

Electronic Supplementary Information

## **Covalent labeling of a chromatin reader domain using proximity-reactive cyclic peptides**

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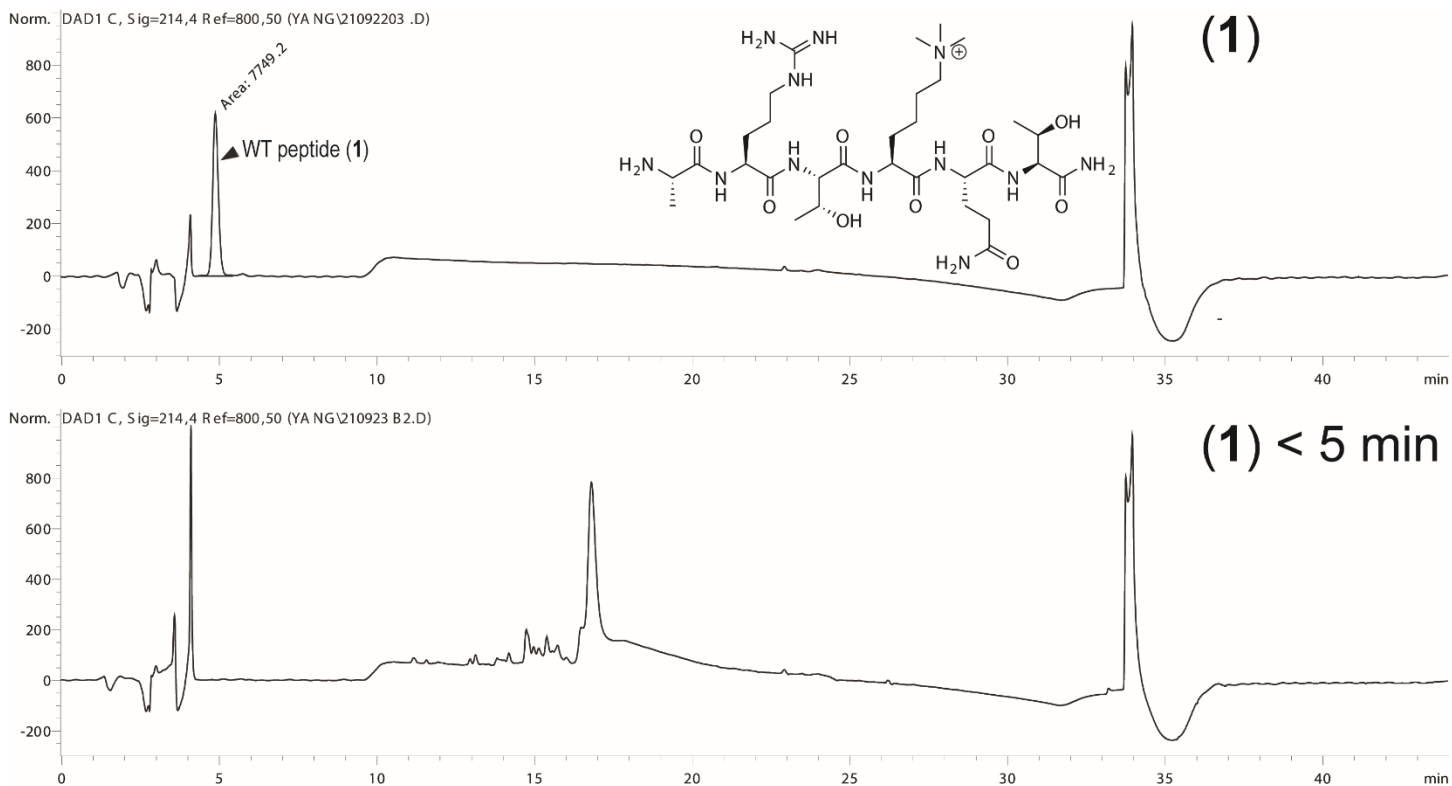
## Supplementary Figure 1

```
ING4 - - - - - PTYCLCHQVSY - - - GEMIGCDNPDCS I EWFHFACVGLTTKPR - - - - - GKWFCPRCSQE - -
ING2 GSEFAIDPNEPTYCLCNQVSY - - - GEMIGCDNEQCP I EWFHFSCVSLTYKPK - - - - - GKWYCPKCRGDNE
→ KDM5A-PHD3 - - - ESDDENAVCAAQNCQRPCDKVDWVQCDGGC - - - DEWFHQVCVGVSPEMAEN - - - - - EDYICINCAKKQ -
KDM5B-PHD3 - - - - - DAICPAVSCLQPEGDEVDWVQCDGSC - - - NQWFHQVCVGVSPEMAEK - - - - - EDYICVRACTVK - -
TAF3 - - - - - IWICPGCNKPDD - - - GSPMIGCDDC - - - DDWYHWPCVGI MTAPPEE - - - - - MQWFCPKCANK - -
MLL5 - - - - - VTRCICGFTHD - - - DGYMICCDKC - - - SVWQHIDCMGIDRQHI - - - - - PDTYLCERCQPR - -
BPTF - - - - - KLYCICKTPYDE - - - SKFYIGCDRC - - - QNWHYHGRCVGI LQSEAELI - - - - - DEYVCPQCQST - -
PHF3 - - - - - SKQCGFCKKPHG - - - NRFMVGCGRC - - - DDWFHGDCVGLSL SQAQQMGEEDKEYVVCVKCCAE - -
DIDO1 - - - - - ALYCICRQPHN - - - NRFMICCDRC - - - EEFW HGDCVGI SEARGRL LERNGEDYICPNCTIL - -
```

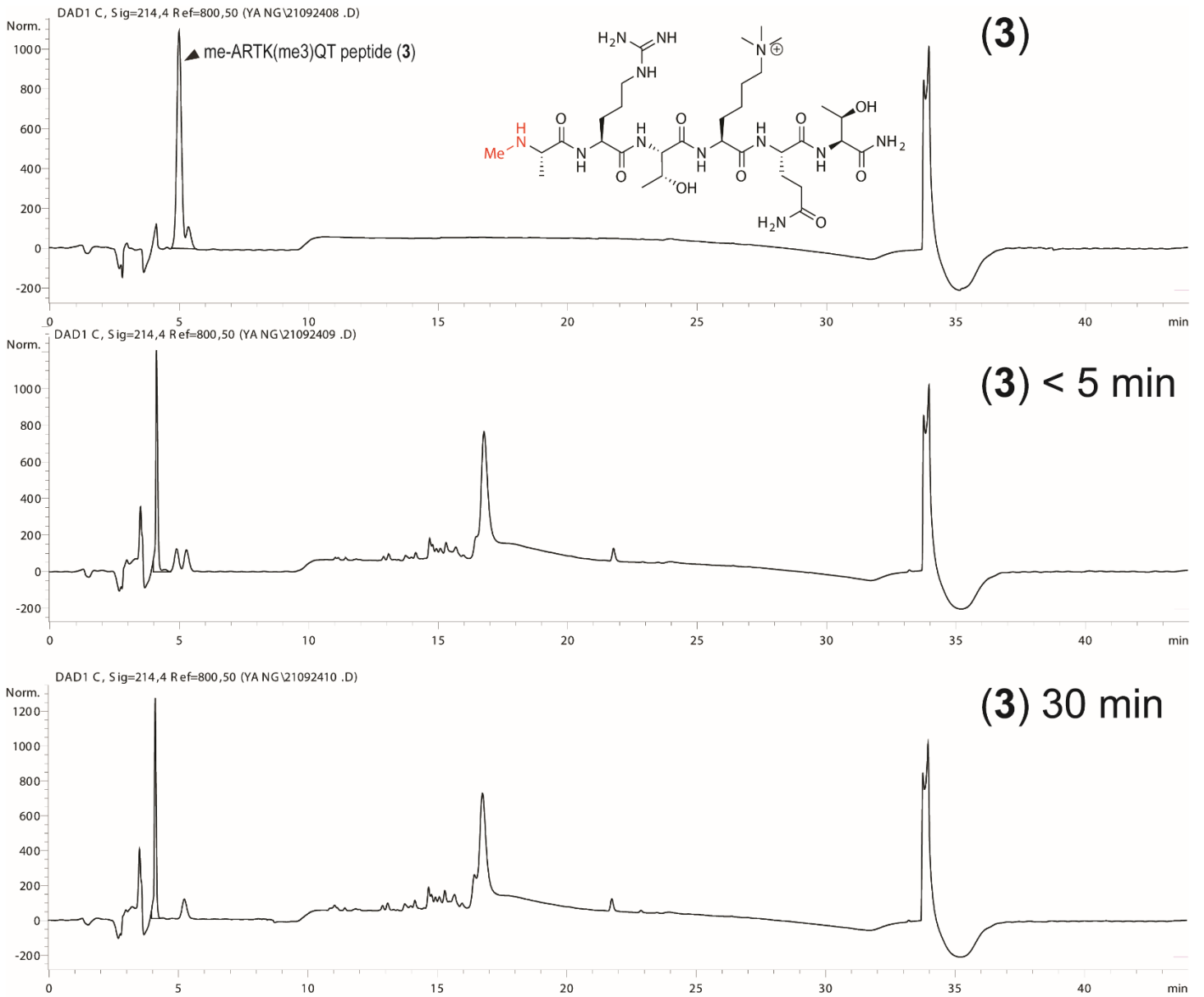
**Fig. S1:** Sequence alignment of KDM5A-PHD3 with other H3K4me3-recognizing PHD domains.

## Supplementary Figure 2

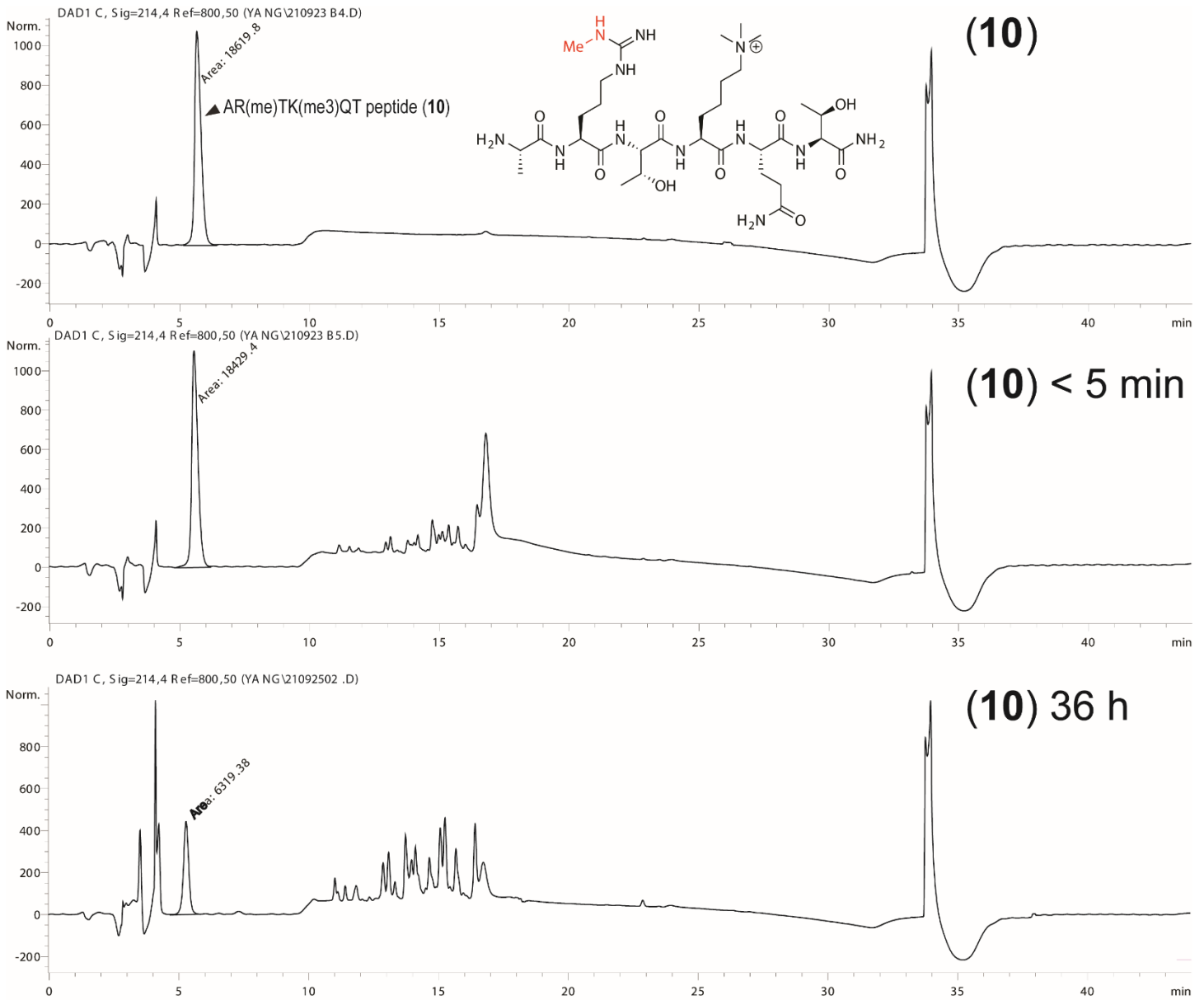
**Trypsin stability assay:** Peptide and trypsin (1:1 w/w) were mixed in 1x PBS buffer and the resulting mixture was incubated at RT. Proteolysis reactions at a given time point was quenched by adding HCl. The proteolysis process was monitored by subjecting the quenched mixture onto reverse-phase HPLC column monitoring at 214 nm [H<sub>2</sub>O (A):MeCN (B); 5% to 100% solvent B over 23 min].



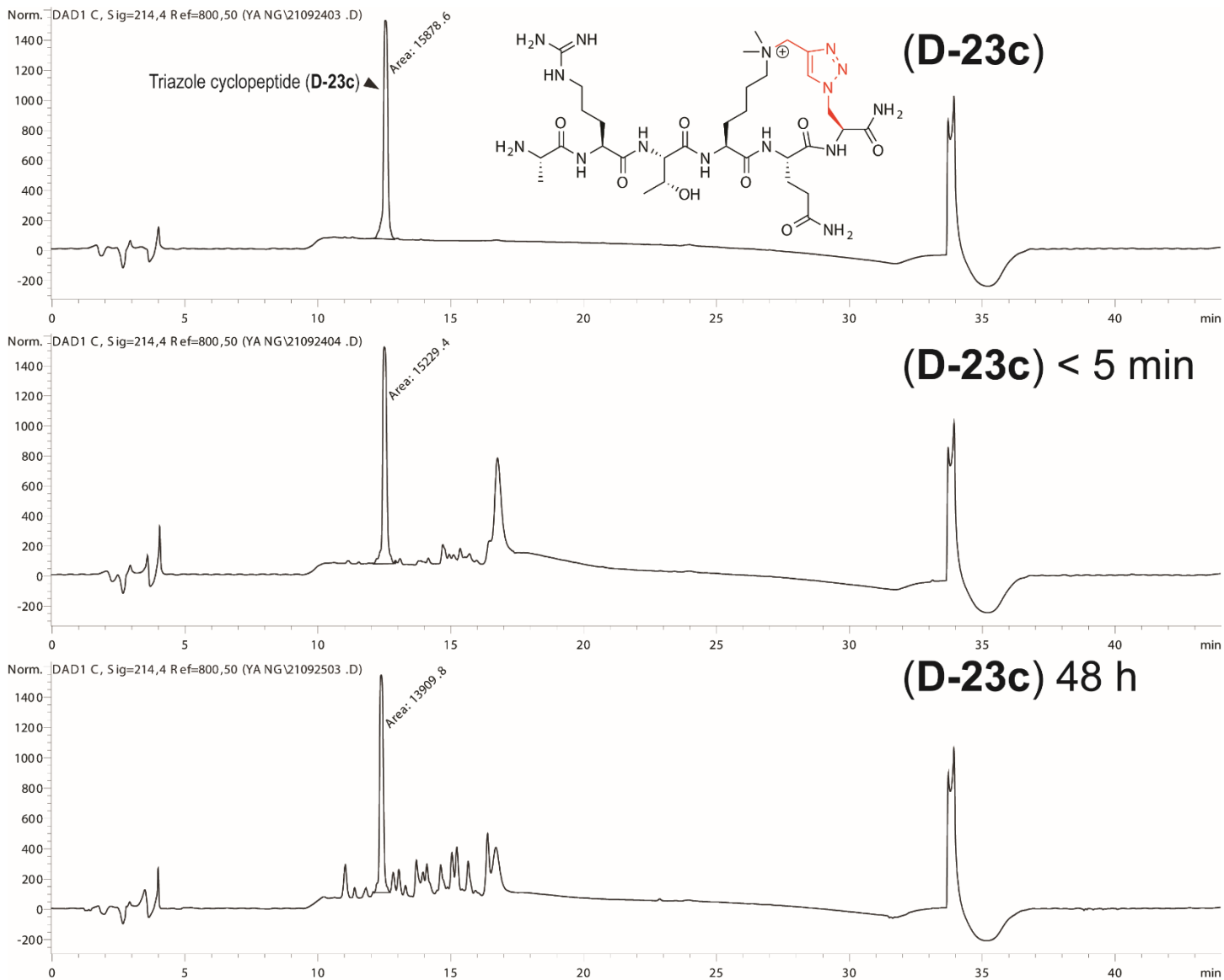
**Fig. S2a:** Chromatograms of WT H3K4me3 hexapeptide (1) before and after incubation with trypsin ( $t = < 5$  min).



**Fig. S2b:** Chromatograms of me-ARTK(me3)QT hexapeptide (3) before and after incubation with trypsin ( $t = < 5$  min, 30 min).

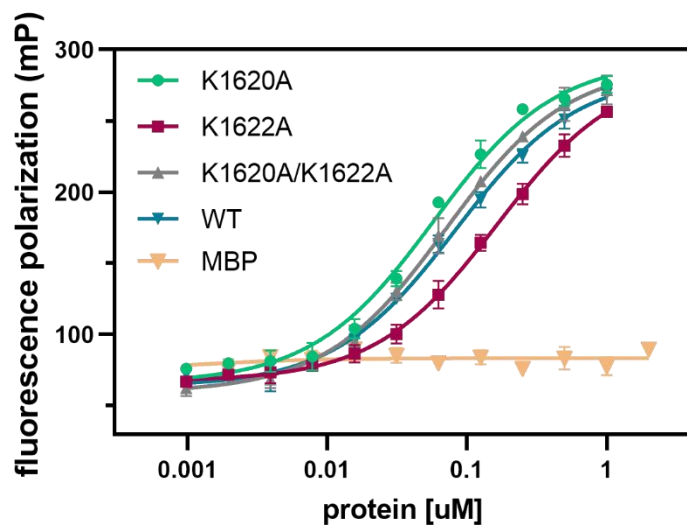


**Fig. S2c:** Chromatograms of AR(me)TK(me3)QT hexapeptide (10) before and after incubation with trypsin ( $t = < 5 \text{ min}, 36 \text{ h}$ ).



**Fig. S2d:** Chromatograms of triazole cyclopeptide (**D-23c**) before and after incubation with trypsin ( $t = < 5$  min, 48 h).

### Supplementary Figure 3



Protein	$K_d$ ( $\mu\text{M} \pm \text{SE}$ )
K1620A	$0.058 \pm 0.0071$
K1622A	$0.16 \pm 0.0054$
K1620A K1622A	$0.071 \pm 0.0042$
WT	$0.083 \pm 0.0055$
His <sub>6</sub> -MBP	NB

**Fig. S3:** Fluorescence polarization binding curves (top) and  $K_d$  values of WT and mutant His<sub>6</sub>-MBP-PHD3 (bottom). Fluorescence polarization binding assays used C-terminally fluorescently labeled H3K4me3 10mer peptide as the tracer, and  $K_d$  values are presented as average  $\pm$  standard error of three replicates.



## Supplementary Figure 4

Peptide	PHD domain	Compound number	$K_i$ ( $\mu\text{M} \pm \text{SE}$ )
Linear (arylfluorosulfate)	PHD3	<b>30</b>	$0.22 \pm 0.039$
Linear (arylfluorosulfate)	PHD3	<b>31</b>	$0.17 \pm 0.020$
Linear (arylfluorosulfate)	PHD3	<b>32</b>	$0.37 \pm 0.077$
Linear (SF)	PHD3	<b>27</b>	$0.13 \pm 0.023$
Linear (SF)	PHD3	<b>28</b>	$0.11 \pm 0.020$
Linear (SF)	PHD3	<b>29</b>	$0.22 \pm 0.048$
Cyclic (SF)	PHD3	Triazole <b>D-34</b>	$0.51 \pm 0.097$
Cyclic (SF)	PHD3	Triazole <b>D-35</b>	$0.38 \pm 0.065$
Cyclic (SF)	PHD3	Thioether <b>C-33</b>	$0.50 \pm 0.068$
Cyclic (SF) biotinylated	PHD3	Thioether <b>C-36</b>	$0.21 \pm 0.040$
Cyclic (SF)	PHD1*	Triazole <b>D-35</b>	$188.8 \pm 42.05$

**Fig. S4:** Competitive fluorescence polarization binding assay data for covalent linear and cyclic peptides. Inhibition constants are presented as average  $\pm$  standard error of three replicates.

\* The inhibition constant for triazole **D-35** binding to PHD1 was obtained using recombinant GST-PHD1 and fluorescently labelled H3K4me0 10-mer peptide, as previously described.<sup>1</sup>

# Supplementary Figure 5

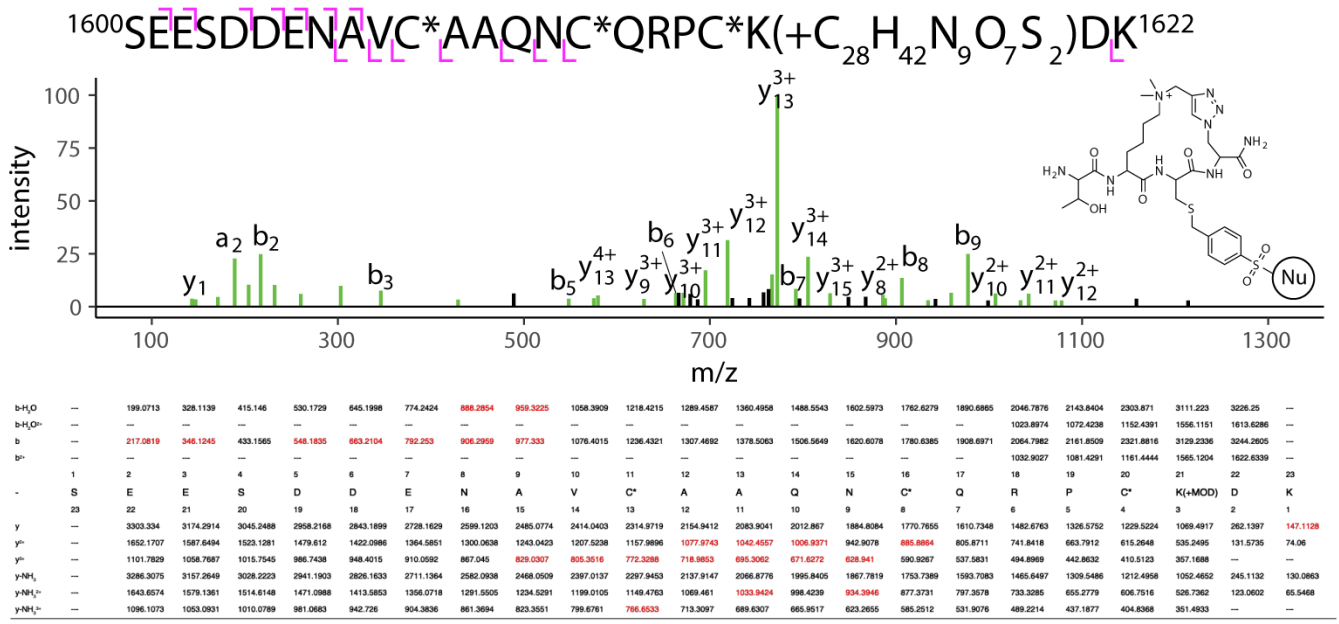


Fig. S5a: HCD product ion spectrum of 678.6790<sup>5+</sup> corresponding to covalent modification at K1620 of PHD3.

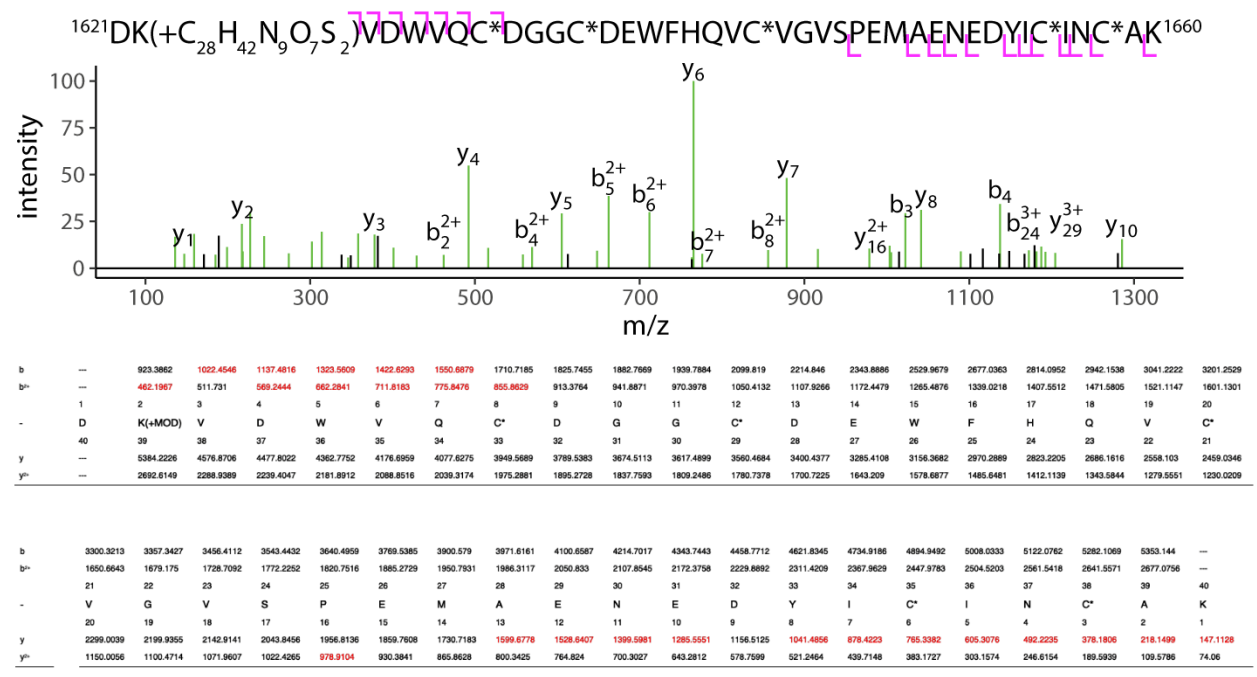


Fig. S5b: HCD product ion spectrum of 1100.6557<sup>5+</sup> corresponding to covalent modification at K1622 of PHD3.

## **Reagents and Materials**

Fmoc-solid phase peptide synthesis was performed using fritted plastic syringes from TORVIQ. Rink amide resin (0.54 mmol<sup>-1</sup>, 100-200 mesh) was manufactured by Novabiochem® and purchased from Sigma-Aldrich. Fmoc-protected amino acids and reagents used for peptide synthesis were purchased from AK Scientific, Iris Biotech, AstaTech, Combi-blocks, Chem-Impex and Sigma-Aldrich, and were used without further purification. Peptides used for the alanine scan, C-terminal truncation scan, Ac-ARTK(me3)QT-NH<sub>2</sub>, SRTK(me3)QT-NH<sub>2</sub>, PRTK(me3)QT-NH<sub>2</sub>, AibRTK(me3)QT-NH<sub>2</sub> were purchased from Genscript.

## **General Experimental Procedures**

Preparative HPLC was performed on a Waters Prep 150Q LC system, with a Waters 2998 Photodiode Array detector, and this system was operated using ChromScope software. All separations used linear gradients of water containing 0.1% trifluoroacetic acid and acetonitrile, at a flow rate of 10 mL/min (XSelect® Peptide CSH™ C18, OBD™ Prep Column, 130Å, 5 µM, 19 mm × 250 mm, 1/pkg). Analytical HPLC was performed on a Varian ProStar 210 Solvent Delivery Module, with a Varian ProStar 335 Photodiode Array Detector, and this system was operated using Galaxy Chromatography Data System. All separations used linear gradients of water containing 0.1% trifluoroacetic acid (solvent A) and acetonitrile (solvent B), at a flow rate of 1 mL/min (Phenomenex Luna® 10 µm C18(2) 100Å, LC column 250 × 4.6 mm, Ea).

Analytical HPLC methods used for peptide characterization:

Method 1: 3% B for 1 minute, 3% to 30% B over 10 minutes, then 30% to 100% B over 1 minute ( $\lambda = 220$  nm).

Method 2: 10% B for 1 minute, 10% to 95% B over 12 minutes and hold at 95% for 3 minutes ( $\lambda = 220$  nm).

Method 3: 10% B for 1 minute, 10% to 95% B over 12 minutes and hold at 95% for 6 minutes ( $\lambda = 220$  nm).

Analytical UPLC was performed on a Waters Acquity system fitted with a TUV detector. Separations for small molecules and peptides used linear gradients of water containing 0.1% formic acid (solvent A1) and acetonitrile containing 0.1% formic acid (solvent B1), at a constant flow rate (0.2 mL/min, BEH, C18, 1.7 µm). Separations for proteins used linear gradients of water containing 0.1% formic acid and 0.05% trifluoroacetic acid (solvent A2), and acetonitrile containing 0.1% formic acid (solvent B2), at a constant flow rate (0.2 mL/min, protein BEH C4, 300Å, 1.7 µm).

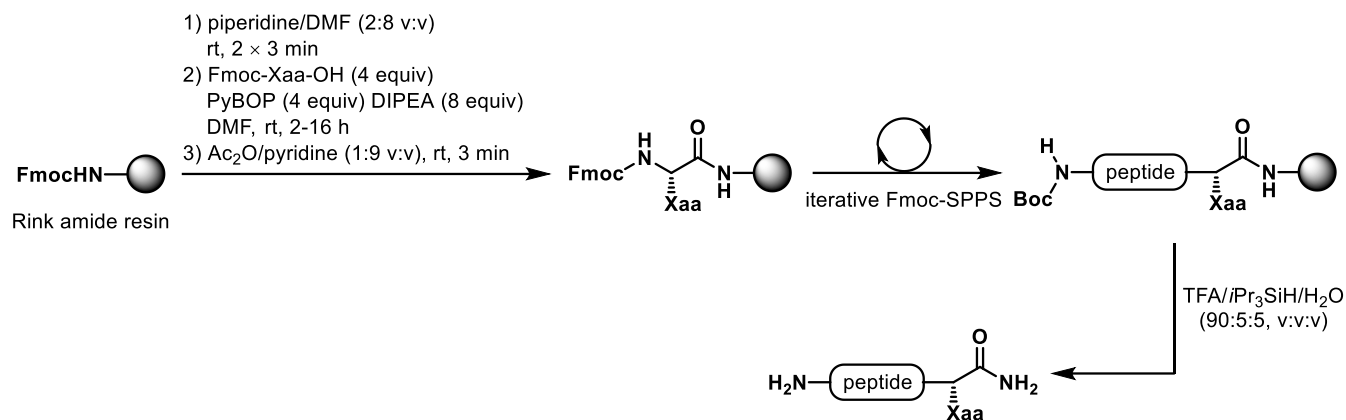
## **Molecular Modelling**

Design feasibility of macrocyclic peptides were assessed using MacroModel. The X-ray crystallographic structure of PHD3-H3K4me3 (PDB 3GL6) was prepared as the initial starting structure. Non-coordinating water molecules found beyond 5 Å from heteroatoms were removed. Macrocyclic peptides were prepared by mutating K4me3 and T6 with a linker which contained amide, thioether, and triazole moieties respectively. The resulting structures were minimized using MMFFs and GB/SA water solvation whilst keeping the PHD3 and amide backbone of H3K4me3 frozen. The minimized model was subsequently searched for energy conformers using the conformational search tool using the MMFFs force field and GV/SA water solvation whilst keeping the PHD3 and

amide backbone of H3K4me3 frozen. The resulting conformers within 4 kJmol<sup>-1</sup> from the lowest-energy conformer were overlayed to assess the rigidity and design feasibility.

# Synthetic Procedures and Peptide Characterization Data

## General Workflow for Fmoc-Solid Phase Peptide Synthesis (Fmoc-SPPS)



### Loading Rink amide resin

In a fritted plastic syringe, Rink amide resin (1.0 equiv, substitution 0.54 mmolg<sup>-1</sup>) was swollen in DMF for 15 min, then washed with DCM (5 × 3 mL) and DMF (5 × 3 mL). The resin-bound Fmoc group was removed by adding a solution of piperidine/DMF (2:8 v:v). The liquid was discarded after three minutes and the deprotection reaction was repeated once more. The resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL) and DMF (5 × 3 mL).

A solution of Fmoc-Xaa-OH (4 equiv), PyBOP (4 equiv) and DIPEA (8 equiv) in DMF was added to the resin and shaken at room temperature for 2-16 hours, at which time the resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL) and DMF (5 × 3 mL).

A solution of Ac<sub>2</sub>O/pyridine (1:9 v:v) was added to the resin. After three minutes, the liquid was discarded and the resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL) and DMF (5 × 3 mL). The resin-bound amino acid was subjected to iterative peptide assembly.

### Iterative Fmoc-SPPS workflow

**Deprotection:** The resin was treated with piperidine/DMF (2:8 v:v, 2 × 3 min) and washed with DMF (5 × 3 mL), DCM (5 × 3 mL) and DMF (5 × 3 mL).

**General amino acid coupling:** A solution of Fmoc-protected amino acid (4 equiv), PyBOP (4 equiv) and DIPEA (8 equiv) in DMF was added to the resin. After 2-16 hours, the resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL) and DMF (5 × 3 mL).

**Capping:** A solution of Ac<sub>2</sub>O/pyridine (1:9 v:v) was added to the resin. After three minutes, the liquid was discarded and the resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL) and DMF (5 × 3 mL).

**Cleavage:** The resin was washed with DCM (10 × 3 mL) to remove any traces of DMF. A mixture of TFA/*i*Pr<sub>3</sub>SiH/H<sub>2</sub>O (90:5:5 v:v:v, 3 mL) was added to the resin and agitated at room temperature for three hours, at which time the resin was washed with TFA (2 × 2 mL) and DCM (2 × 2 mL).

**Workup/purification:** The cleavage and wash solutions were concentrated by gently blowing over the combined solutions with air. Ice-cold diethyl ether was added to the residue, and the precipitated crude peptide was pelleted by centrifugation. The diethyl ether solution was removed, and the pellet was dried to afford a colorless-yellow powder. The crude peptide was subsequently purified by reverse phase HPLC.

Loading and/or coupling of Fmoc-protected cysteine amino acids: A solution of Fmoc-protected cysteine amino acid (3 equiv), Oxyma pure (3 equiv) and *N,N'*-diisopropylcarbodiimide (3 equiv) in DMF was added to the resin at 4 °C. The coupling reaction was performed at 4 °C for 2-16 hours, at which time the resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL) and DMF (5 × 3 mL).

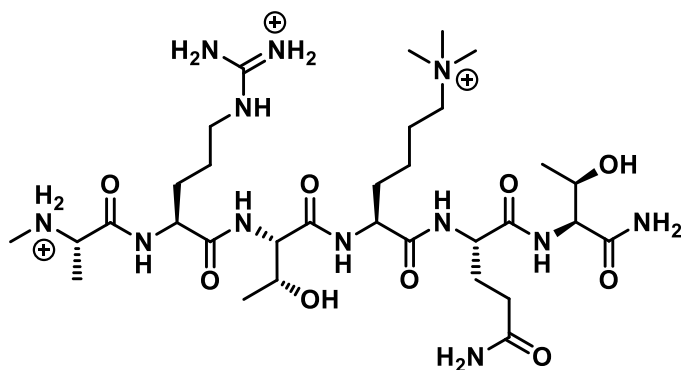
### **Deprotection procedures**

Alloc and Allyl protecting groups: The resin (1 equiv) was treated with a solution of Pd(PPh<sub>3</sub>)<sub>4</sub> (0.5 equiv) and PhSiH<sub>3</sub> (2 equiv) in DCM for one hour, at which time the reaction vessel was carefully vented and the dark liquid was discarded. The deprotection reaction was monitored by LCMS and repeated if necessary.

Mmt protecting group: The resin was washed with DCM (5 × 3 mL). The resin was treated with a solution of DCM/TFA/*i*PrSi<sub>3</sub>H (95:1:4 v:v:v) and agitated for two minutes at room temperature, at which time the liquid is discarded. A small portion of resin was treated with TFA to monitor the deprotection. The cleavage reaction was repeated until the Mmt cation was not detected (intense orange color).

S-(*t*Bu) protecting group: The resin was washed with DMF (5 × 3 mL) and was agitated with a solution of DTT (5 equiv) dissolved in DMF/DIPEA/H<sub>2</sub>O (95:2.5:2.5 v:v:v) for 10 minutes at RT, at which time the liquid was discarded. The reaction was repeated three more times or until the deprotection was complete, indicated by LCMS analysis.

