Range: 2.348e+2
BocHN
NHBoc
O
O
O
O
EtO
O
9
Chemical Formula: $C_{33}H_{34}N_2O_9$
Exact Mass: 602,23
8.04
0.78
7.86
AU
1.5e+2
1.0e+2
5.0e+1
0.0
2.00
4.00
6.00
8.00
10.00
12.00


150805PB1__1b
1: TOF MS ES+
TIC
2.30e5
0.81
0.44
8.10
1.55
7.92
4.59
7.25
%
13
Time
2.00
4.00
6.00
8.00
10.00
12.00

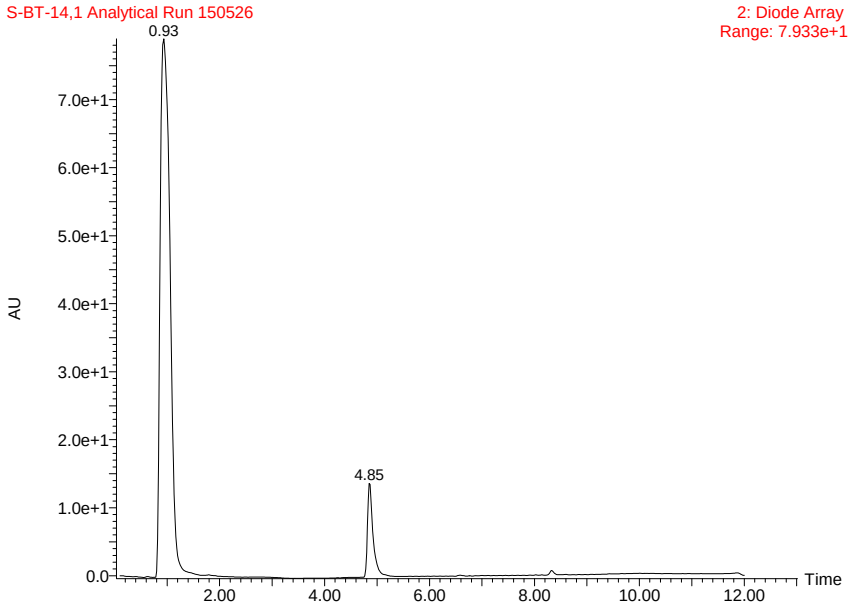

150805PB1__1b 219 (8.103) Cm (212:235)
1: TOF MS ES+
5.07e4
603.2
100
%
605.2
171.1
302.0
391.3
1205.5
0
m/z
200
400
600
800
1000
1200
1400

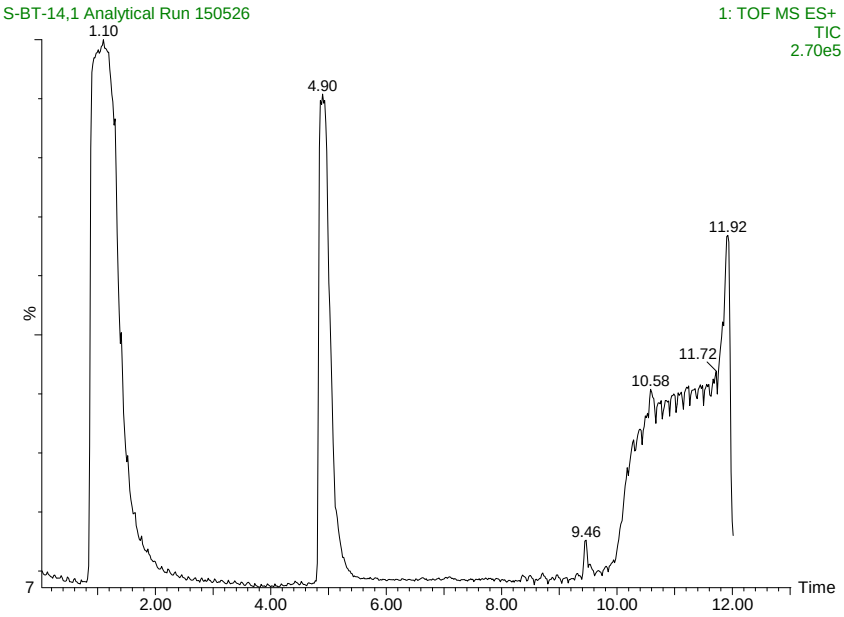

150803PB1__1a
3: Diode Array
Range: 1.968e+2
BocHN
NHBoc
HO
10
Chemical Formula: $C_{31}H_{30}N_2O_9$
Exact Mass: 574,20
AU
6.88
0.84
0.11

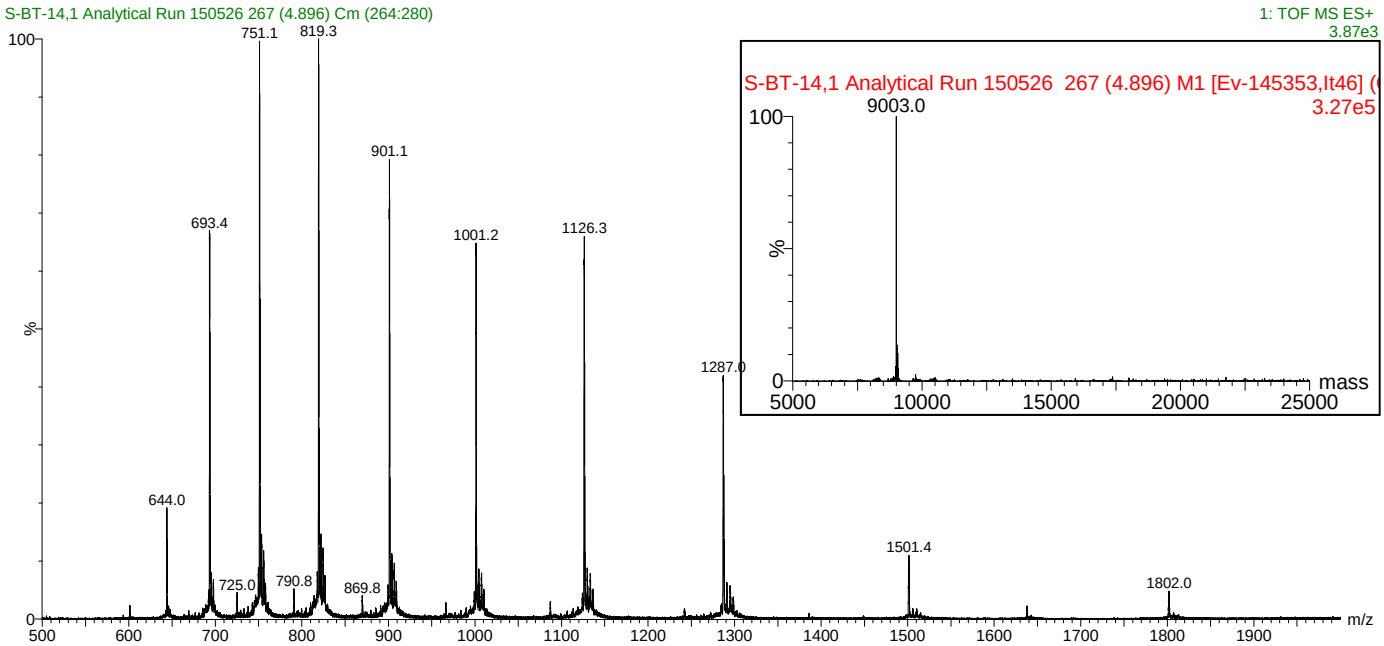

150803PB1__1a
1: TOF MS ES+
TIC
1.12e5
%
6.96
0.22
0.96
1.29
3.96
4.51
4.81
5.51
6.73
8.66
9.77
10.55
10.99
11.88
Time


150803PB1__1a 188 (6.956) Cm (186:194)
1: TOF MS ES+
2.38e4
575.2
576.2
302.0
391.3
%
m/z

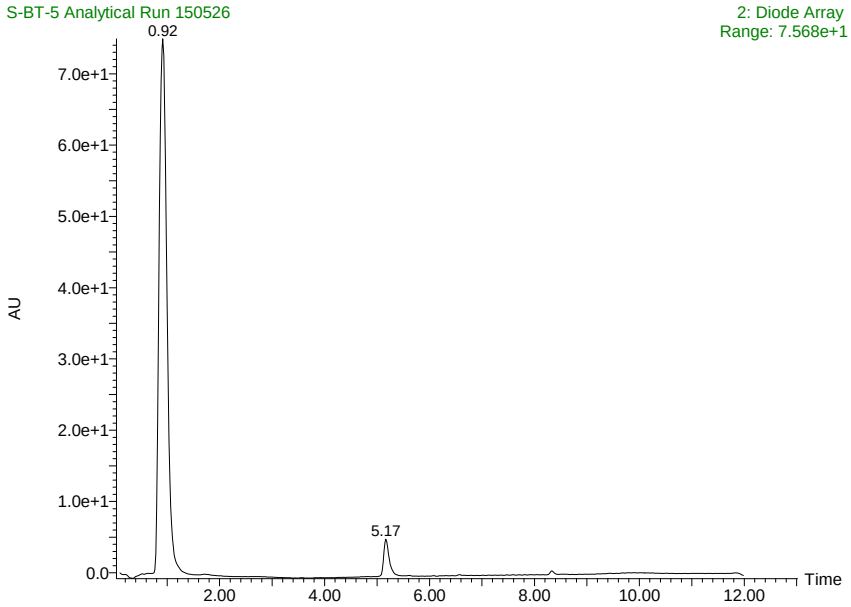
Rh-Ub(1-76, Nle_1)-S-$(CH_2)_2$-CO_2-Me (14)

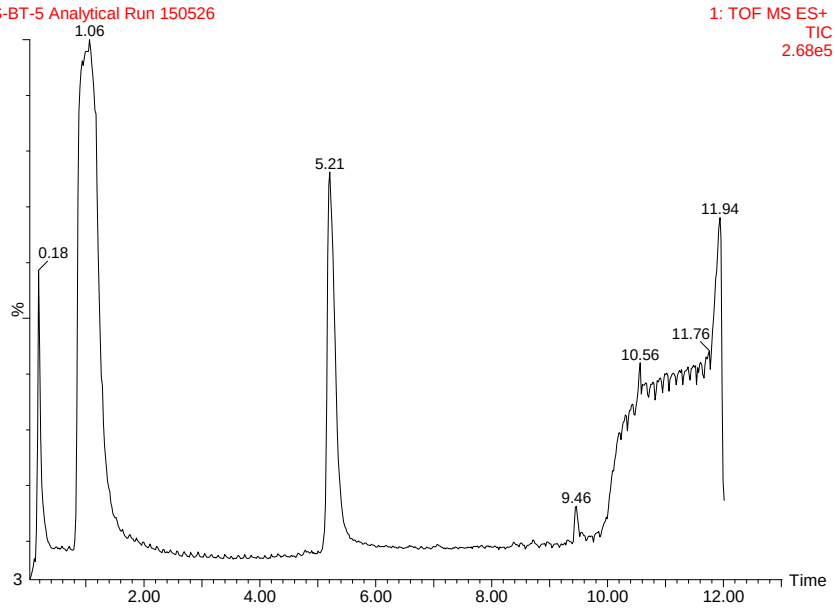


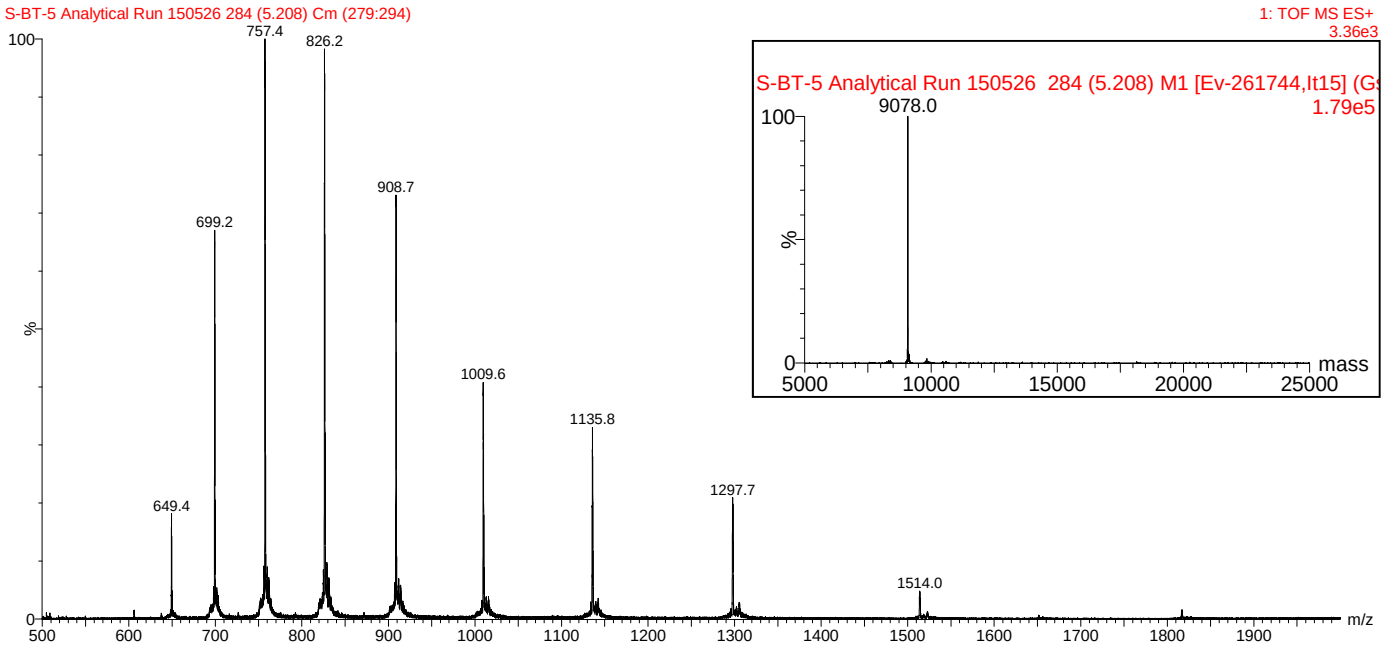




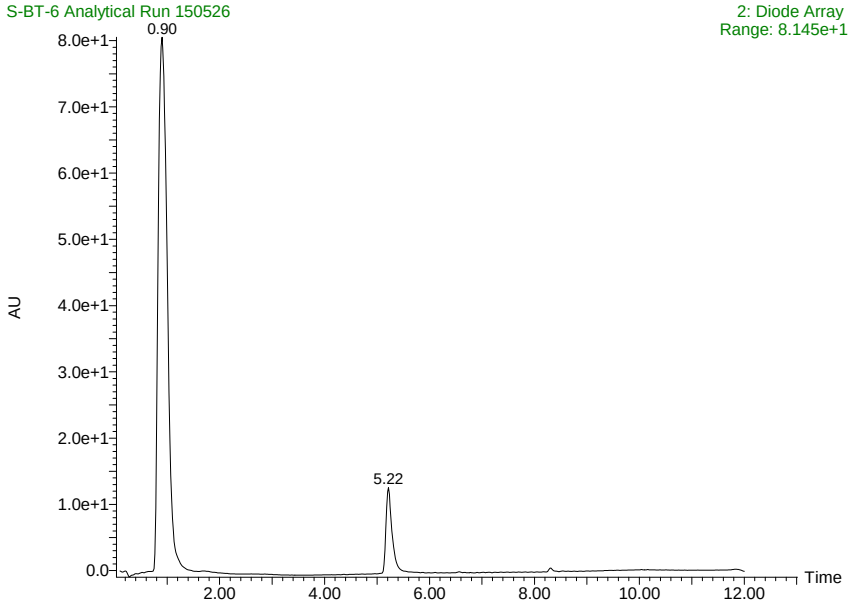
TAMRA-Ub(1-76, Nle_1, γ-thioLys_6) (16a)

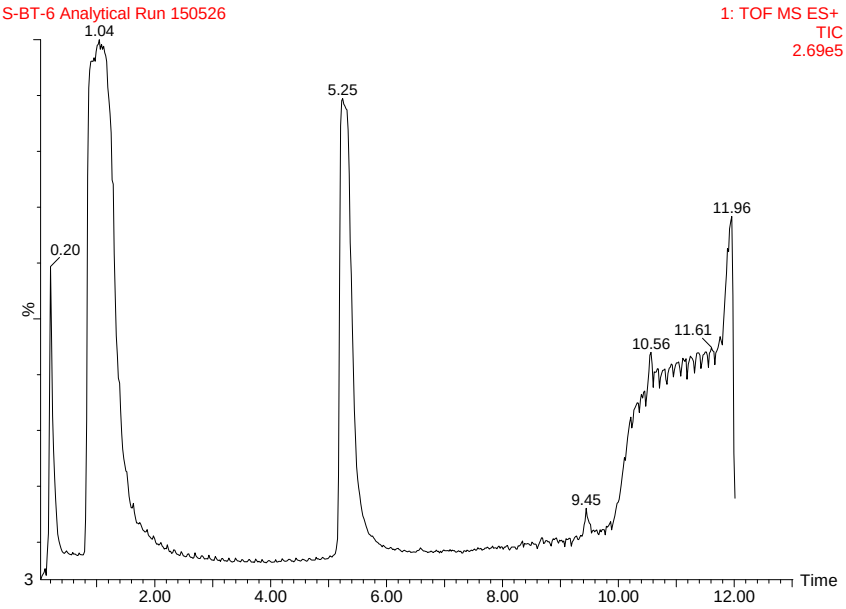


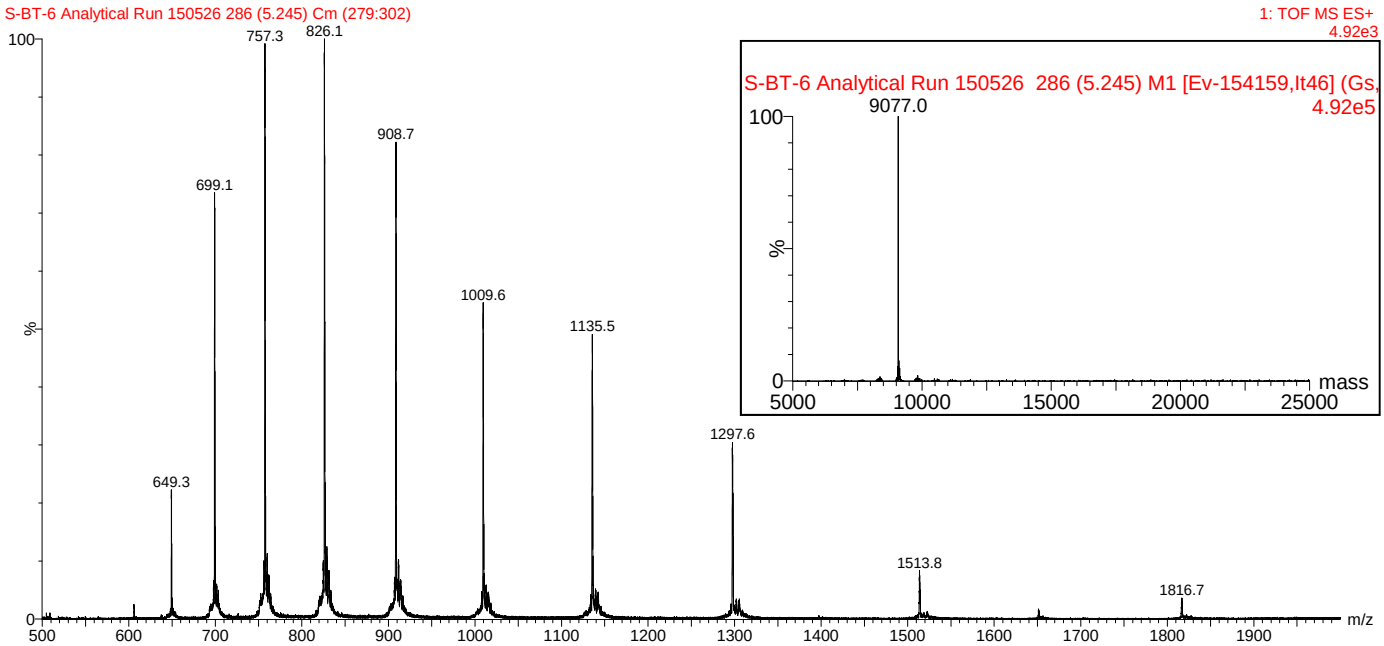




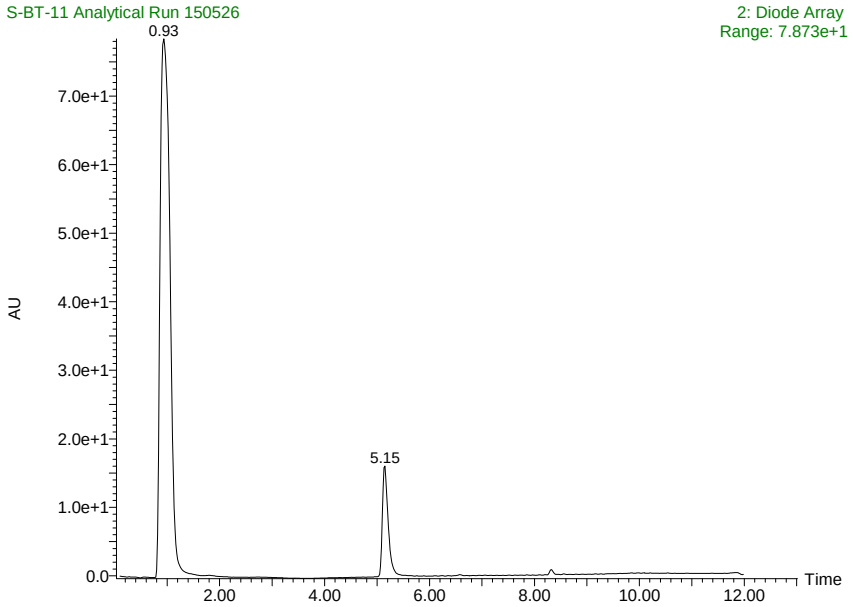
TAMRA-Ub(1-76, Nle_1, γ-$thioLys_{11}$) (16b)

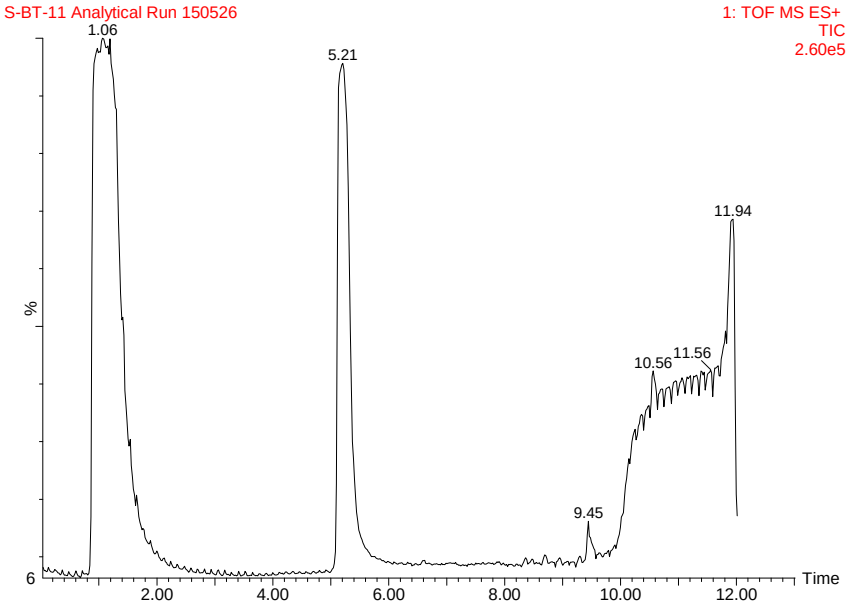


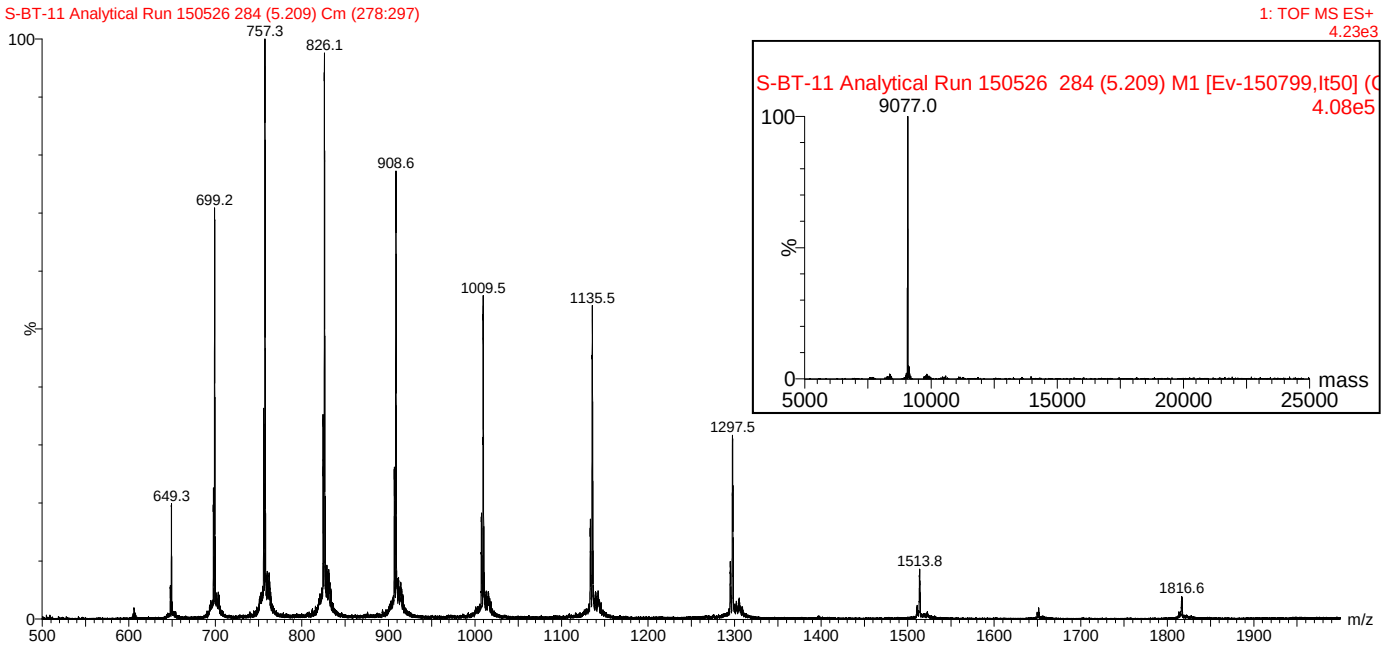




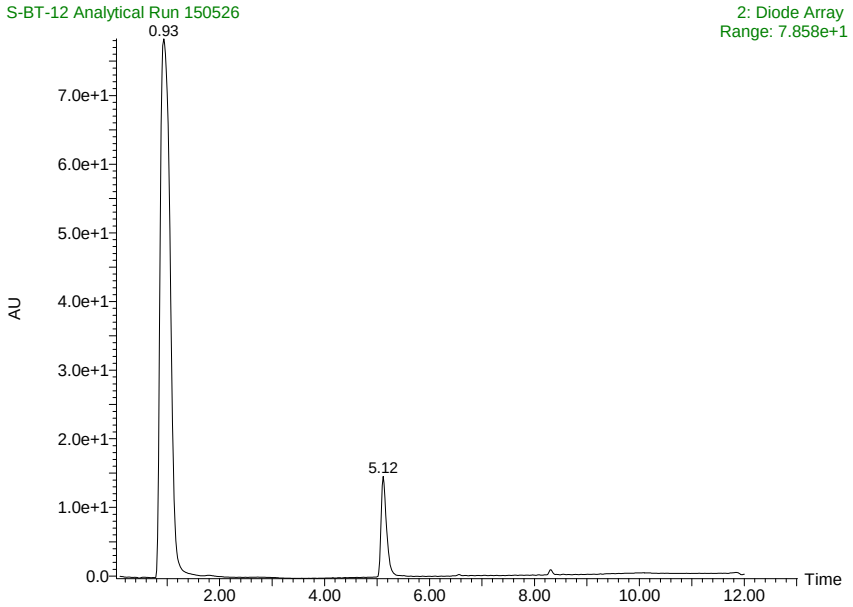
TAMRA-Ub(1-76, Nle_1, γ-thioLys_{27}) (16c)