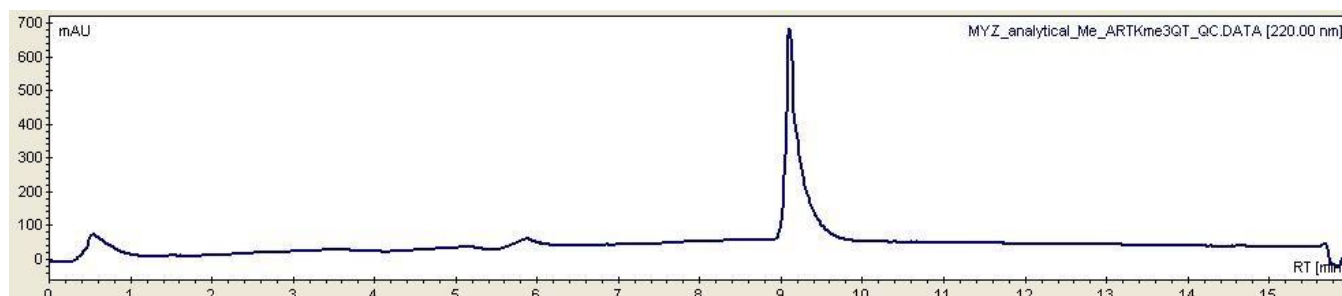
### Compound 3



Yield: 8%

Exact mass: 761.50 [C<sub>32</sub>H<sub>65</sub>N<sub>12</sub>O<sub>9</sub>]<sup>3+</sup>

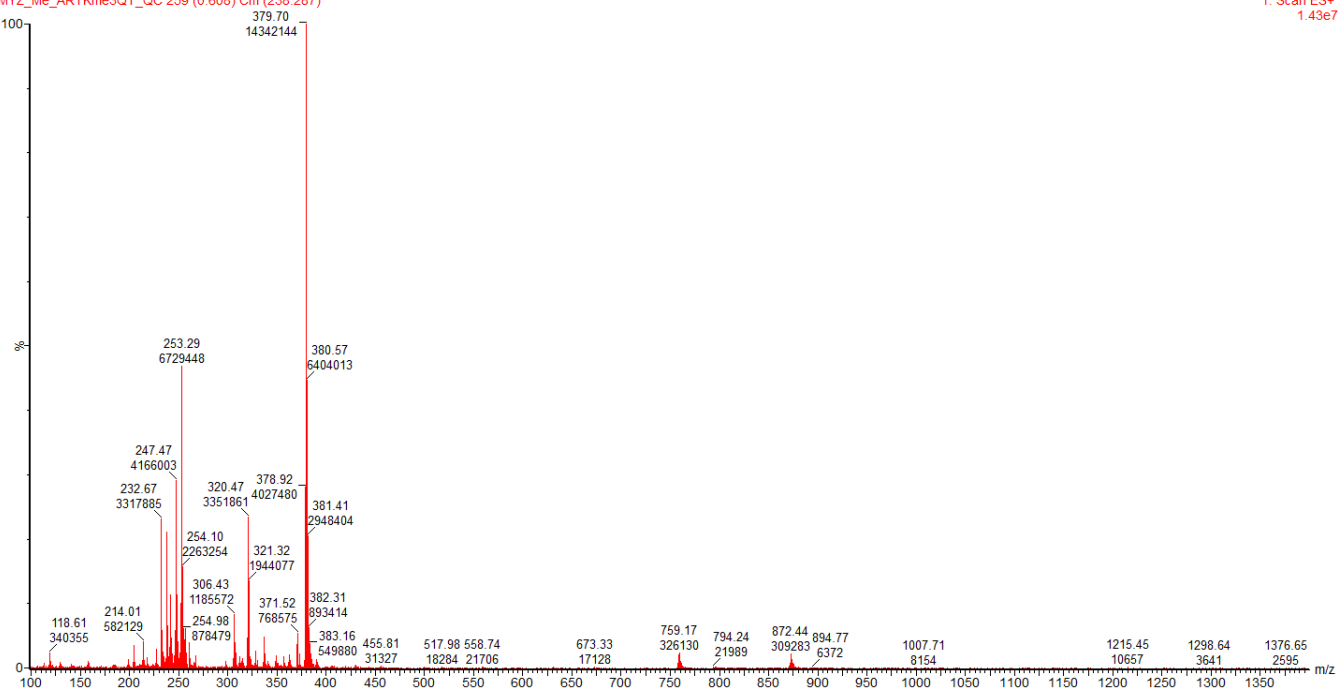
HPLC trace (Method 1):



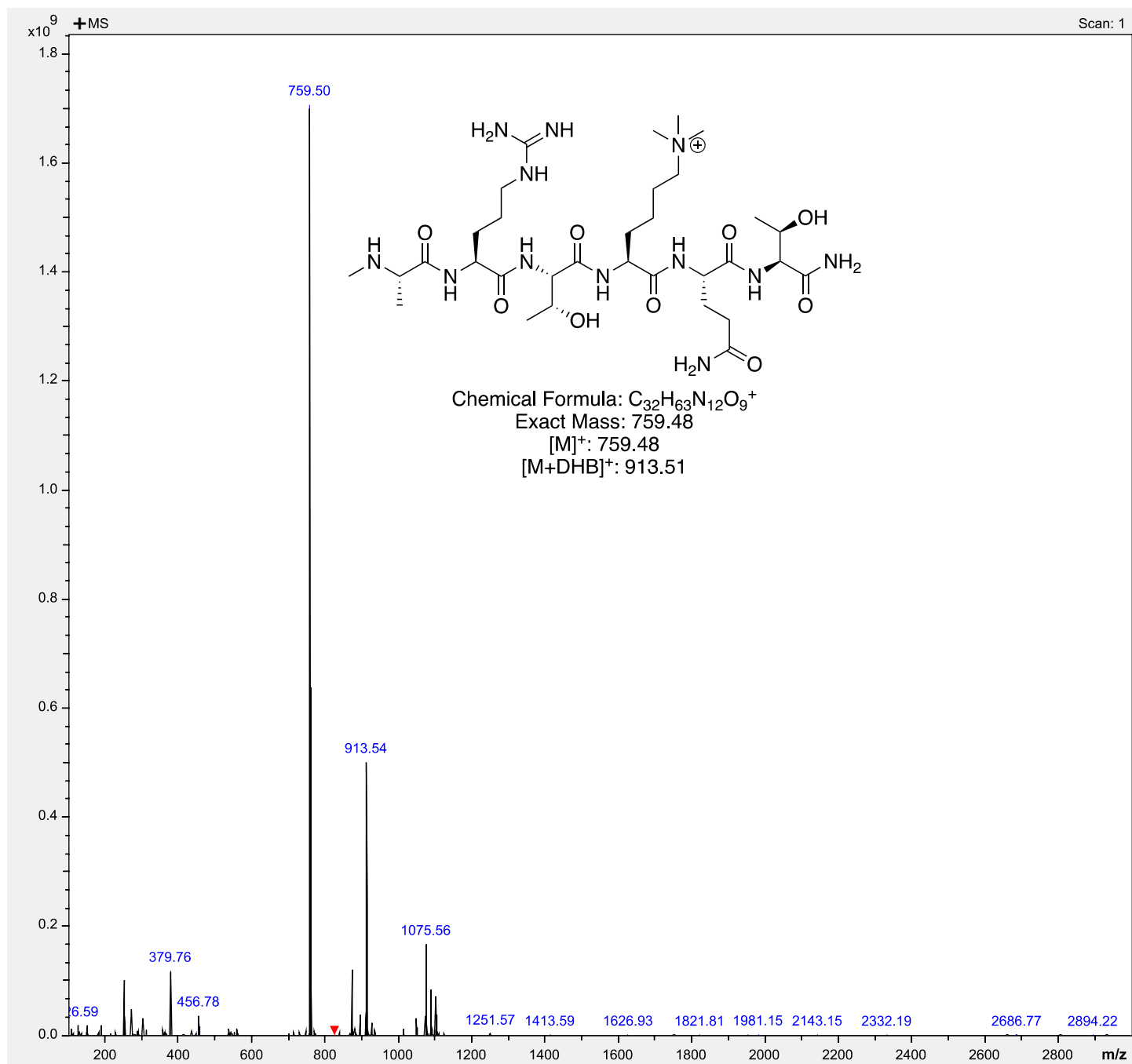
### LCMS

11/10/21  
MYZ\_Me\_ARTKme3QT\_QC 259 (0.608) Cm (238.287)

1: Scan ES+  
1.43e7

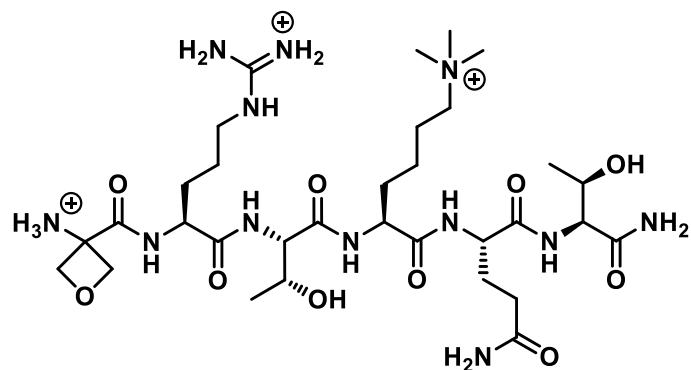


## MALDI





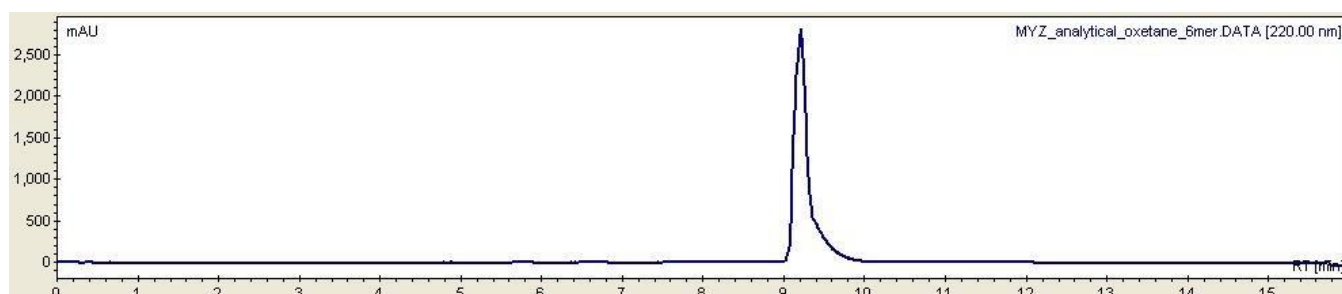
## Compound 4



Yield: 8%

Exact mass: 775.48 [C<sub>32</sub>H<sub>63</sub>N<sub>12</sub>O<sub>10</sub>]<sup>3+</sup>

HPLC trace (Method 1):

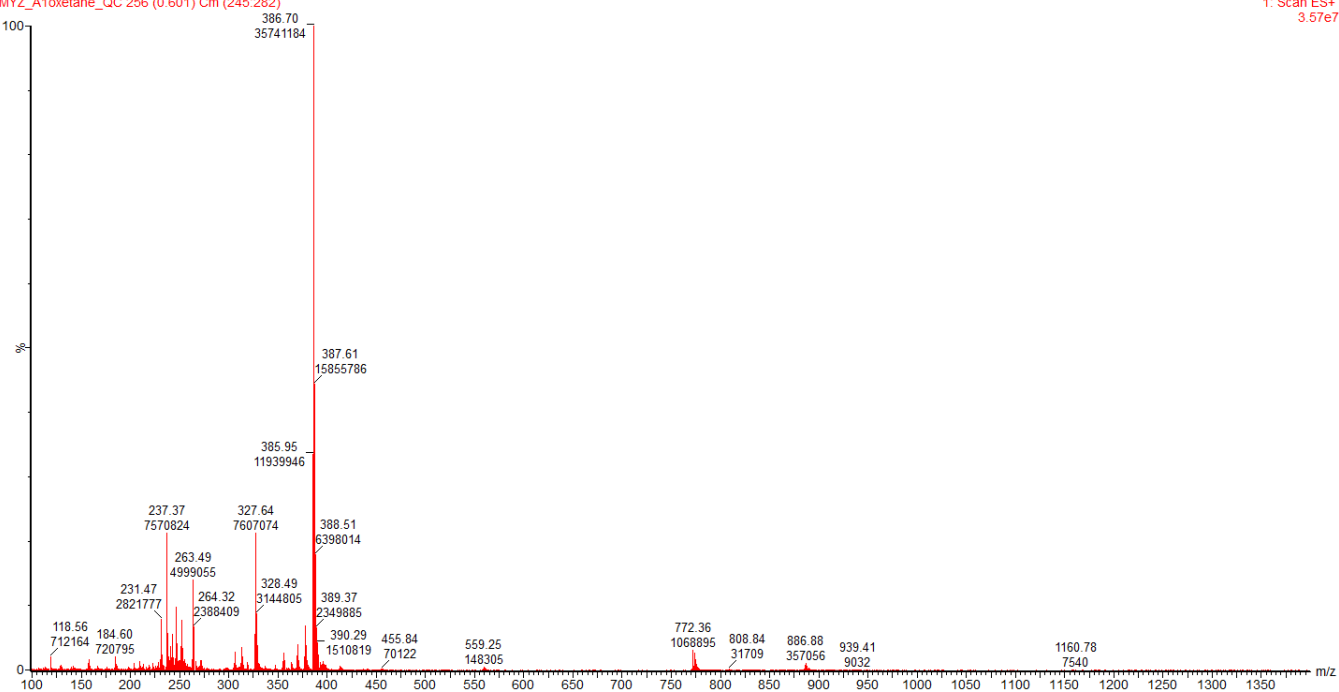


## LCMS

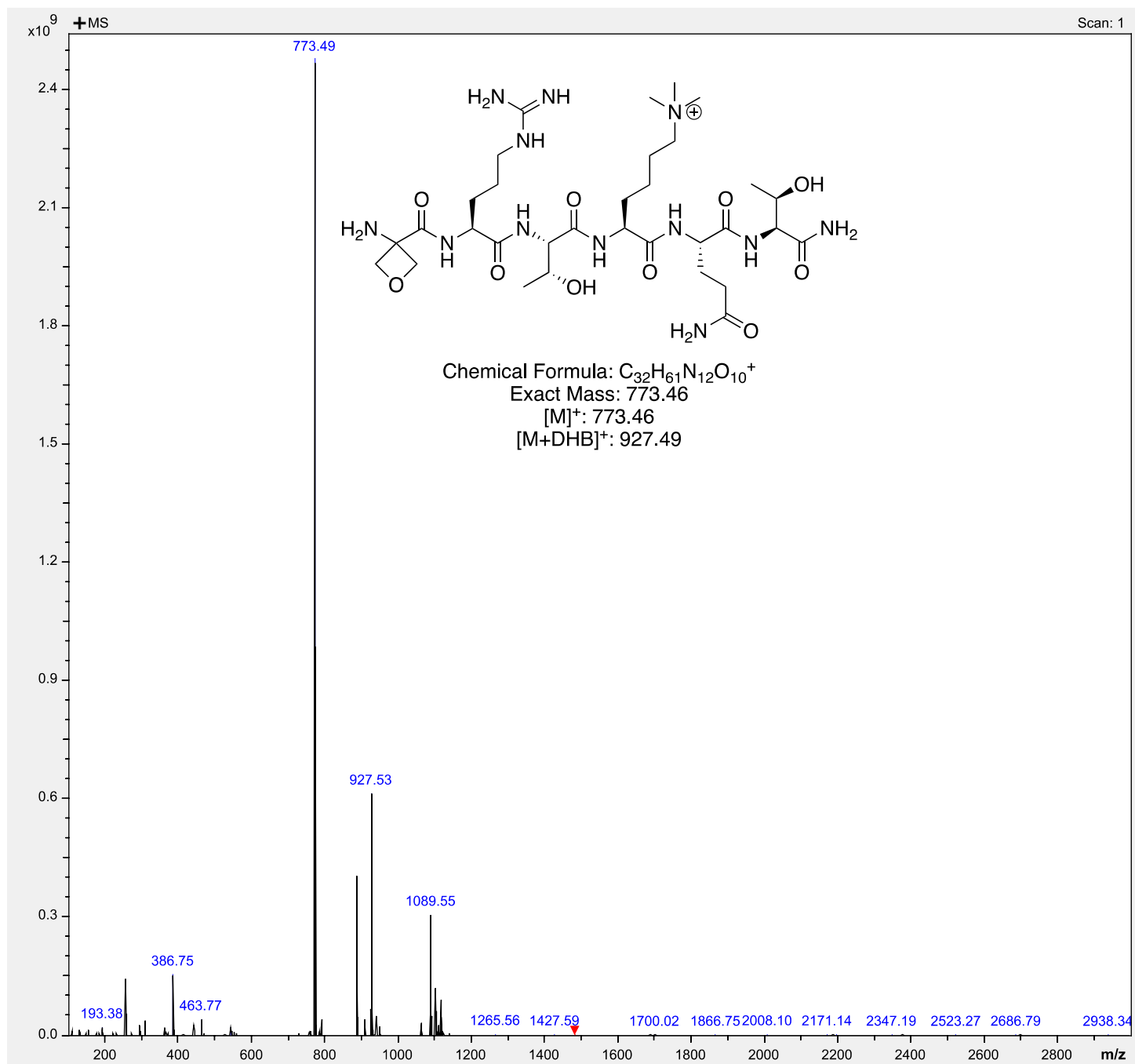
11/10/21

MYZ\_A1oxetane\_QC 256 (0.601) Cm (245.282)

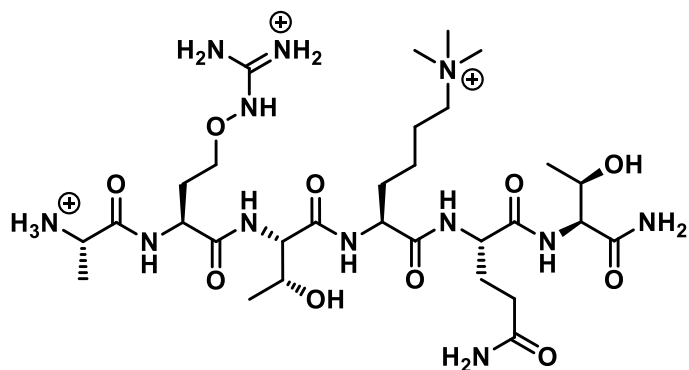
1: Scan ES+  
3.57e7



## MALDI



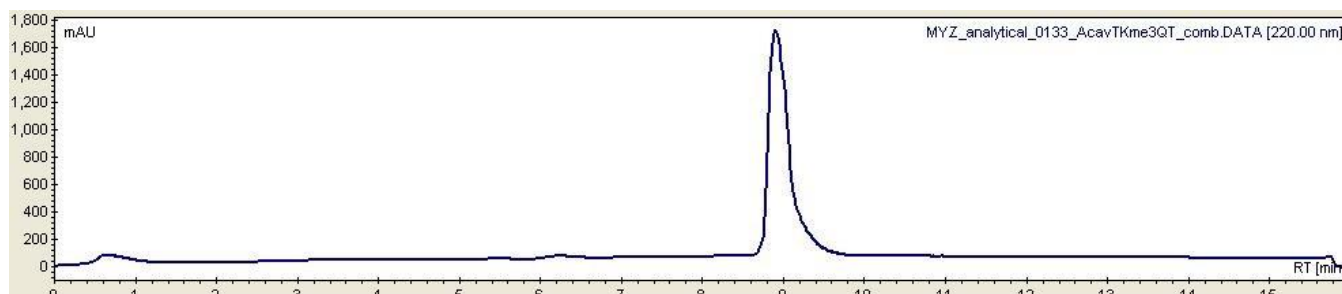
## Compound 8



Yield: 17%

Exact mass: 749.46  $[\text{C}_{30}\text{H}_{61}\text{N}_{12}\text{O}_{10}]^{3+}$

HPLC trace (Method 1):

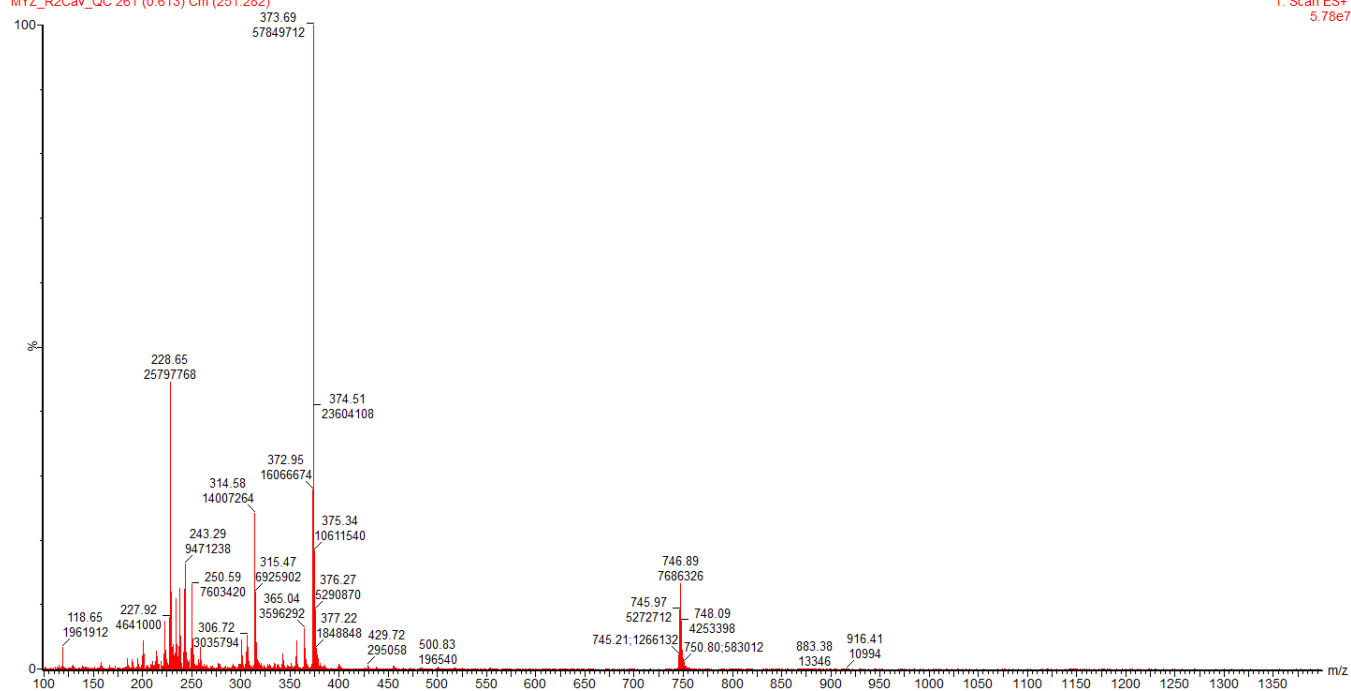


## LCMS

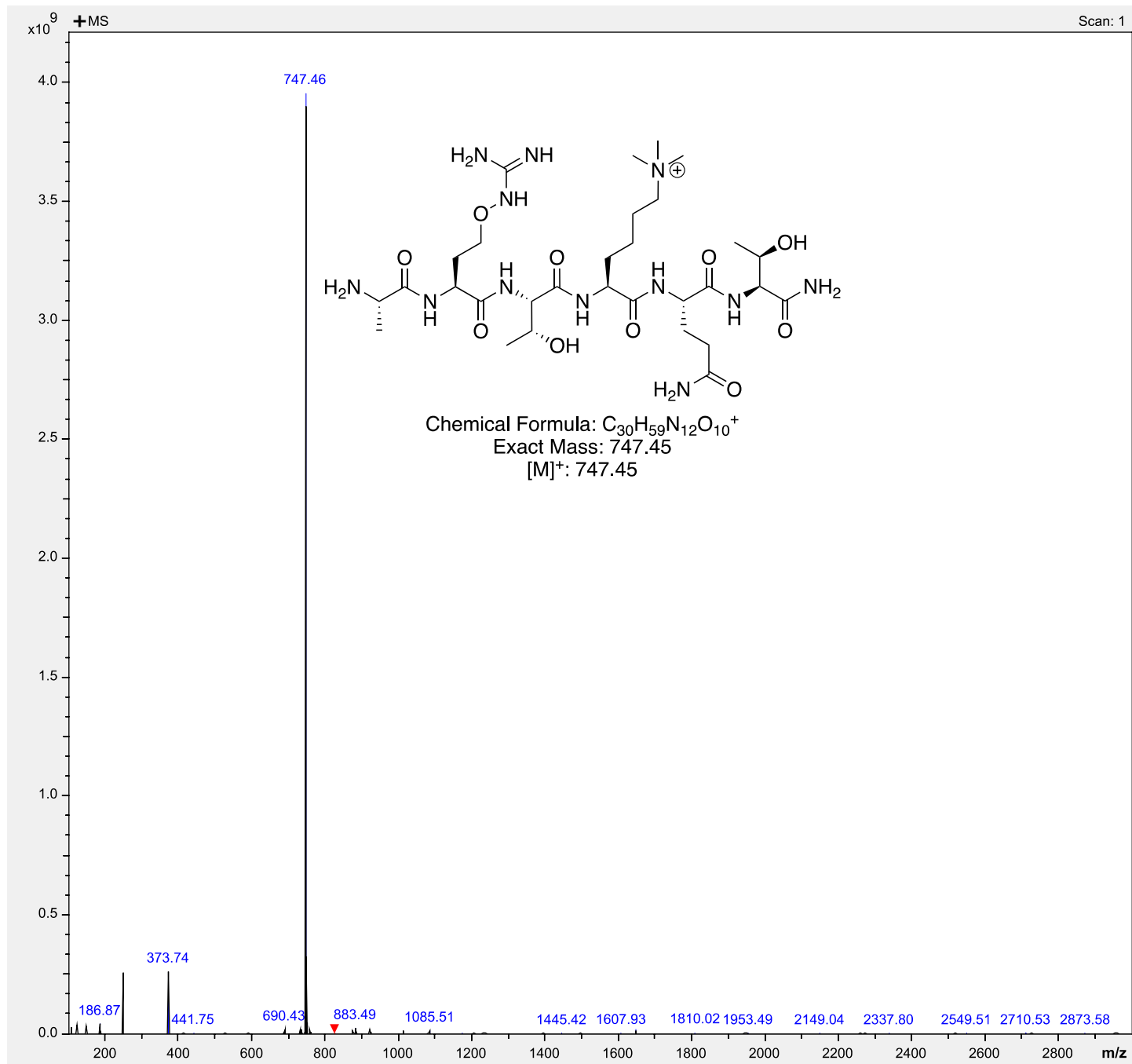
11/10/21

MYZ\_R2Cav\_QC 261 (0.613) Cm (251:282)

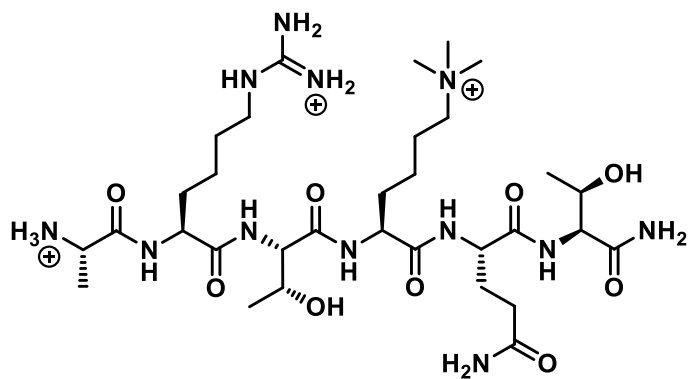
1: Scan ES+  
5.78e7



# MALDI



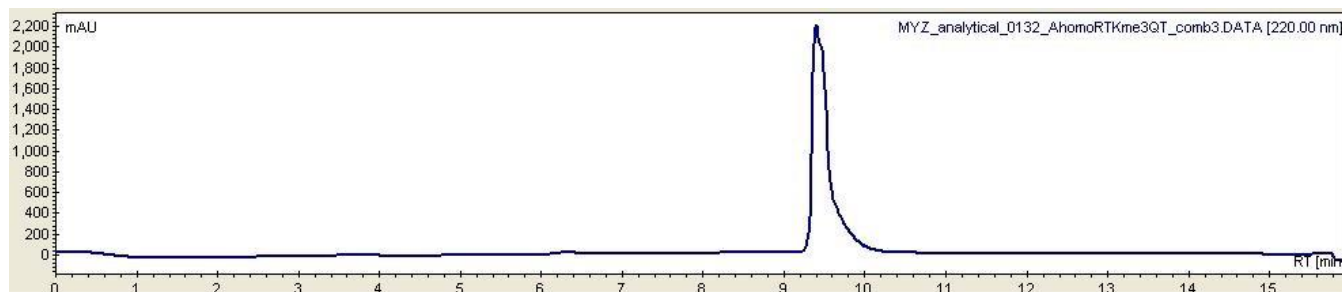
## Compound 9



Yield: 12%

Exact mass: 761.50 [C<sub>32</sub>H<sub>65</sub>N<sub>12</sub>O<sub>9</sub>]<sup>3+</sup>

HPLC trace (Method 1):



## LCMS

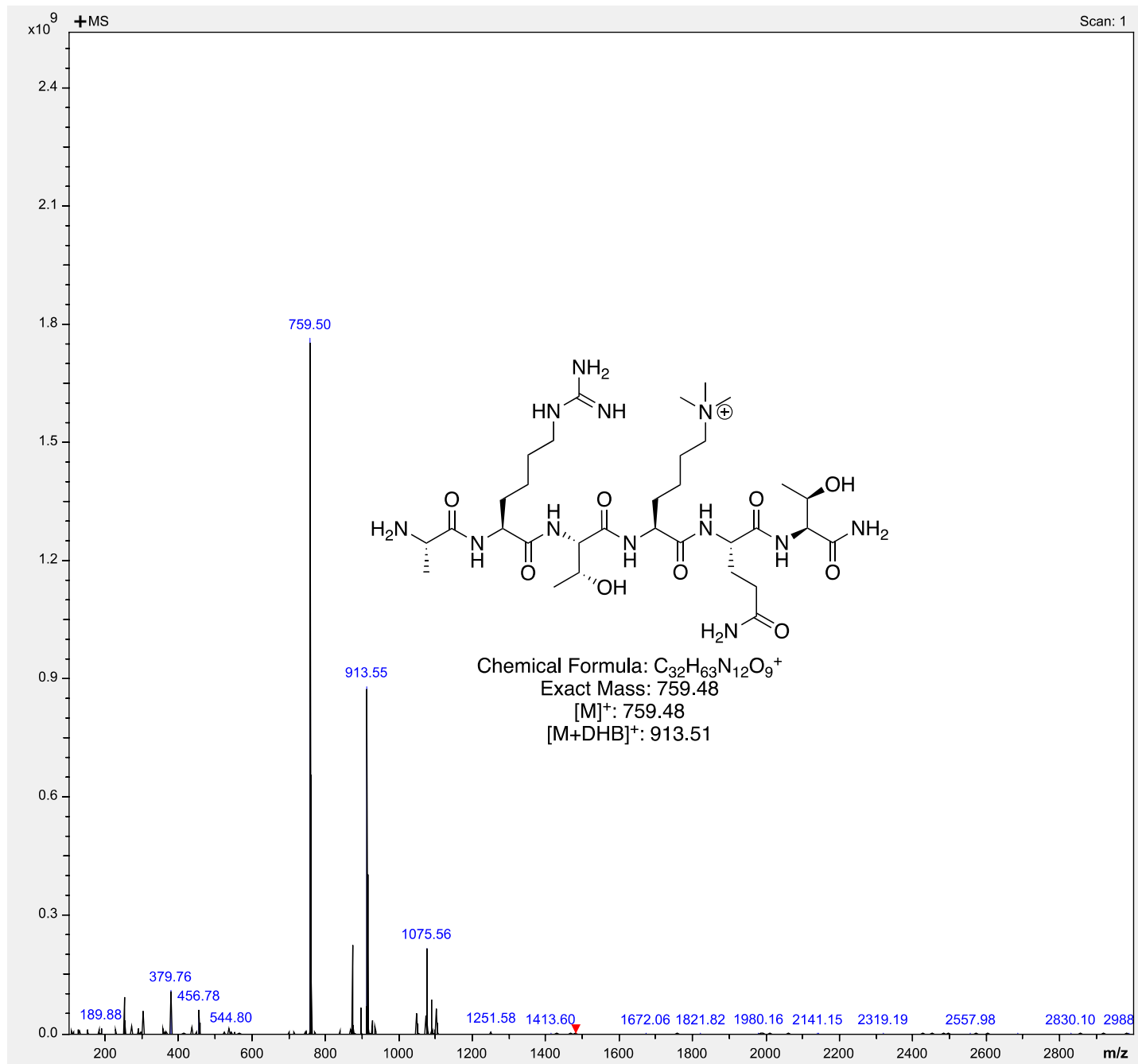
11/10/21

MYZ\_R2hR\_QC 257 (0.604) Cm (256.276)

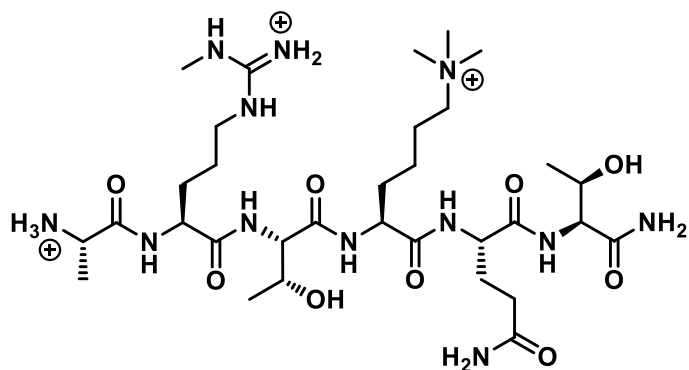
1: Scan ES+  
7.10e7



# MALDI



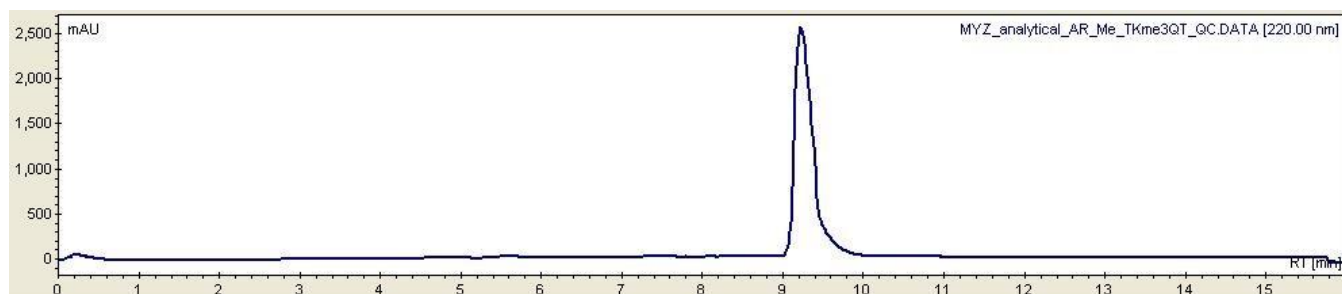
## Compound 10



Yield: 22%

Exact mass: 761.50 [C<sub>32</sub>H<sub>65</sub>N<sub>12</sub>O<sub>9</sub>]<sup>3+</sup>

HPLC trace (Method 1):

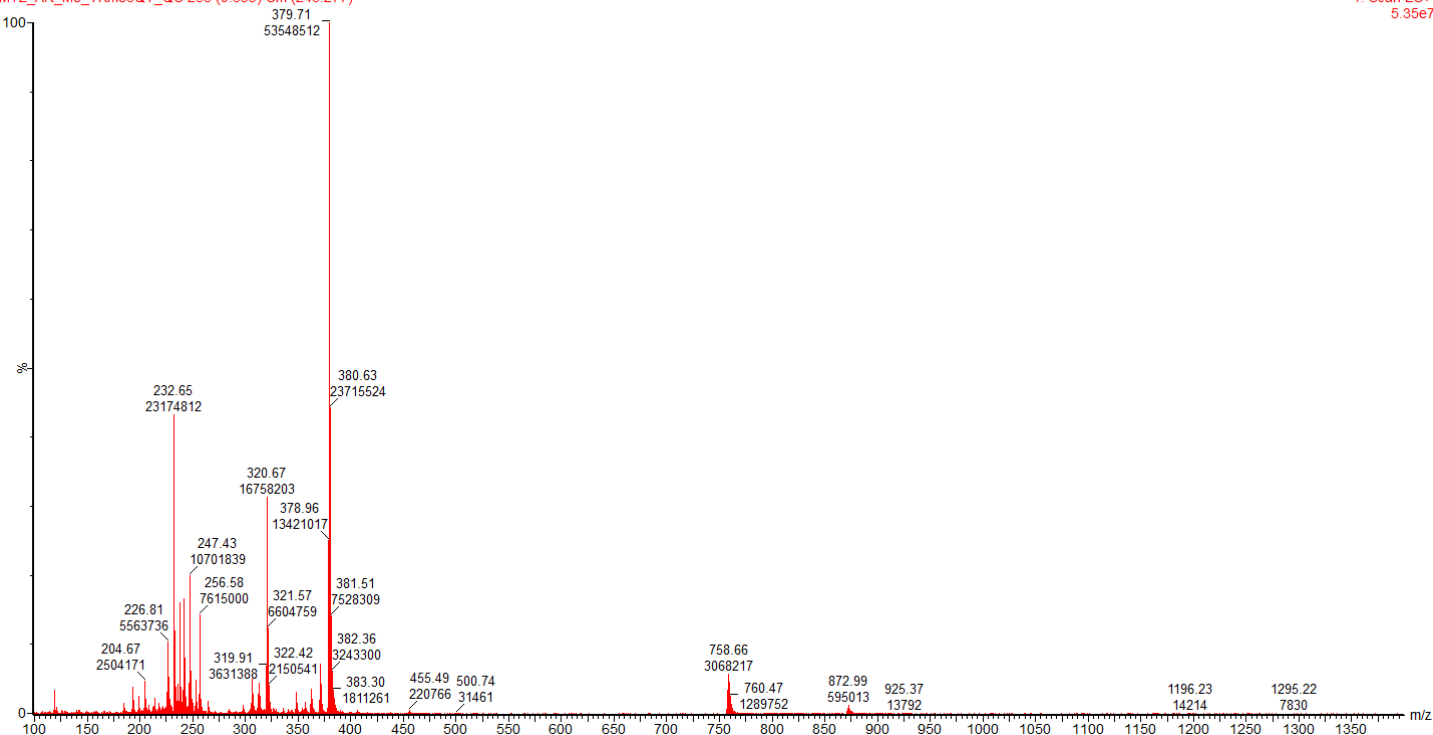


## LCMS

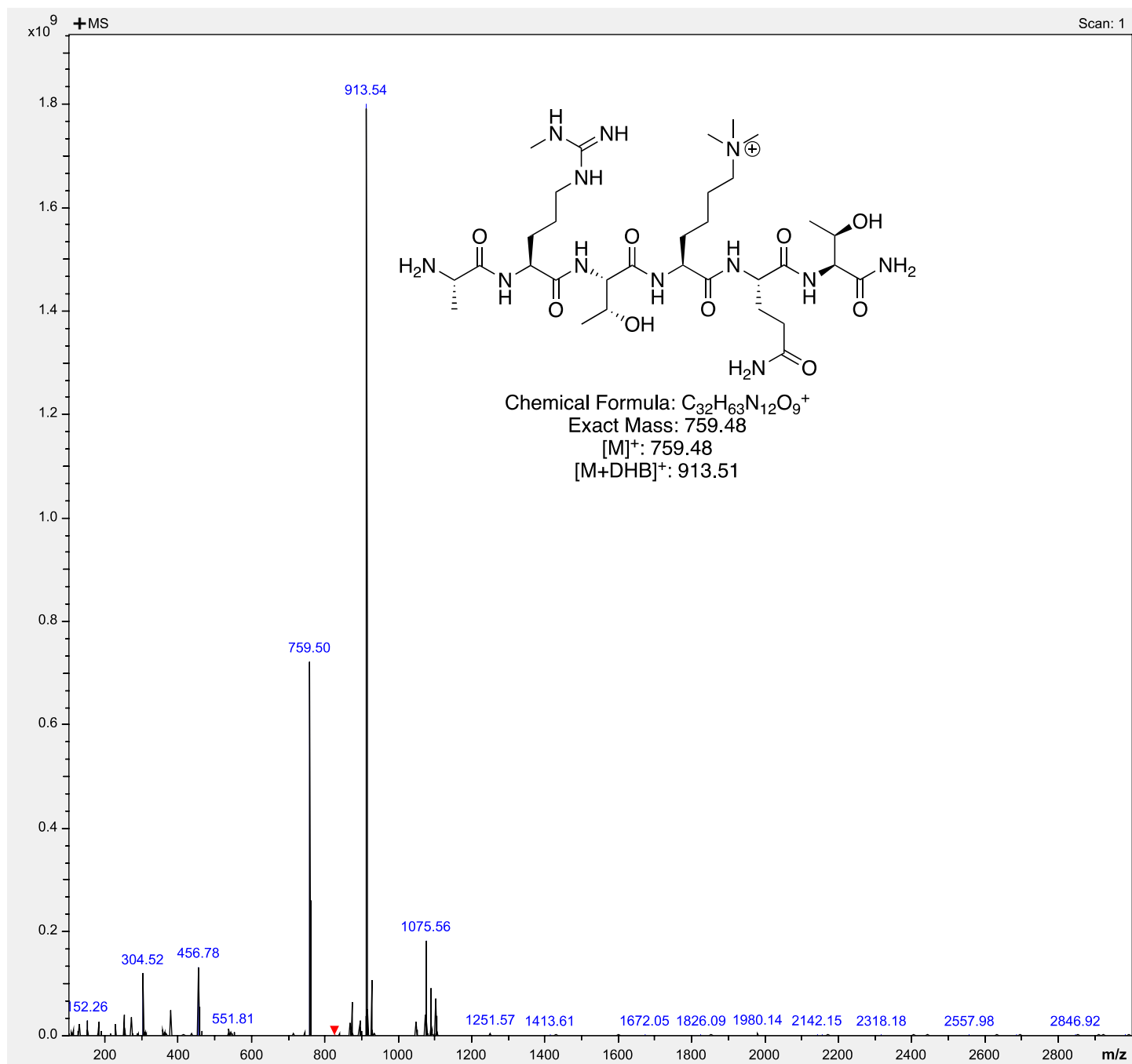
11/10/21

MYZ\_AR\_Me\_TKme3QT\_QC 255 (0.599) Cm (243:277)