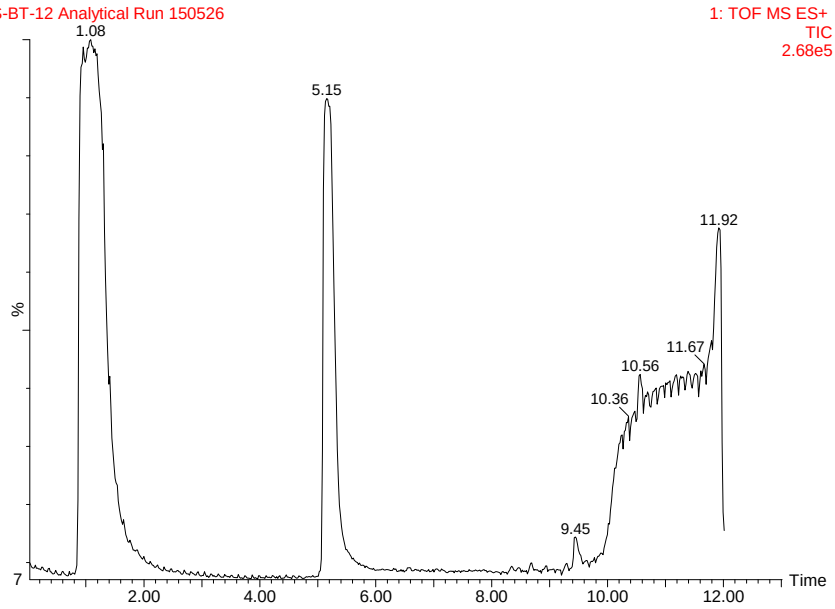


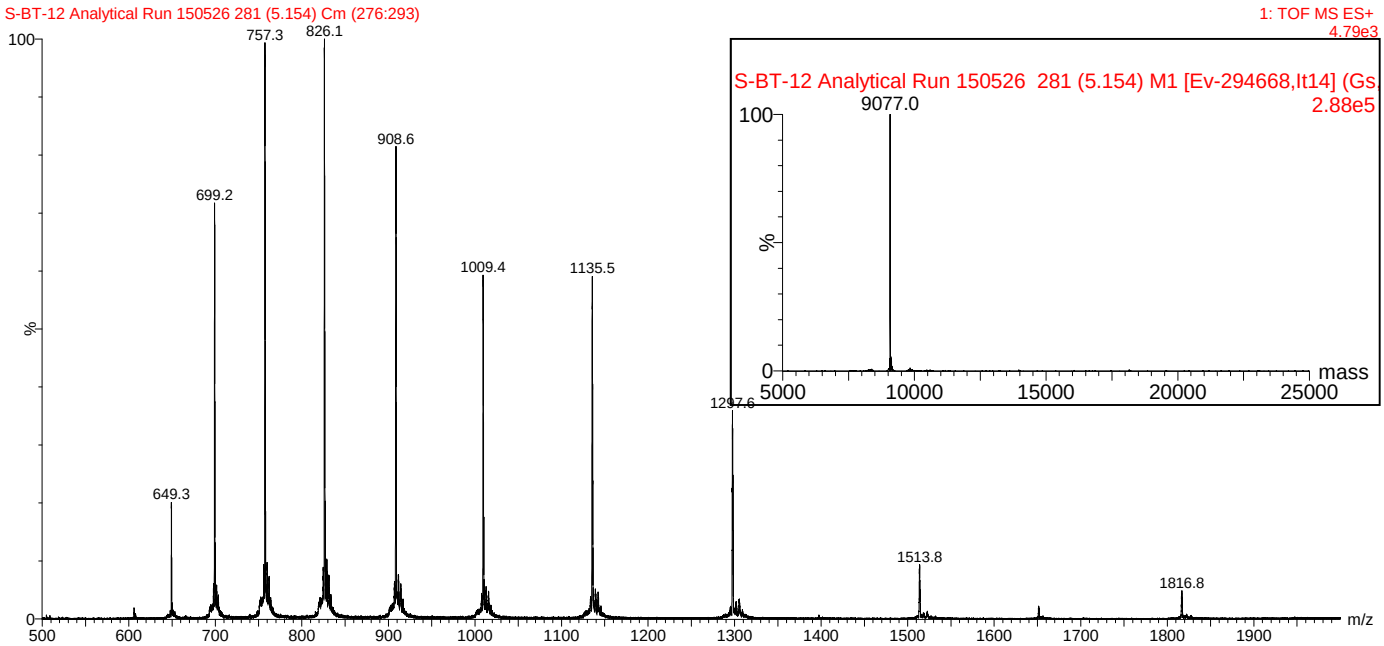




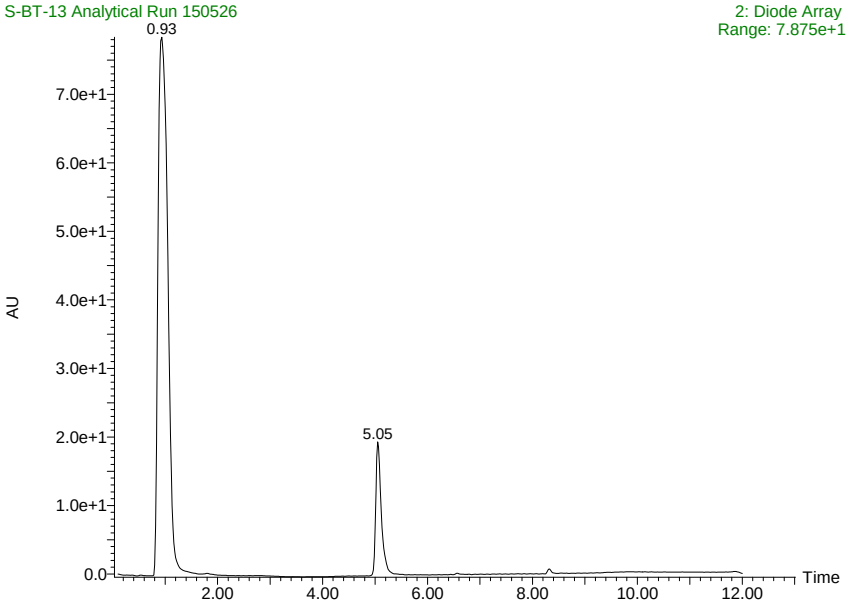
TAMRA-Ub(1-76, Nle_1, γ-thioLys_{29}) (16d)

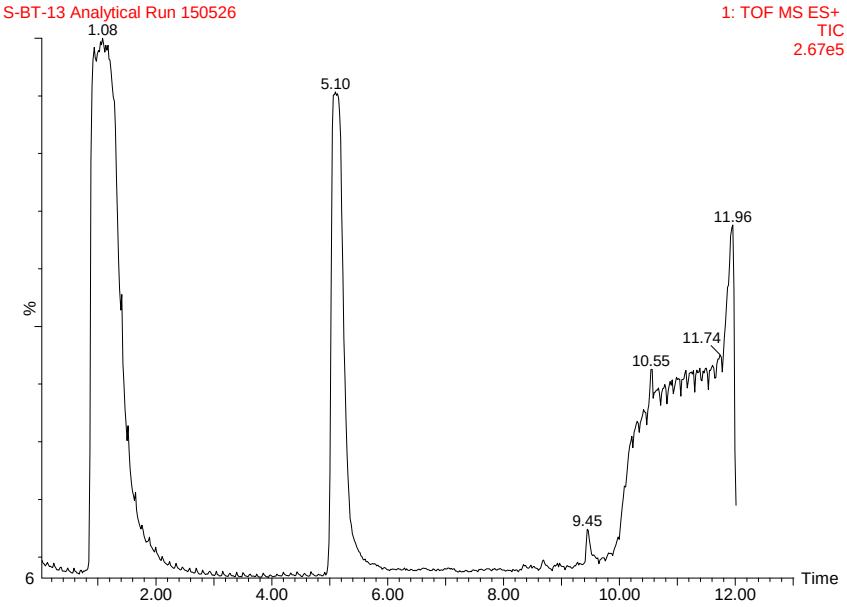


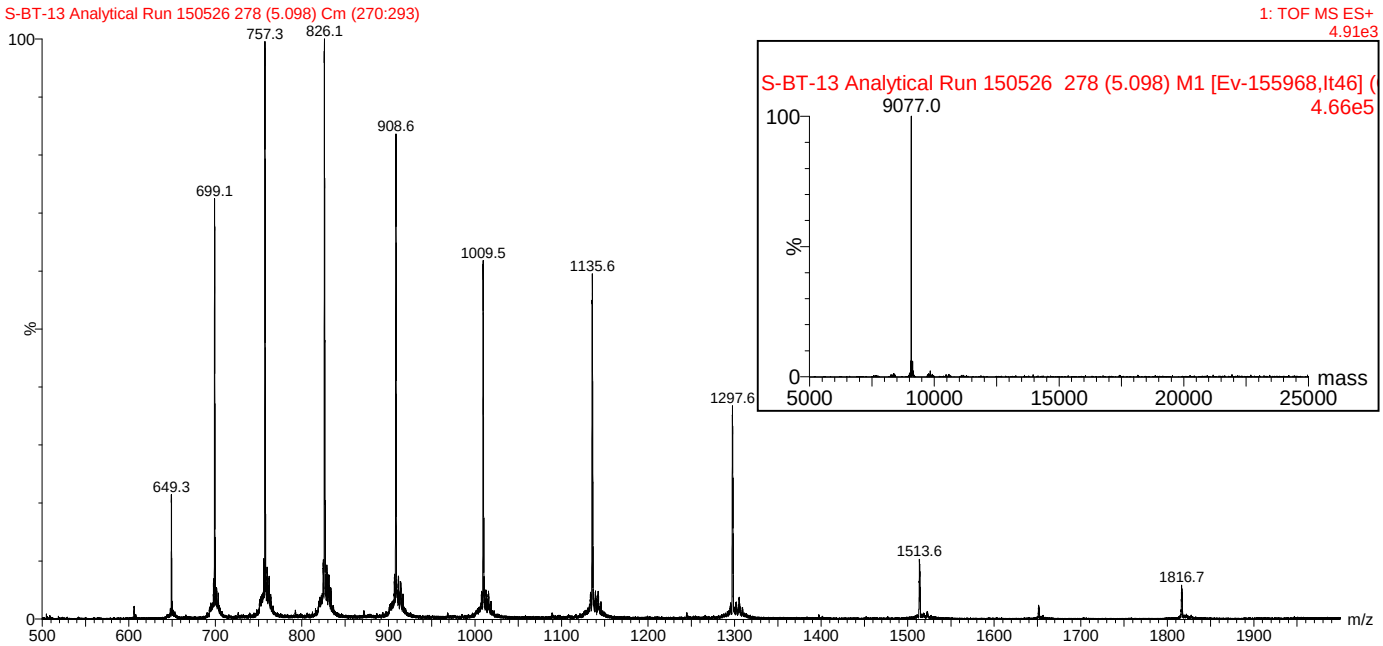




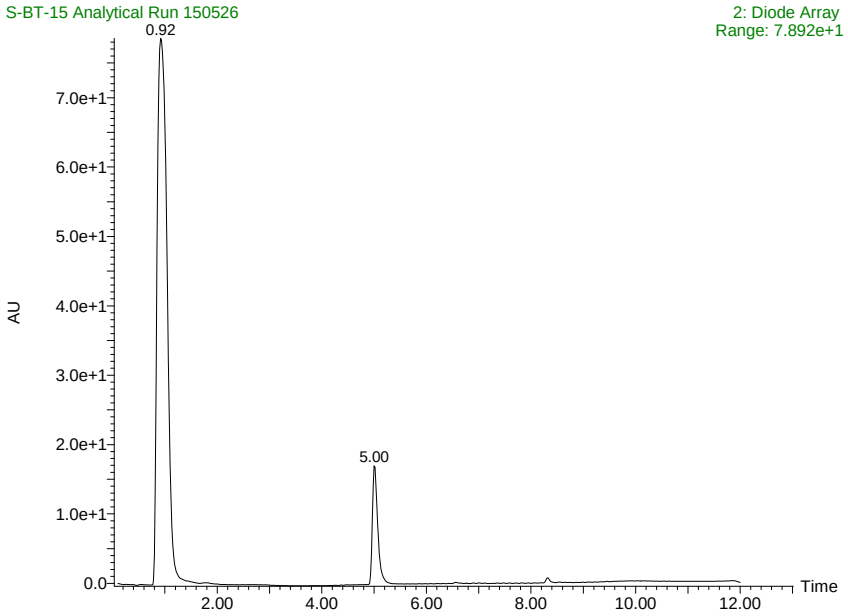
TAMRA-Ub(1-76, Nle_1, γ-thioLys_{33}) (16e)