1: Scan ES+  
5.35e7

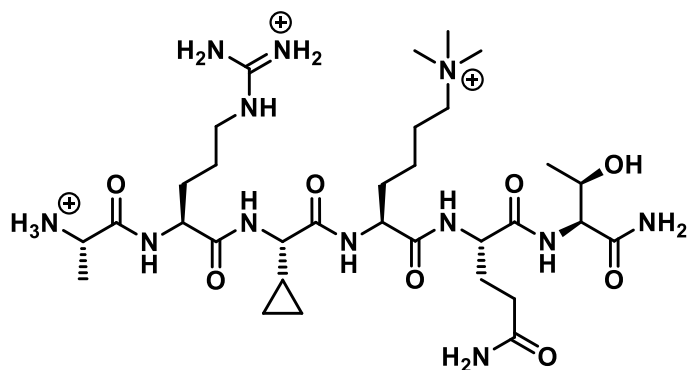


## MALDI





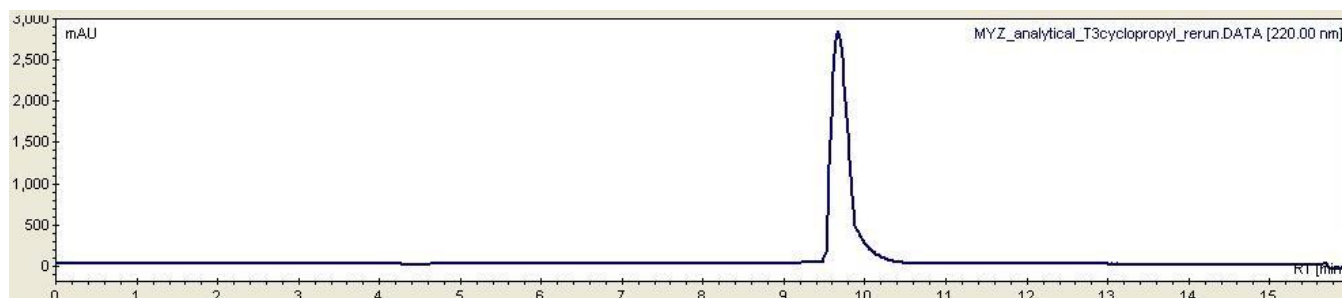
# Compound 11



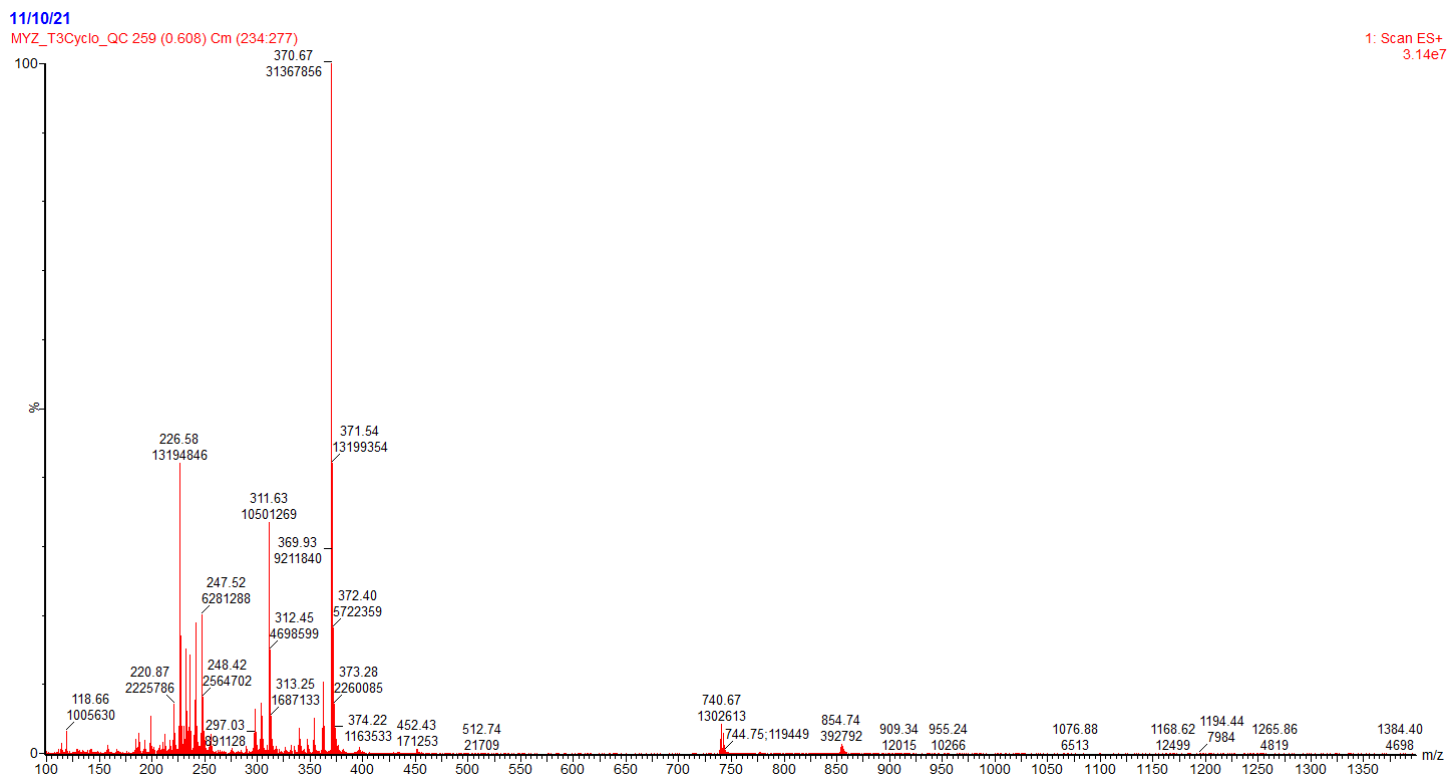
Yield: 6%

Exact mass: 743.39 [C<sub>32</sub>H<sub>63</sub>N<sub>12</sub>O<sub>8</sub>]<sup>3+</sup>

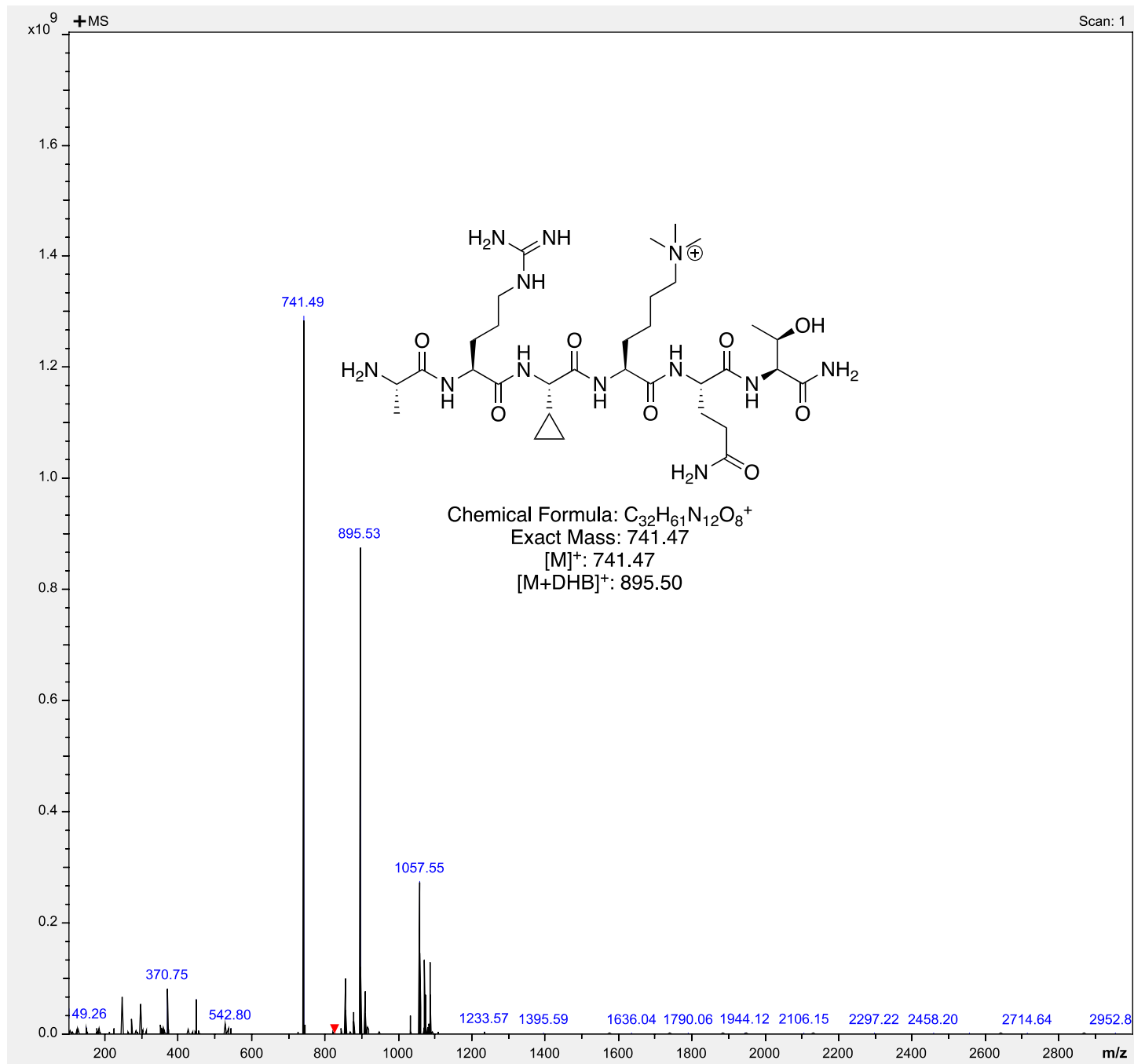
HPLC trace (Method 2):



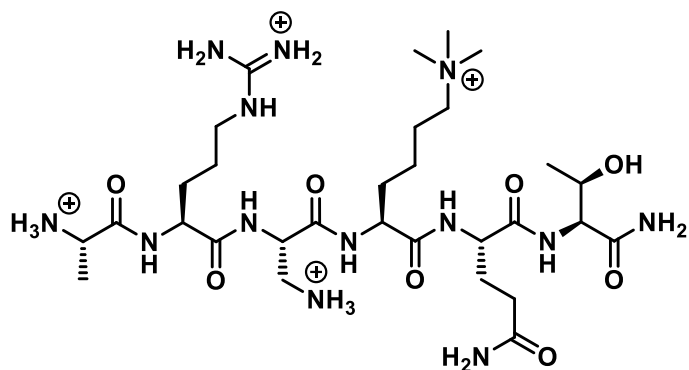
## LCMS



# MALDI



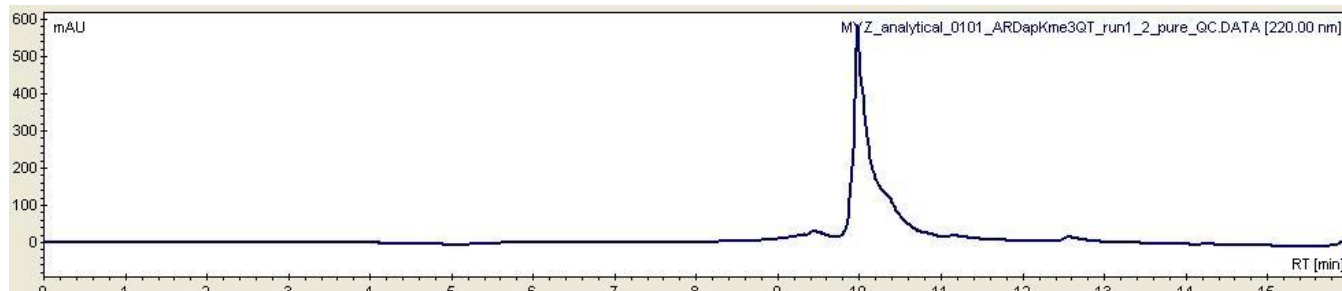
## Compound 12



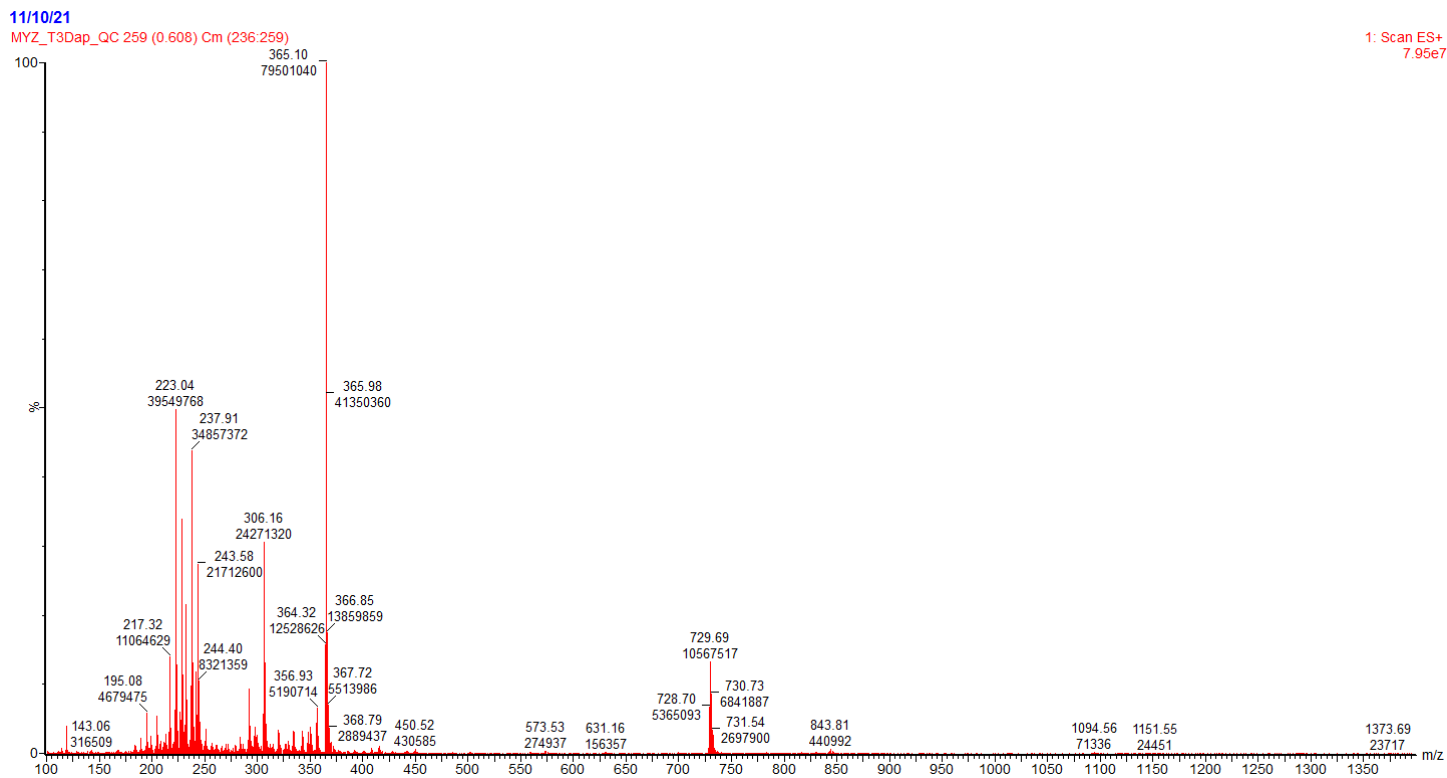
Yield: 15%

Exact mass: 733.49  $[\text{C}_{30}\text{H}_{63}\text{N}_{13}\text{O}_8]^{4+}$

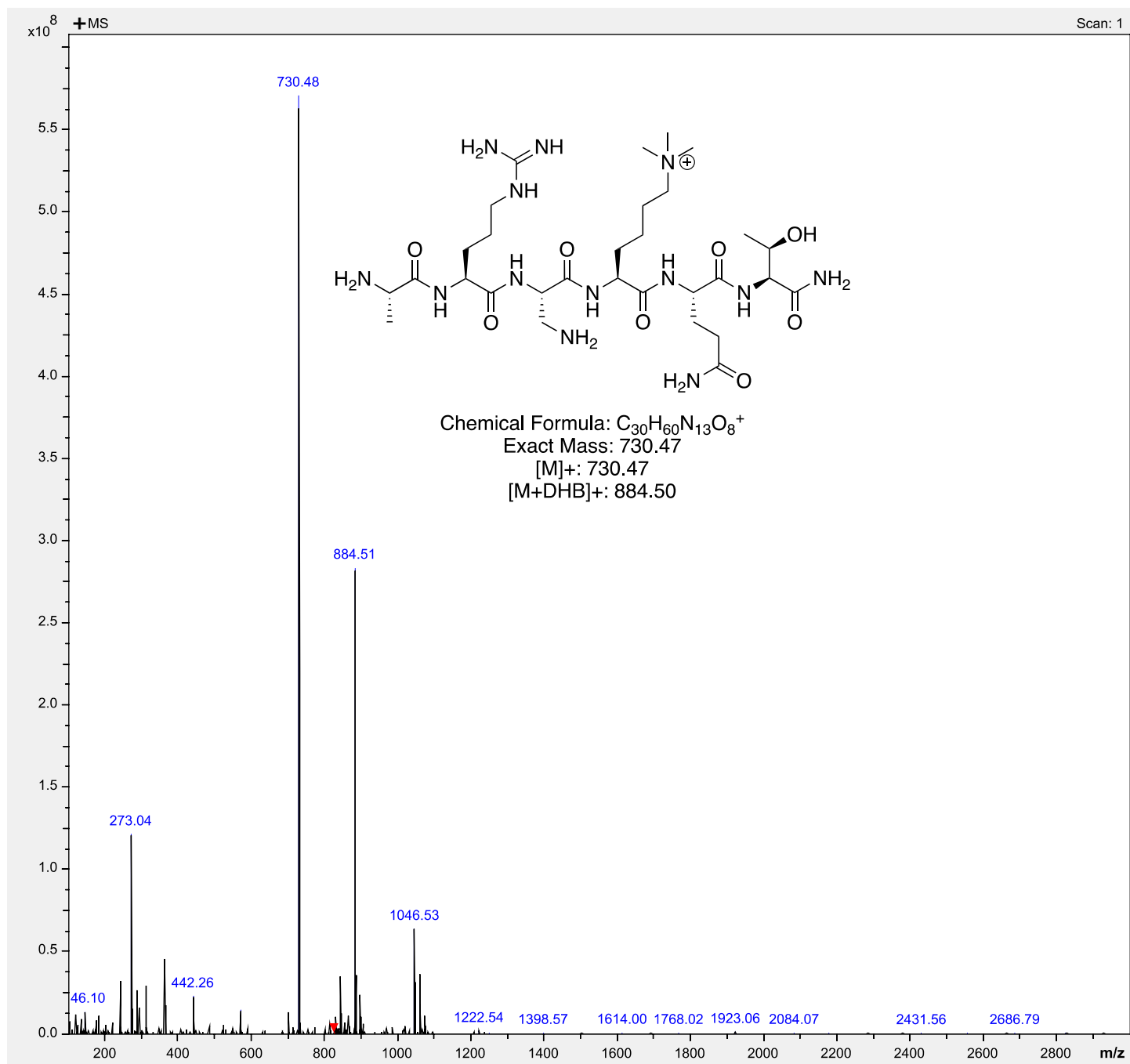
HPLC trace (Method 1):



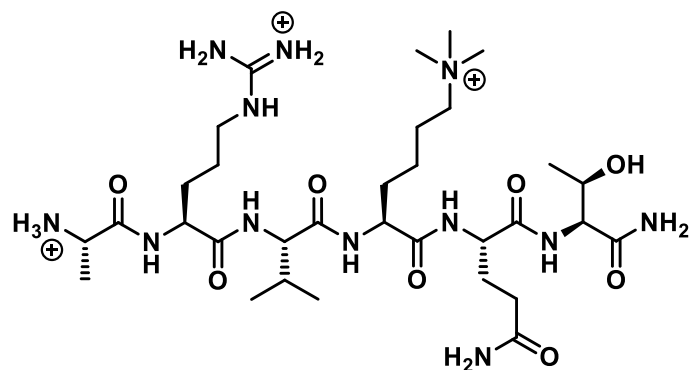
## LCMS



## MALDI



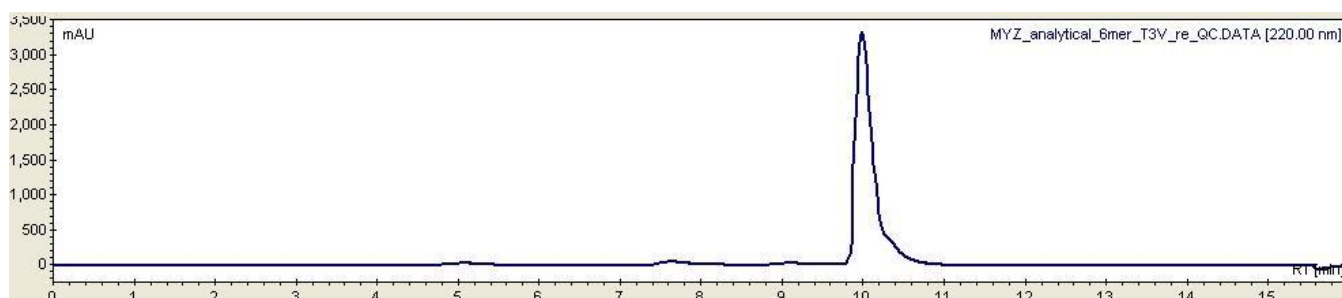
## Compound 13



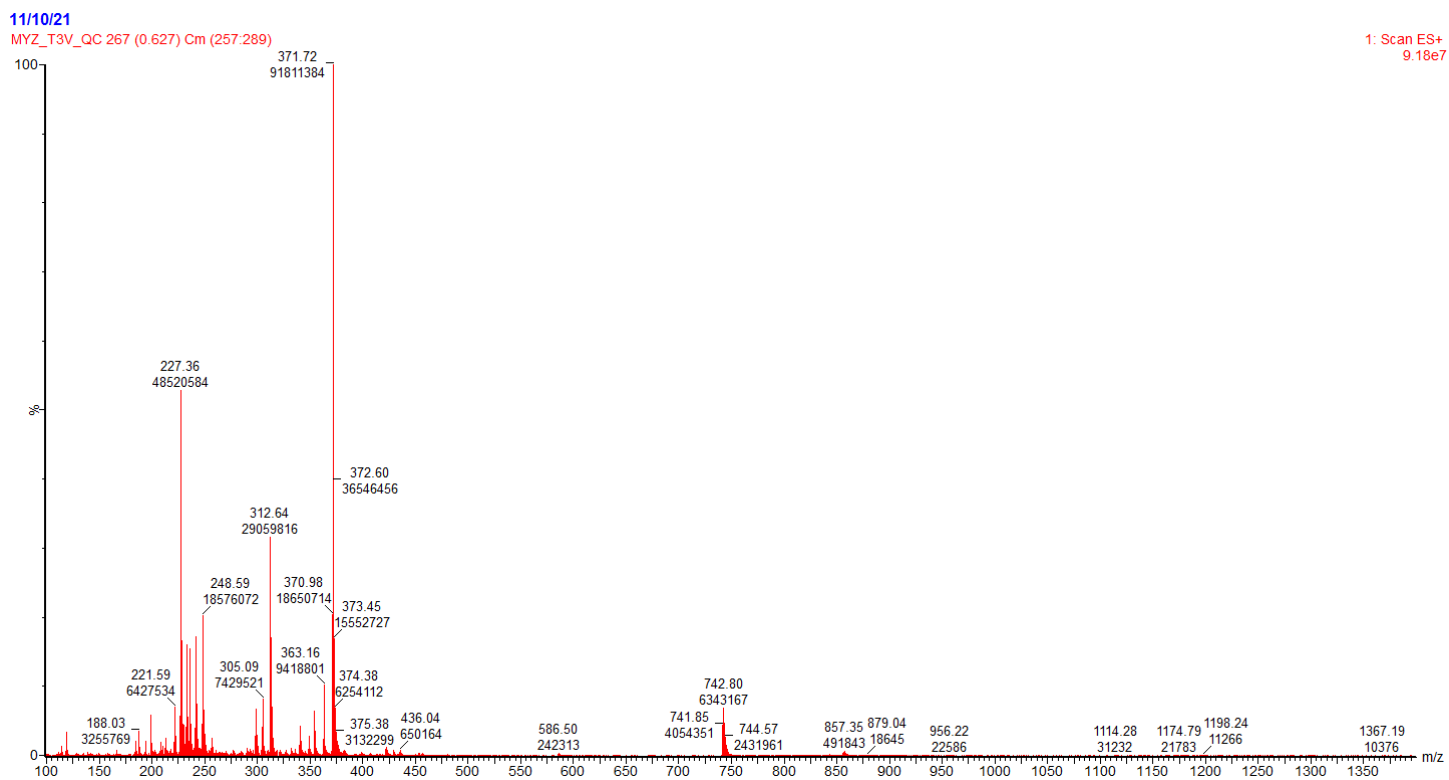
Yield: 17%

Exact mass: 745.50 [C<sub>32</sub>H<sub>65</sub>N<sub>12</sub>O<sub>8</sub>]<sup>3+</sup>

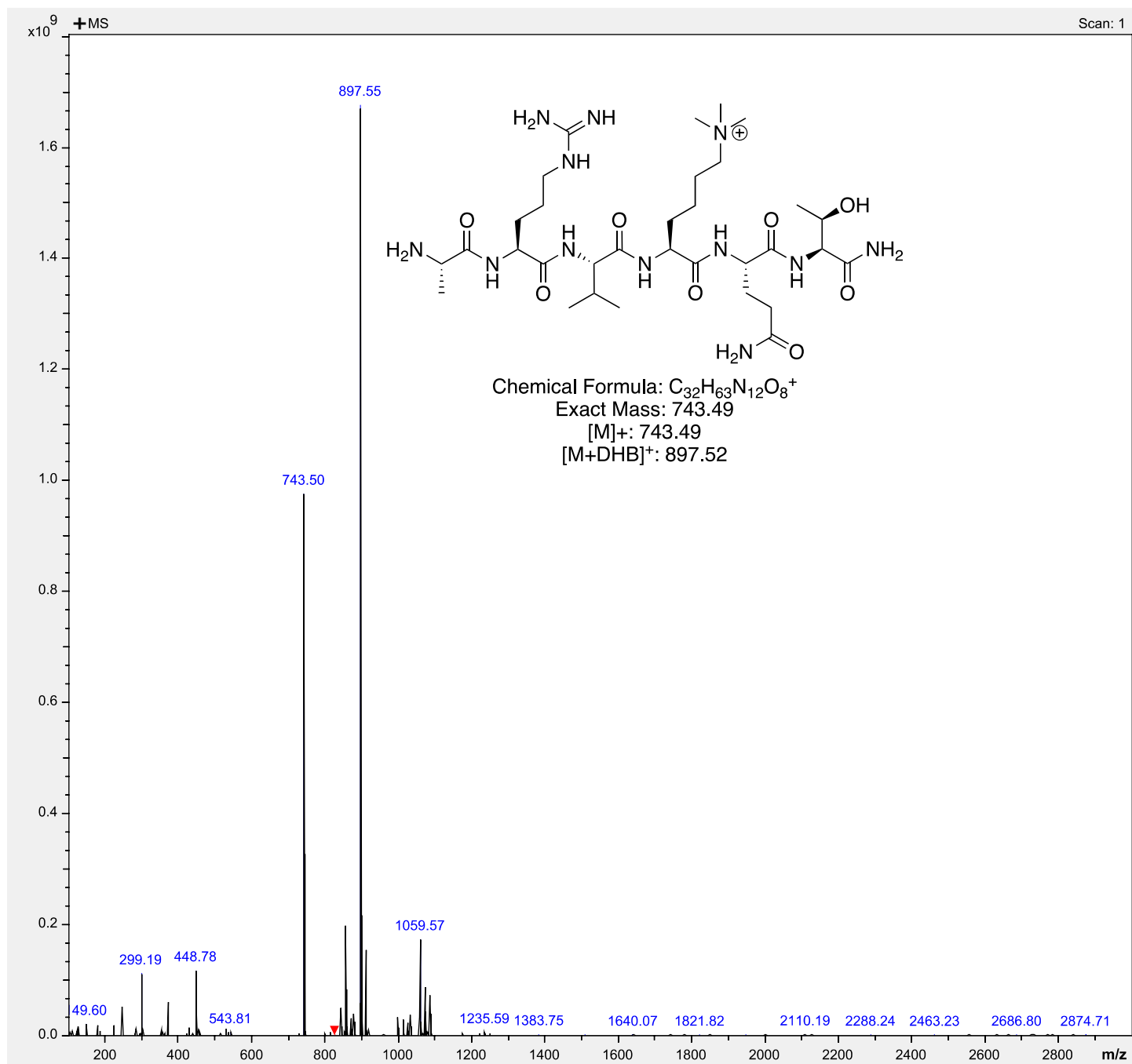
HPLC trace (Method 1):



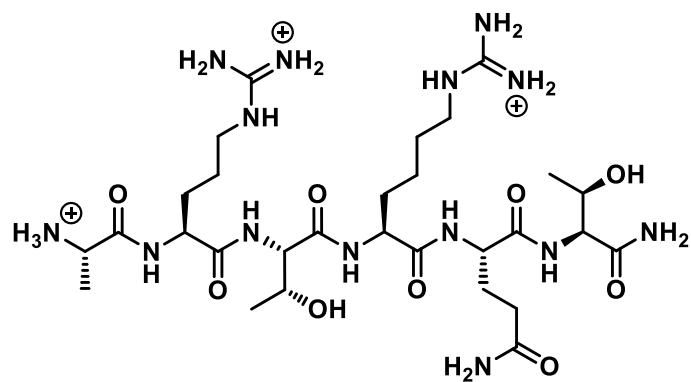
## LCMS



# MALDI



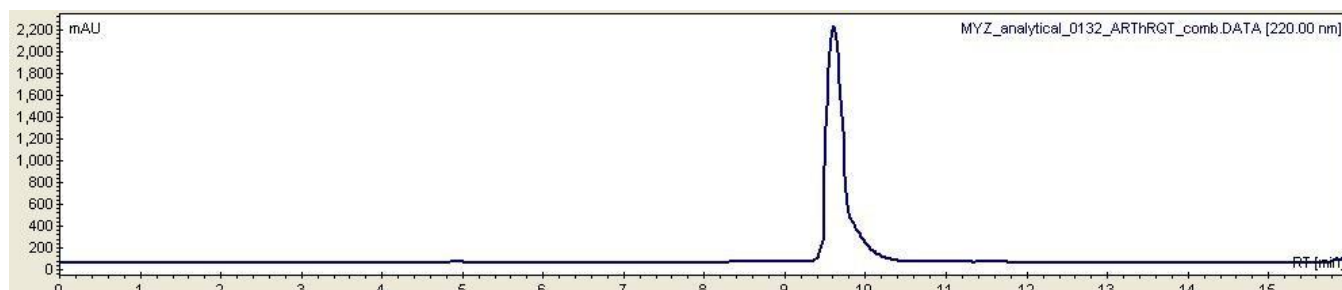
# Compound 14



Yield: 26%

Exact mass: 747.46  $[\text{C}_{29}\text{H}_{59}\text{N}_{14}\text{O}_9]^{3+}$

HPLC trace (Method 1):

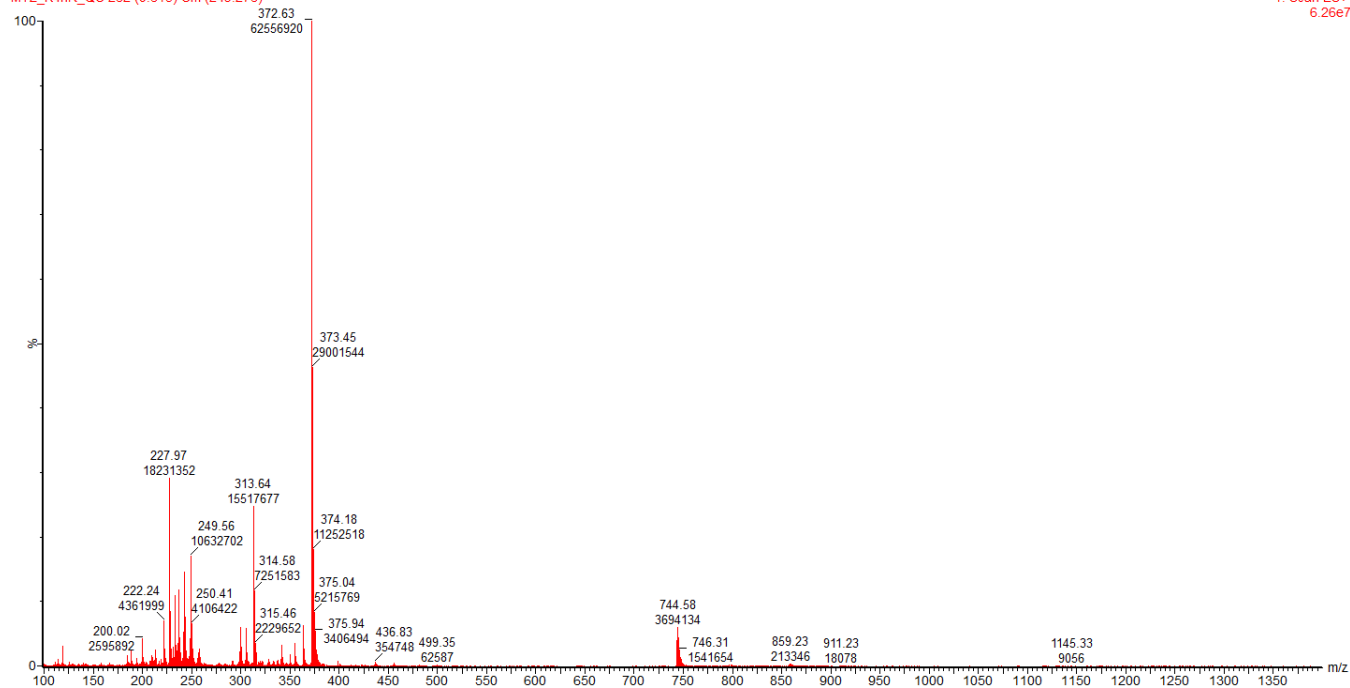


## LCMS

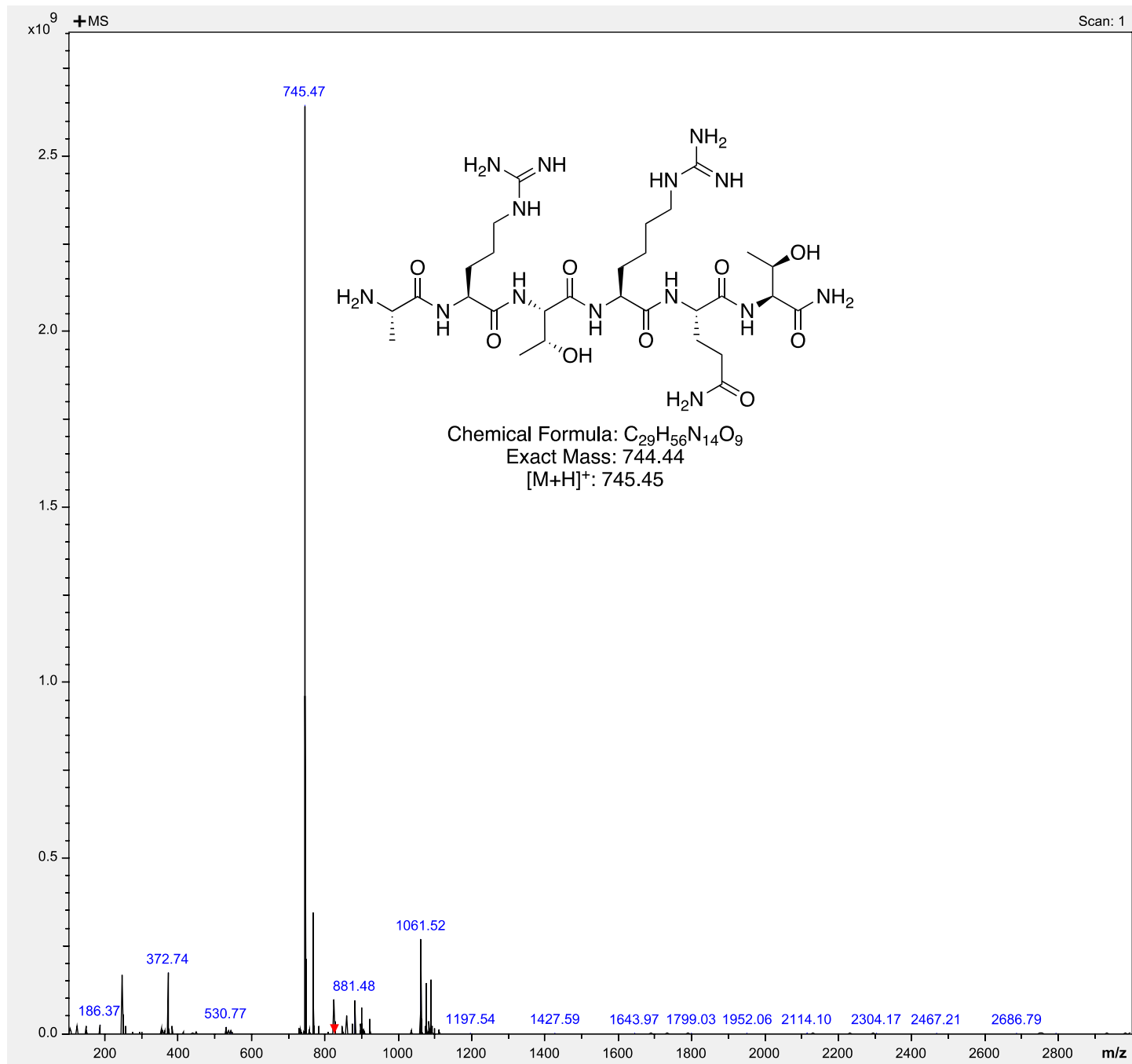
11/10/21

MYZ\_K4hR\_QC 262 (0.615) Cm (249:276)

1: Scan ES+  
6.26e7

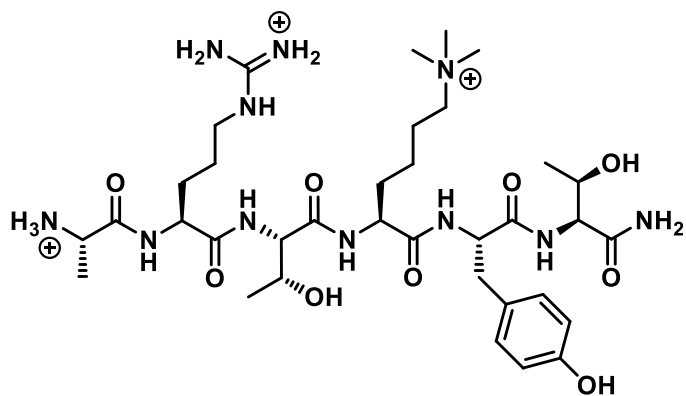


# MALDI





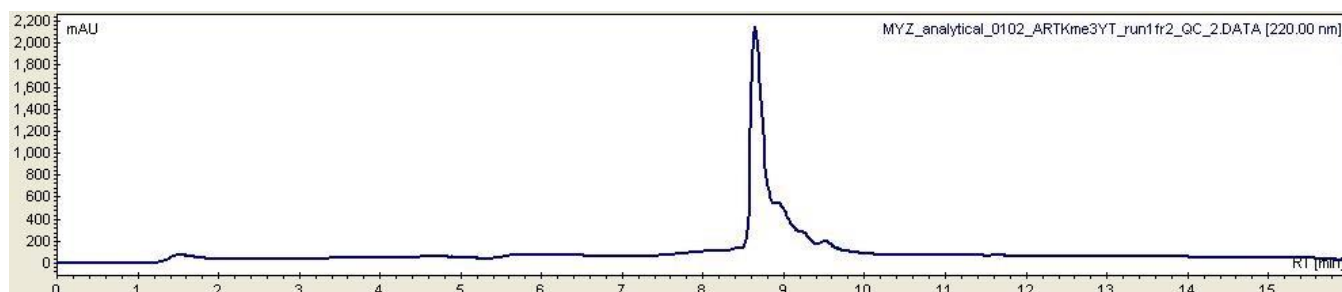
# Compound 15



Yield: 8%

Exact mass: 782.49 [C<sub>35</sub>H<sub>64</sub>N<sub>11</sub>O<sub>9</sub>]<sup>3+</sup>

HPLC trace (Method 1):

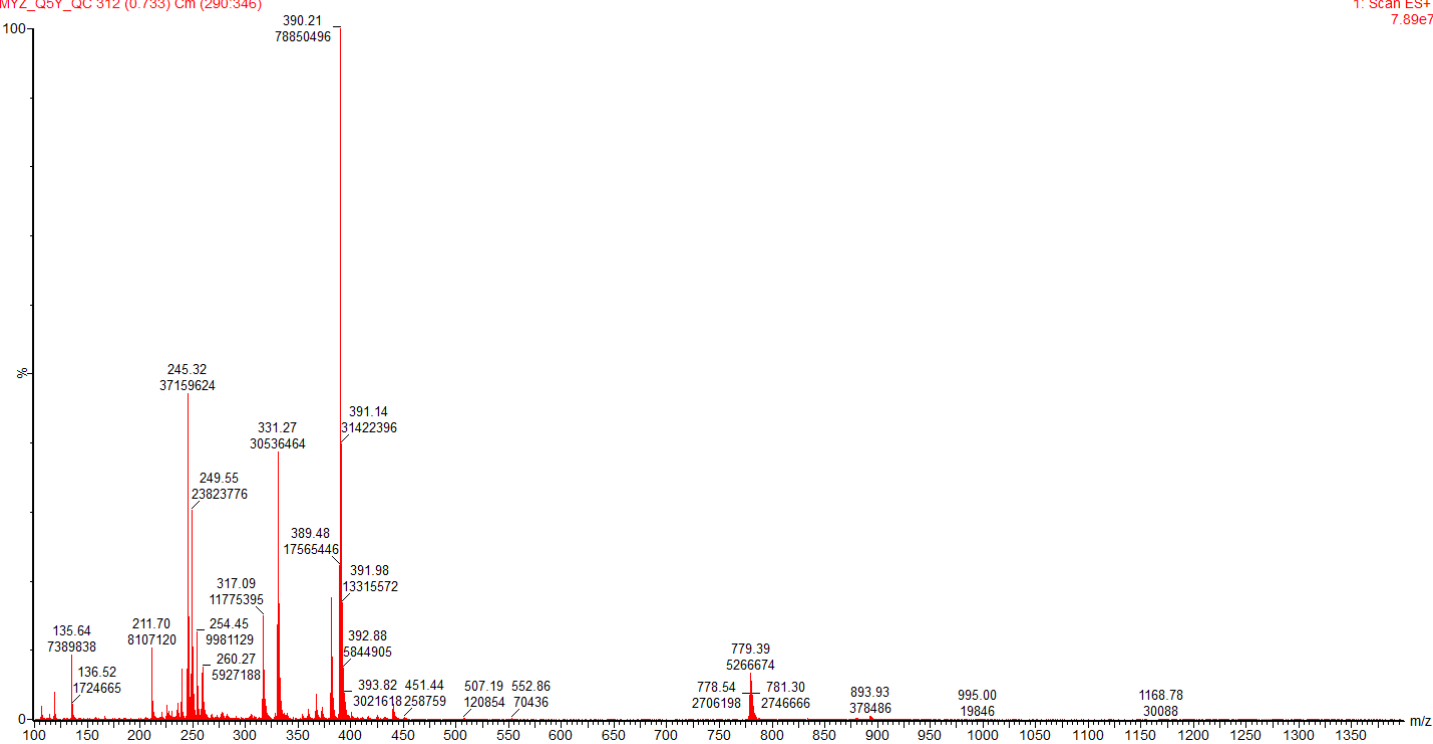


## LCMS

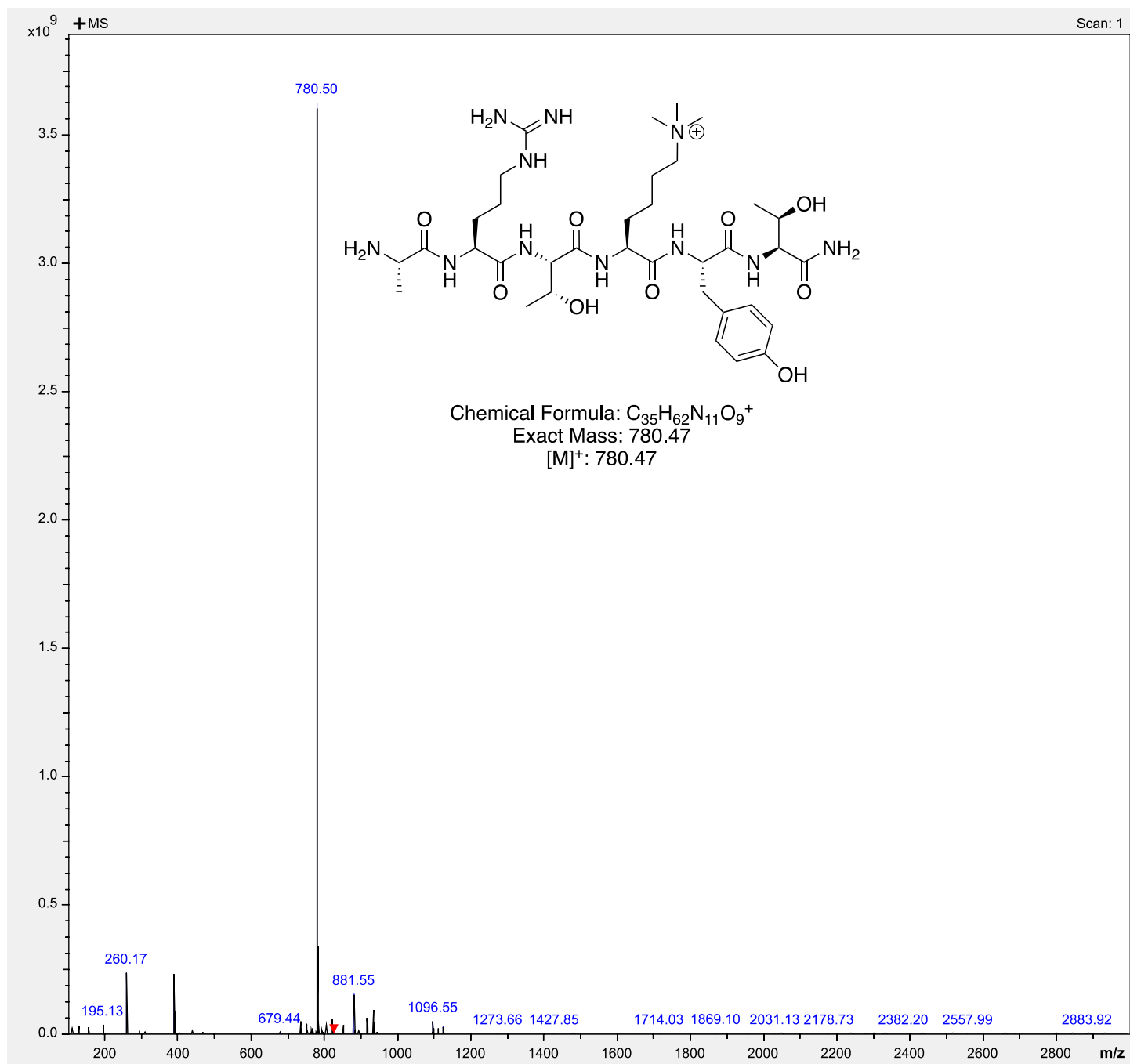
11/10/21

MYZ\_Q5Y\_QC 312 (0.733) Cm (290:346)

1: Scan ES+  
7.89e7

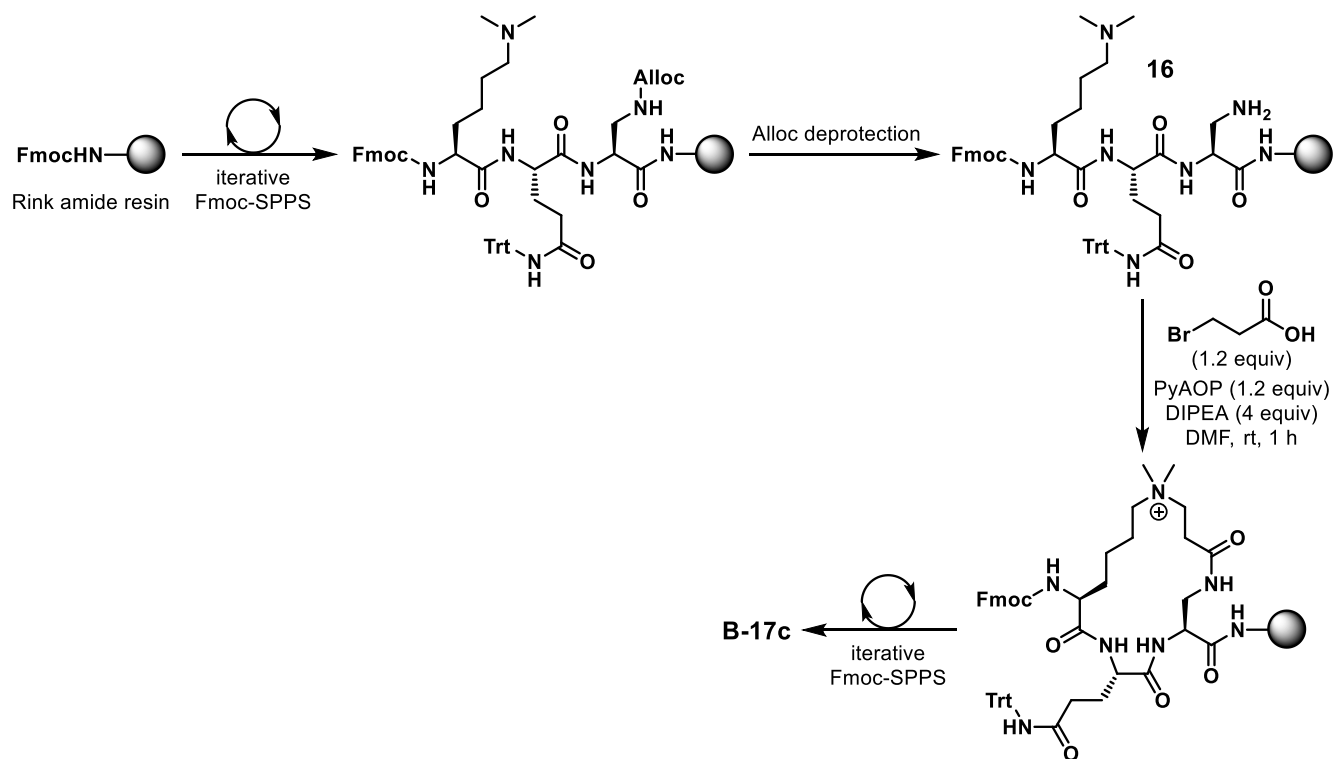


# MALDI



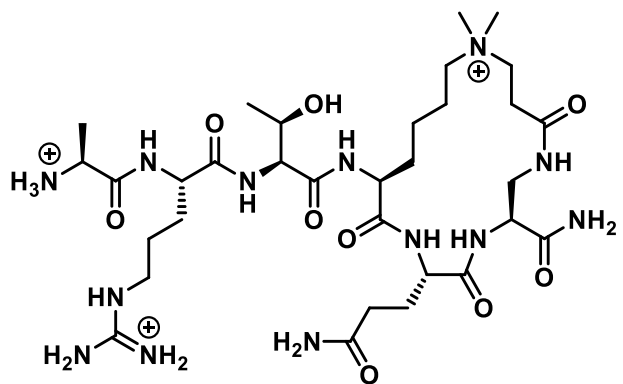
## Macrocyclization Synthetic Procedures

### Series B: macrolactamization



Tripeptide Fmoc-Lys(me<sub>2</sub>)-Gln(Trt)-Dap(Alloc)-CONH-Rink amide resin (1 equiv) was prepared on Rink amide resin according to the procedures outlined in the **Iterative Fmoc-SPPS workflow**. The Alloc group was deprotected (see **Deprotection procedures**) and the resin-bound tripeptide intermediate **16** was treated with a solution of 3-bromopropionic acid (1.2 equiv), PyAOP (1.2 equiv) and DIPEA (2.4 equiv) in DMF and agitated at room temperature for one hour, at which time consumption of starting material was observed by LCMS analysis. The resin-bound peptide was subsequently elongated following the **Iterative Fmoc-SPPS workflow** to afford lactam **B-17c**.

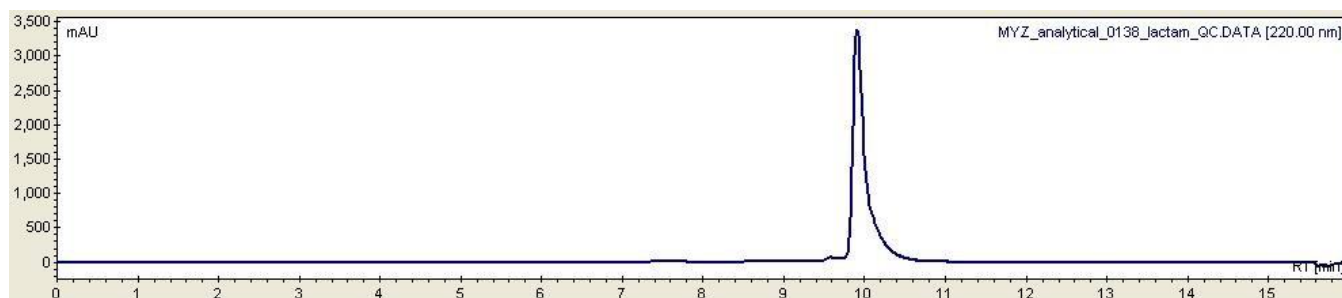
## Compound B-17c



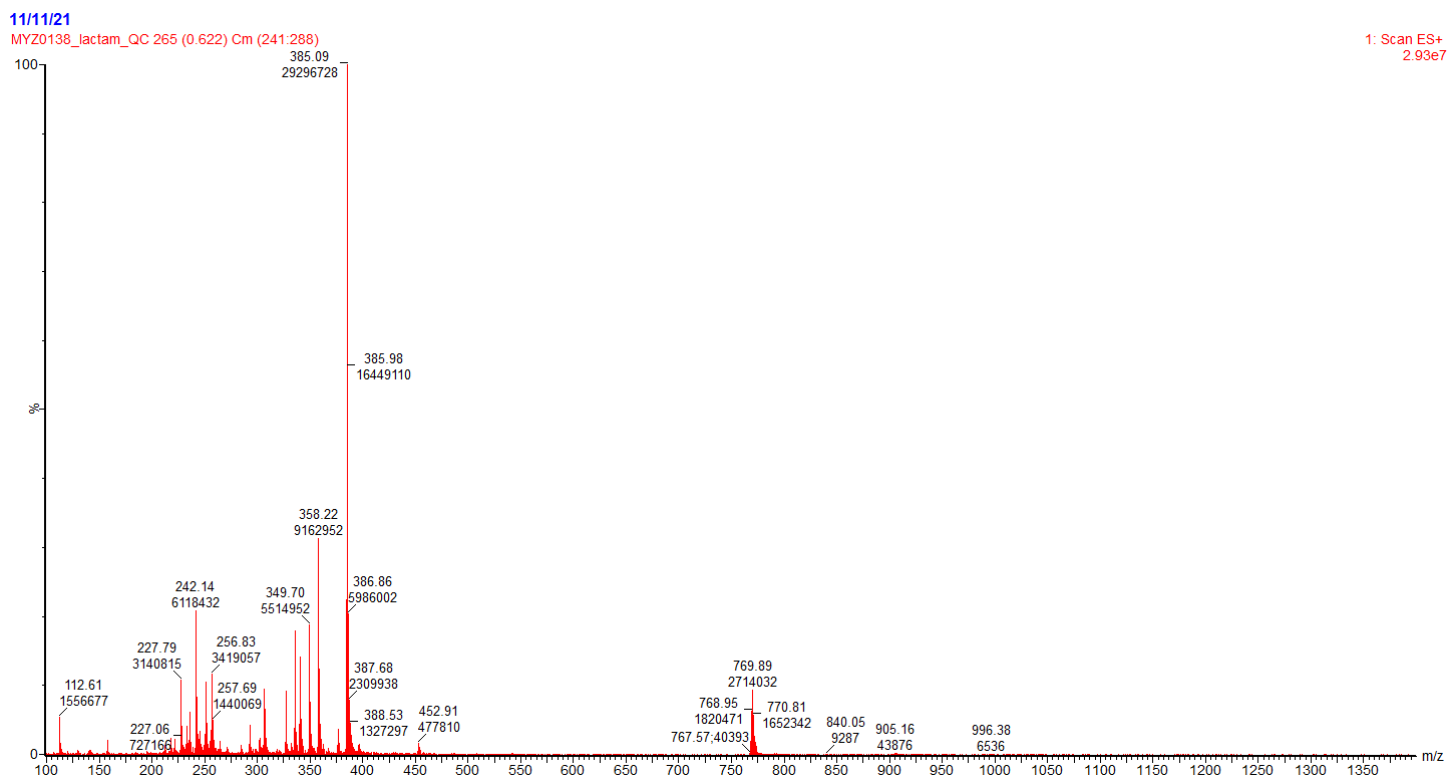
Yield: 8%

Exact mass: 772.48 [C<sub>32</sub>H<sub>62</sub>N<sub>13</sub>O<sub>9</sub>]<sup>3+</sup>

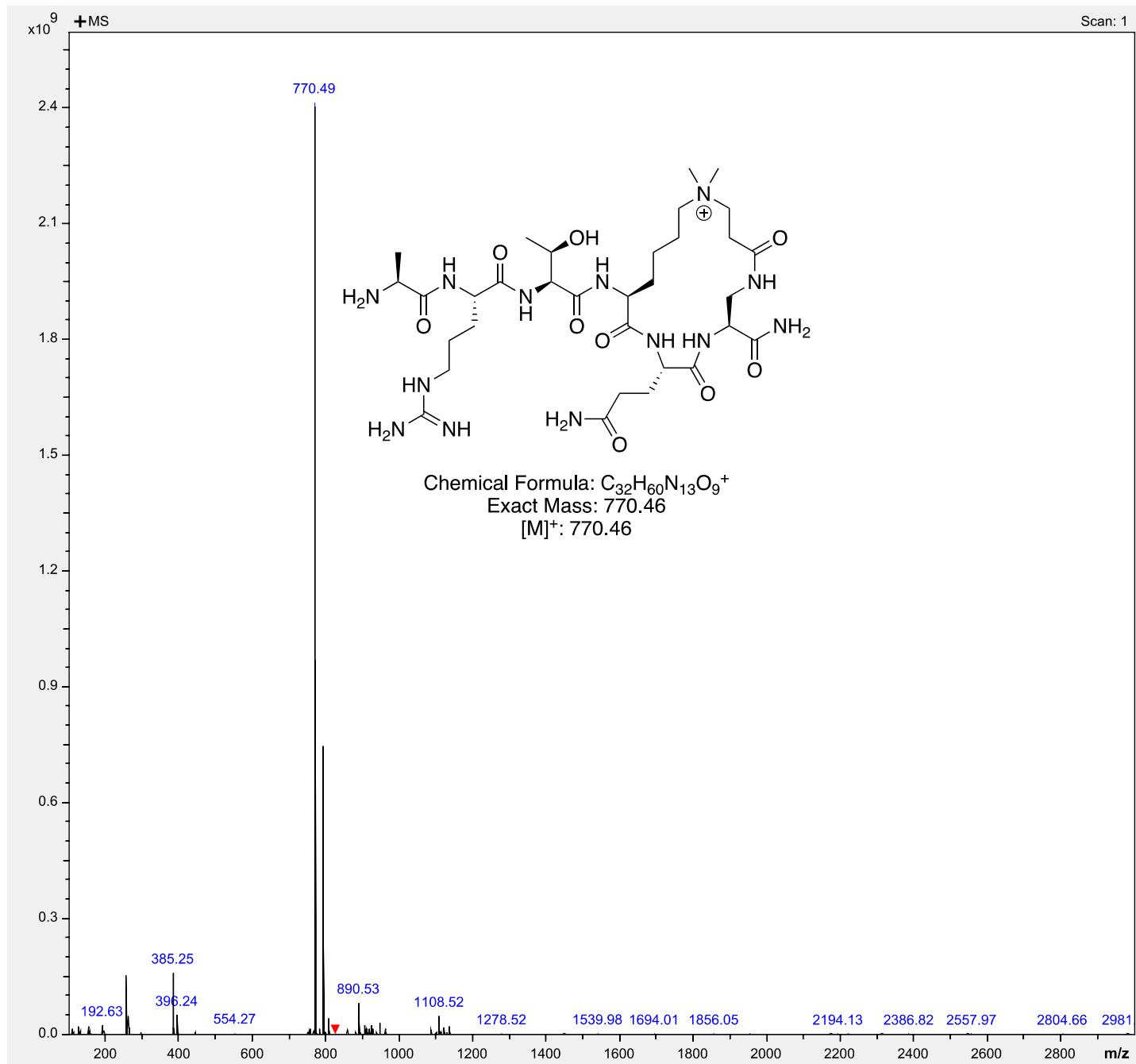
HPLC trace (Method 1):



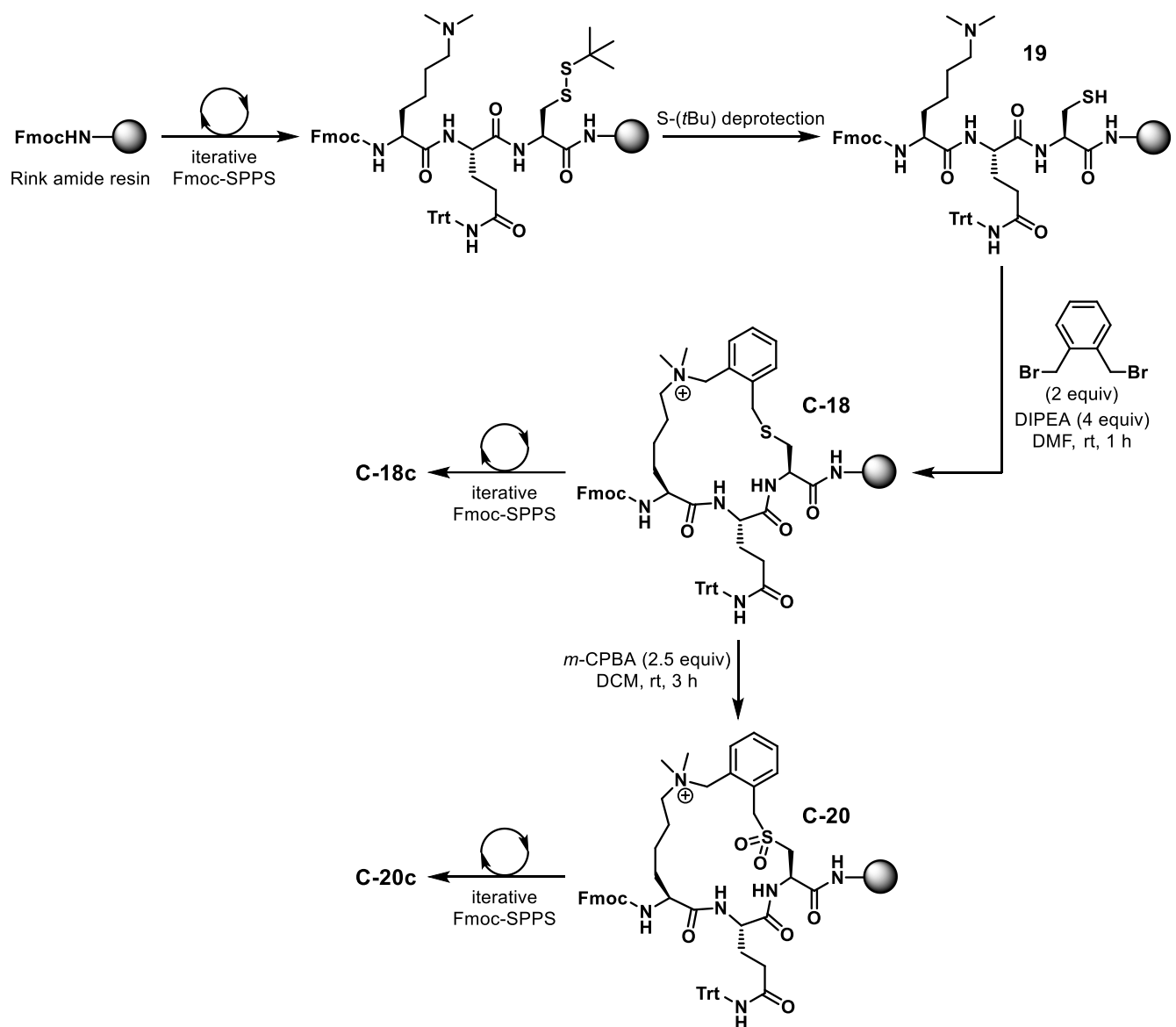
## LCMS



# MALDI



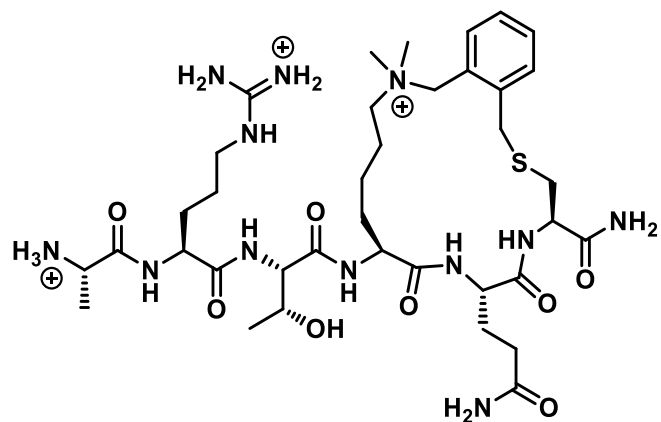
## Series C: thioether macrocyclization



Tripeptide Fmoc-Lys(Me<sub>2</sub>)-Gln(Trt)-Cys(S-tBu)-CONH-rink amide resin (1 equiv) was prepared on Rink amide resin according to the procedures outlined in the **Iterative Fmoc-SPPS workflow**. The resin was washed with DMF (5 × 3 mL) and the S-(tBu) group was deprotected (see **Deprotection procedures**). The resin-bound tripeptide intermediate **19** was washed with DMF (5 × 3 mL) and treated with a solution of DIPEA (4 equiv) in DMF, followed by a solution of 1,2-bis(bromomethyl)benzene (2 equiv) in DMF. The resin was agitated at room temperature for one hour, whilst monitoring the reaction using LCMS analysis. Once the formation of **C-18** and consumption of starting material was confirmed, the resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL) and DMF (5 × 3 mL), and the peptide was elongated following the **Iterative Fmoc-SPPS workflow** to afford thioether cyclopeptide **C-18c**.

**Sulfone C-20c synthesis:** Following the completion of the macrocyclization reaction using 1,2-bis(bromomethyl)benzene and DIPEA in DMF, the resin-bound thioether macrocycle **C-18** was washed with DMF (5 × 3 mL), DCM (5 × 3 mL), DMF (5 × 3 mL) and DCM (5 × 3 mL). The resin was subsequently treated with a solution of *m*-CPBA (2.5 equiv) in DCM and agitated at room temperature for three hours. Once complete oxidation of **C-18** to **C-20** was confirmed by LCMS, the liquid was discarded and the resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL) and DMF (5 × 3 mL). The peptide was elongated following the **Iterative Fmoc-SPPS workflow** to afford sulfone cyclopeptide **C-20c**.

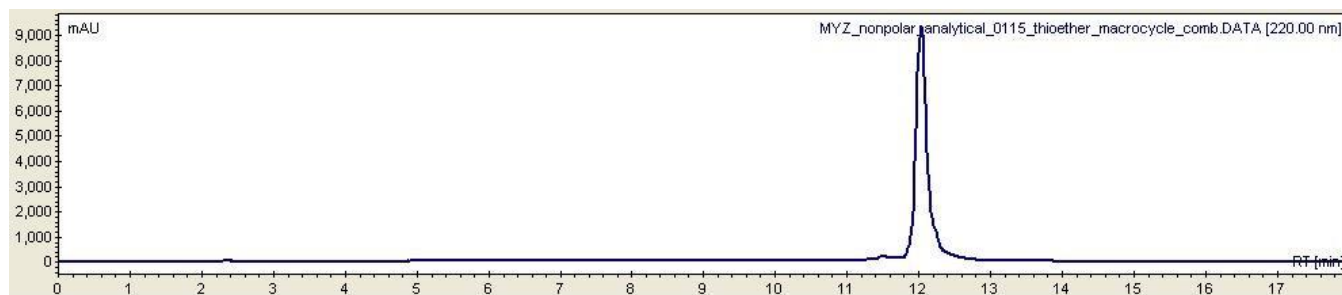
## Compound C-18c



Yield: 10%

Exact mass: 837.48 [C<sub>37</sub>H<sub>65</sub>N<sub>12</sub>O<sub>8</sub>S]<sup>3+</sup>

HPLC trace (Method 2):

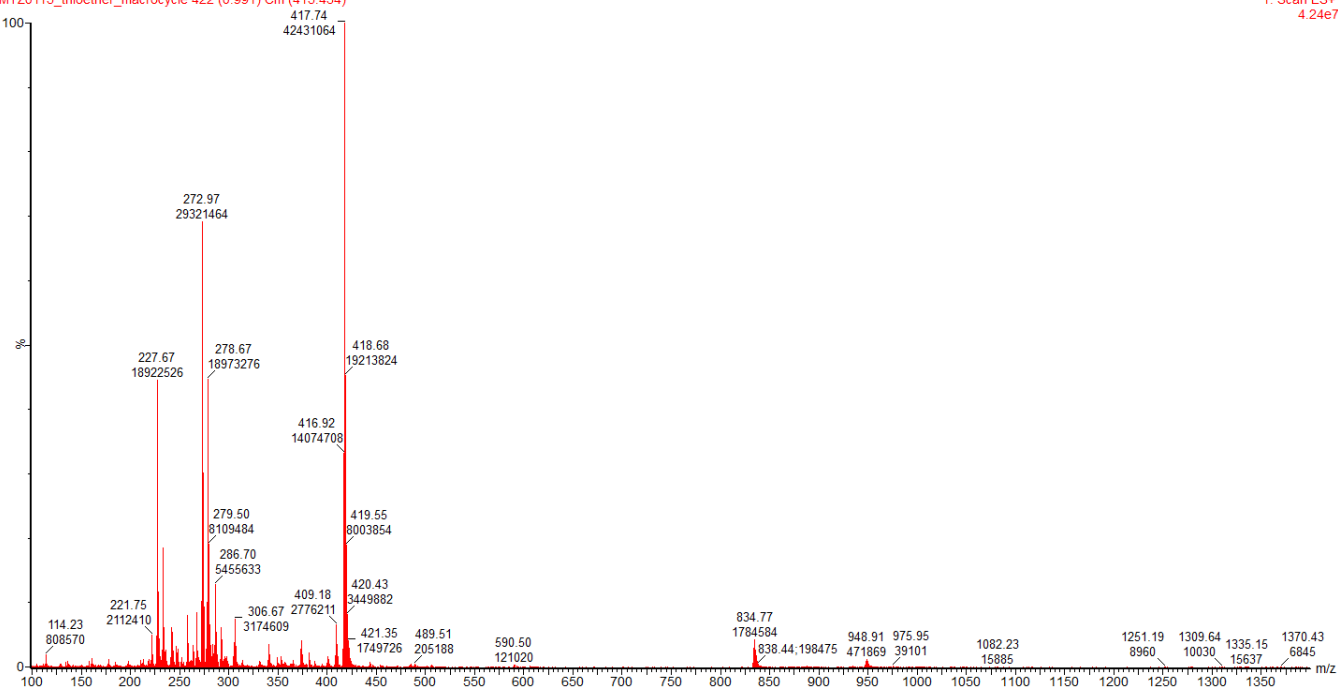


## LCMS

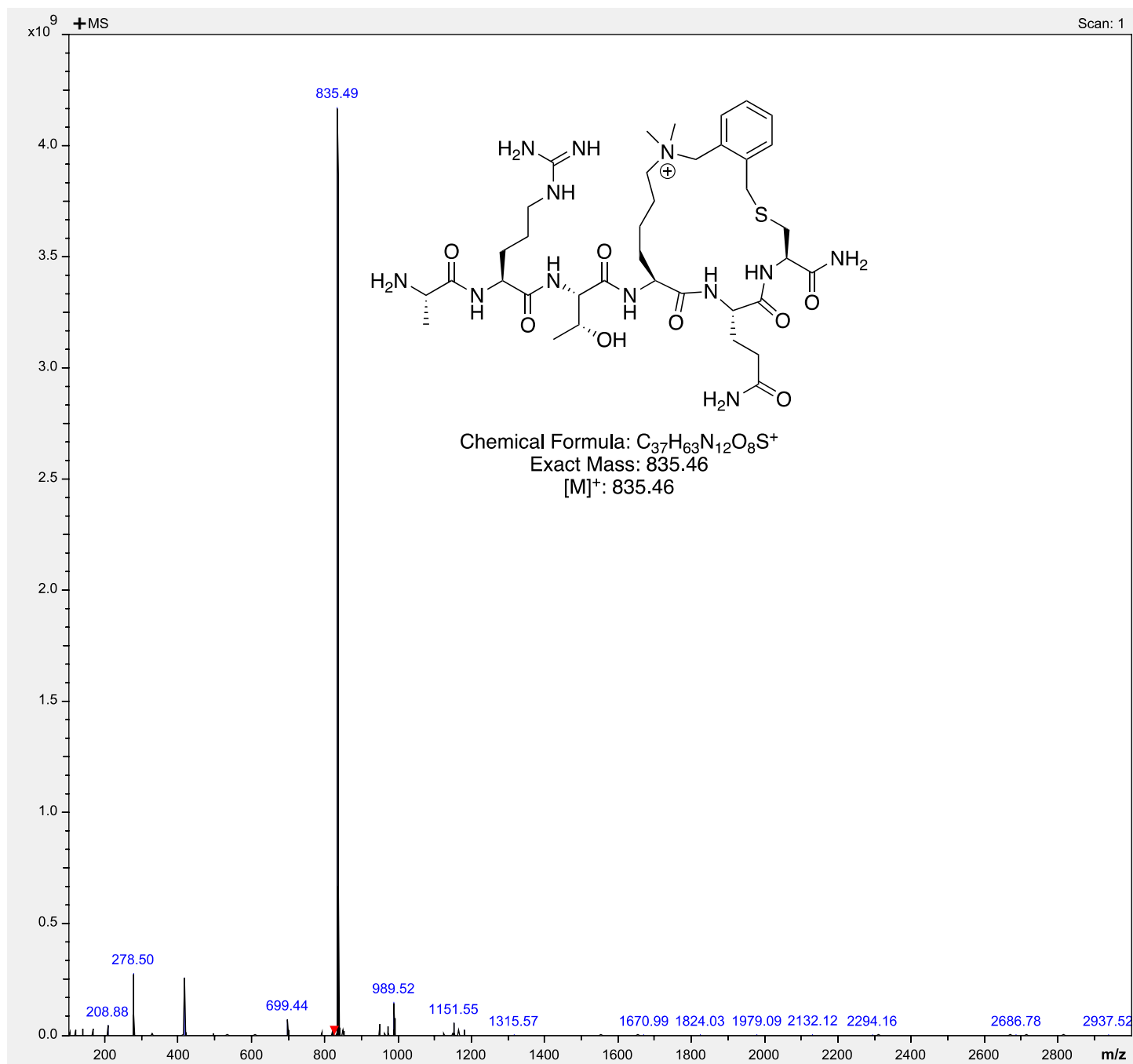
11/10/21

MYZ0115\_thioether\_macrocycle 422 (0.991) Cm (415.434)