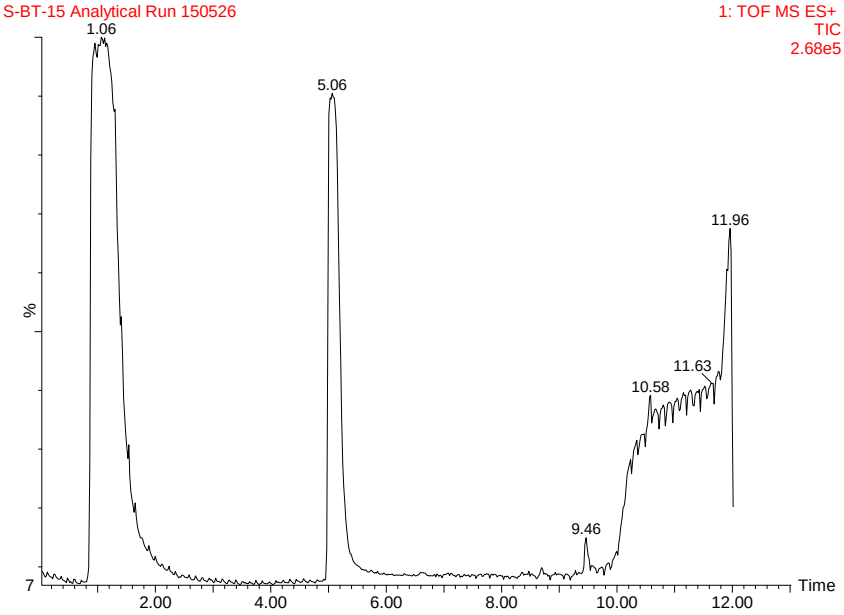


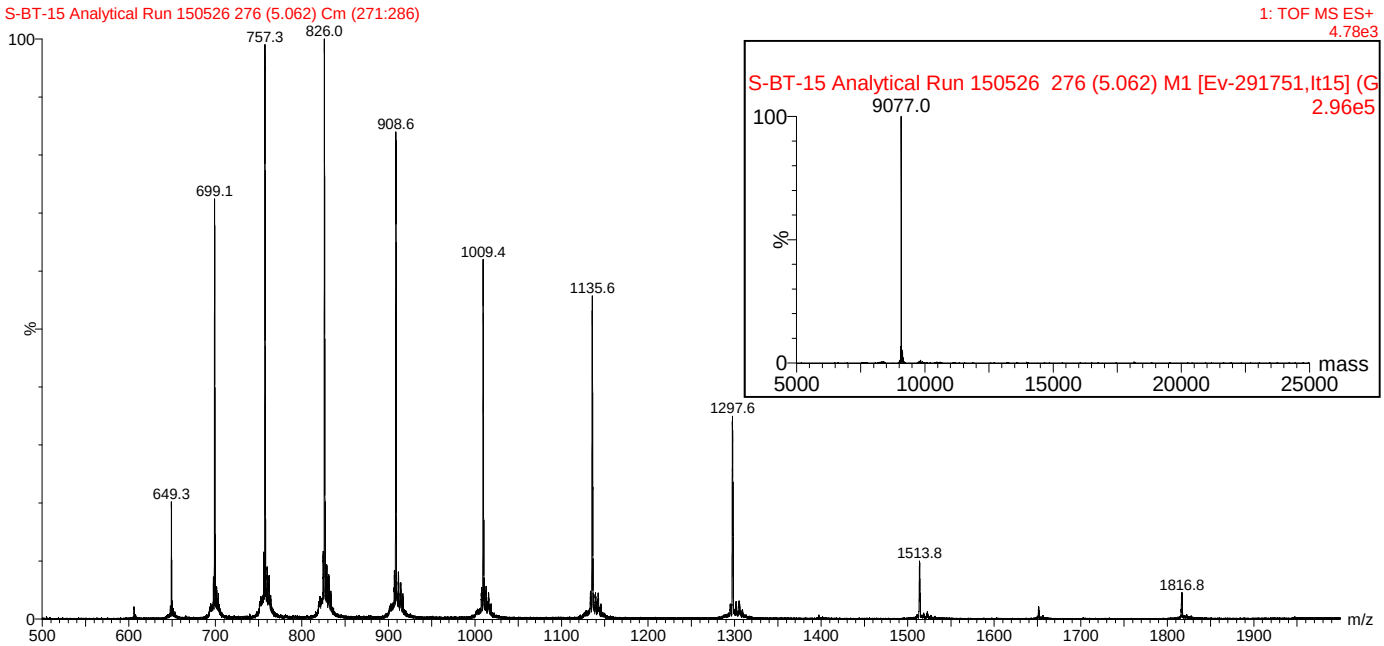




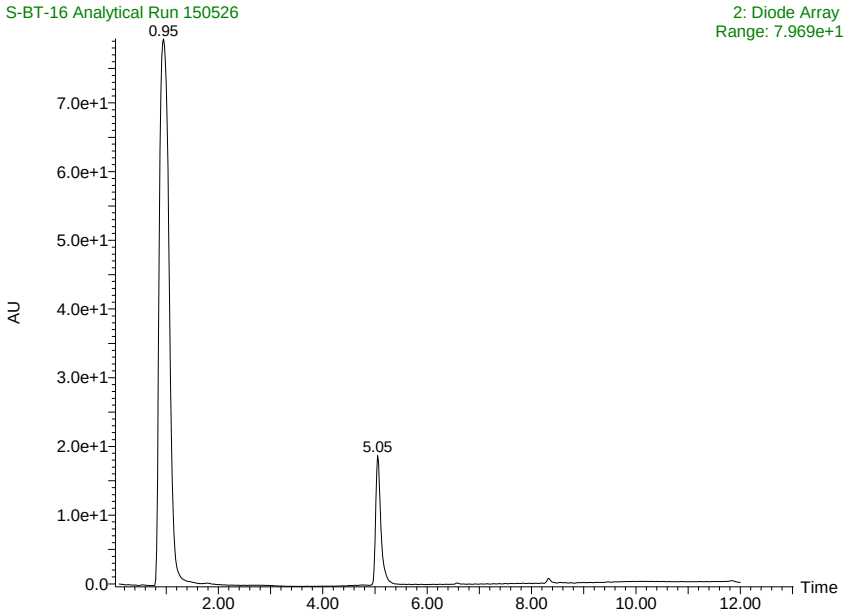
TAMRA-Ub(1-76, Nle_1, γ-thioLys_{48}) (16f)

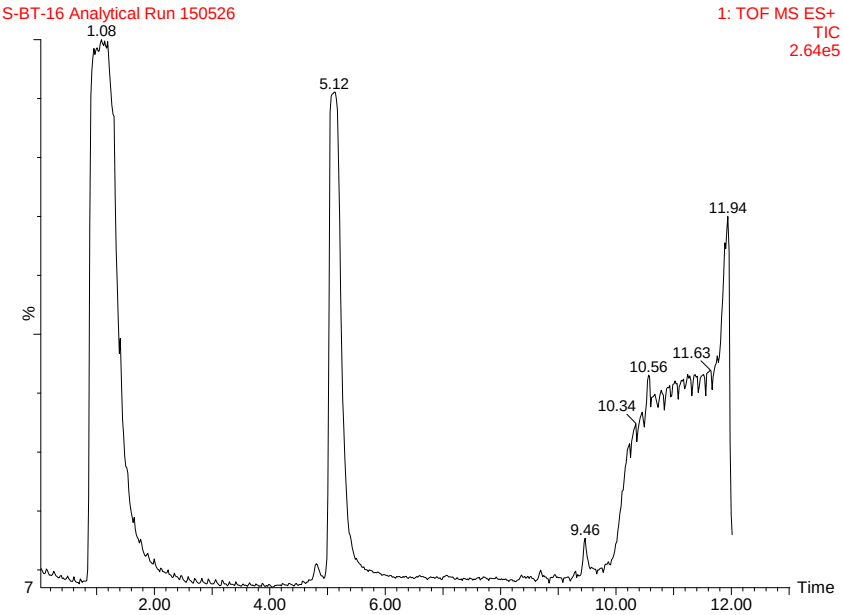


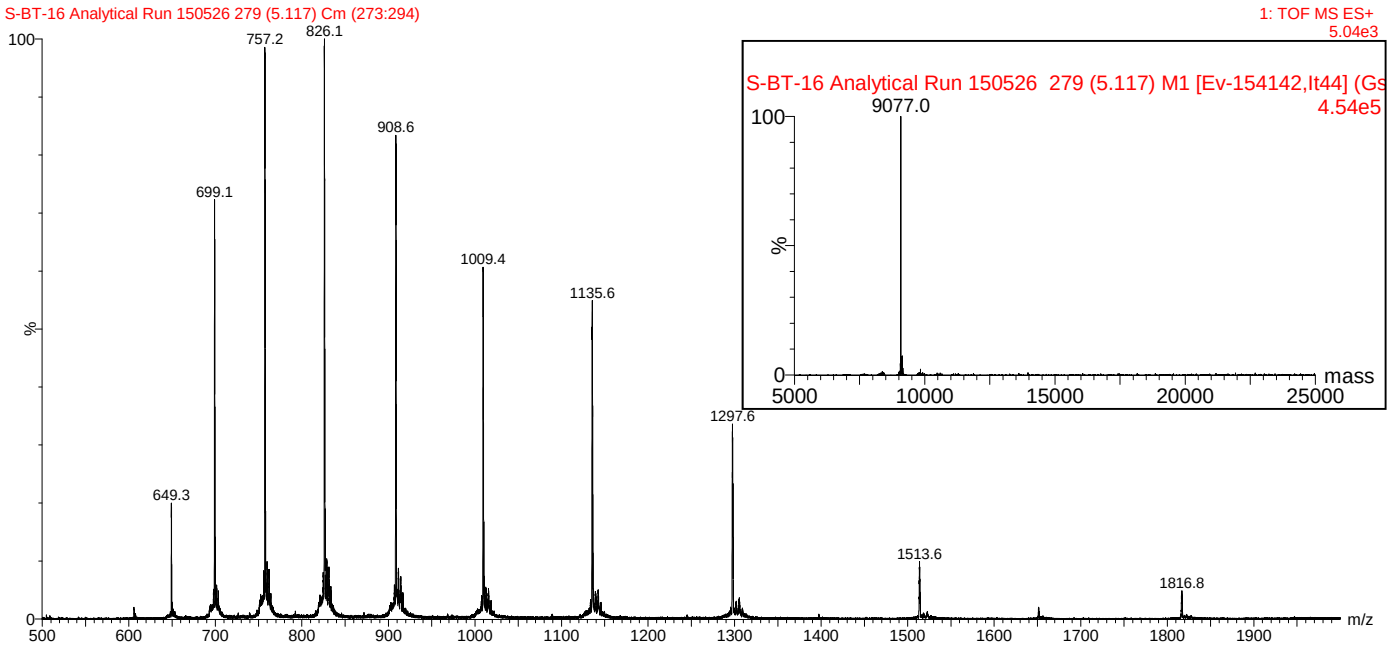




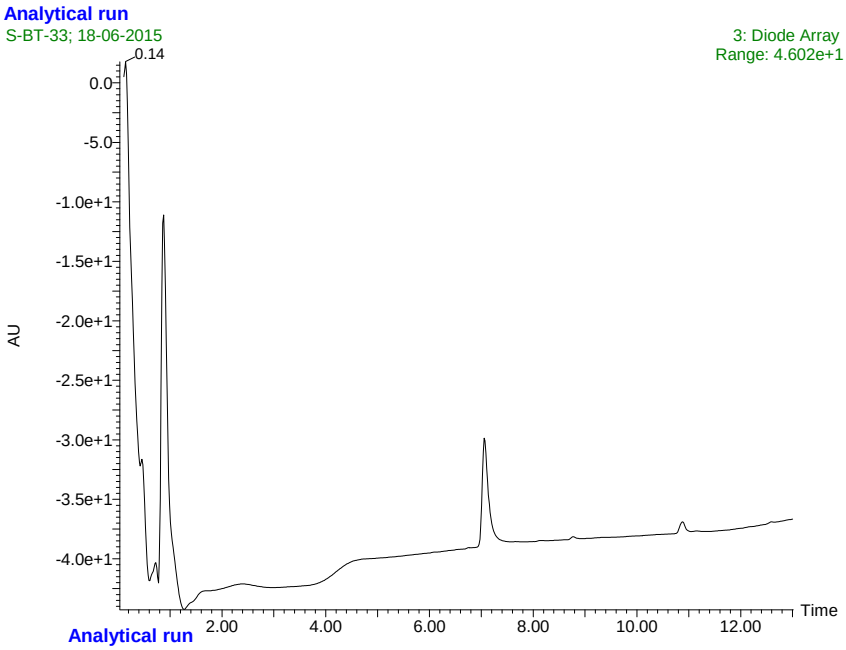
TAMRA-Ub(1-76, Nle_1, γ-thioLys_{63}) (16g)

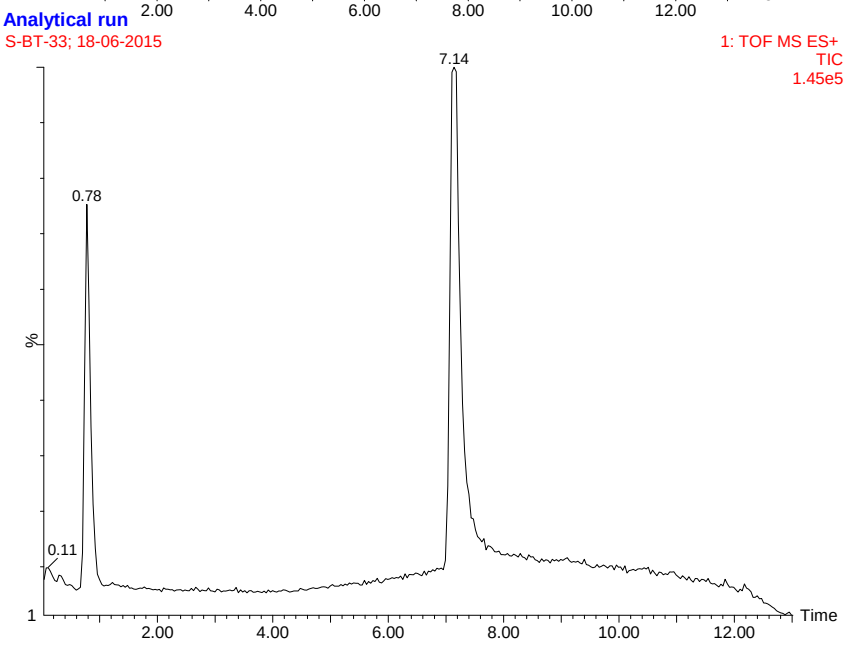


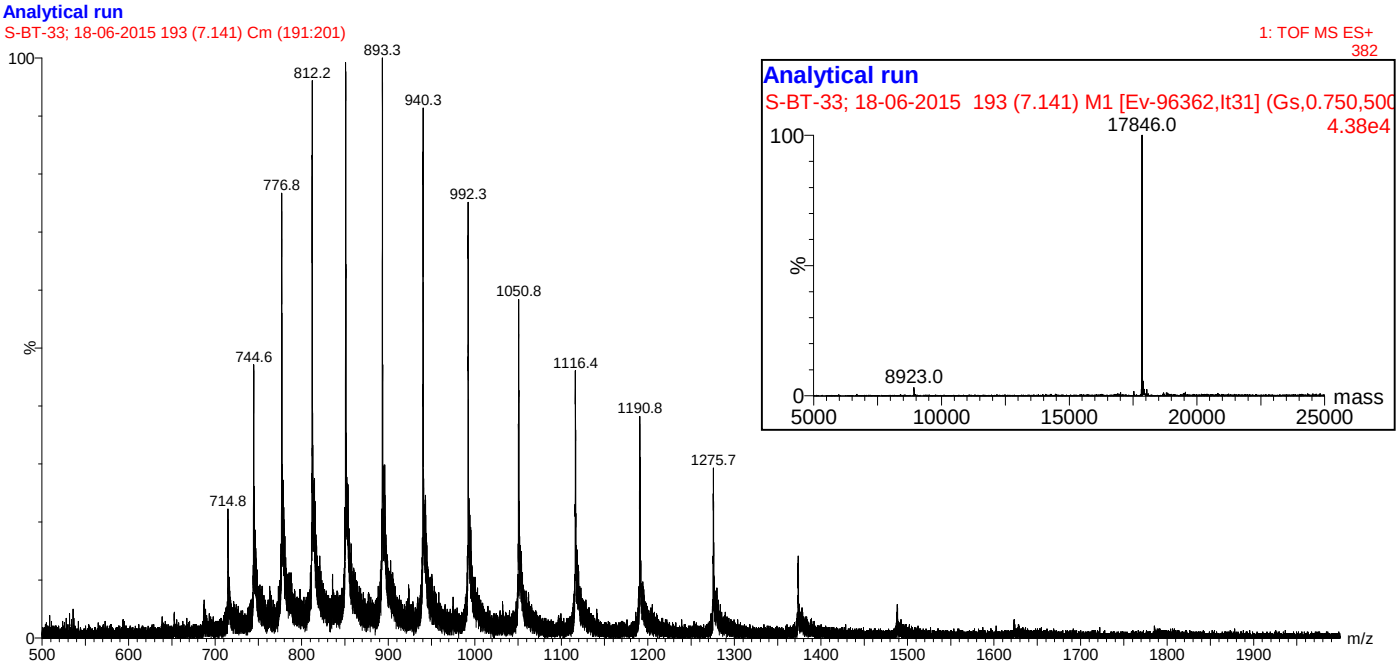




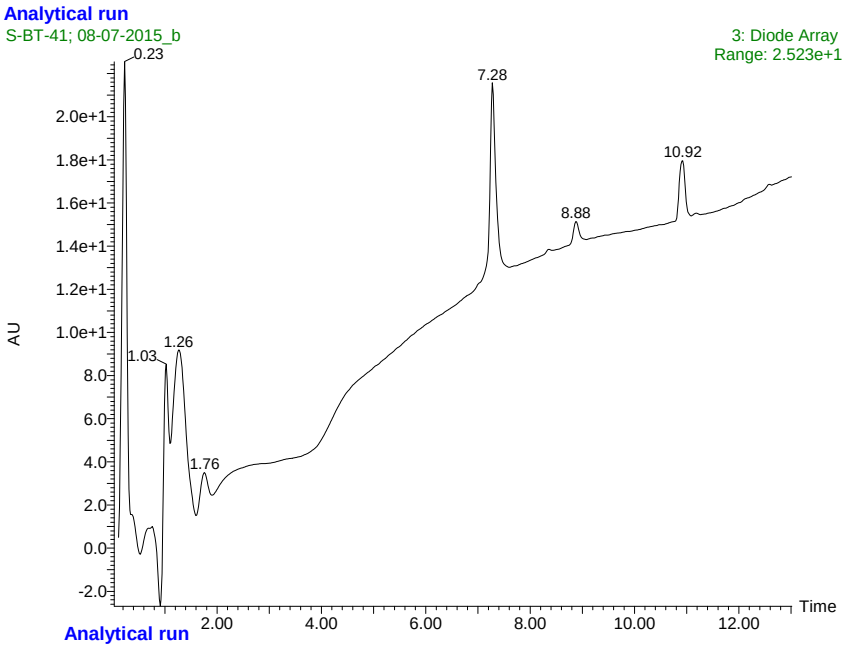
K6-linked diUb FRET pair (17a)