1: Scan ES+  
4.24e7

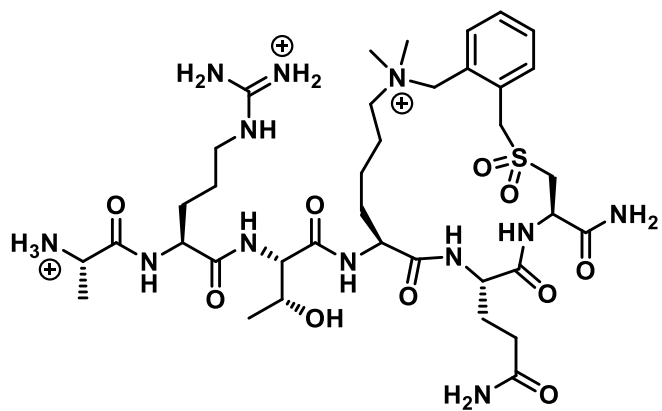


# MALDI





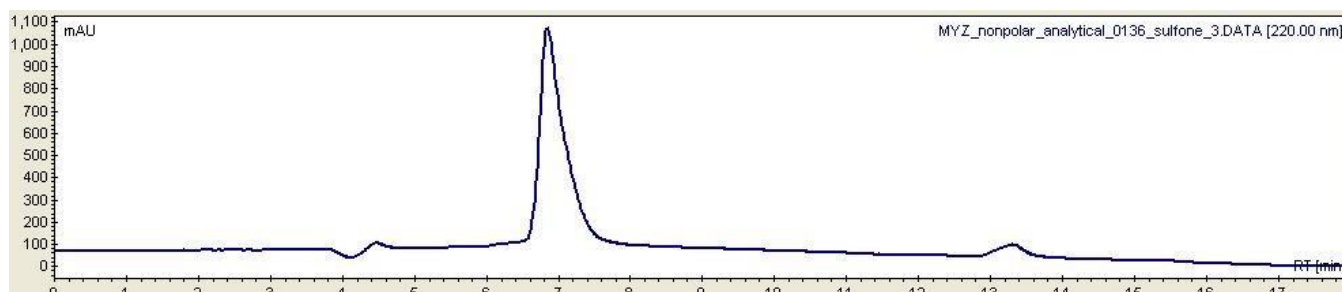
# Compound C-20c



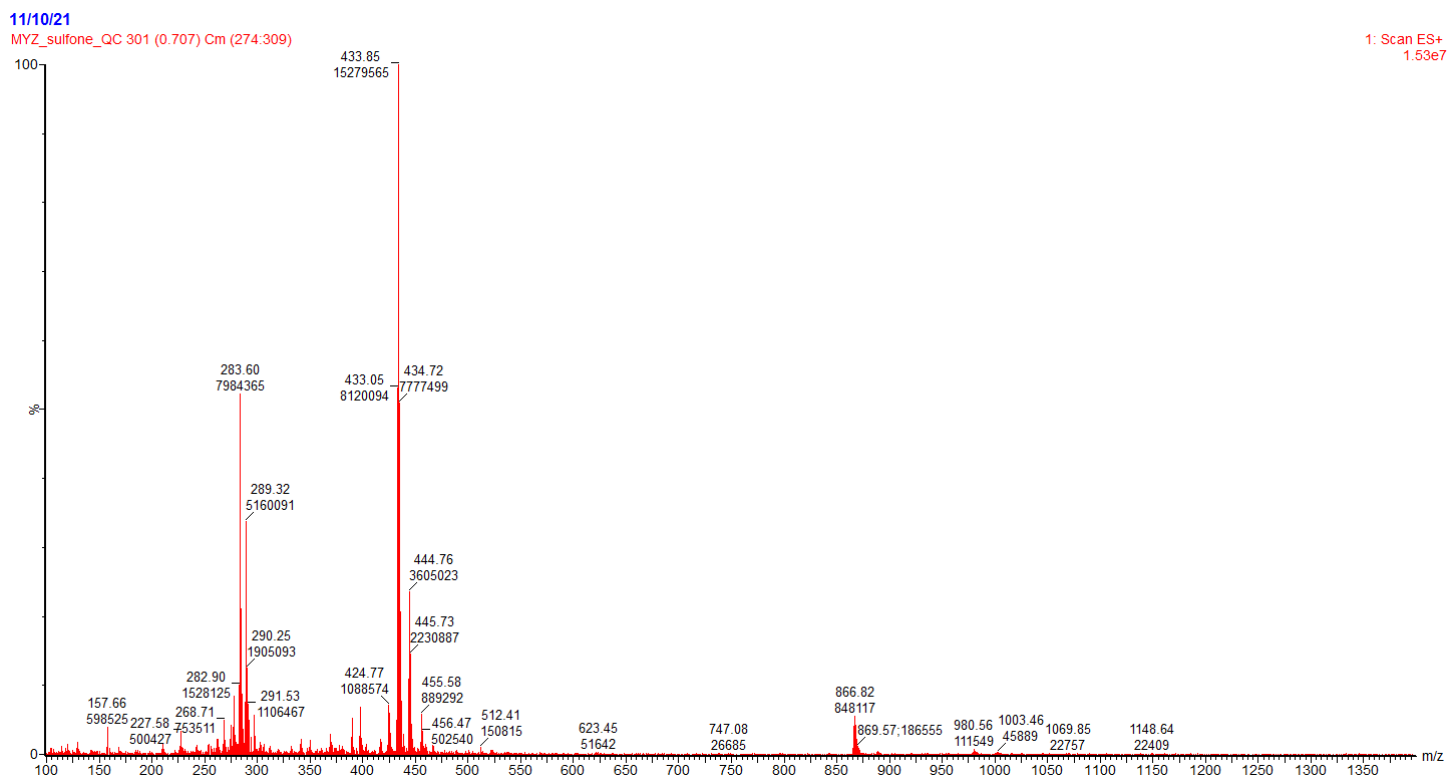
Yield: 1%

Exact mass: 869.47 [C<sub>37</sub>H<sub>65</sub>N<sub>12</sub>O<sub>10</sub>S]<sup>3+</sup>

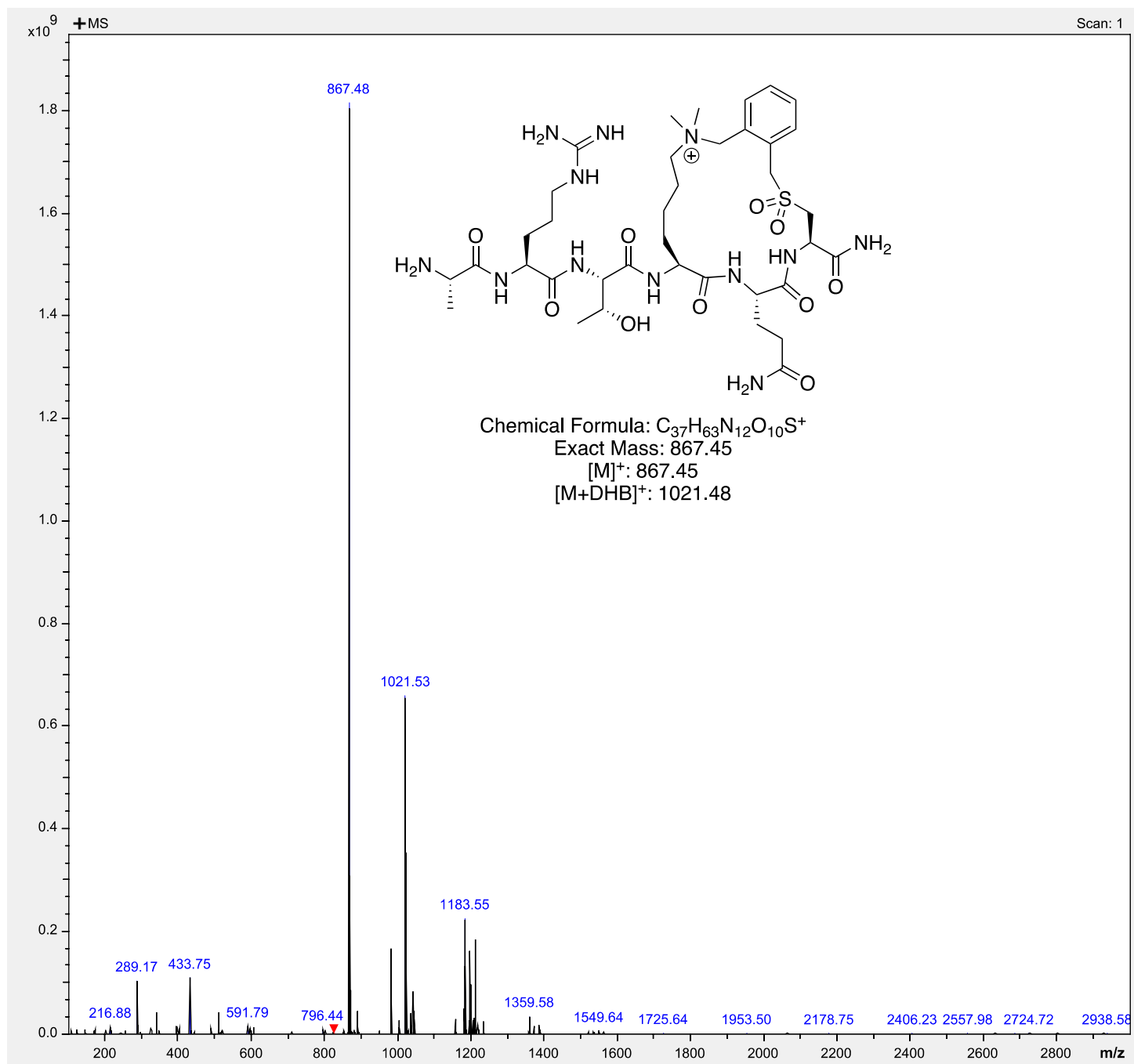
HPLC trace (Method 2):



## LCMS

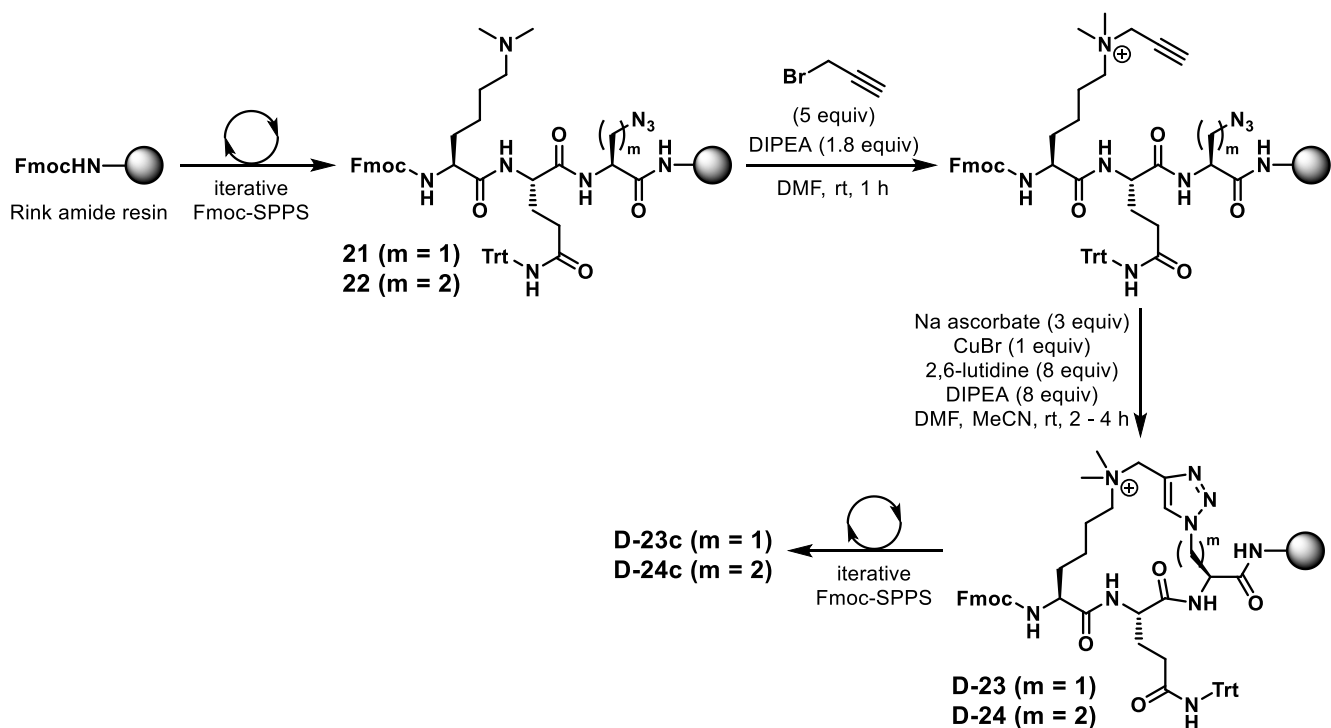


# MALDI



## Series D: CuAAC reaction

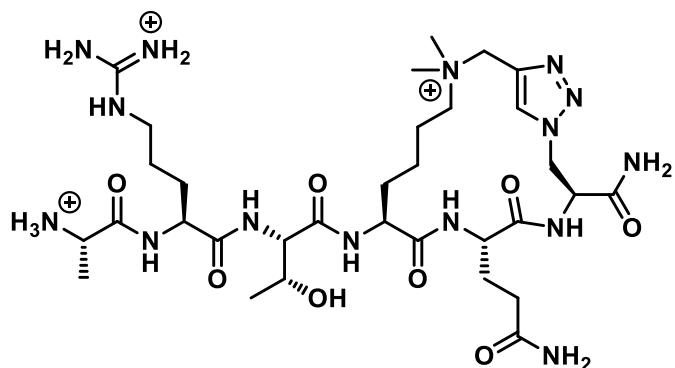
### Condition a:



Tripeptide Fmoc-Lys(me<sub>2</sub>)-Gln(Trt)-Dap(N<sub>3</sub>)-CONH-rink amide resin (**21**, 1 equiv) or Fmoc-Lys(me<sub>2</sub>)-Gln(Trt)-Dab(N<sub>3</sub>)-CONH-rink amide resin (**22**, 1 equiv) was prepared on Rink amide resin according to the procedures outlined in the **Iterative Fmoc-SPPS workflow**. The resin was washed with DMF (5 × 3 mL) and treated with a solution of propargyl bromide (5 equiv) and DIPEA (1.8 equiv) in DMF at room temperature for one hour. The alkylation reaction was monitored by LCMS, and upon consumption of starting material the liquid was discarded, and the resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL) and DMF (5 × 3 mL).

The subsequent CuAAC reaction followed a modified literature procedure.<sup>2</sup> The resin was transferred to a Synthware™ cylindrical pressure vessel (15 mL O.D.) sealed with a rubber septum using 1 mL of DMF, and the reaction flask was degassed and refilled with nitrogen. 2,6-Lutidine (8 equiv) and DIPEA (8 equiv) were added to the resin followed by the addition of sodium ascorbate (3 equiv as a 1% solution in degassed DMF) and copper(I) bromide (1 equiv as a 1% solution in degassed acetonitrile) was added last. The resin mixture was stirred at room temperature under nitrogen for 2-4 hours whilst monitoring the reaction using analytical HPLC. Following consumption of starting material, the reaction was transferred to a plastic fritted syringe and washed with DMF (5 × 3 mL), DCM (5 × 3 mL) and DMF (5 × 3 mL). The cyclized tripeptide intermediates **D-23** and **D-24** were elongated following the procedures outlined in the **Iterative Fmoc-SPPS workflow** to afford triazoles **D-23c** and **D-24c**.

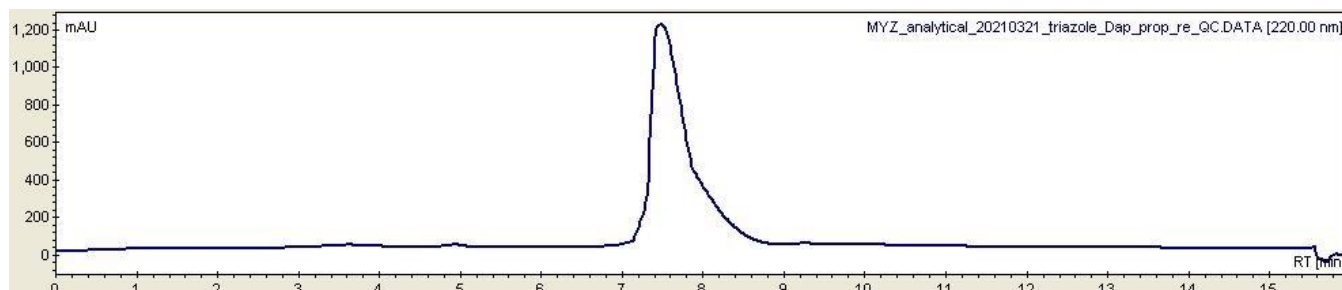
## Compound D-23c



Yield: 2%

Exact mass: 782.47 [C<sub>32</sub>H<sub>60</sub>N<sub>15</sub>O<sub>8</sub>]<sup>3+</sup>

HPLC trace (Method 1):

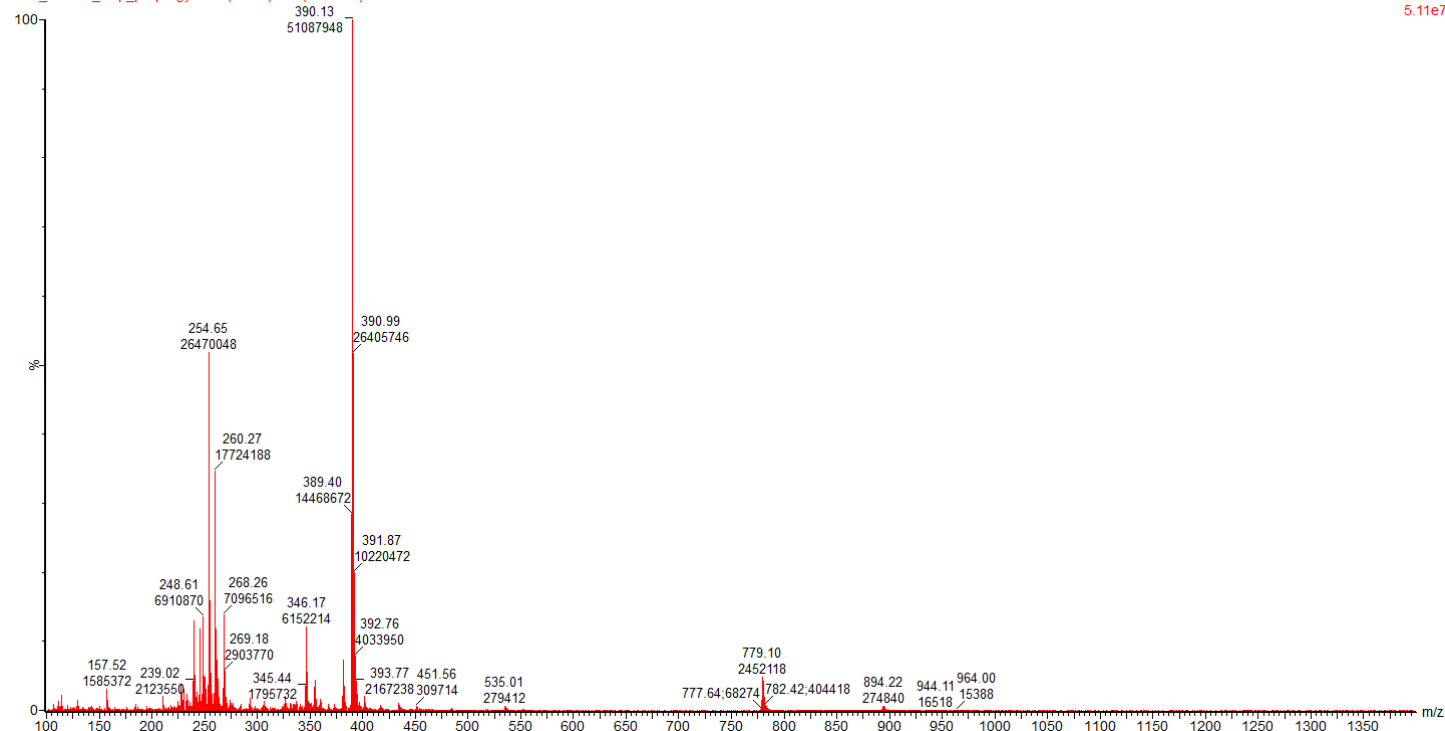


## LCMS

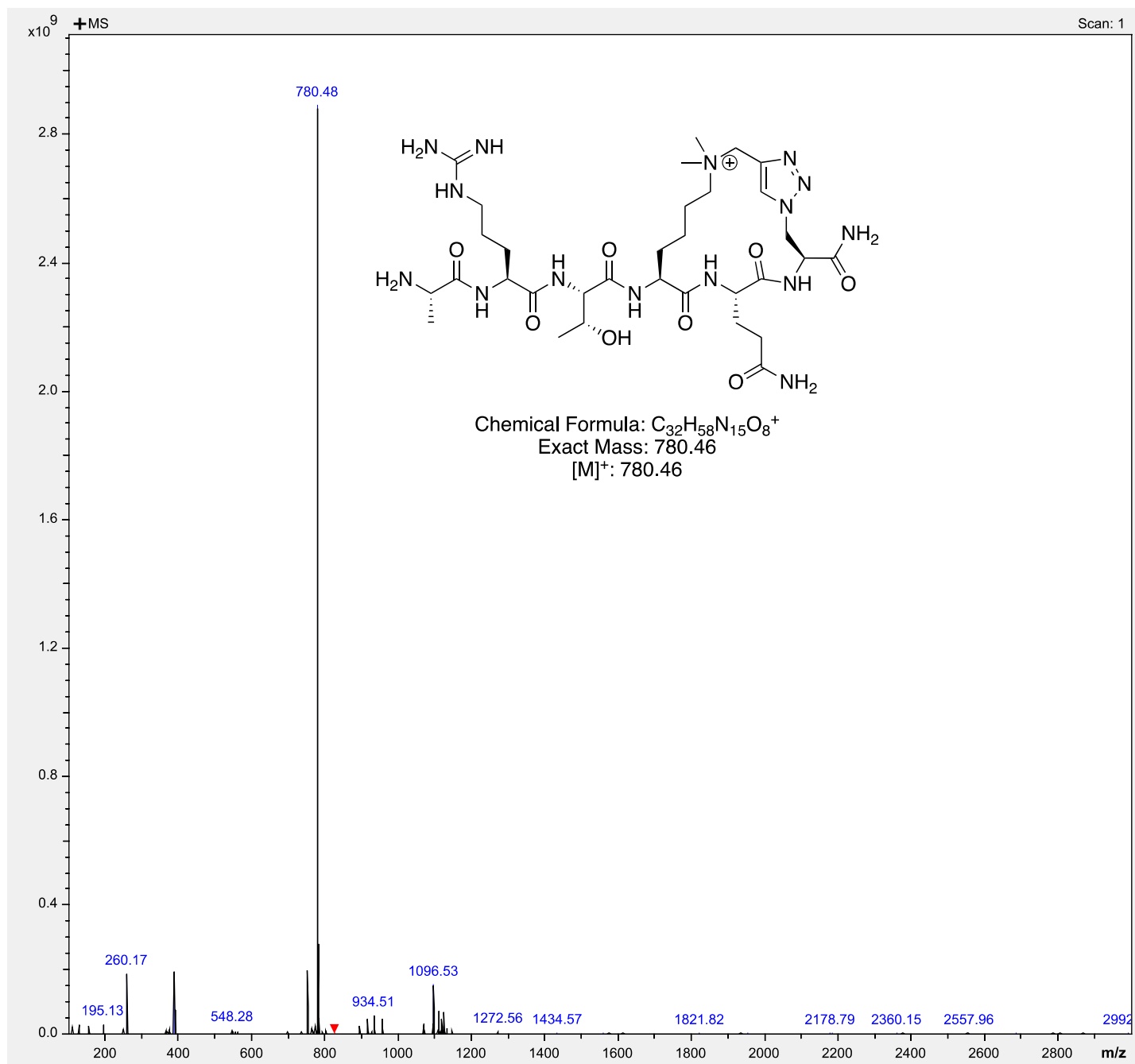
11/10/21

MYZ\_triazole\_Dap\_propargyl 259 (0.608) Cm (248:279)

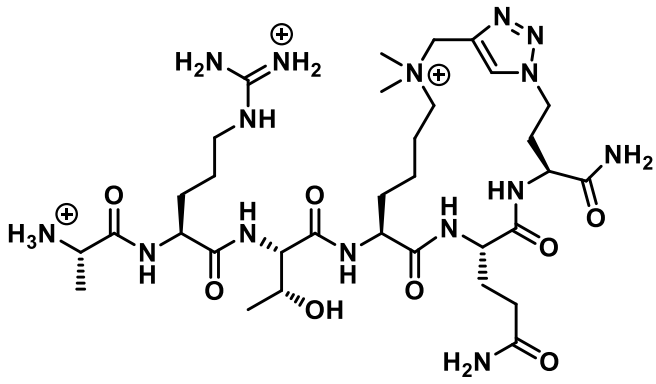
1: Scan ES+  
5.11e7



# MALDI



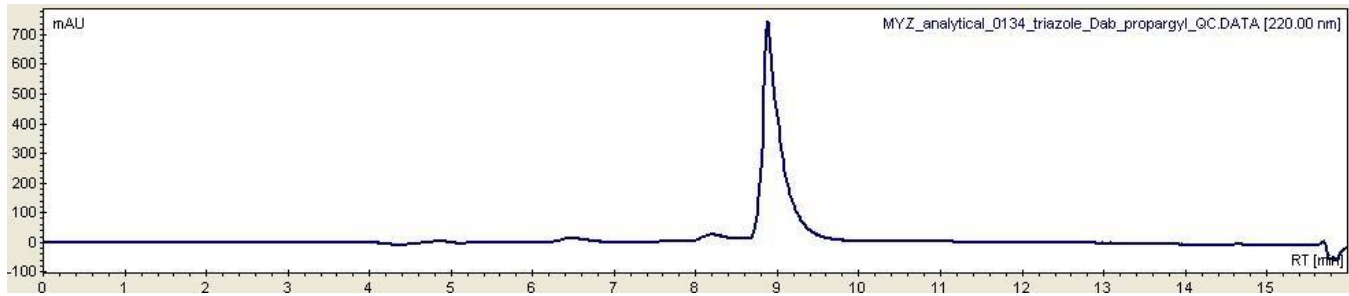
## Compound D-24c



Yield: 5%

Exact mass: 796.49 [C<sub>33</sub>H<sub>62</sub>N<sub>15</sub>O<sub>8</sub>]<sup>3+</sup>

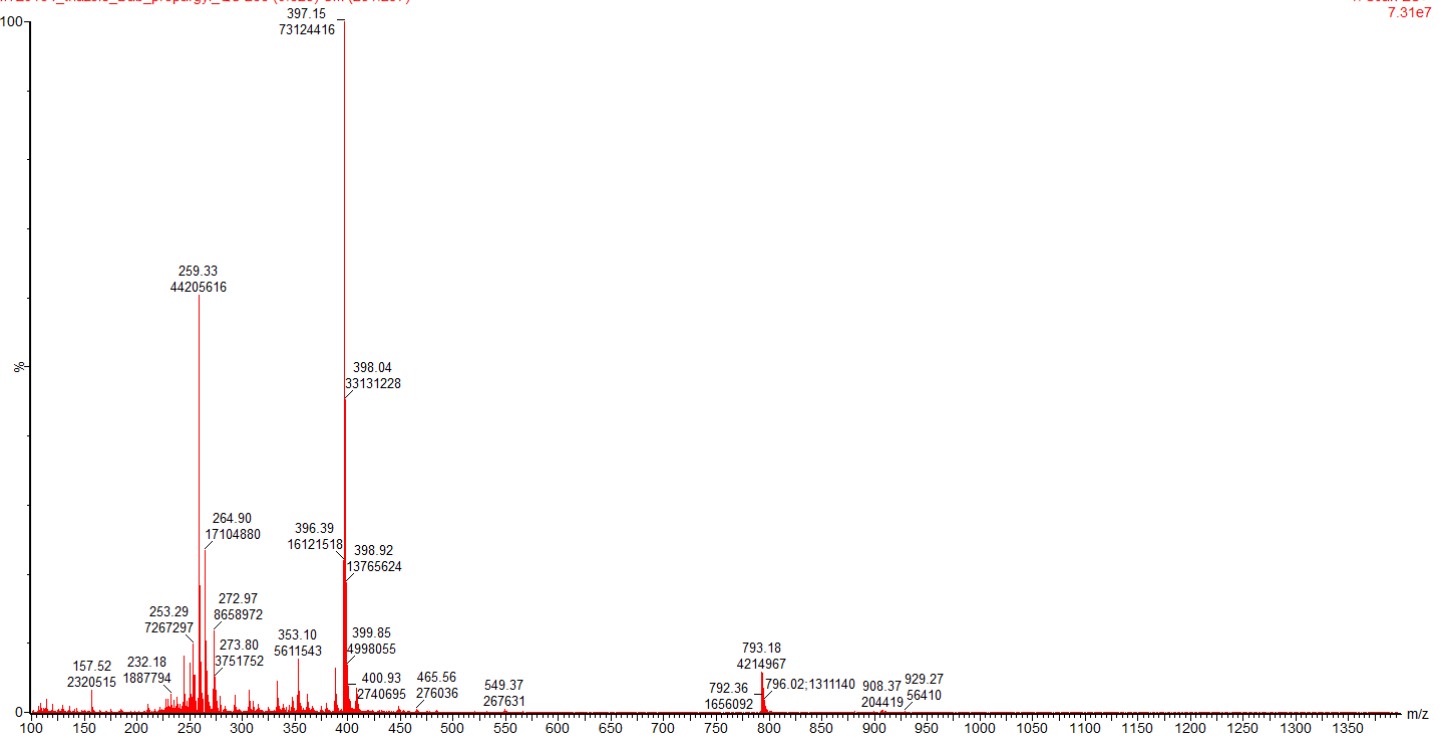
HPLC trace (Method 1):



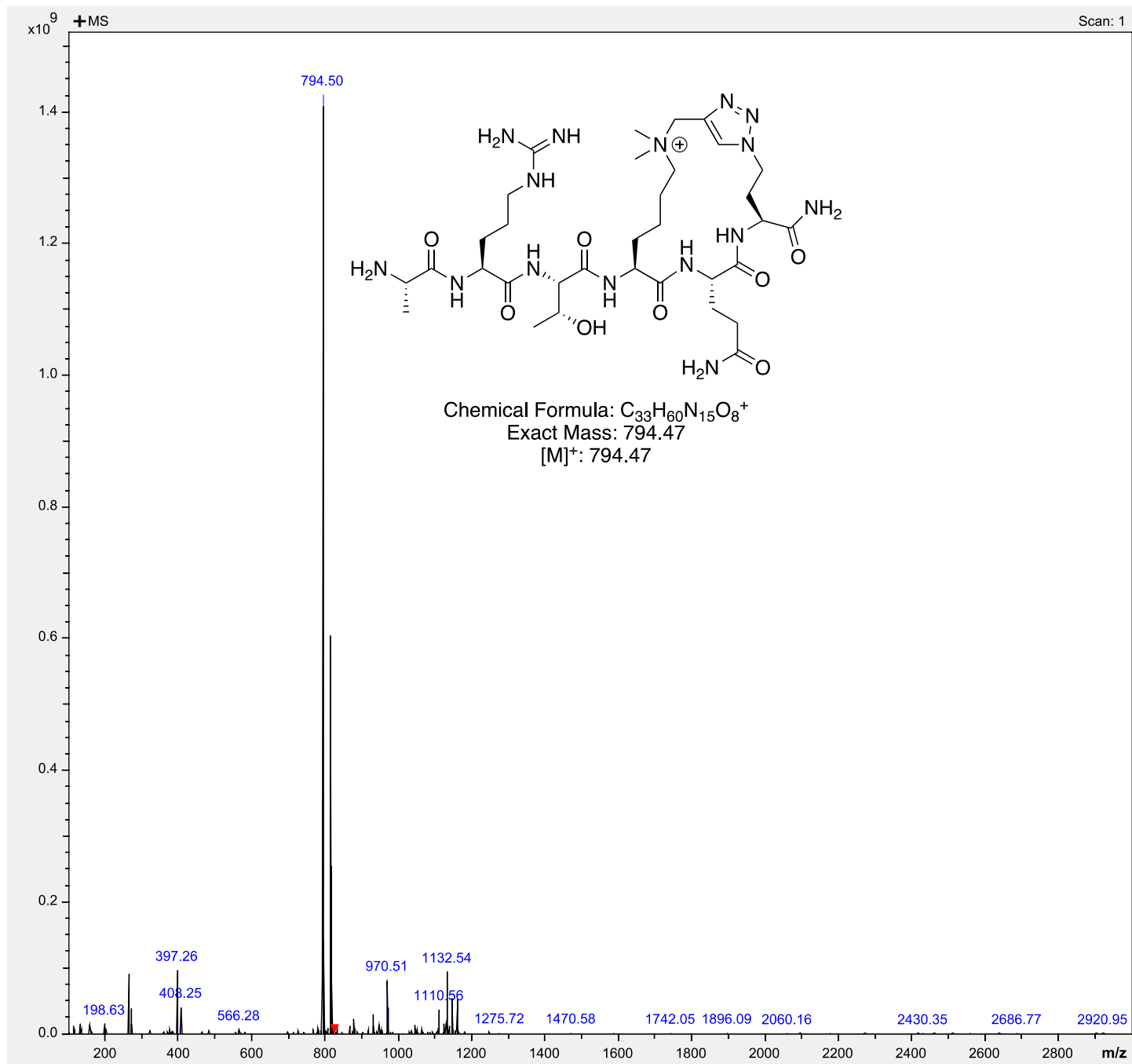
## LCMS

11/10/21  
MYZ0134\_tripargyl\_GC 266 (0.625) Cm (251.297)

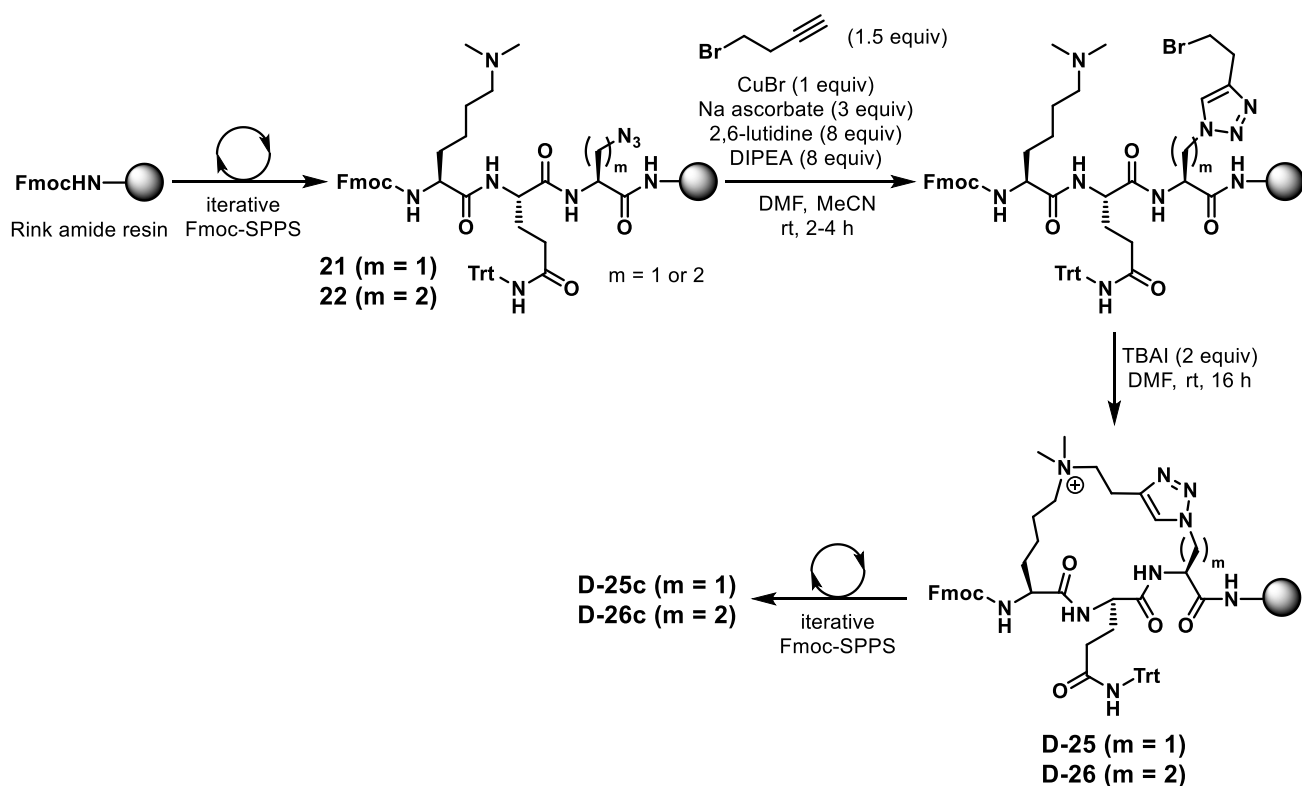
1: Scan ES+  
7.31e7



# MALDI



## Condition b:

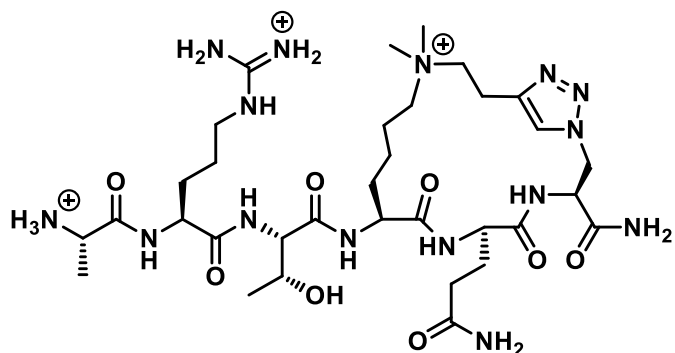


Tripeptide **Fmoc-Lys(me<sub>2</sub>)-Gln(Trt)-Dap(N<sub>3</sub>)-CONH-rink amide resin** (**21**, 1 equiv) or **Fmoc-Lys(me<sub>2</sub>)-Gln(Trt)-Dab(N<sub>3</sub>)-CONH-rink amide resin** (**22**, 1 equiv) was prepared on Rink amide resin according to the procedures outlined in the **Iterative Fmoc-SPPS workflow**. The resin was washed with DMF (5 × 3 mL).

The CuAAC reaction procedures from Condition a was used with the addition of 4-bromo-1-butyne (1.5 equiv). Following the consumption of starting material (monitored by LCMS analysis), the resin was transferred to a plastic fritted syringe and washed with DMF (5 × 3 mL), DCM (5 × 3 mL) and DMF (5 × 3 mL). The resin was treated with a solution of tetrabutylammonium iodide (2 equiv) in DMF and agitated at room temperature for 16 hours, at which time the liquid was expelled and the resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL) and DMF (5 × 3 mL). The cyclized tripeptide intermediates **D-25** and **D-26** were elongated following the **Iterative Fmoc-SPPS workflow** to afford triazoles **D-25c** and **D-26c**.



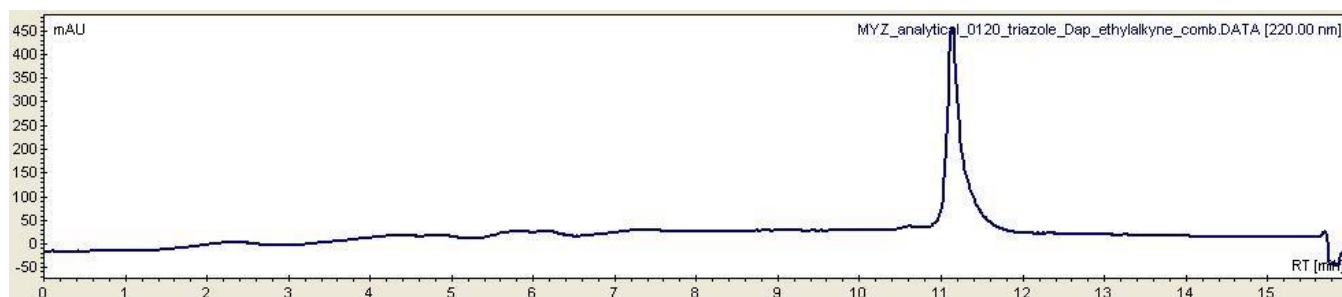
## Compound D-25c



Yield: 2%

Exact mass: 796.49 [C<sub>33</sub>H<sub>62</sub>N<sub>15</sub>O<sub>8</sub>]<sup>3+</sup>

HPLC trace (Method 1):

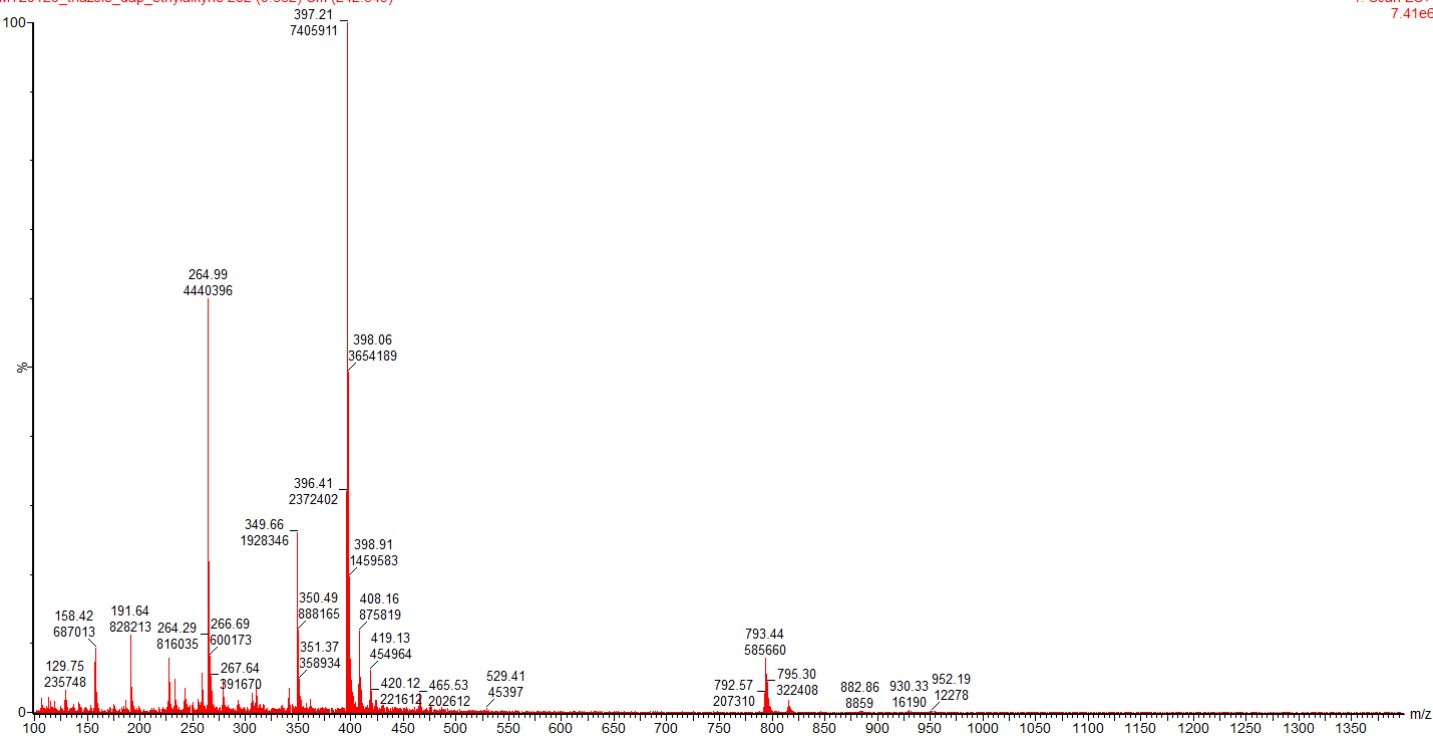


## LCMS

11/10/21

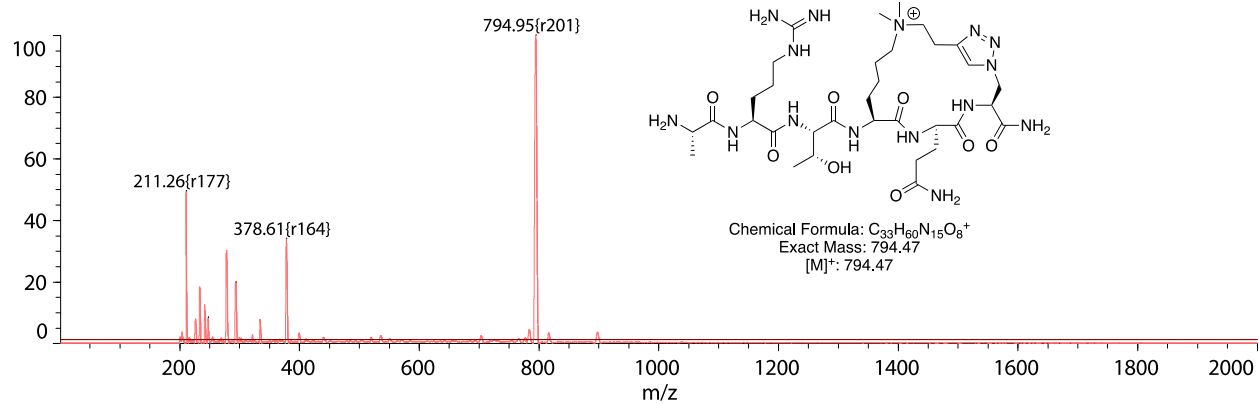
MYZ0120\_triazole\_dap\_ethylalkyne 282 (0.662) Cm (242:349)

1: Scan ES+  
7.41e6

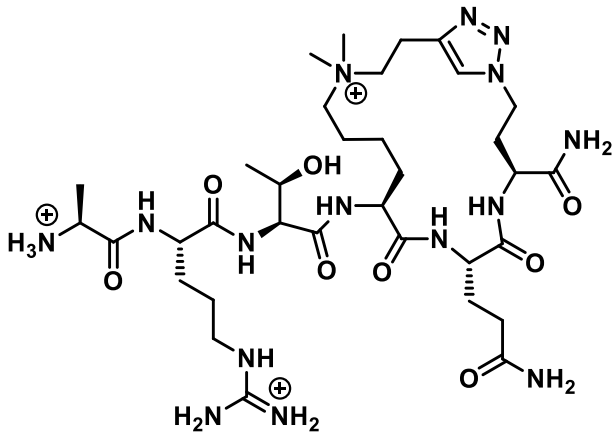


# MALDI

%Int. 160 mV[sum= 14561 mV] Profiles 1-91 Smooth Sv-GI 50 -Baseline 150



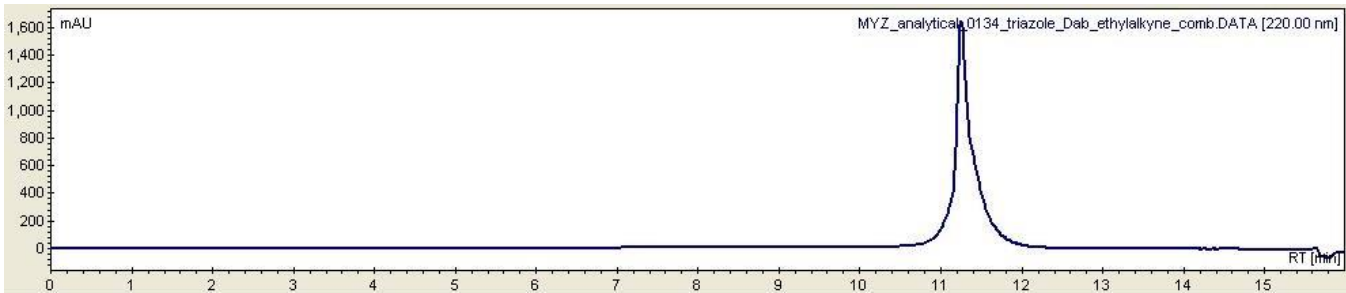
# Compound D-26c



Yield: 2%

Exact mass: 810.50 [C<sub>34</sub>H<sub>64</sub>N<sub>15</sub>O<sub>8</sub>]<sup>3+</sup>

HPLC trace (Method 1):

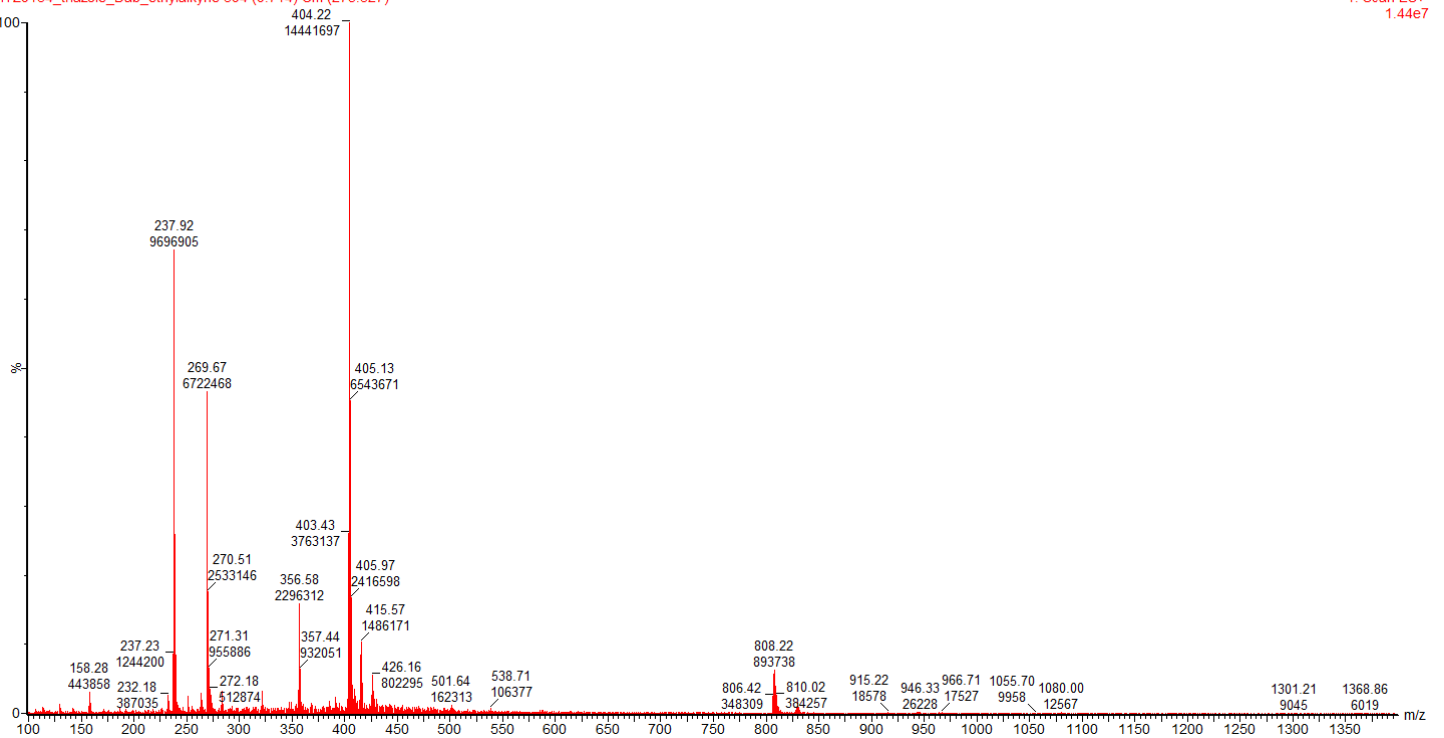


## LCMS

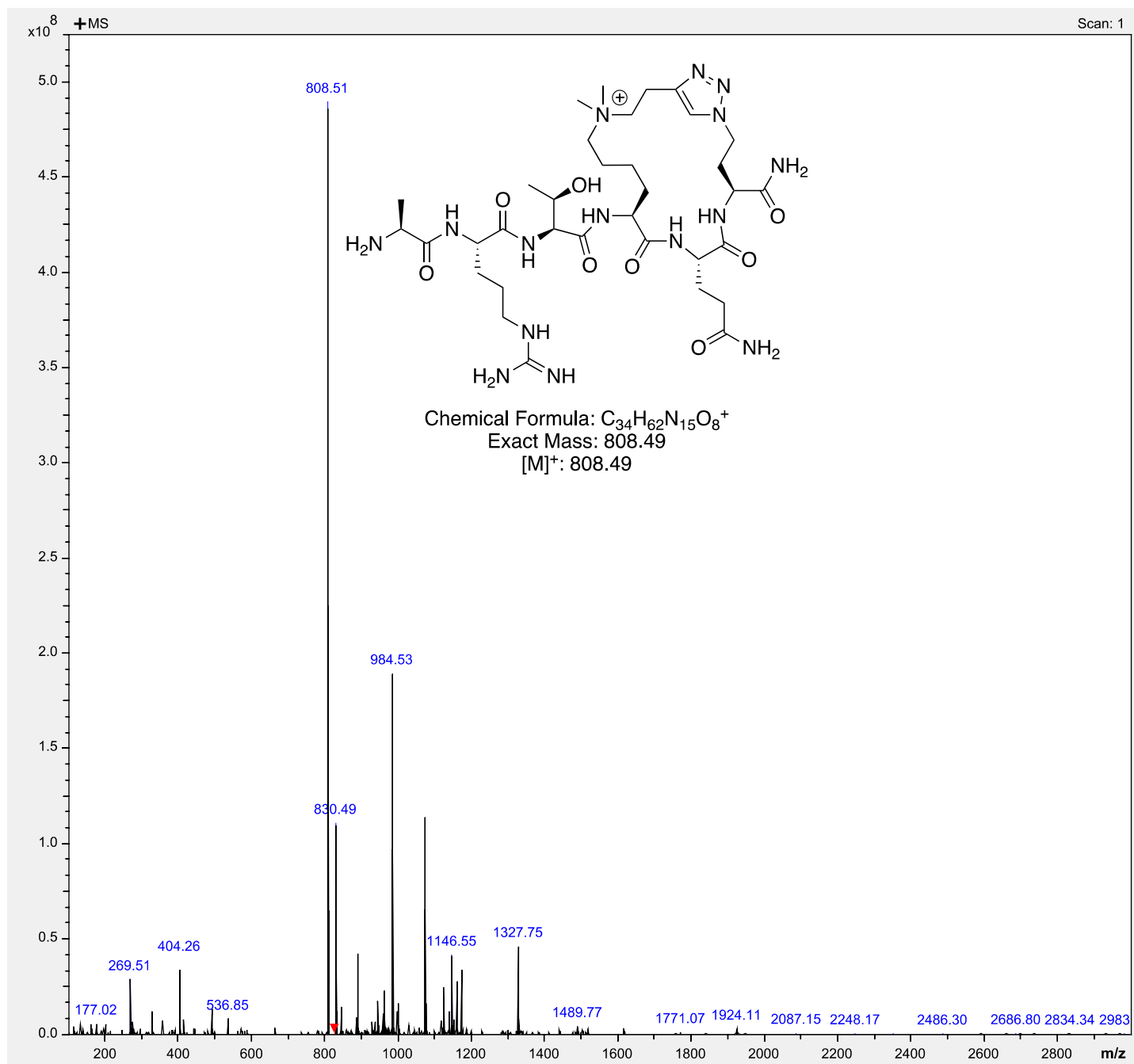
11/10/21

MYZ0134\_triazole\_Dab\_ethylalkyne 304 (0.714) Cm (275:327)

1: Scan ES+  
1.44e7

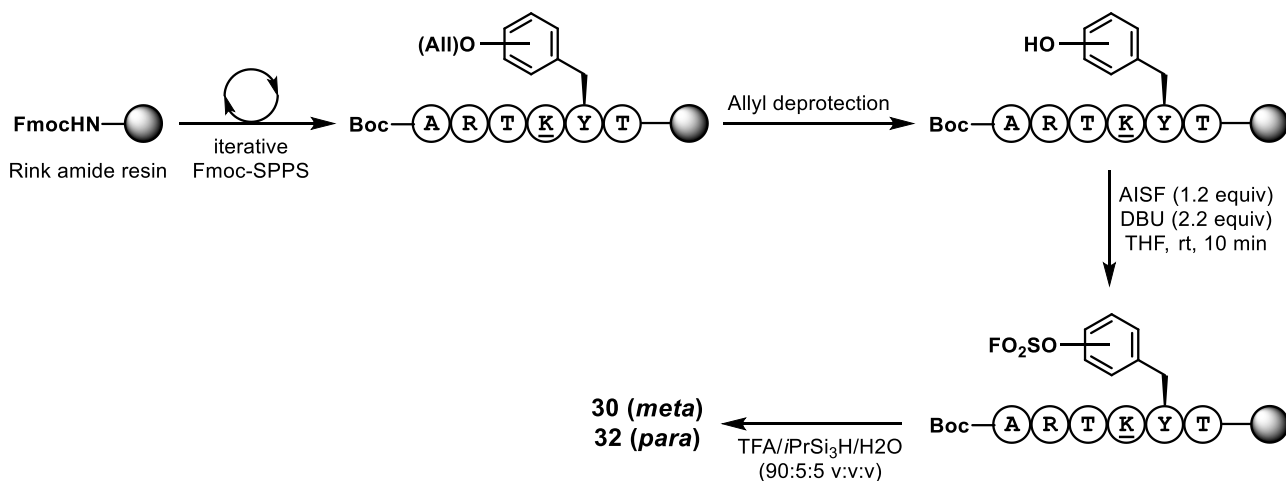


# MALDI



## Covalent Warhead Synthetic Procedures

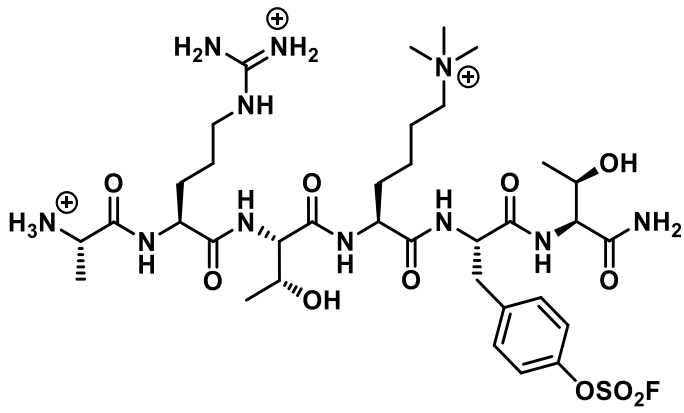
### Installation of the arylfluorosulfate covalent warhead using 4-(acetylamino)phenyl]imidodisulfuryl difluoride (AISF)



Hexapeptide Boc-Ala-Arg(Pbf)-Thr(*t*Bu)-K(*me*<sub>3</sub>)-Tyr(All)-Thr(*t*Bu)-CONH-Rink amide resin (1 equiv) was prepared on Rink amide resin according to the procedures outlined in the **Iterative Fmoc-SPPS workflow**. The resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL), DMF (5 × 3 mL) and DCM (5 × 3 mL). The Allyl group was deprotected according to the protocols outlined in the **Deprotection procedures**.

The arylfluorosulfate peptide synthesis followed a slightly modified literature procedure.<sup>3</sup> A solution of AISF (1.2 equiv) in THF was added to resin, followed by the addition of a solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (2.2 equiv) in THF. The resin mixture was shaken at room temperature for 10 minutes at which time the liquid was expelled and the resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL), DMF (5 × 3 mL) and DCM (5 × 3 mL). Incorporation of the fluorosulfate functionality was confirmed by LCMS analysis. The peptide was cleaved from resin and purified according to the procedures outlined in the **Iterative Fmoc-SPPS workflow**.

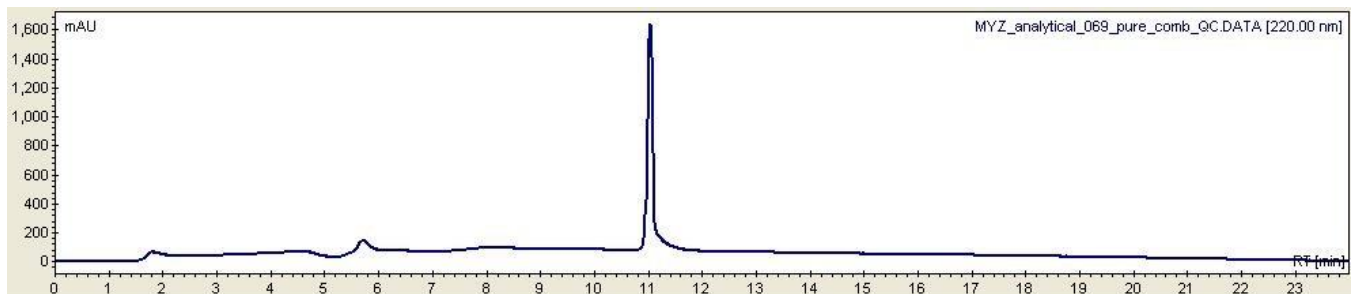
Compound 32



Yield: 11%

Exact mass: 864.44 [C<sub>35</sub>H<sub>63</sub>FN<sub>11</sub>O<sub>11</sub>S]<sup>3+</sup>

HPLC trace (Method 3):

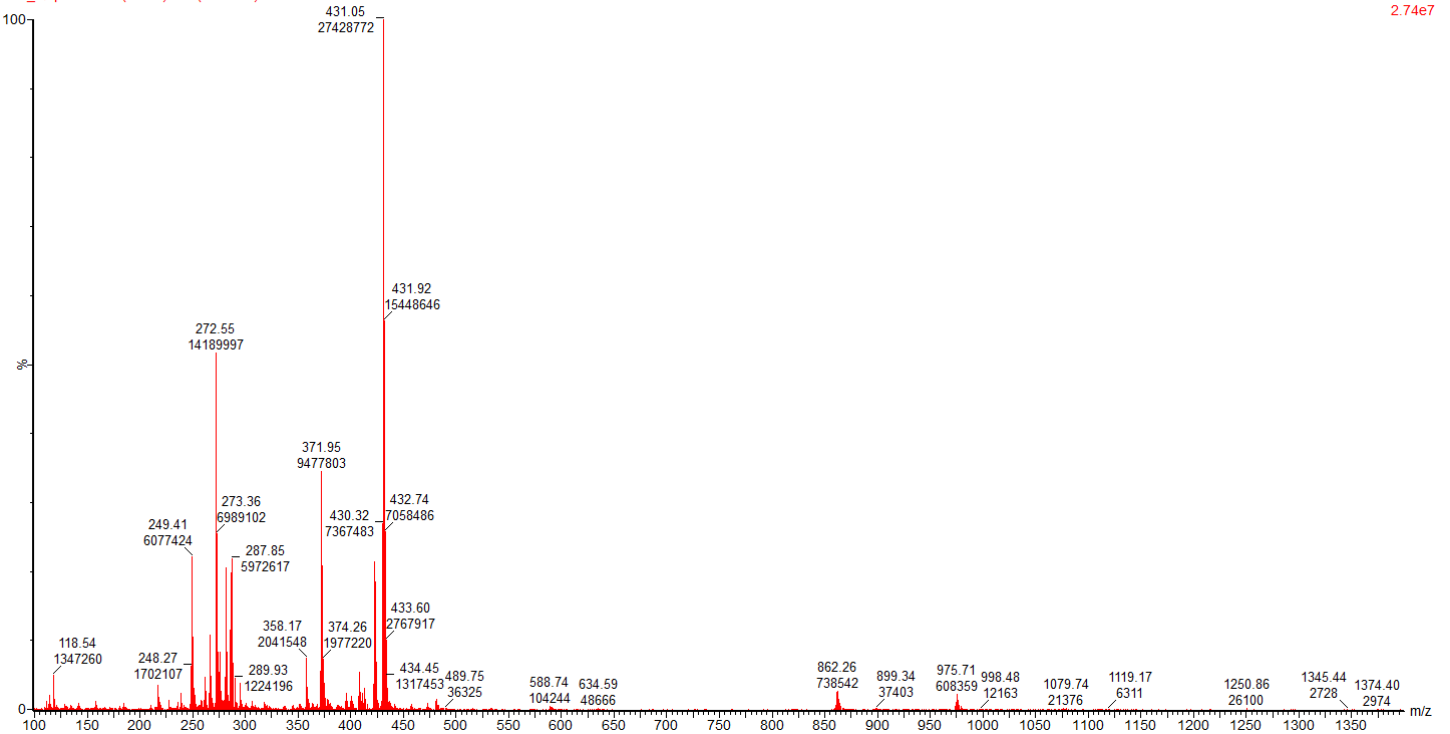


LCMS

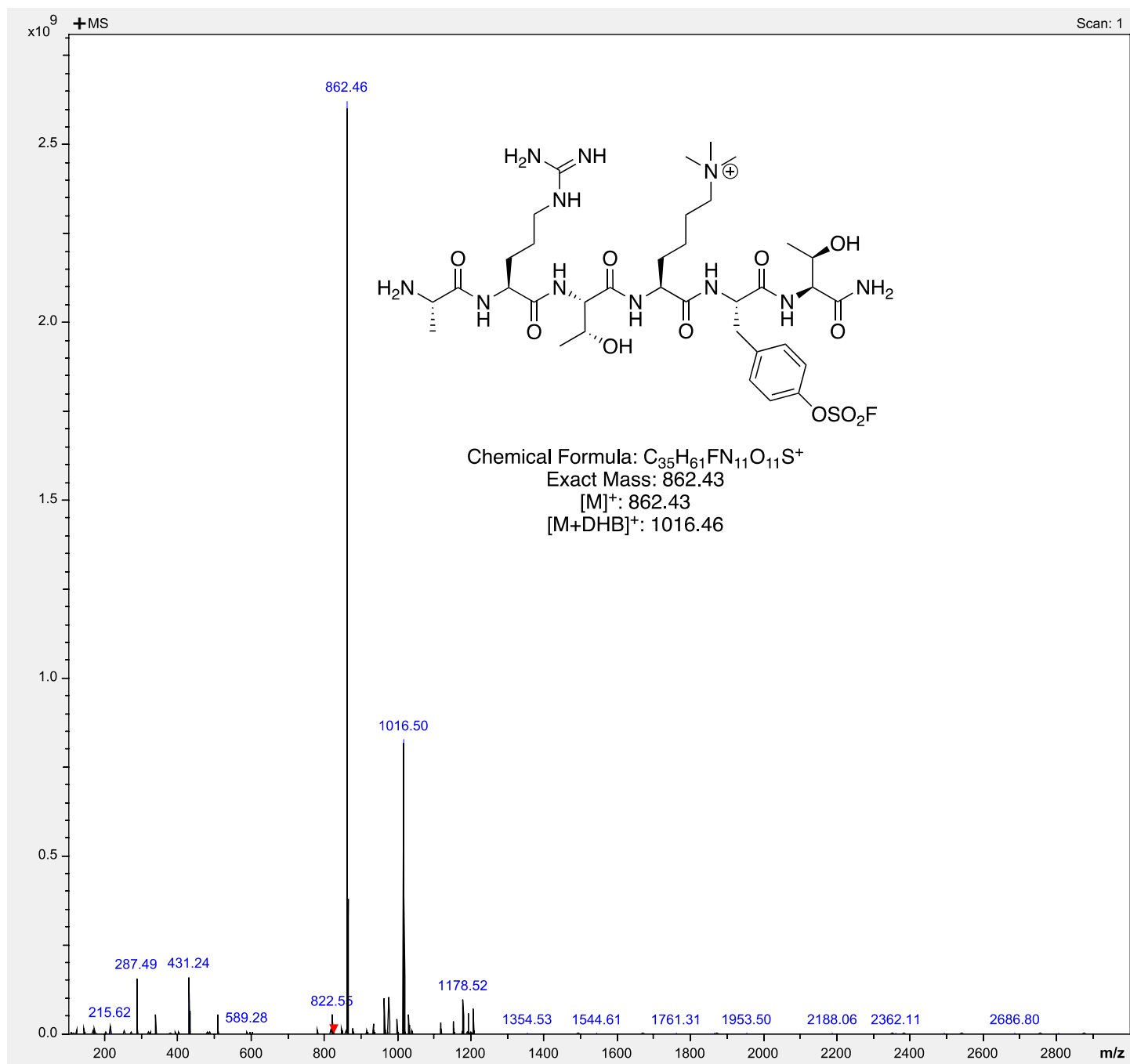
11/10/21

MYZ\_Q5pFSY 649 (1.524) Cm (640.660)

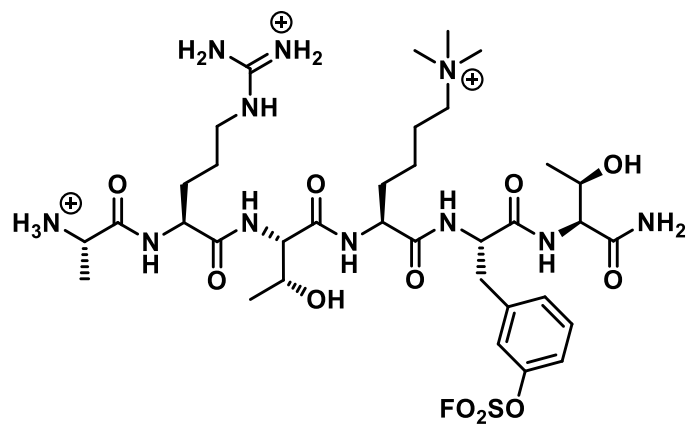
1: Scan ES+  
2.74e7



# MALDI



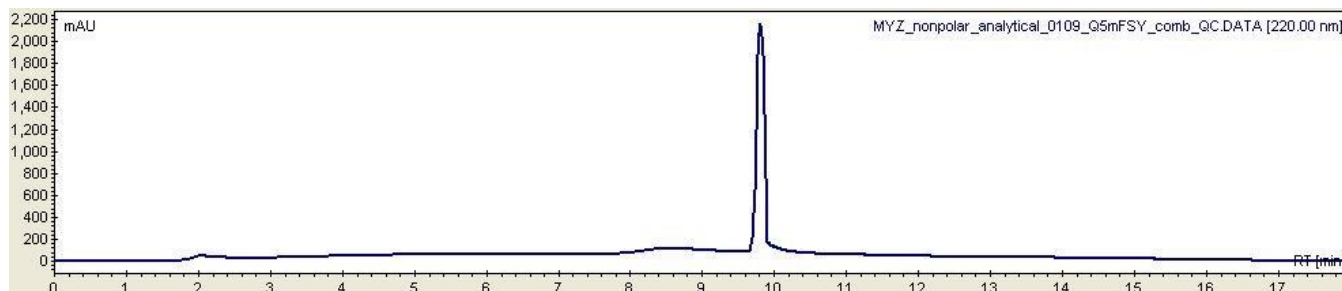
# Compound 30



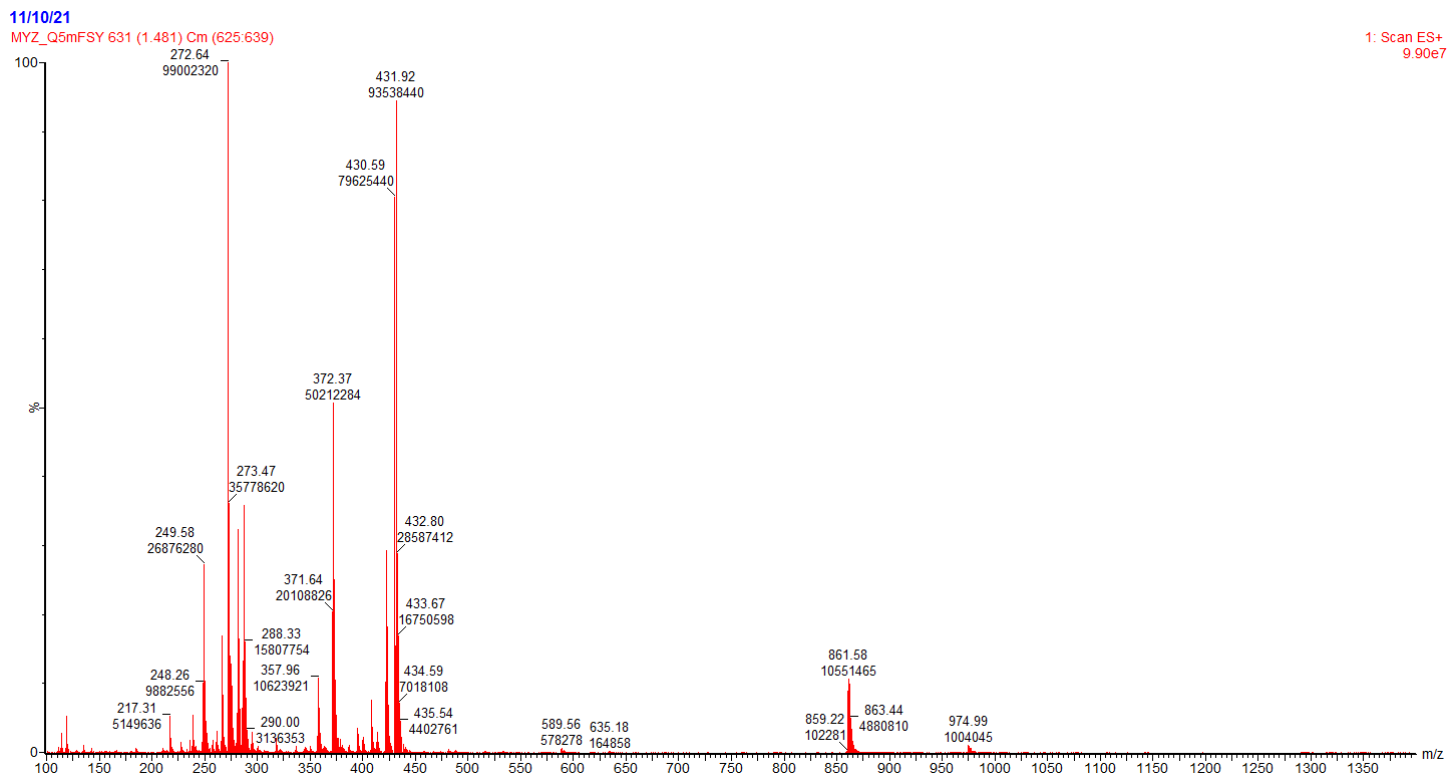
Yield: 27%

Exact mass: 864.44 [C<sub>35</sub>H<sub>63</sub>FN<sub>11</sub>O<sub>11</sub>S]<sup>3+</sup>

HPLC trace (Method 2):