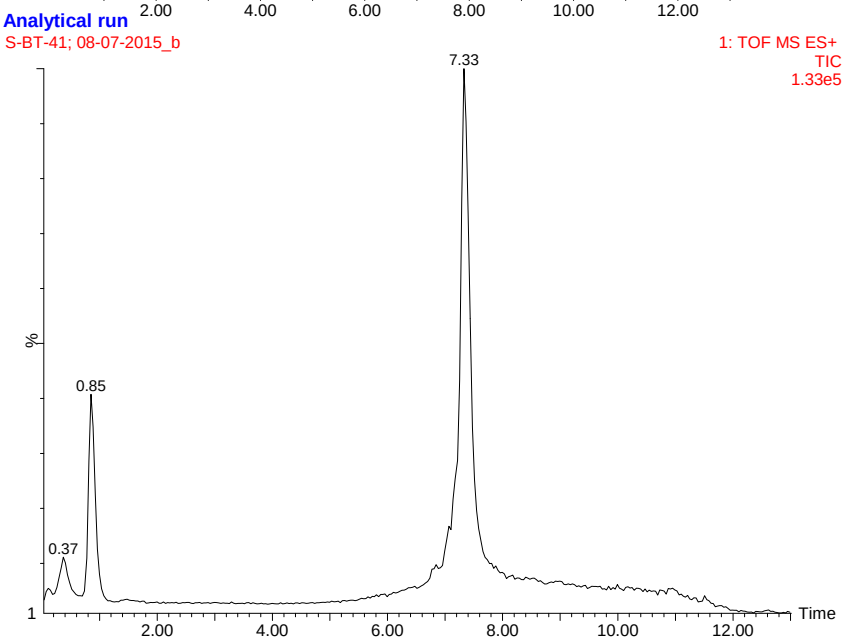


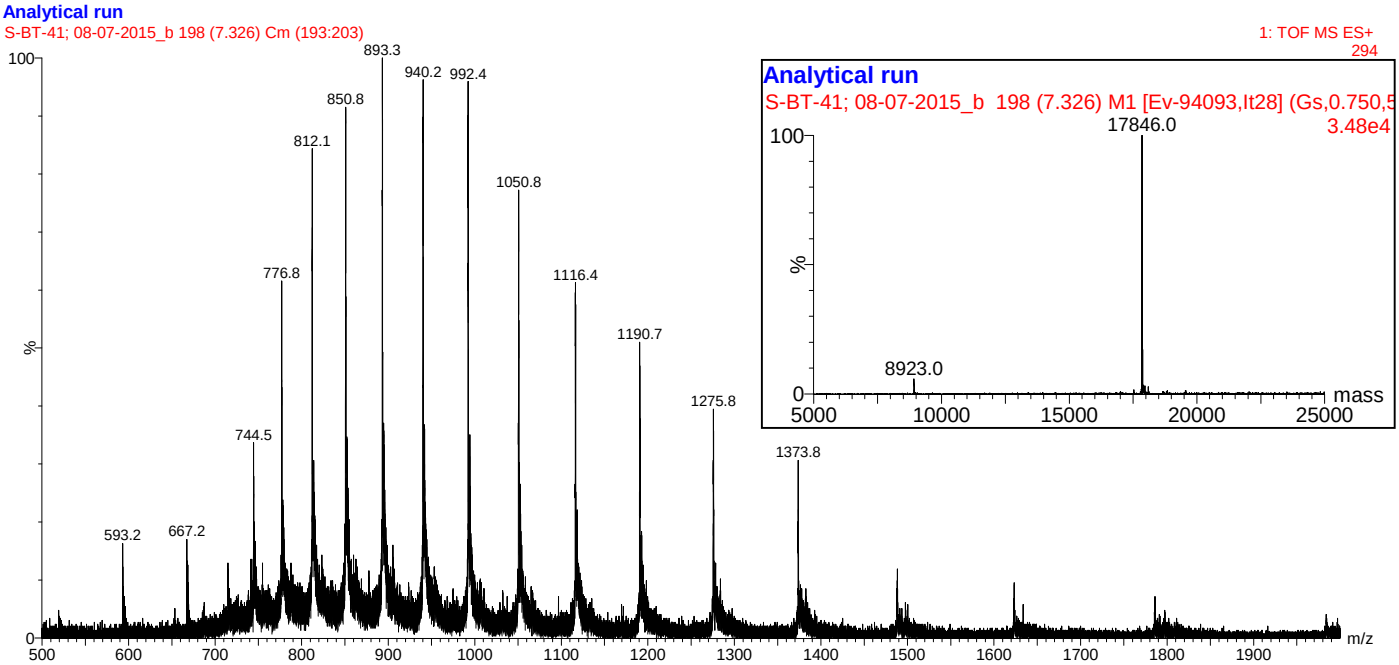




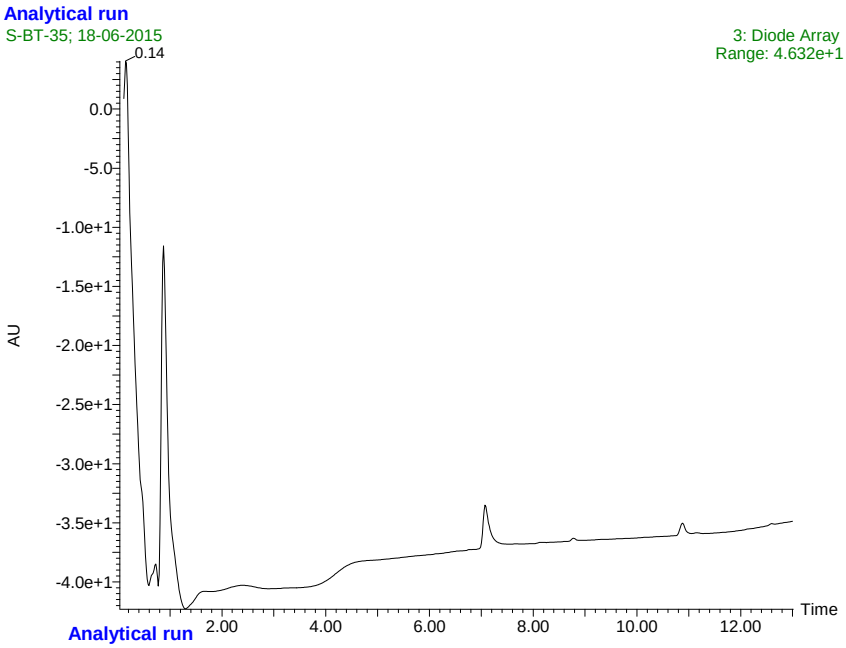
K11-linked diUb FRET pair (17b)

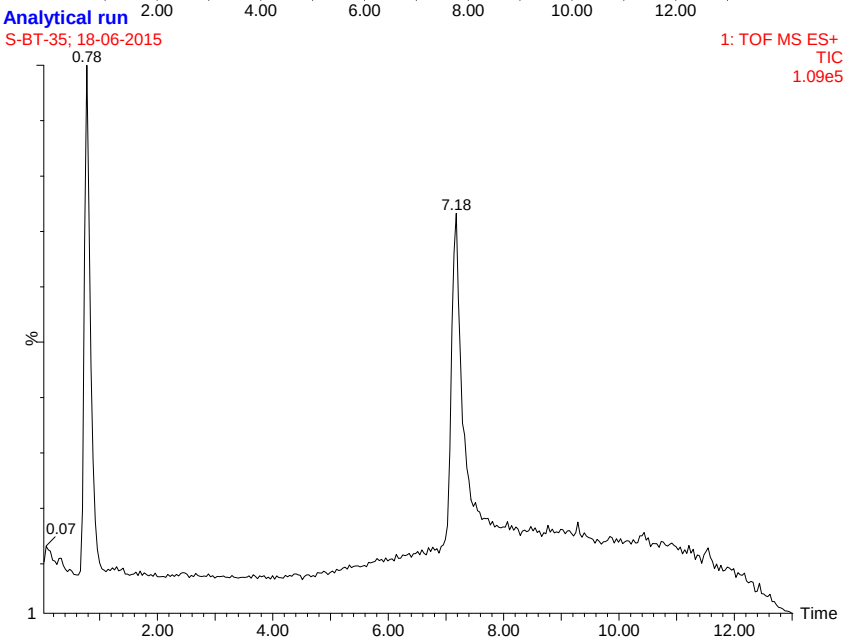


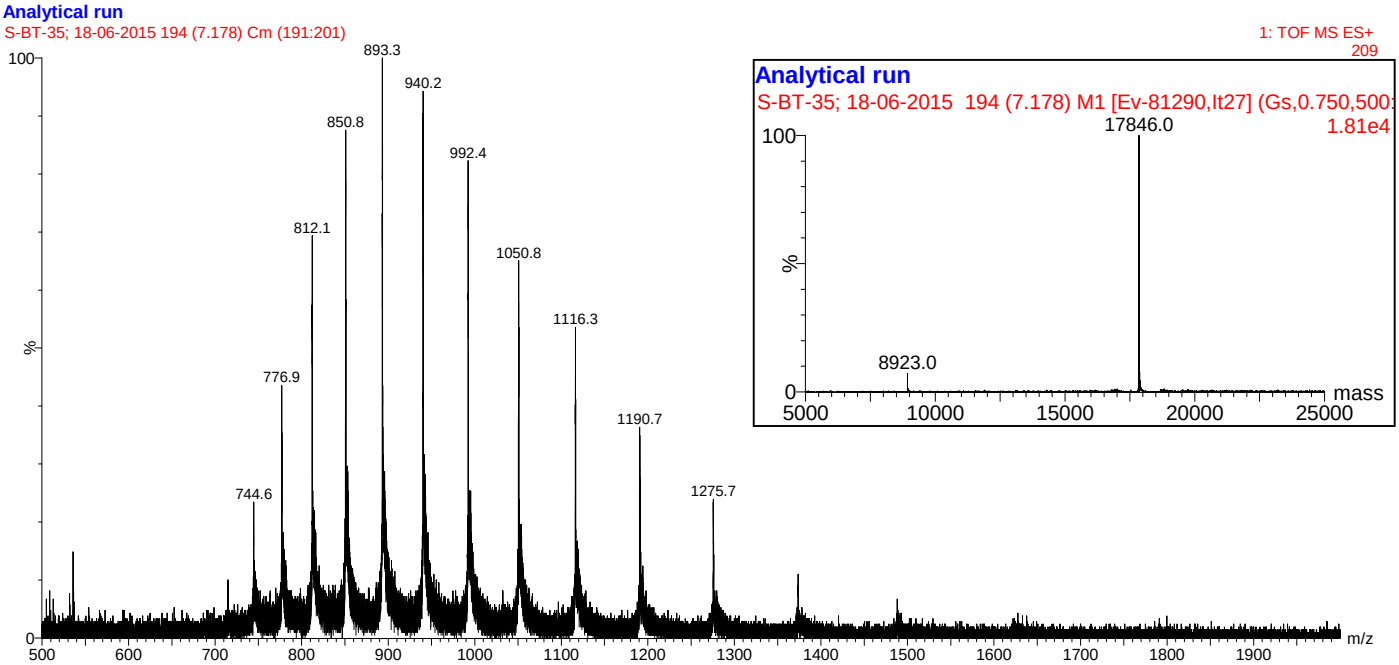




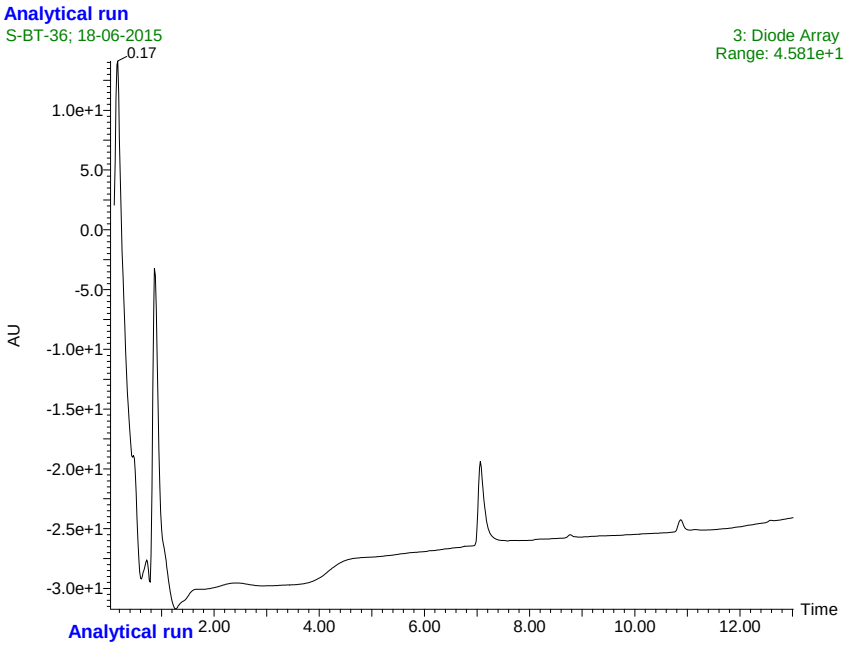
K27-linked diUb FRET pair (17c)