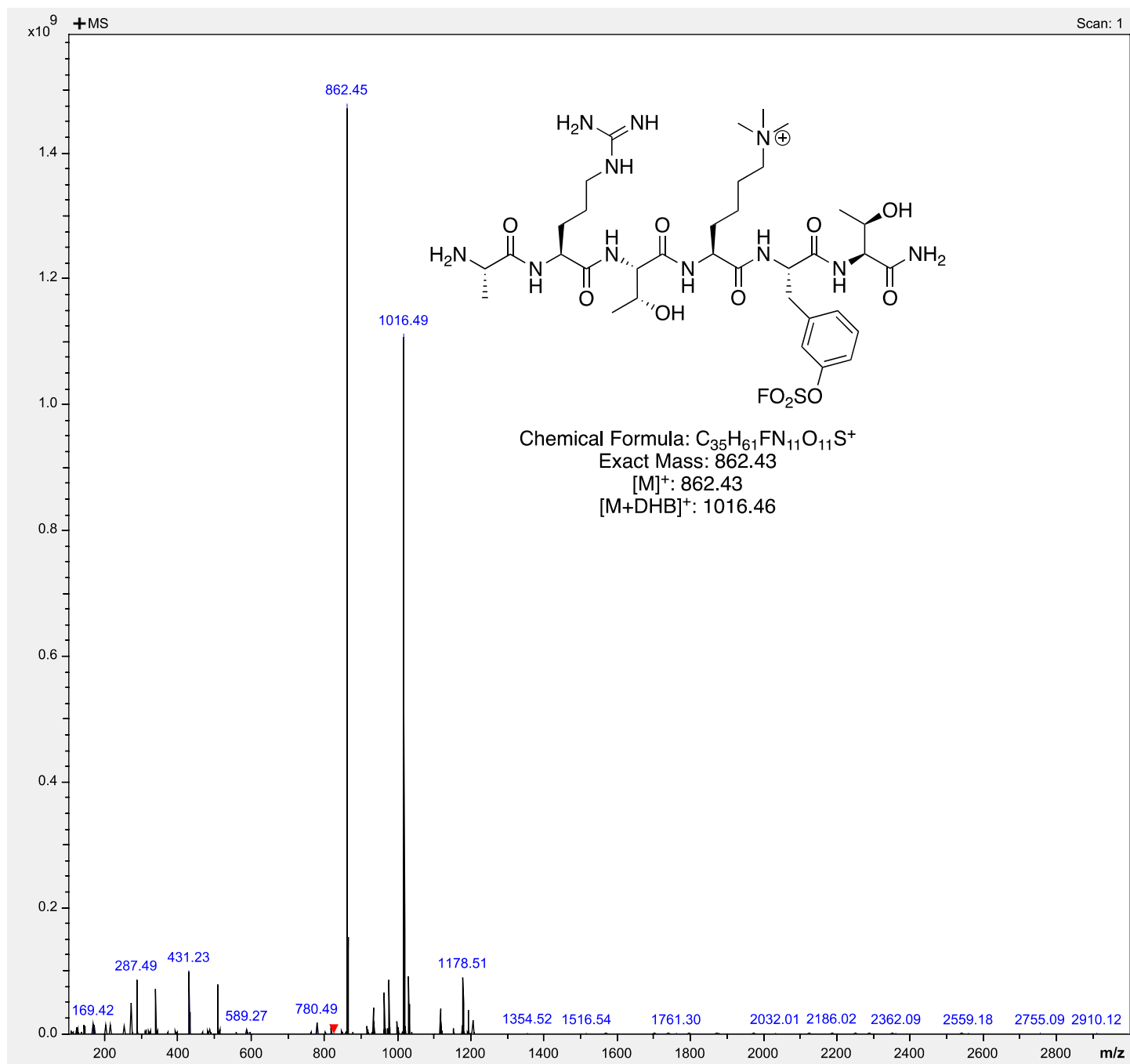


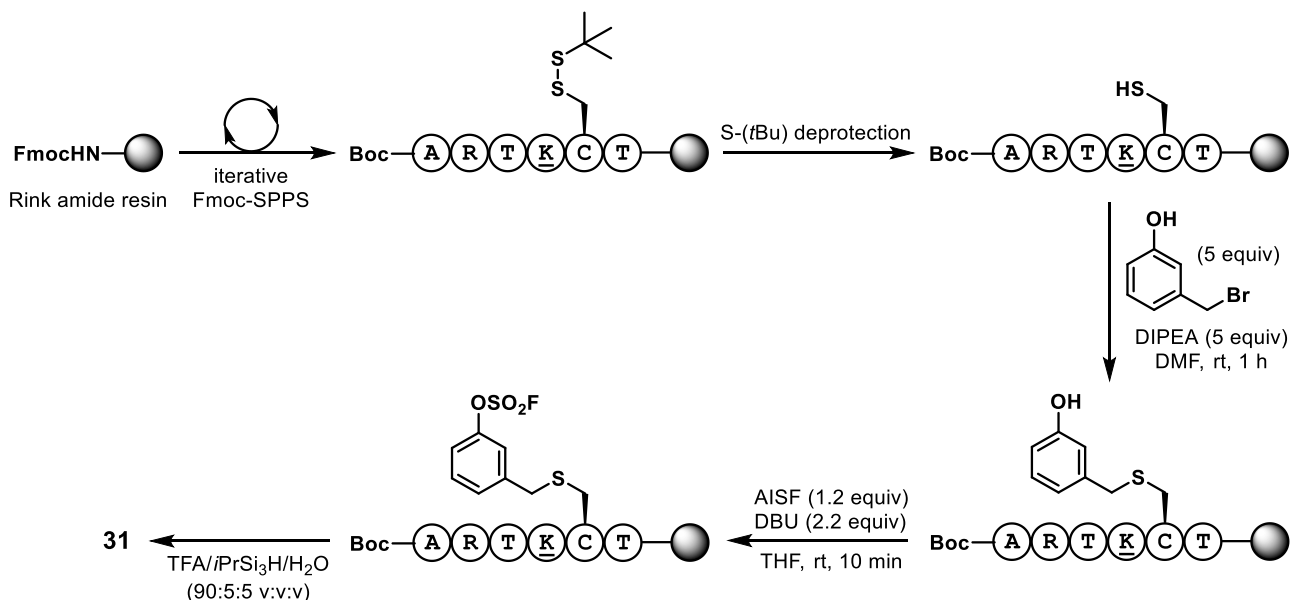
## LCMS





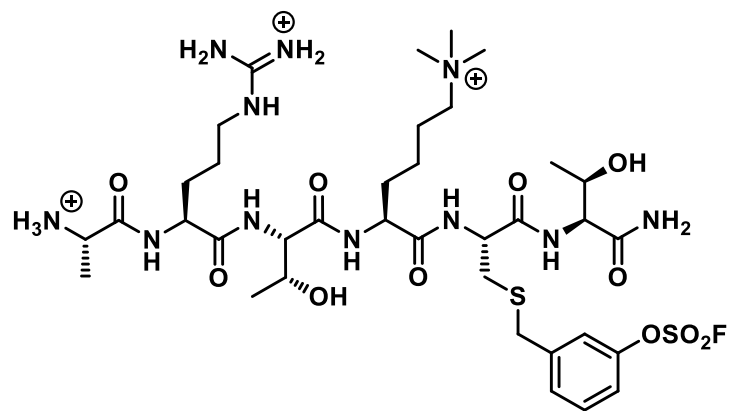
# MALDI





Hexapeptide Boc-Ala-Arg(Pbf)-Thr(*t*Bu)-K(*me*<sub>3</sub>)-Cys(S*t*Bu)-Thr(*t*Bu)-CONH-Rink amide resin (1 equiv) was prepared on Rink amide resin according to the procedures outlined in the **Iterative Fmoc-SPPS workflow**. The S-(*t*Bu) group was deprotected according to the protocols outlined in the **Deprotection procedures**. The resin was washed with DMF (5 × 3 mL) and treated with a solution of DIPEA (5 equiv) and 3-(bromomethyl)phenol (5 equiv) in DMF and agitated at room temperature for 30 minutes or until consumption of starting material was confirmed by LCMS analysis. The liquid was discarded, and the resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL), DMF (5 × 3 mL) and DCM (5 × 3 mL). Installation of the fluorosulfate group was achieved using the synthetic procedures outlined for arylfluorosulfate peptides **30** and **32**. The peptide was cleaved from resin and purified according to the procedures outlined in the **Iterative Fmoc-SPPS workflow** to afford arylfluorosulfate peptide **31**.

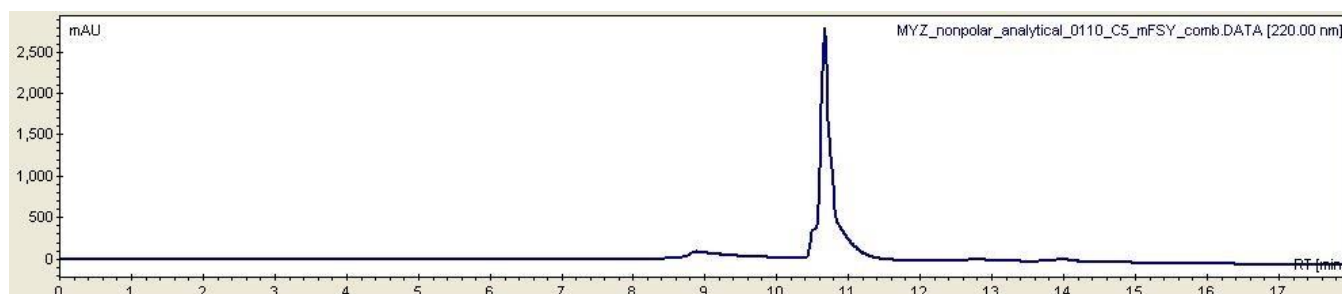
## Compound 31



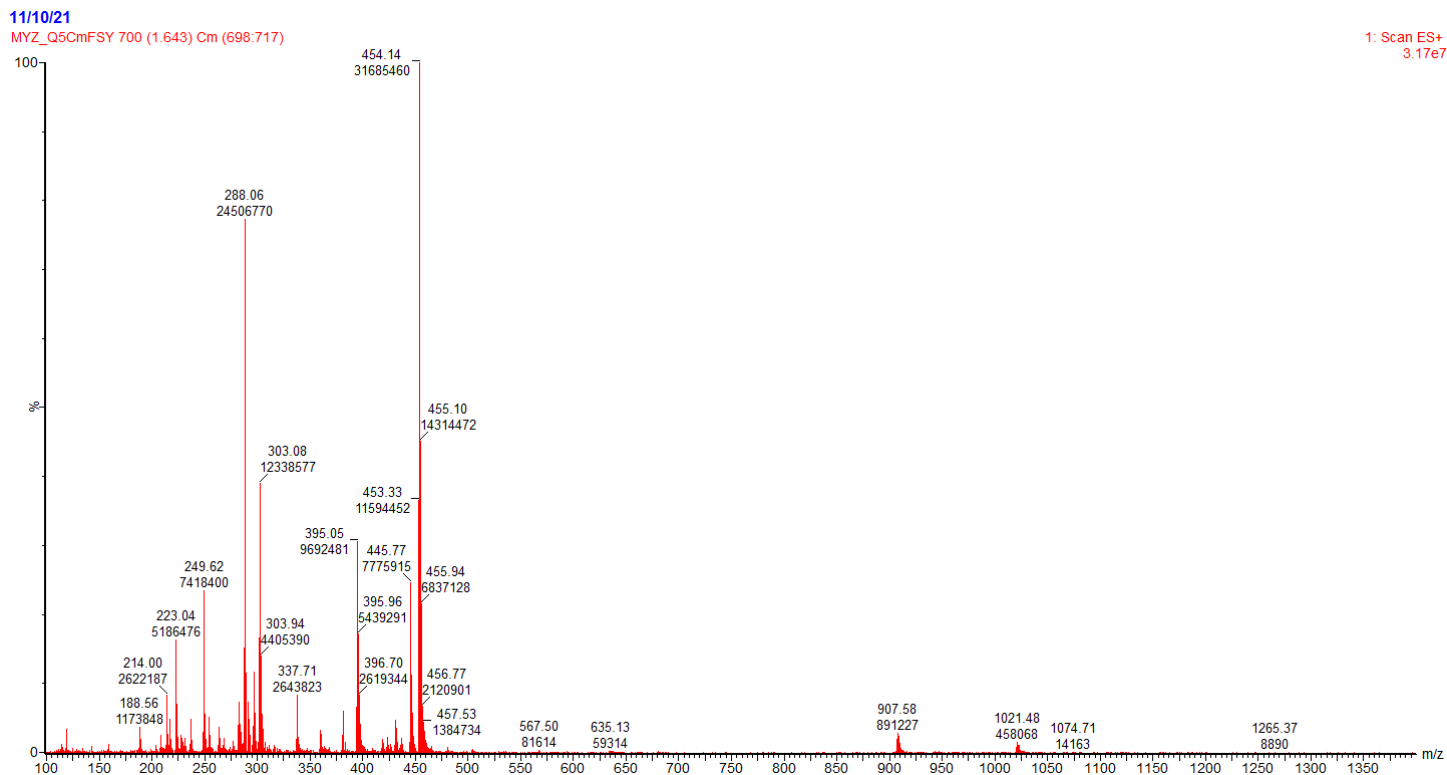
Yield: 16%

Exact mass: 910.43 [C<sub>36</sub>H<sub>65</sub>FN<sub>11</sub>O<sub>11</sub>S<sub>2</sub>]<sup>3+</sup>

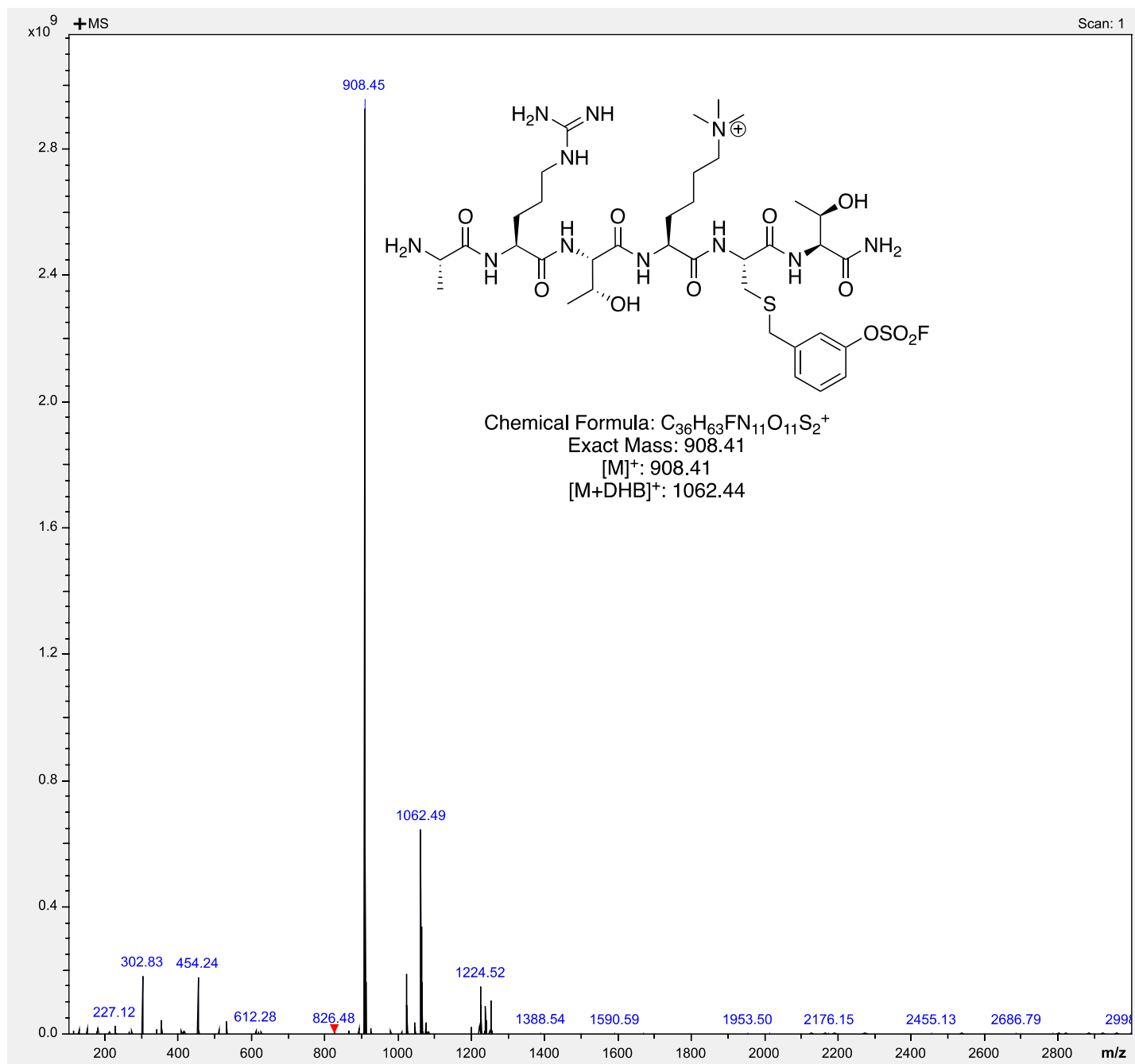
HPLC trace (Method 2):



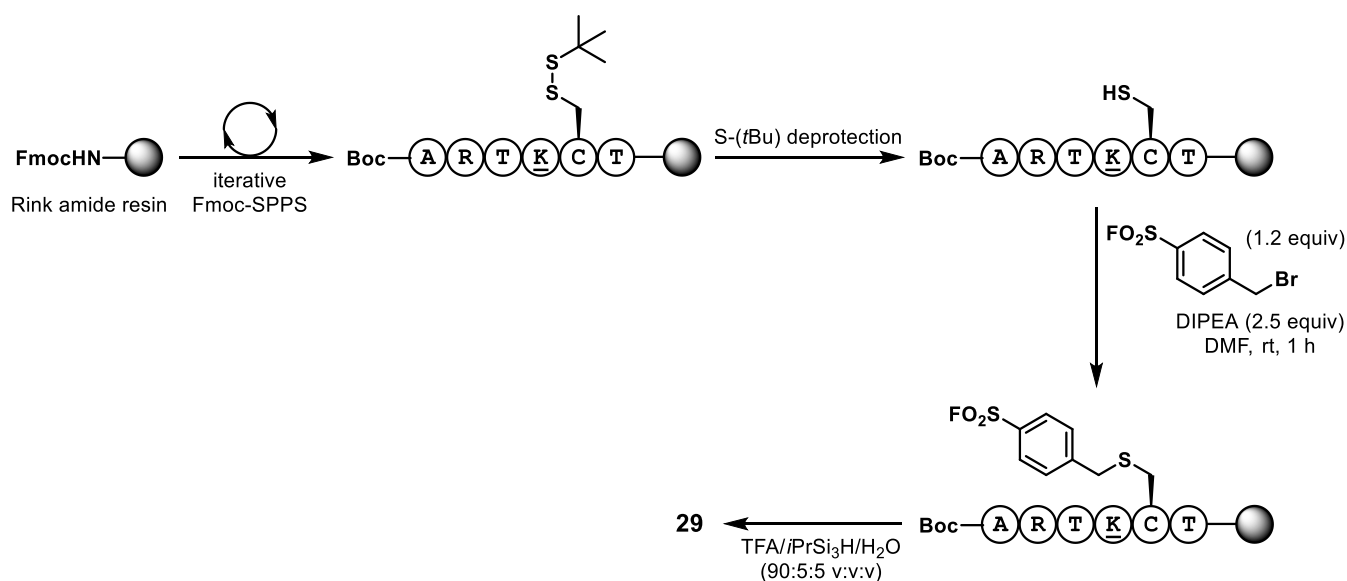
## LCMS



# MALDI

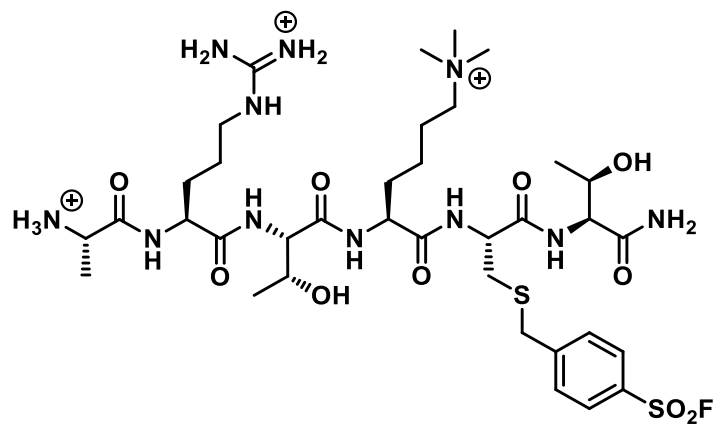


## Installation of the 4-benzylsulfonyl fluoride covalent warhead



Hexapeptide Boc-Ala-Arg(Pbf)-Thr(*t*Bu)-K(*me*<sub>3</sub>)-Cys(S*t*Bu)-Thr(*t*Bu)-CONH-Rink amide resin (1 equiv) was prepared on Rink amide resin according to the procedures outlined in the **Iterative Fmoc-SPPS workflow**. The S-(*t*Bu) group was deprotected according to the protocols outlined in **Deprotection procedures**. The resin was washed with DMF (5 × 3 mL) and treated with a solution of DIPEA (2.5 equiv) and 4-(bromomethyl)benzenesulfonyl fluoride (1.2 equiv) in DMF and agitated at room temperature for 30 minutes or until consumption of starting material was confirmed (monitored by LCMS analysis). The liquid was discarded, and the resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL), DMF (5 × 3 mL) and DCM (5 × 3 mL). The peptide was cleaved and purified according to the procedures outlined in the **Iterative Fmoc-SPPS workflow** to afford sulfonyl fluoride peptide **29**.

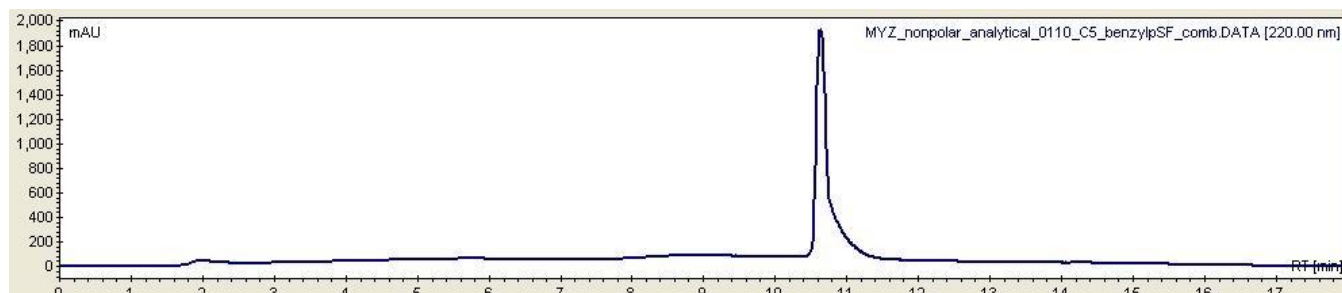
# Compound 29



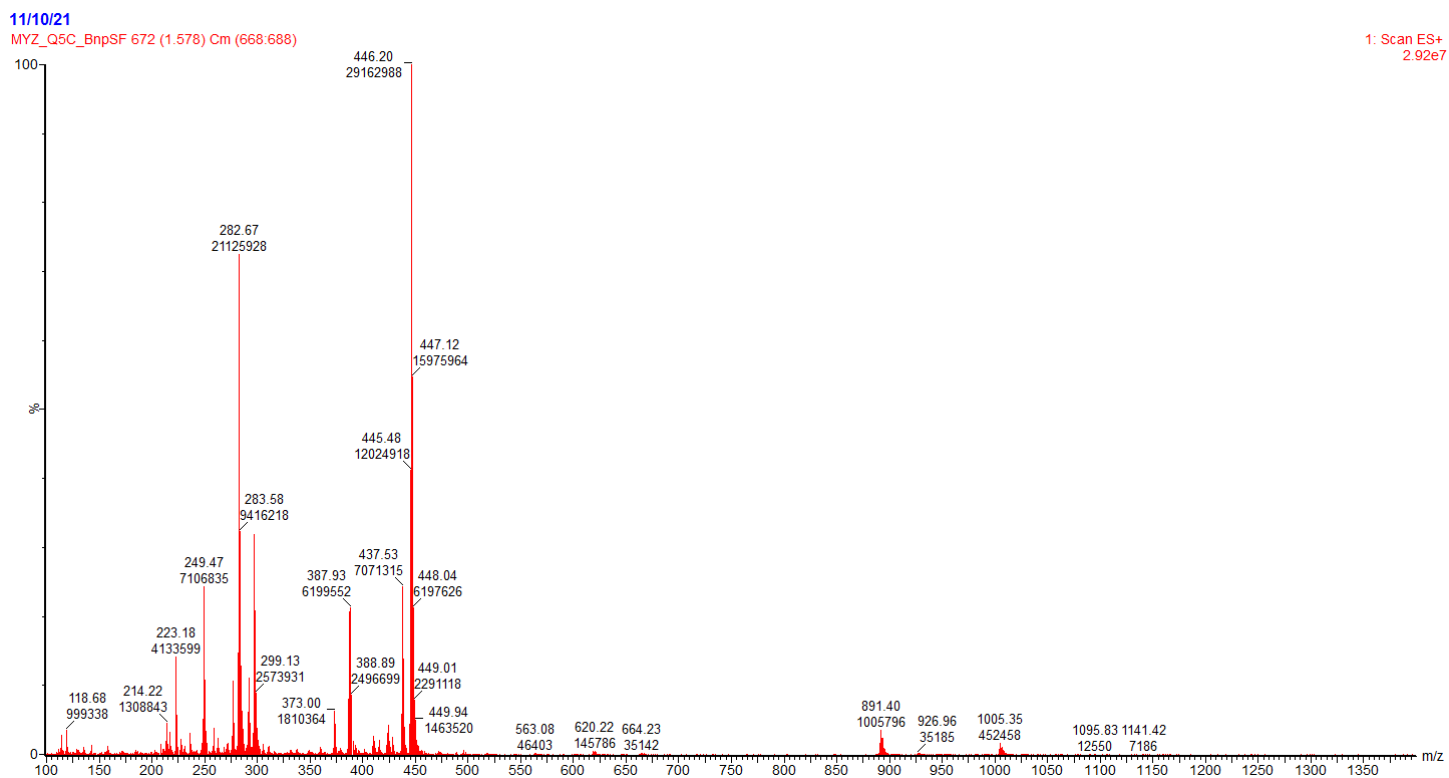
Yield: 14%

Exact mass: 894.43 [C<sub>36</sub>H<sub>65</sub>FN<sub>11</sub>O<sub>10</sub>S<sub>2</sub>]<sup>3+</sup>

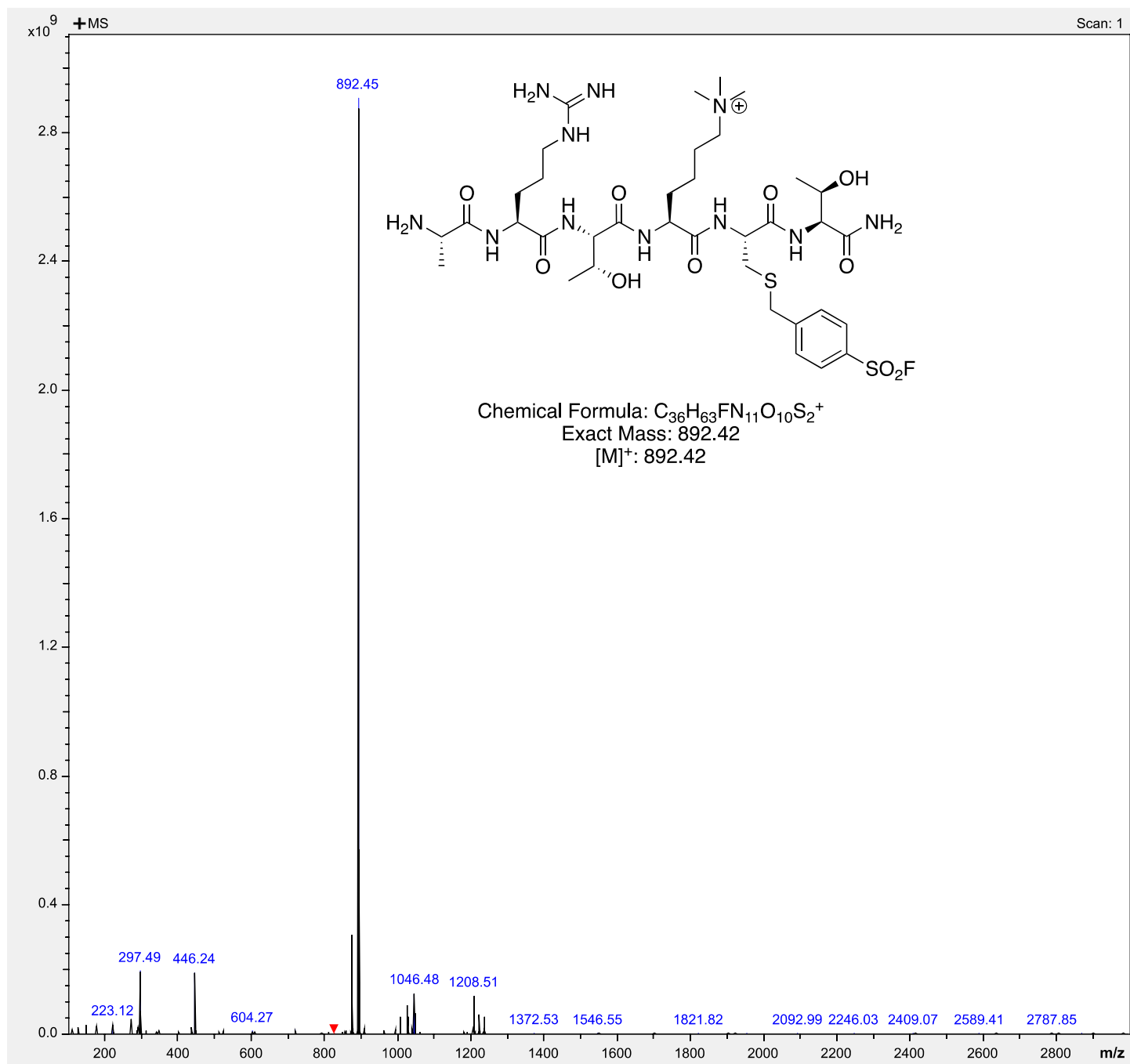
HPLC trace (Method 2):



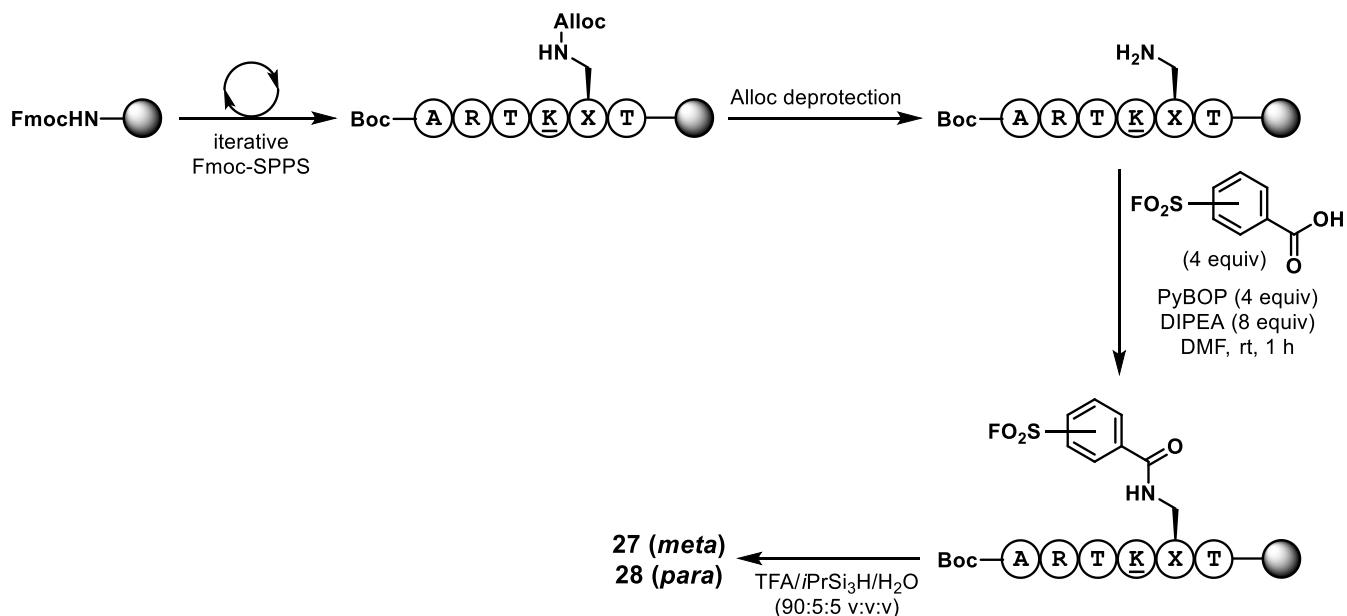
## LCMS



## MALDI



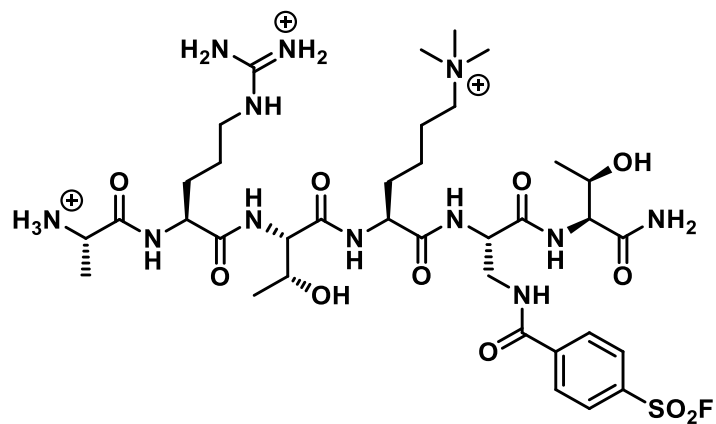
## Installation of the fluorosulfonylbenzamide covalent warhead



Hexapeptide Boc-Ala-Arg(Pbf)-Thr(*t*Bu)-K(*me*<sub>3</sub>)-Dap(Alloc)-Thr(*t*Bu)-CONH-Rink amide resin (1 equiv) was prepared on Rink amide resin according to the procedures outlined in the **Iterative Fmoc-SPPS workflow**. The Alloc group was deprotected according to the protocols outlined in **Deprotection procedures**. The resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL), DMF (5 × 3 mL). The Alloc-deprotected resin was treated with a solution of *para*, or *meta*-fluorosulfonylbenzoic acid (4 equiv), PyBOP (4 equiv) and DIPEA (8 equiv) in DMF and agitated at room temperature for one hour or until consumption of starting material was confirmed (monitored by LCMS analysis), at which time the liquid was discarded and the resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL), DMF (5 × 3 mL) and DCM (5 × 3 mL). The peptide was cleaved from resin and purified according to the procedures outlined in the **Iterative Fmoc-SPPS workflow** to afford **27** and **28**.