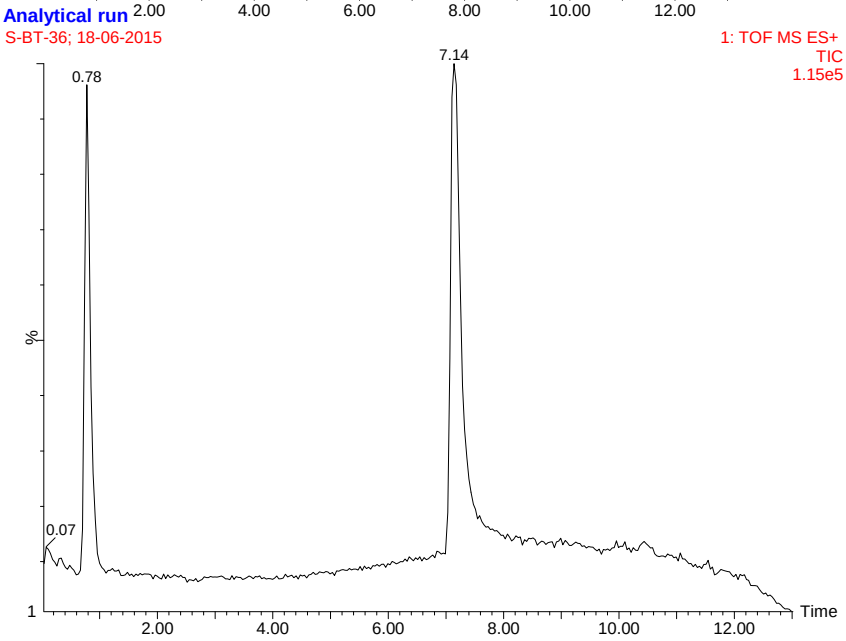


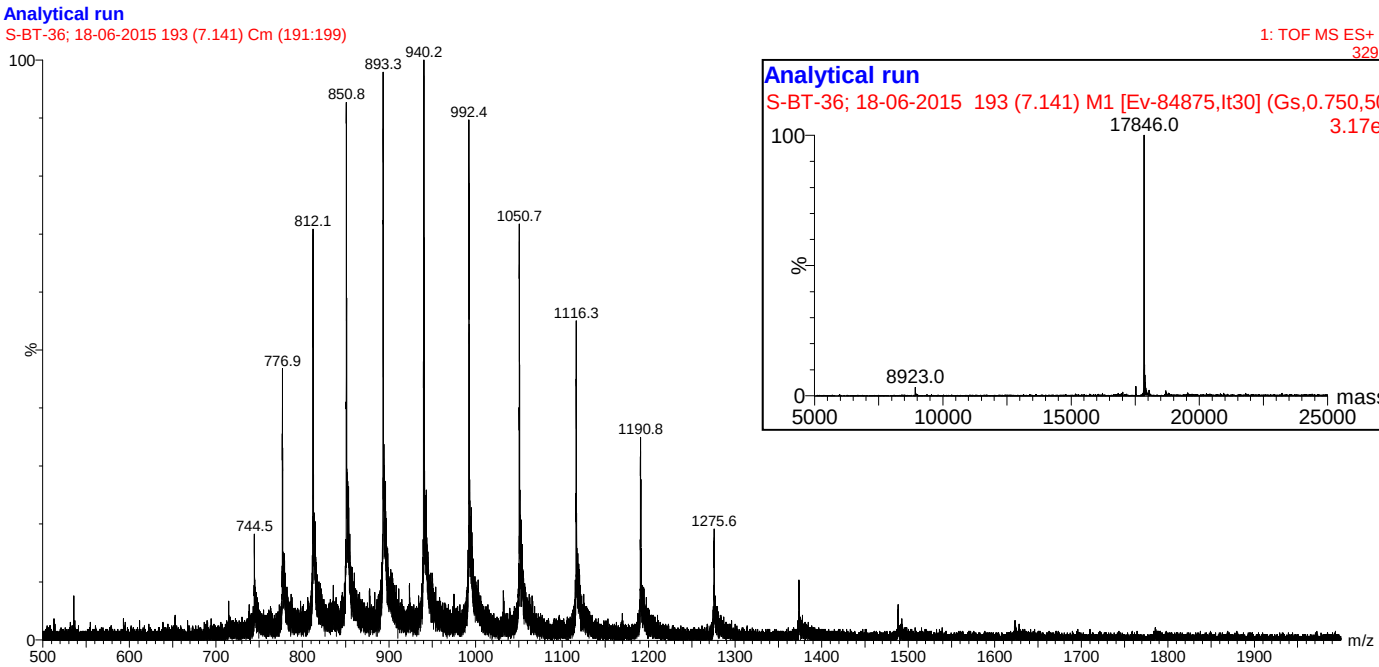




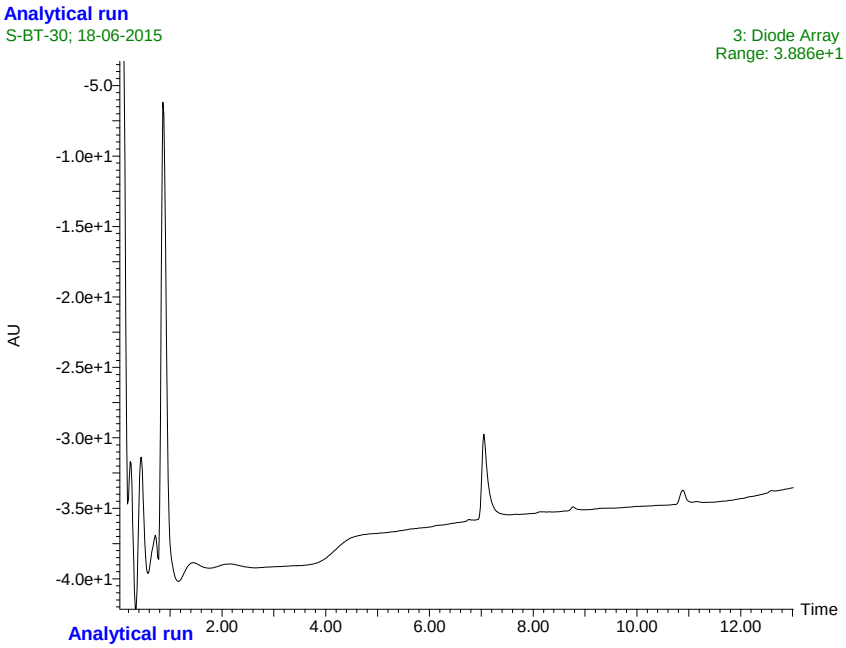
K29-linked diUb FRET pair (17d)

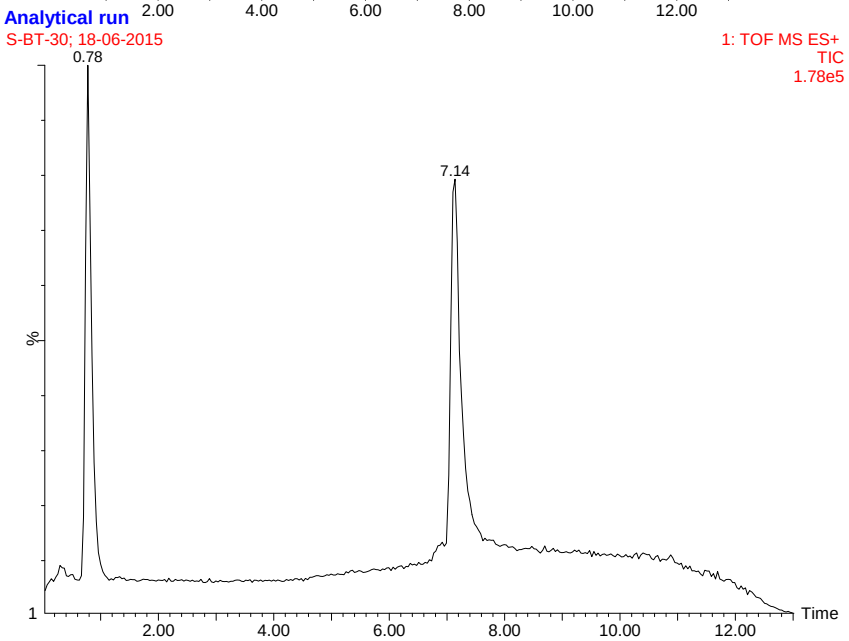


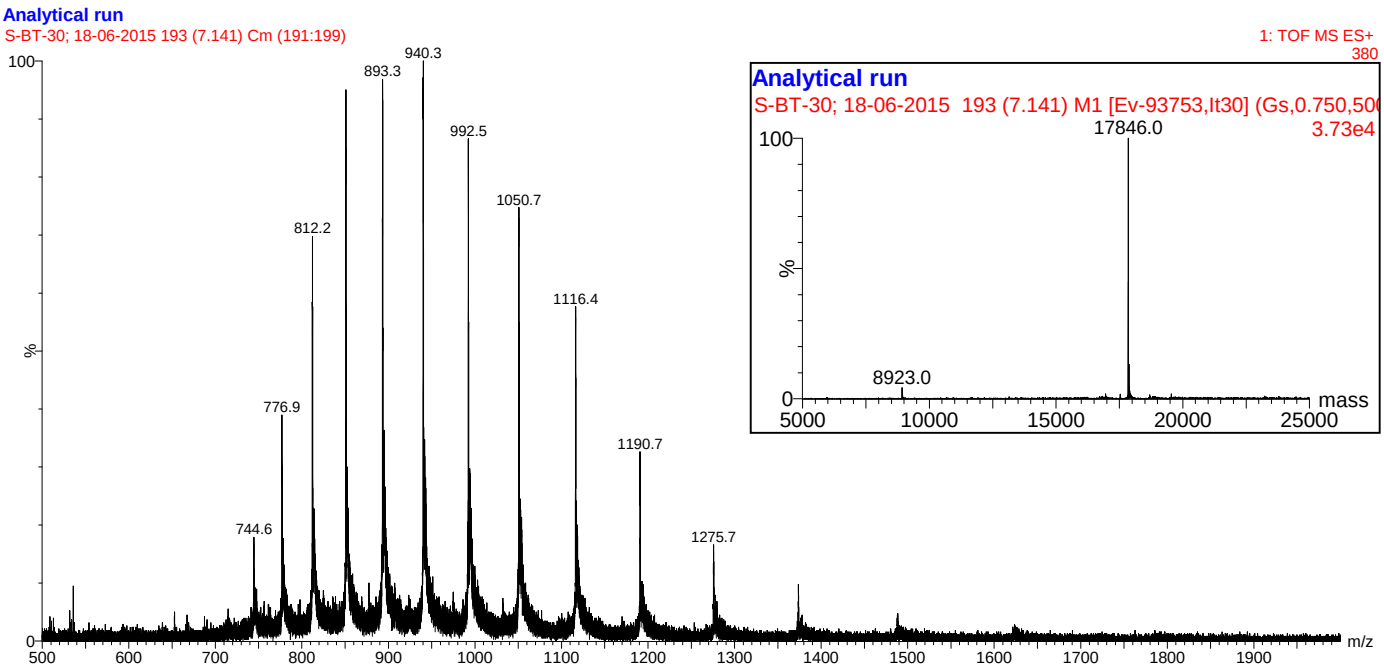




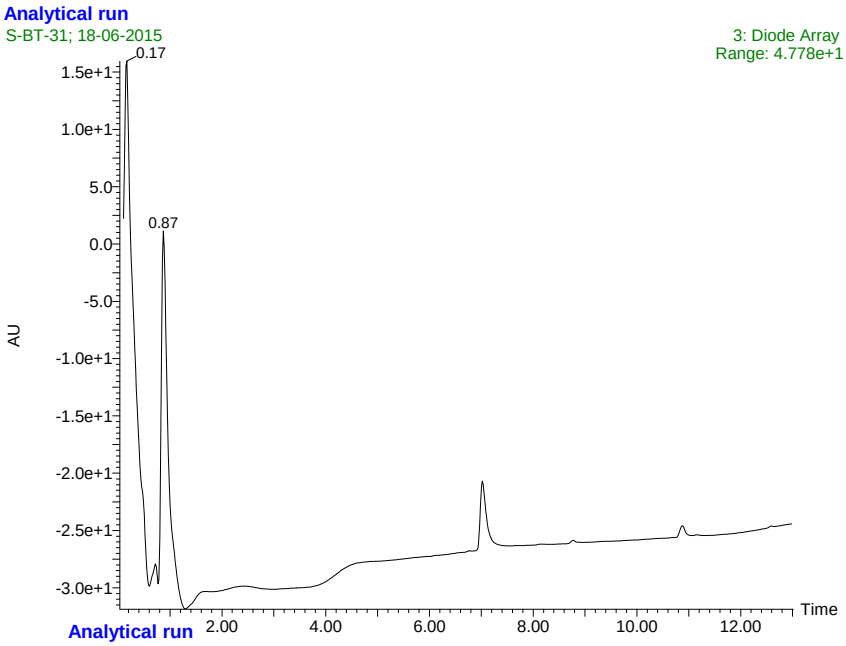
K33-linked diUb FRET pair (17e)

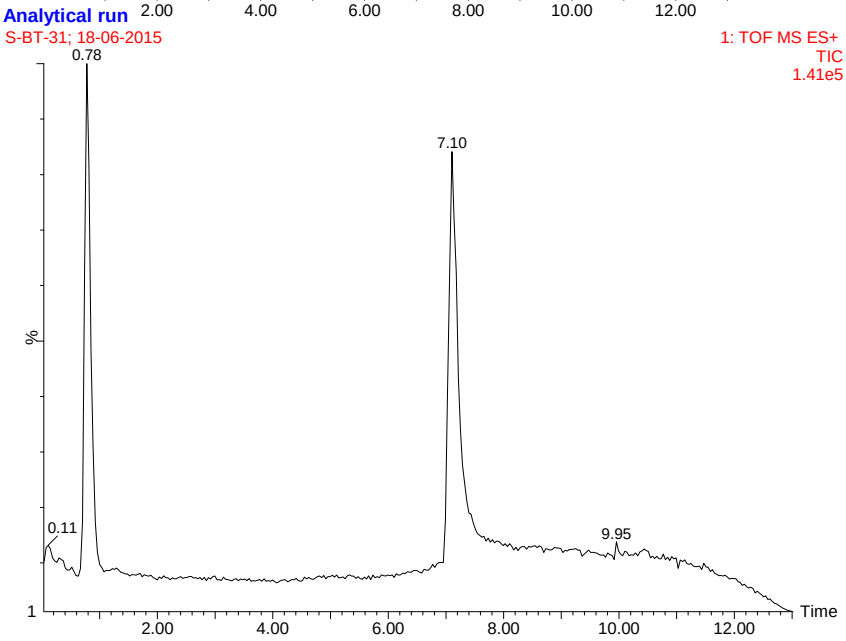


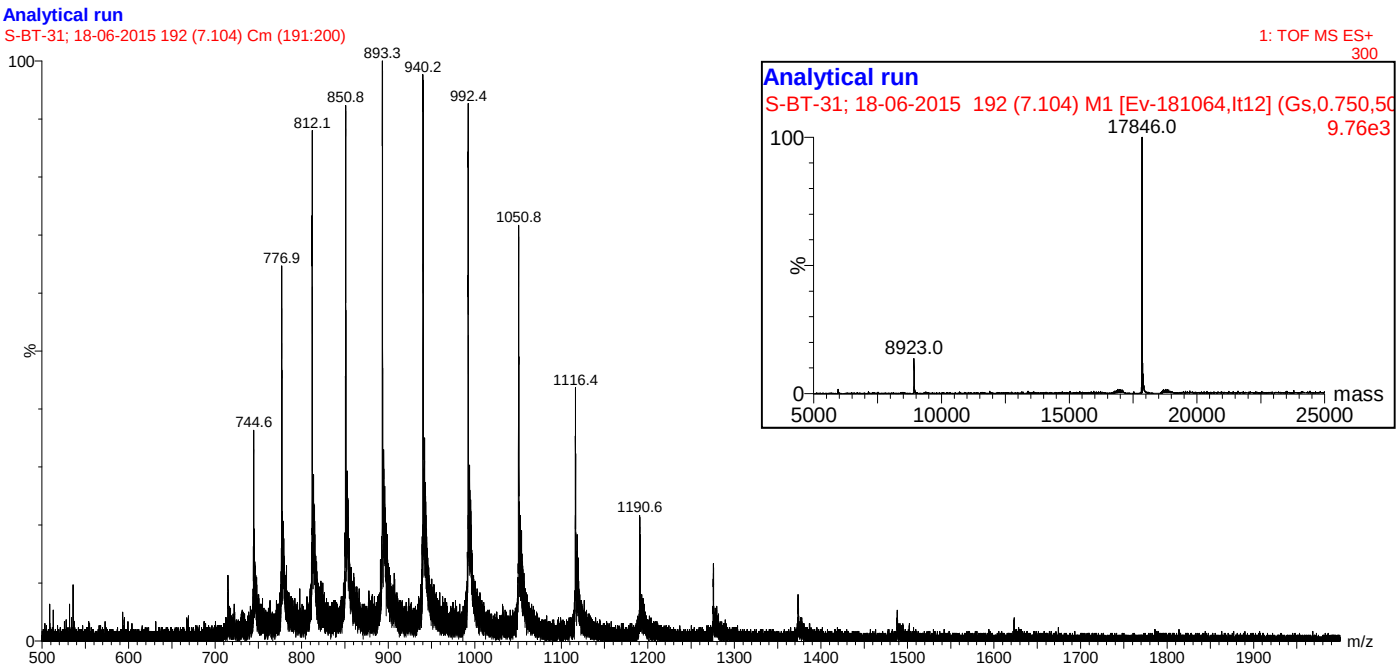




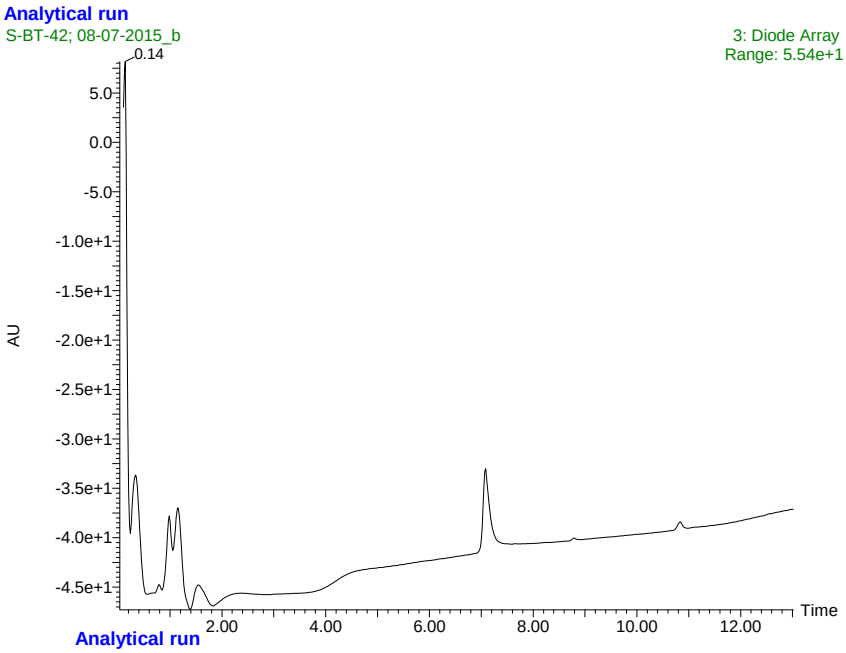
K48-linked diUb FRET pair (17f)

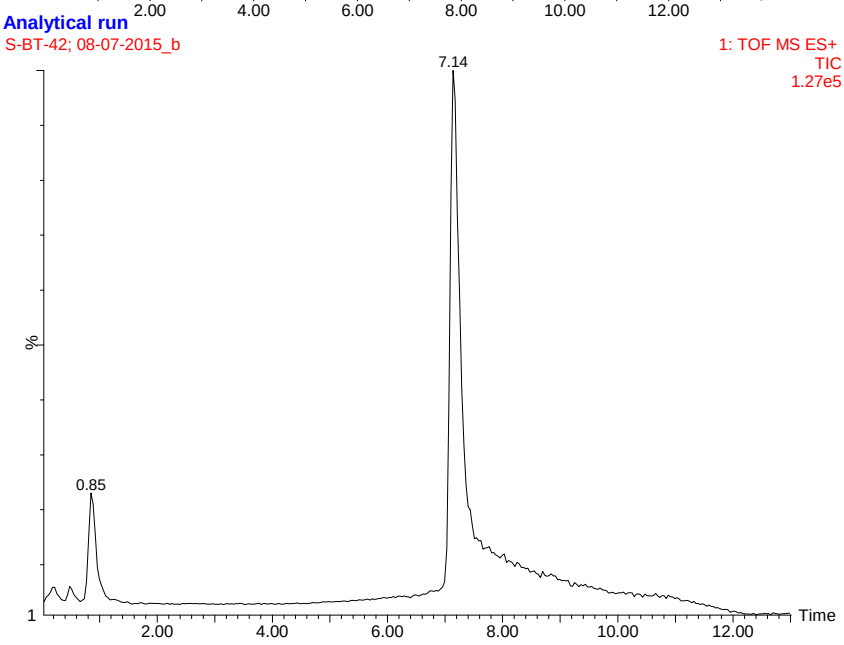


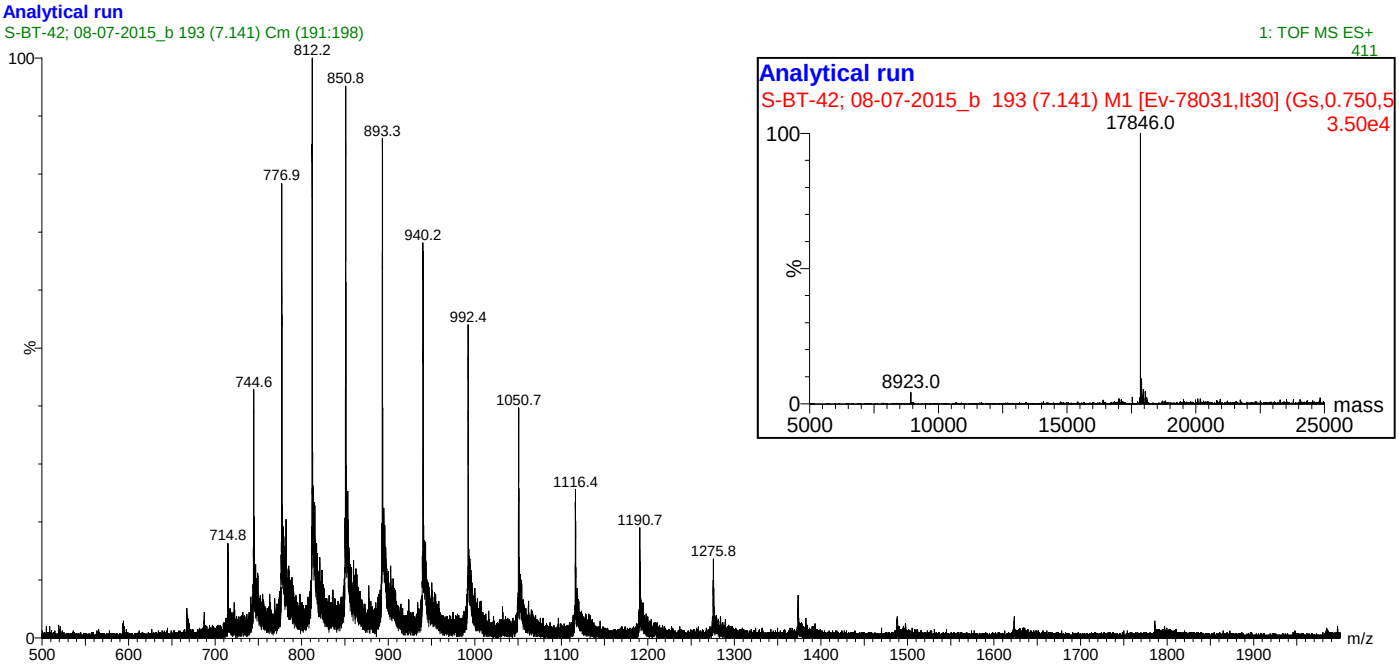




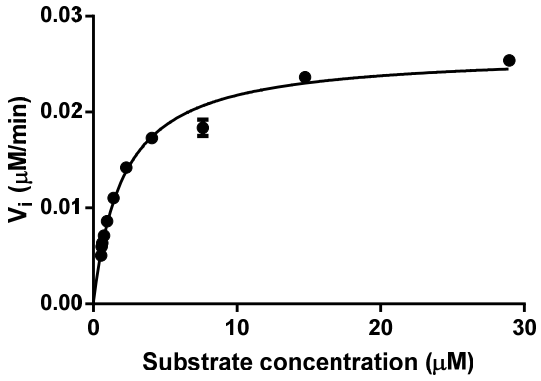
K63-linked diUb FRET pair (17g)



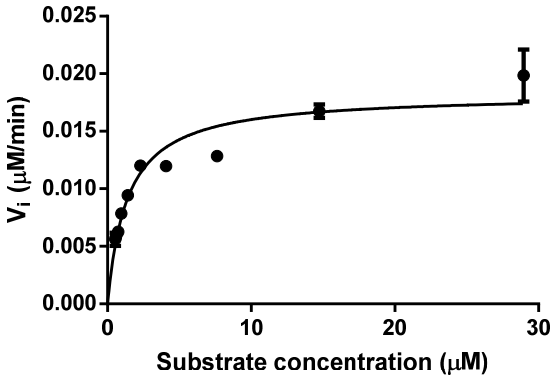




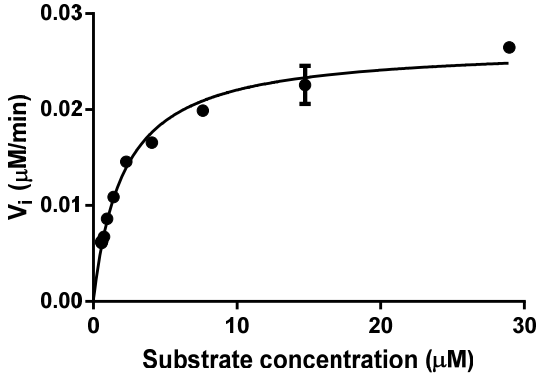
USP 21 (3.5 nM)
K6-linked diUb



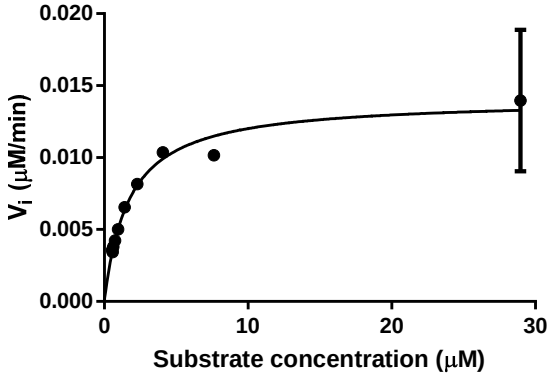
USP 21 (3.5 nM)
K11-linked diUb



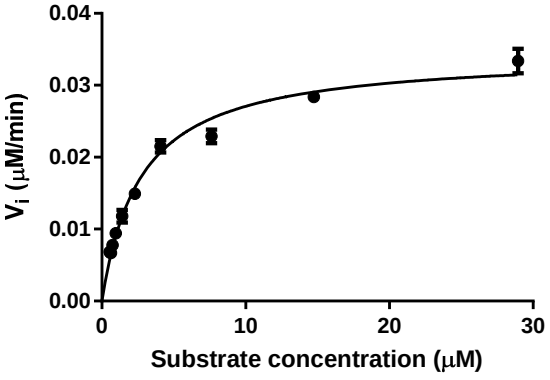
USP 21 (3.5 nM)
K33-linked diUb



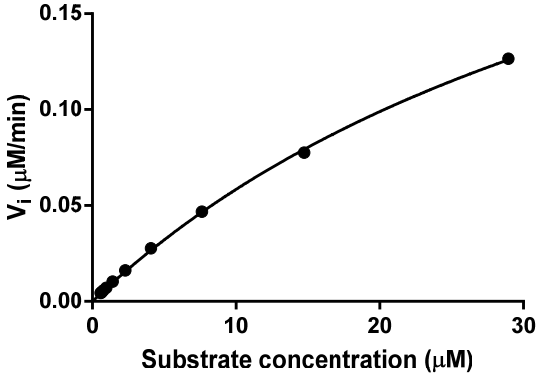
USP 21 (3.5 nM)
K48-linked diUb



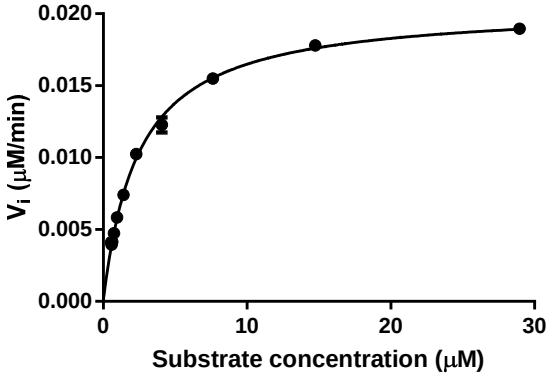
USP 21 (3.5 nM)
K63-linked diUb



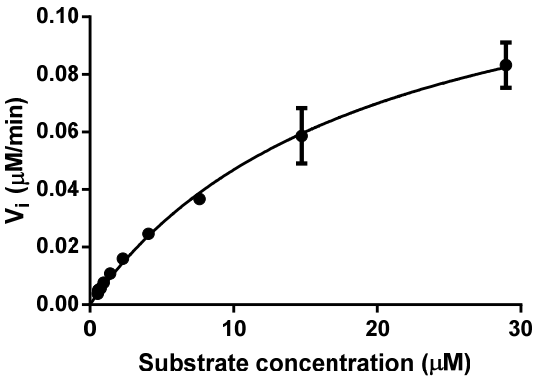
AMSH (2000 nM)
K63-linked diUb



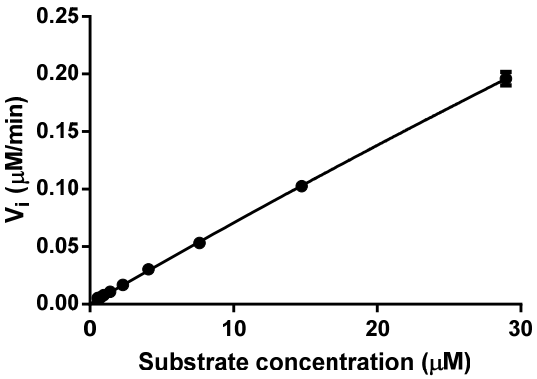
AMSH* (2 nM)
K63-linked diUb

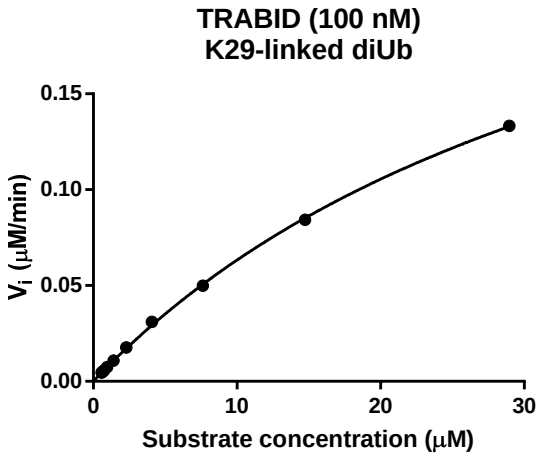


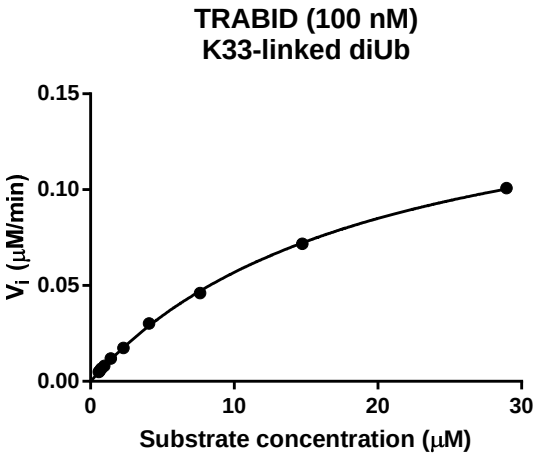
Cezanne (1.5 nM)
K11-linked diUb

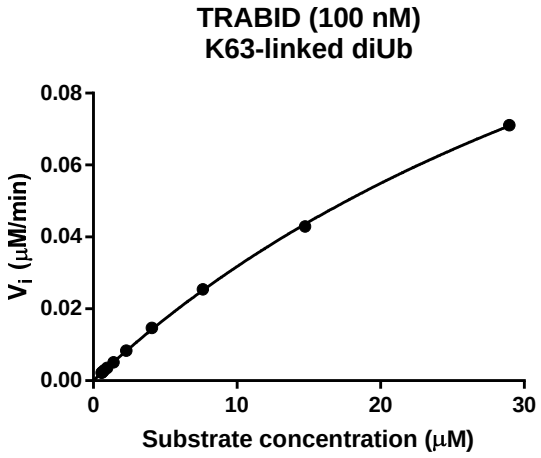


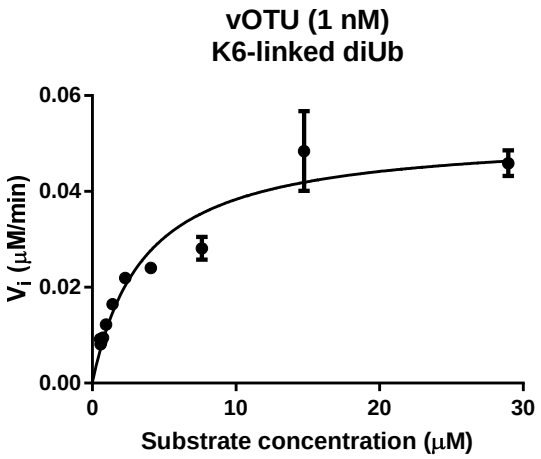
OTUD1 (30 nM)
K63-linked diUb

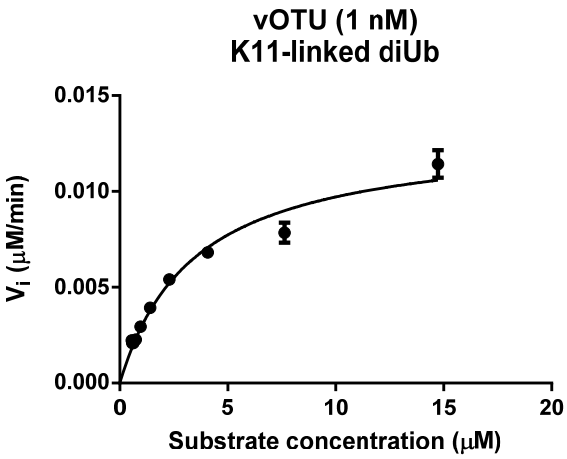


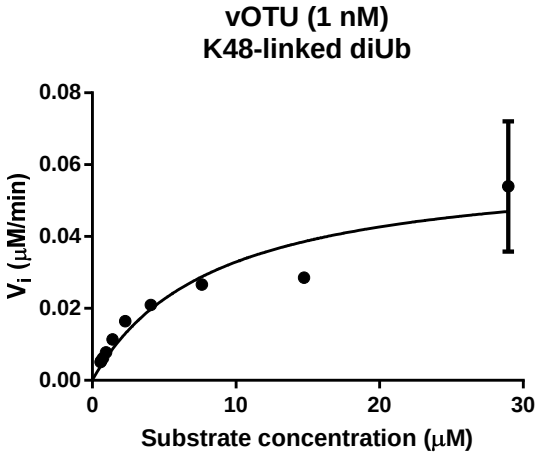

TRABID (100 nM)
K29-linked diUb
0.15
0.10
0.05
0.00
V_i (μM/min)
0
10
20
30
Substrate concentration (μM)

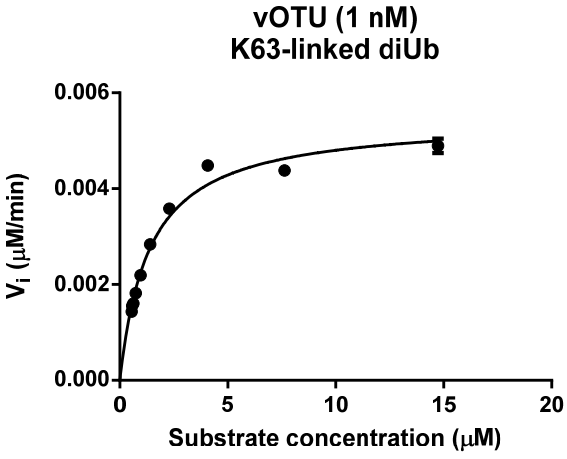

TRABID (100 nM)
K33-linked diUb
0.15
0.10
0.05
0.00
V_i (μM/min)
0
10
20
30
Substrate concentration (μM)

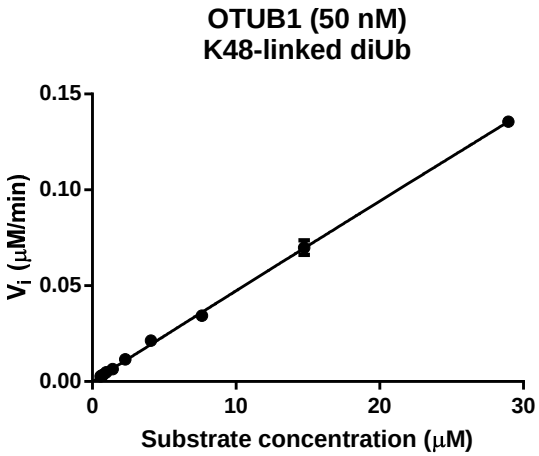

TRABID (100 nM)
K63-linked diUb
0.08
0.06
0.04
0.02
0.00
V_i (μM/min)
0
10
20
30
Substrate concentration (μM)

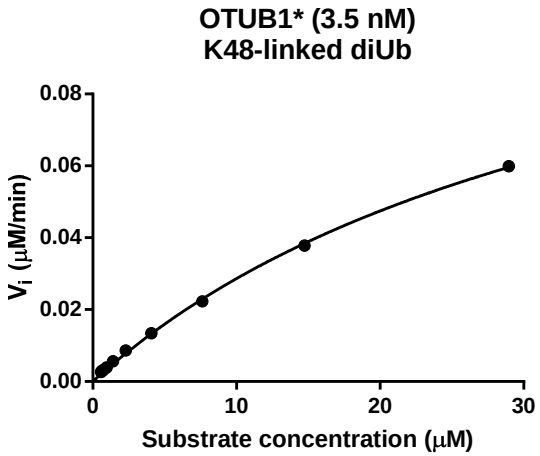

vOTU (1 nM)
K6-linked diUb
0.06
0.04
0.02
0.00
V_i (μM/min)
0
10
20
30
Substrate concentration (μM)

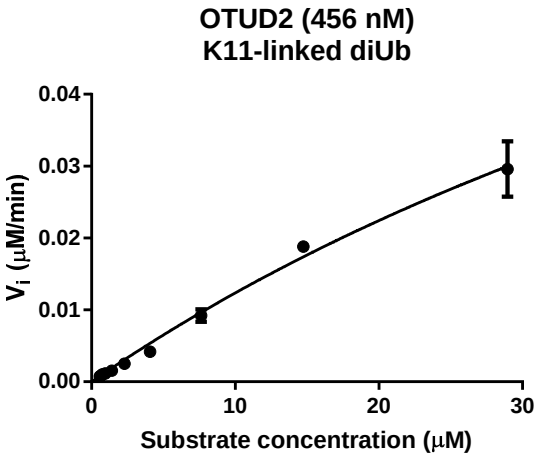

vOTU (1 nM)
K11-linked diUb
0.015
0.010
0.005
0.000
V_i (μM/min)
0
5
10
15
20
Substrate concentration (μM)

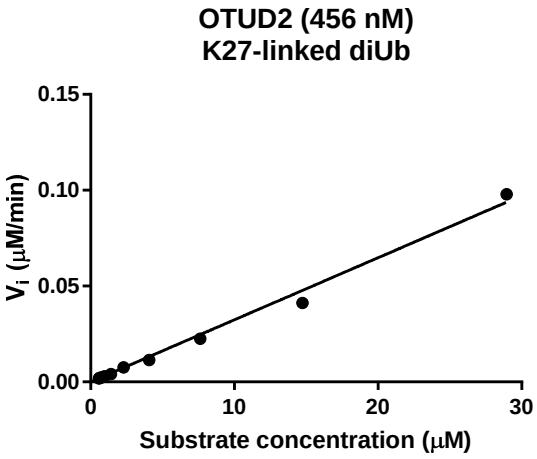

vOTU (1 nM)
K48-linked diUb
0.08
0.06
0.04
0.02
0.00
V_i (μM/min)
0
10
20
30
Substrate concentration (μM)

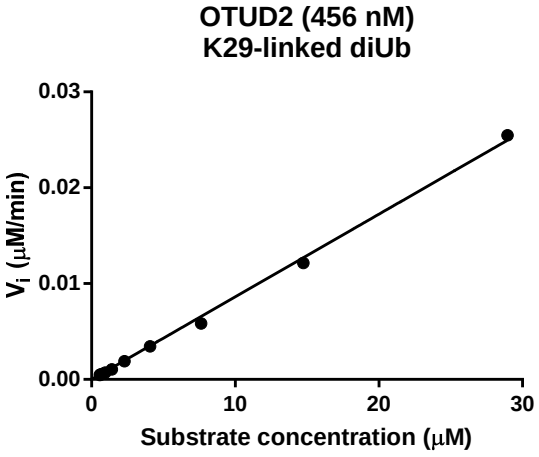

vOTU (1 nM)
K63-linked diUb
0.006
0.004
0.002
0.000
V_i (μM/min)
0
5
10
15
20
Substrate concentration (μM)

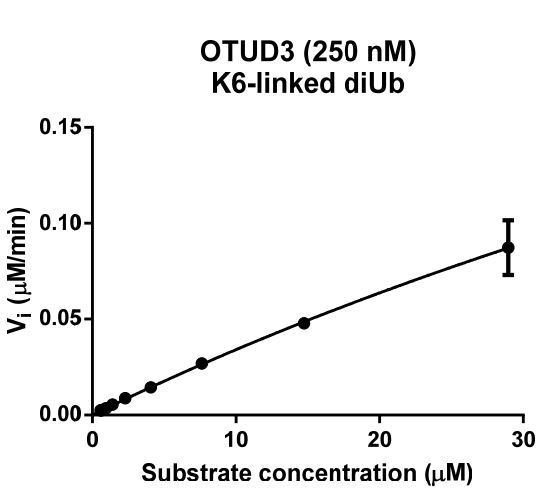

OTUB1 (50 nM)
K48-linked diUb
0.15
0.10
0.05
0.00
V_i (μM/min)
0
10
20
30
Substrate concentration (μM)

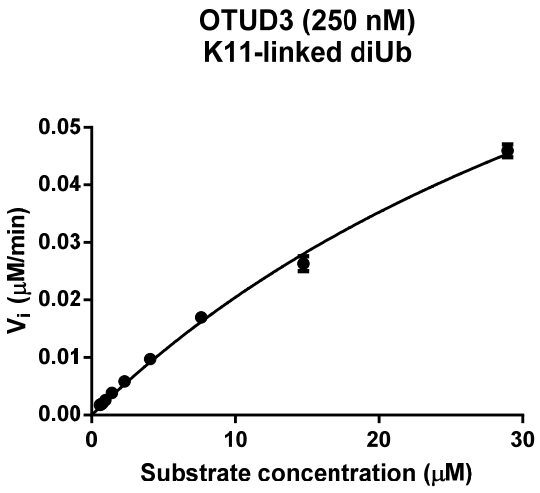

OTUB1* (3.5 nM)
K48-linked diUb
0.08
0.06
0.04
0.02
0.00
V_i (μM/min)
0
10
20
30
Substrate concentration (μM)

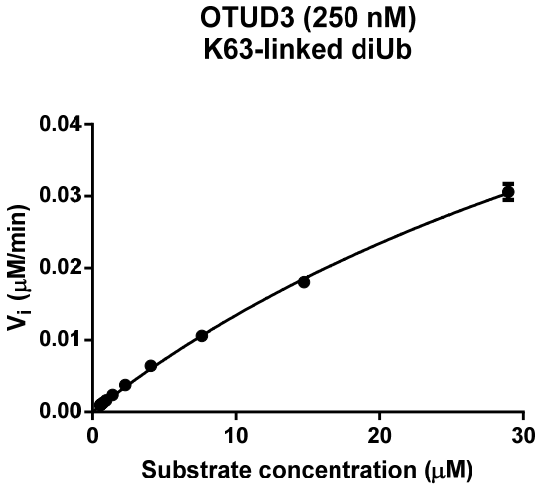

OTUD2 (456 nM)
K11-linked diUb
0.04
0.03
0.02
0.01
0.00
V_i (μM/min)
0
10
20
30
Substrate concentration (μM)


OTUD2 (456 nM)
K27-linked diUb
0.15
0.10
0.05
0.00
V_i (μM/min)
0
10
20
30
Substrate concentration (μM)


OTUD2 (456 nM)
K29-linked diUb
0.03
0.02
0.01
0.00
V_i (μM/min)
0
10
20
30
Substrate concentration (μM)


OTUD3 (250 nM)
K6-linked diUb
0.15
0.10
0.05
0.00
V_i (μM/min)
0
10
20
30
Substrate concentration (μM)


OTUD3 (250 nM)
K11-linked diUb
0.05
0.04
0.03
0.02
0.01
0.00
V_i (μM/min)
0
10
20
30
Substrate concentration (μM)


OTUD3 (250 nM)
K63-linked diUb
0.04
0.03
0.02
0.01
0.00
V_i (μM/min)
0
10
20
30
Substrate concentration (μM)