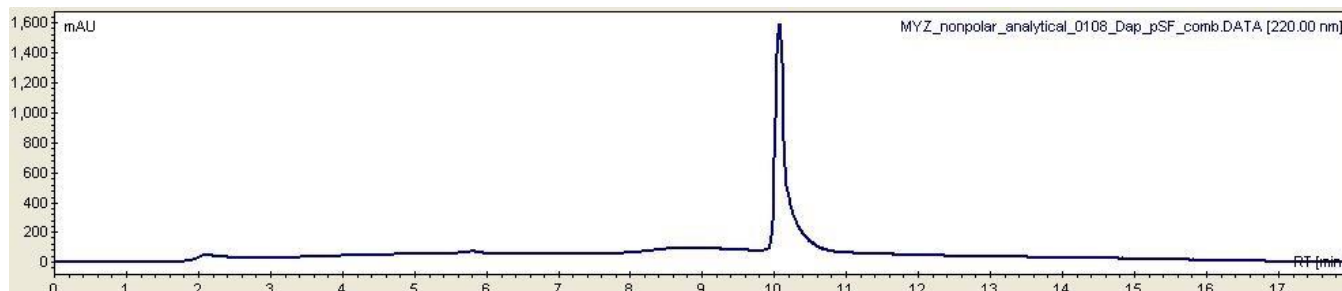
# Compound 28



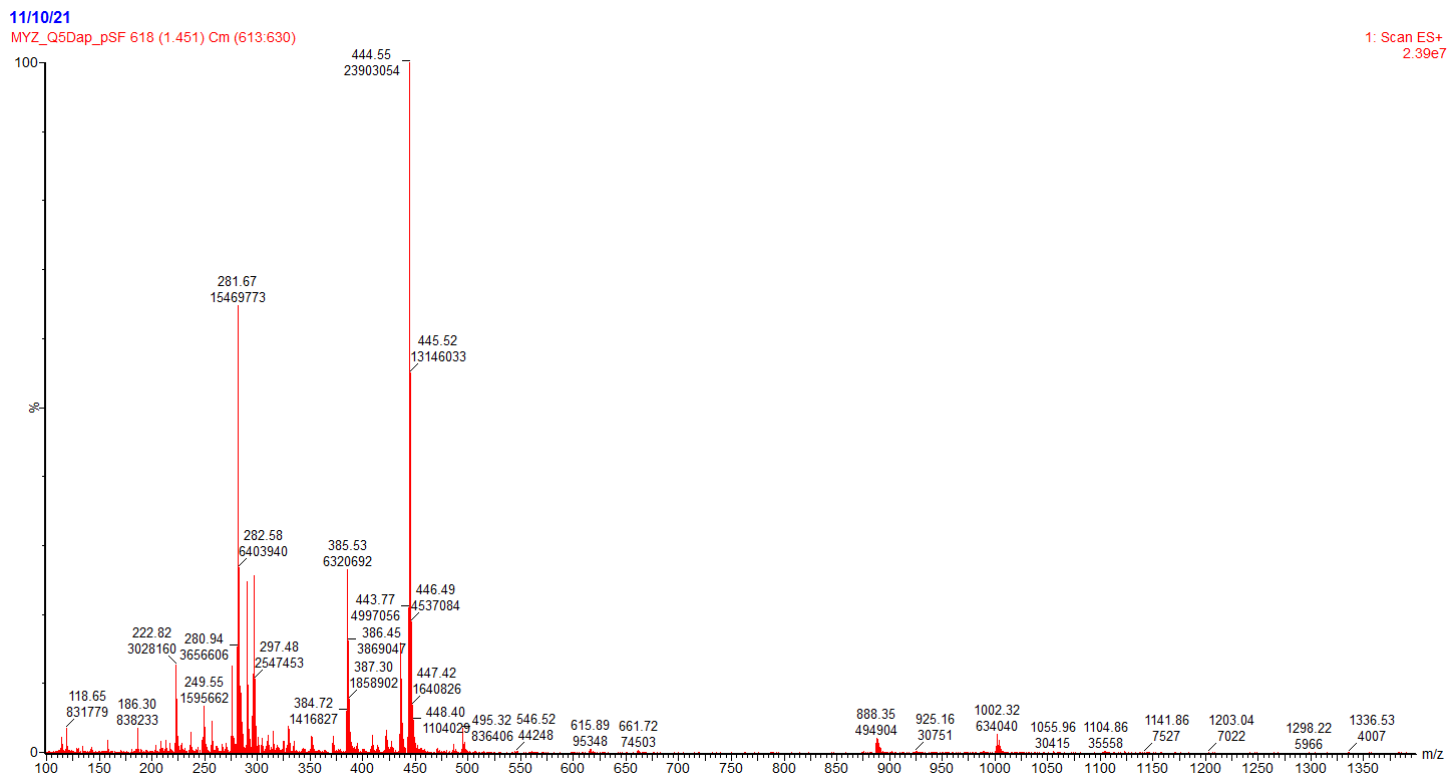
Yield: 20%

Exact mass: 891.45 [C<sub>36</sub>H<sub>64</sub>FN<sub>12</sub>O<sub>11</sub>S]<sup>3+</sup>

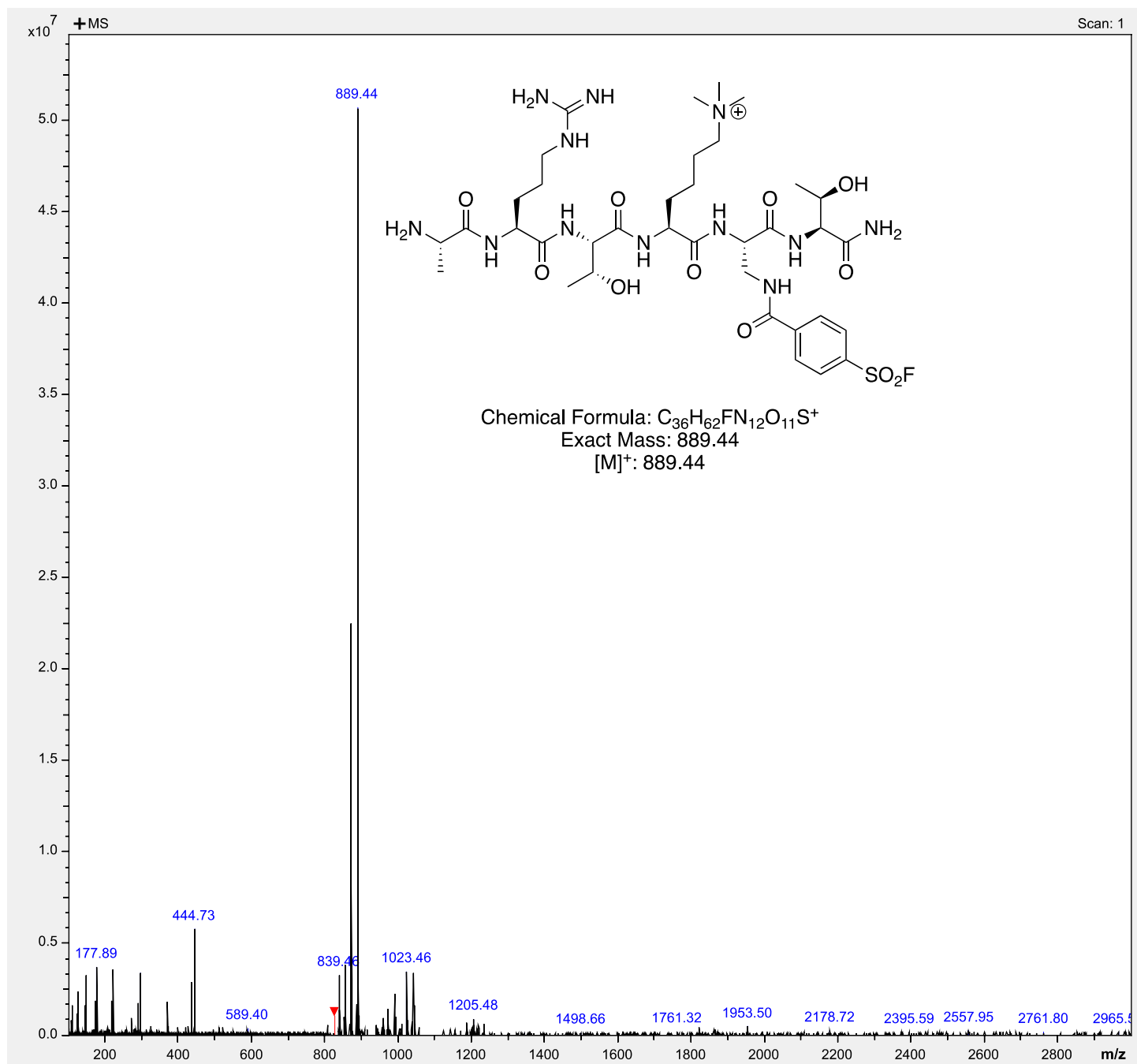
HPLC trace (Method 2):



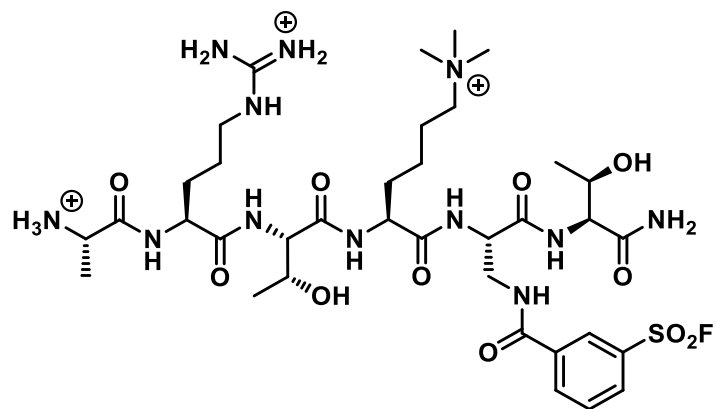
## LCMS



## MALDI



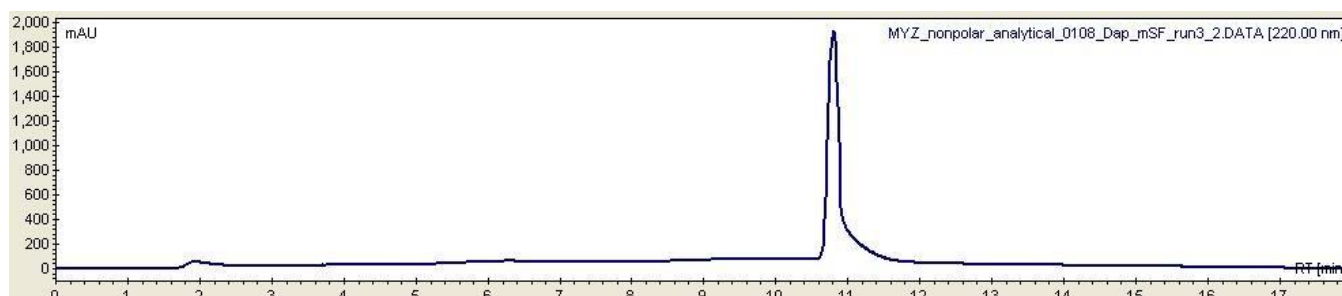
# Compound 27



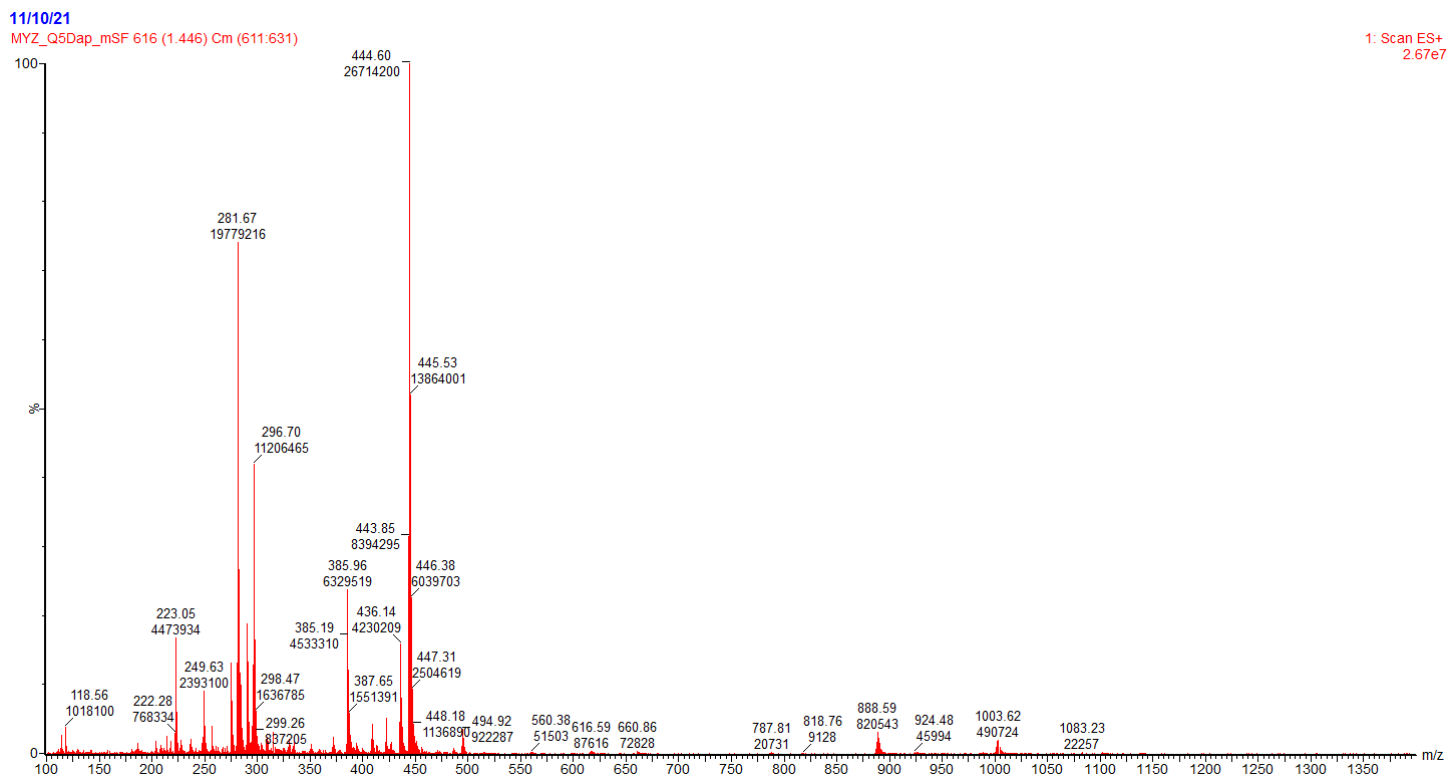
Yield: 10%

Exact mass: 891.45 [C<sub>36</sub>H<sub>64</sub>FN<sub>12</sub>O<sub>11</sub>S]<sup>3+</sup>

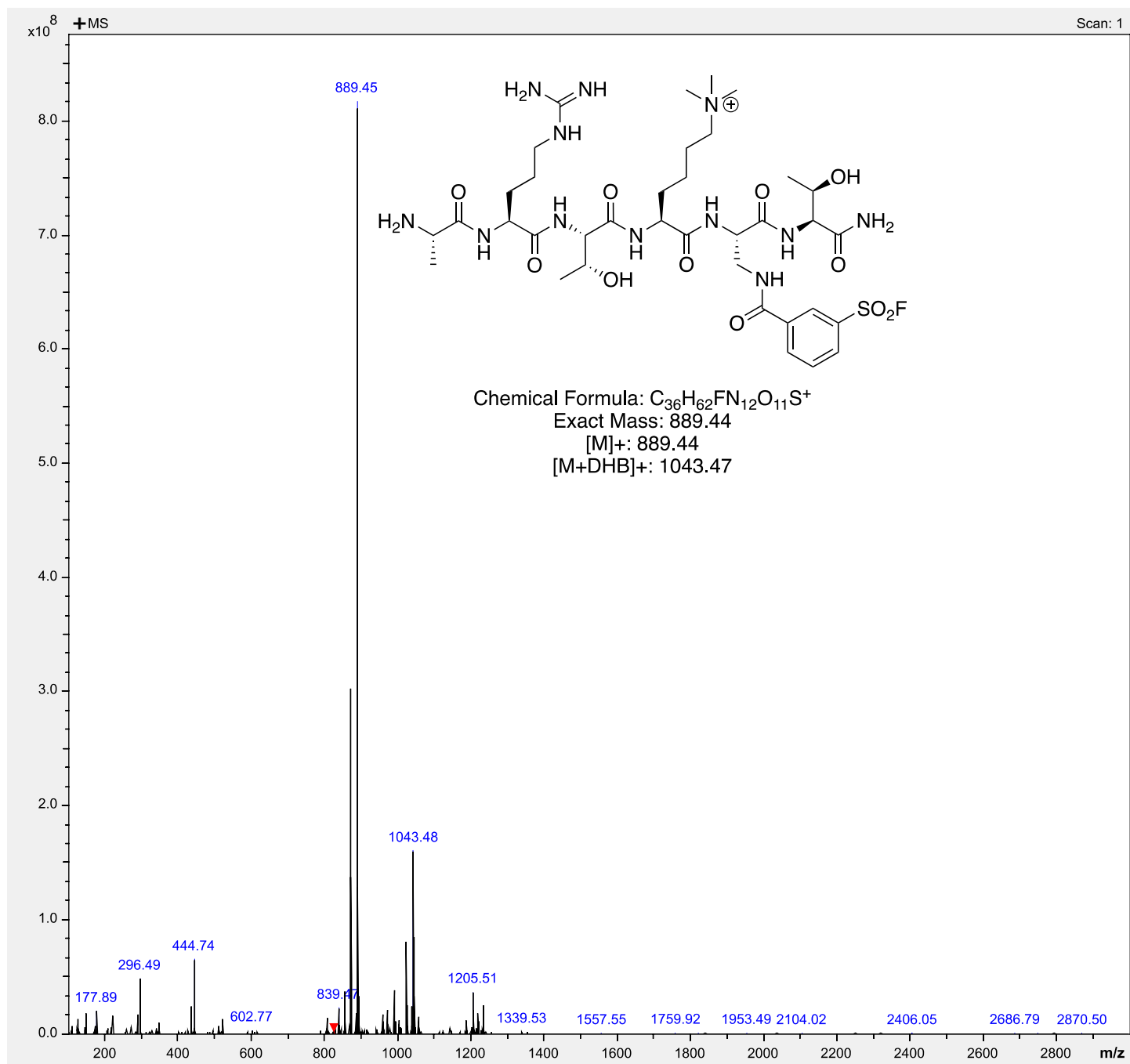
HPLC trace (Method 2):



## LCMS

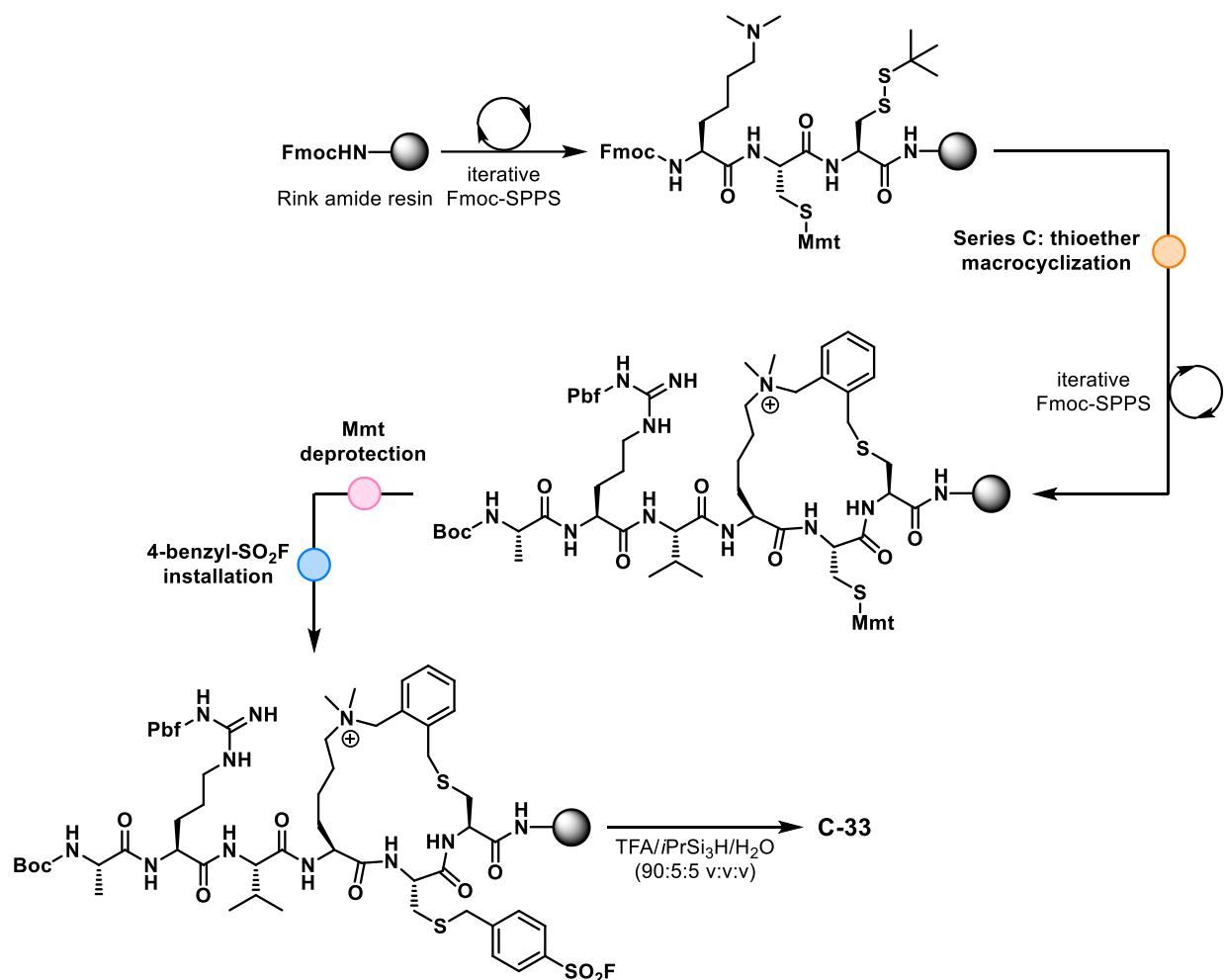


# MALDI



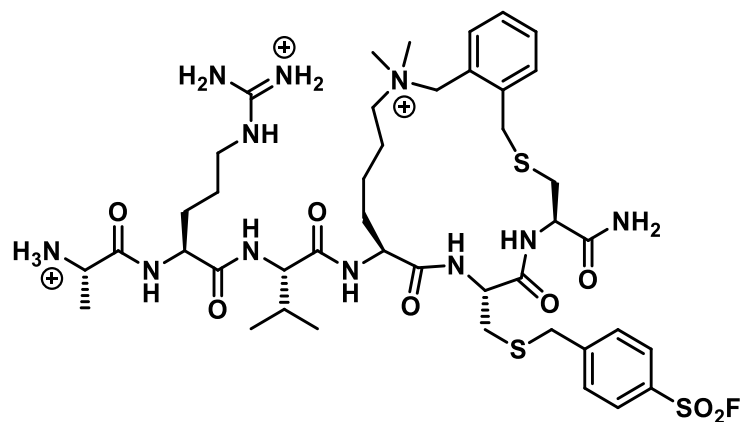
## Synthesis of covalent cyclopeptides

### Synthesis of covalent thioether cyclopeptide C-33



Tripeptide Fmoc-Lys(me<sub>2</sub>)-Cys(Mmt)-Cys(S*t*Bu)-CONH-Rink amide resin (1 equiv) was prepared on Rink amide resin according to the procedures outlined in the **Iterative Fmoc-SPPS workflow**. The tripeptide intermediate was subjected to **Series C: thioether macrocyclization** conditions and elongated using the procedures outlined in the **Iterative Fmoc-SPPS workflow**. The Mmt group was cleaved using the procedures described in **Deprotection procedures**, and the 4-benzylsulfonyl fluoride functionality was introduced according to the protocols outlined in **Installation of the 4-benzylsulfonyl fluoride covalent warhead**. The resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL), DMF (5 × 3 mL) and DCM (5 × 3 mL). The peptide was cleaved from resin and purified according to the procedures outlined in the **Iterative Fmoc-SPPS workflow** to afford **C-33**.

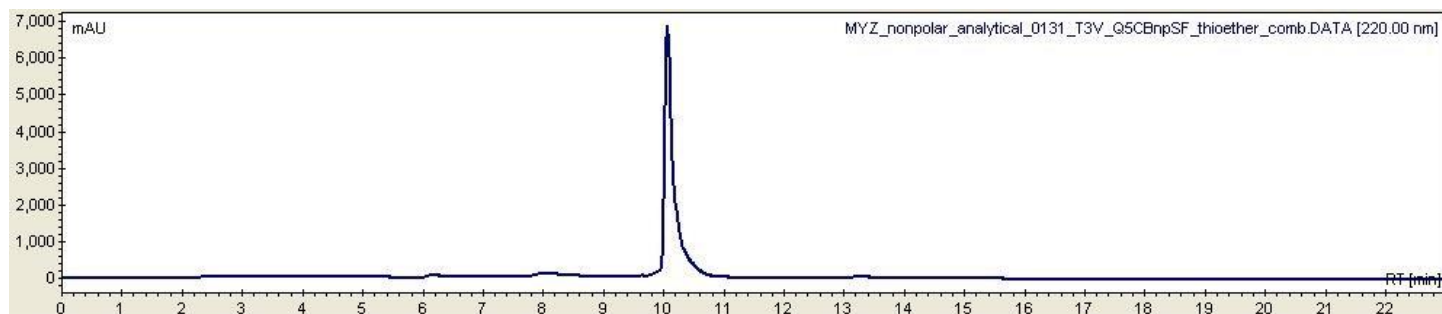
# Compound C-33



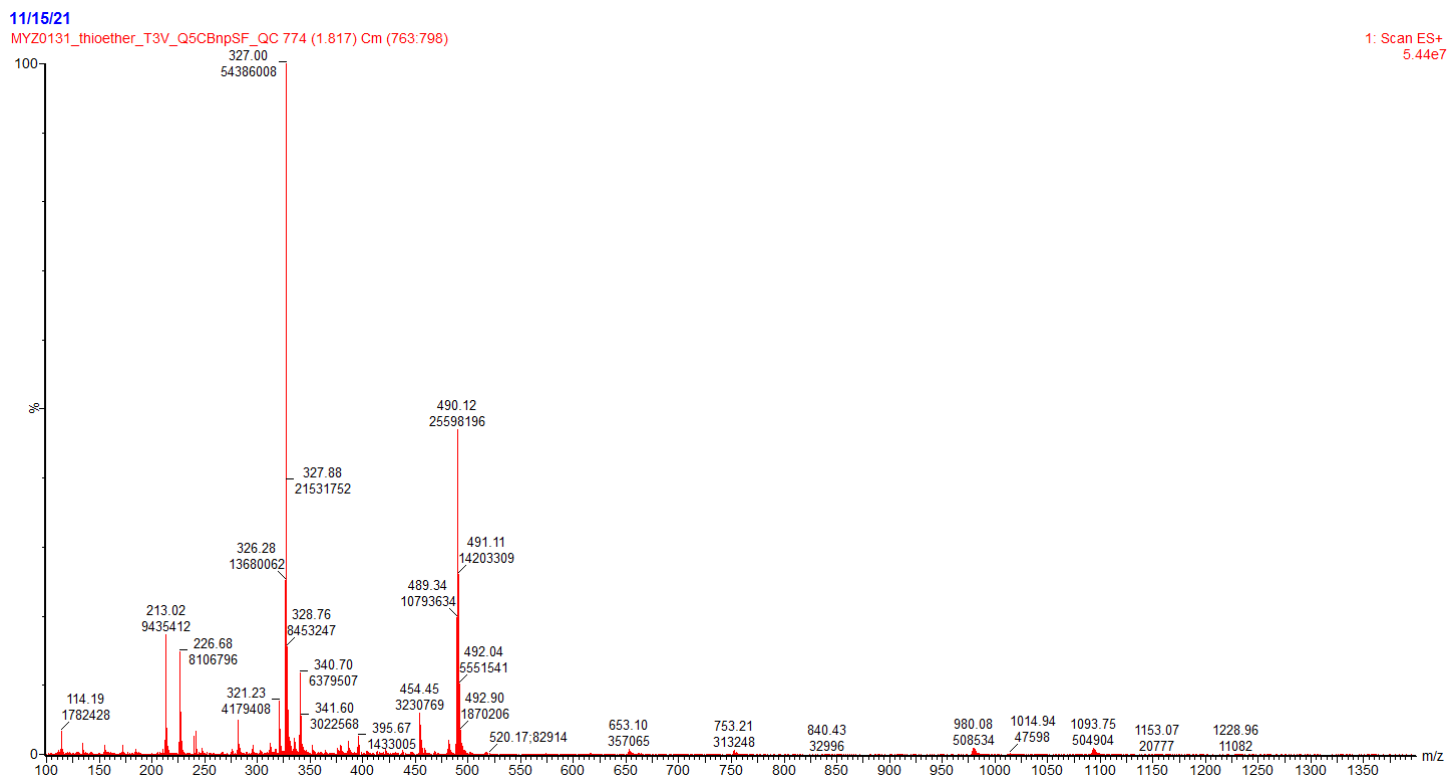
Yield: 6%

Exact mass: 982.45 [C<sub>43</sub>H<sub>69</sub>FN<sub>11</sub>O<sub>8</sub>S<sub>3</sub>]<sup>3+</sup>

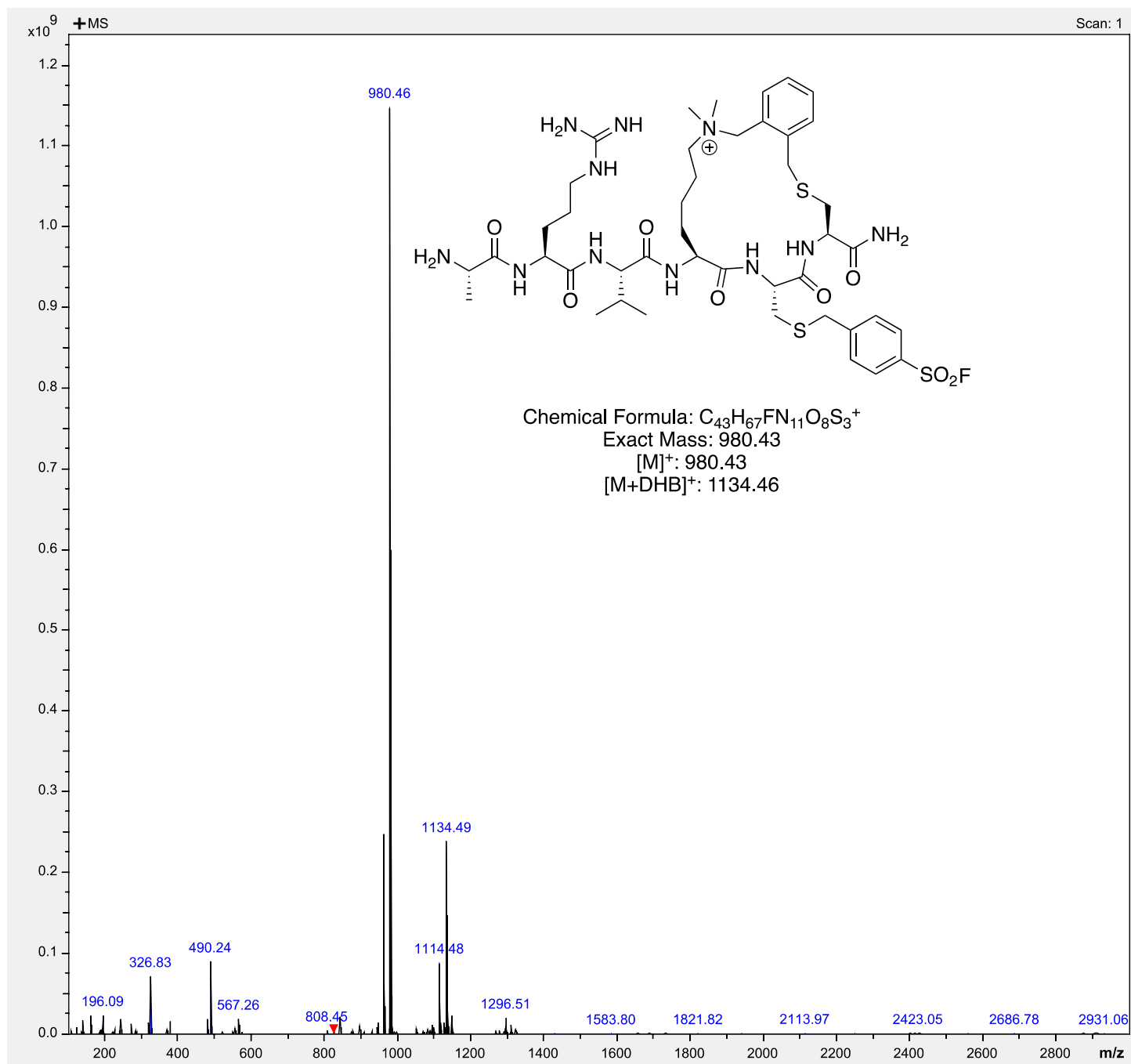
HPLC trace (Method 3):



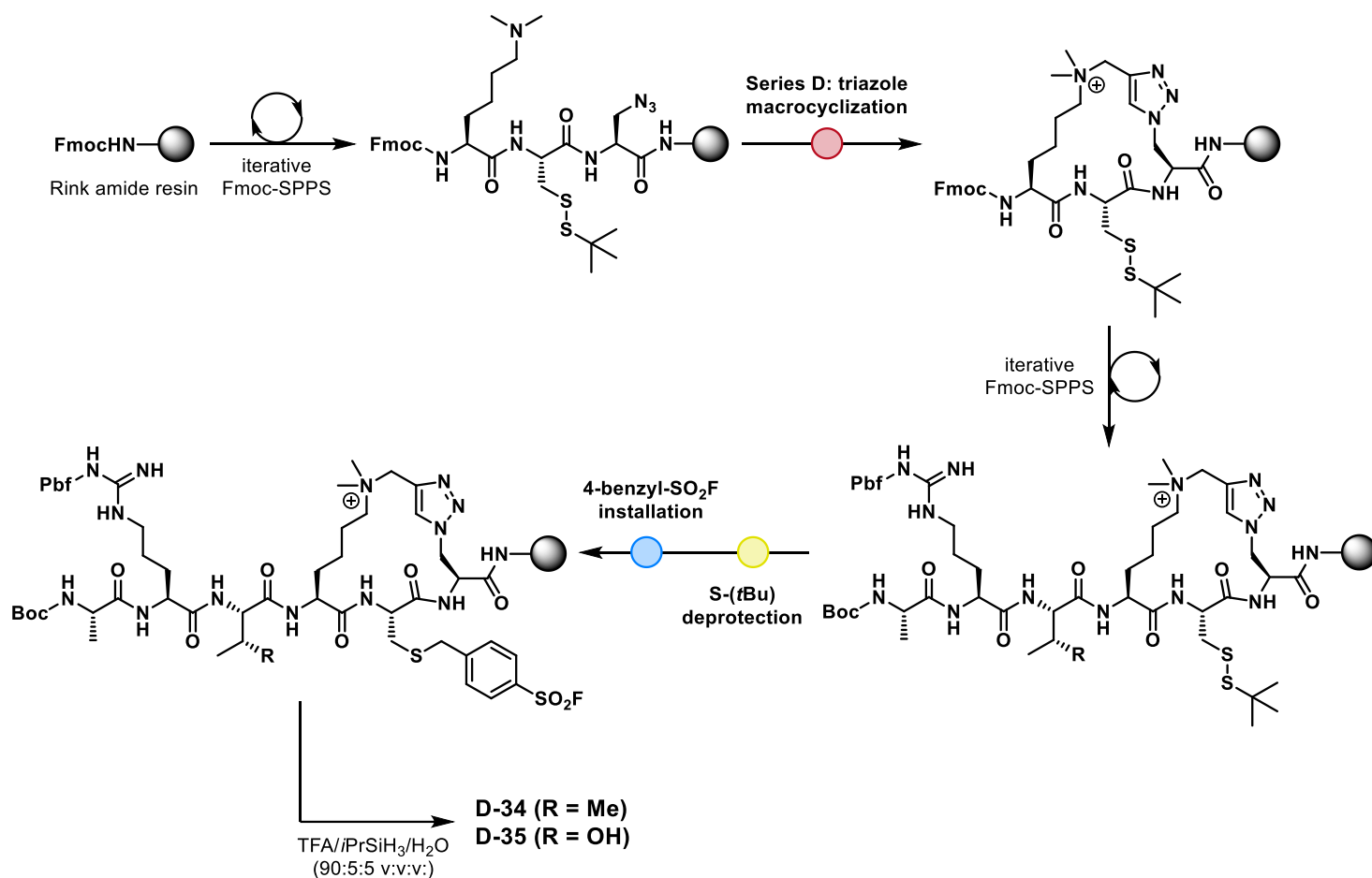
## LCMS



# MALDI



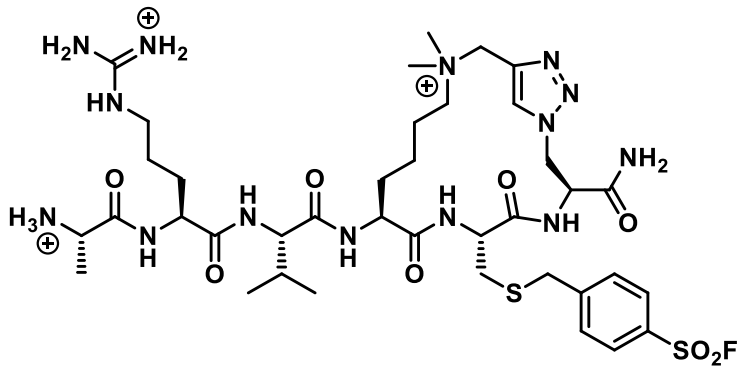
## Synthesis of covalent thioether peptide D-34 and D-35



Tripeptide Fmoc-Lys(me<sub>2</sub>)-Cys(S*t*Bu)-Dap(N<sub>3</sub>)-CONH-Rink amide resin (1 equiv) was prepared on Rink amide resin according to the procedures outlined in the **Iterative Fmoc-SPPS workflow**. The tripeptide intermediate was subjected to **Series D: triazole macrocyclization** conditions and elongated using the procedures outlined in the **Iterative Fmoc-SPPS workflow**. The S-(*t*Bu) group was cleaved using the procedures described in **Deprotection procedures**, and the 4-benzylsulfonyl fluoride functionality was introduced according to the protocols outlined in **Installation of the 4-benzylsulfonyl fluoride covalent warhead**. The resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL), DMF (5 × 3 mL) and DCM (5 × 3 mL). The peptide was cleaved from resin and purified according to the procedures outlined in the **Iterative Fmoc-SPPS workflow** to afford **D-34** and **D-35**.



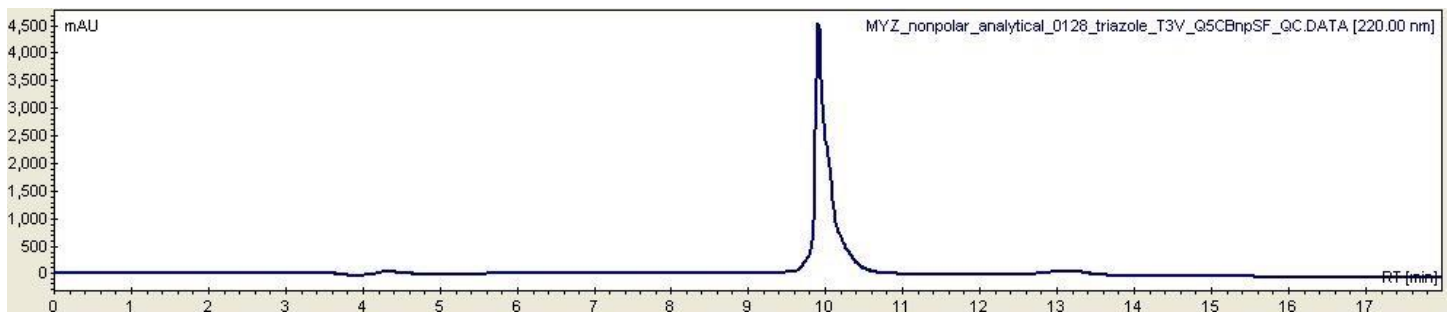
# Compound D-34



Yield: 2%

Exact mass: 927.44 [C<sub>38</sub>H<sub>64</sub>FN<sub>14</sub>O<sub>8</sub>S<sub>2</sub>]<sup>3+</sup>

HPLC trace (Method 2):

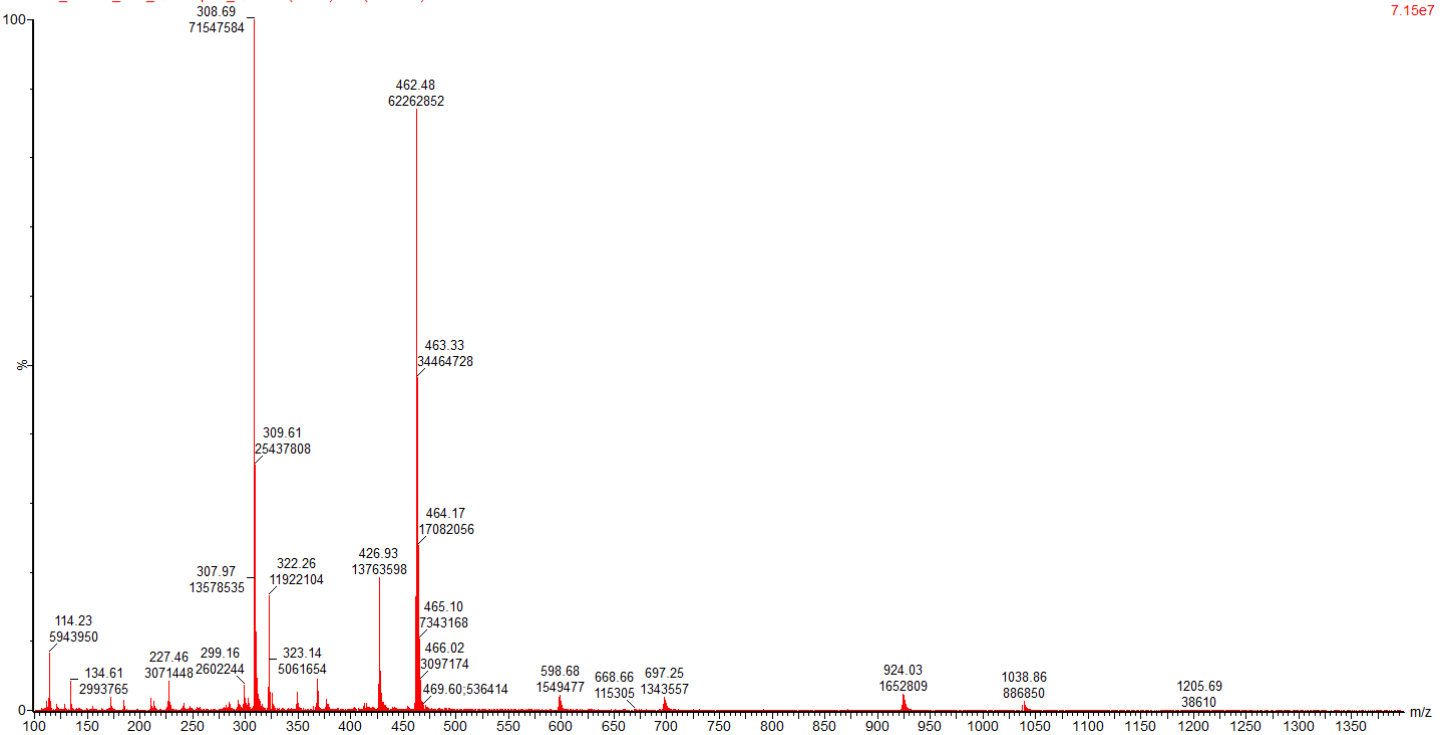


## LCMS

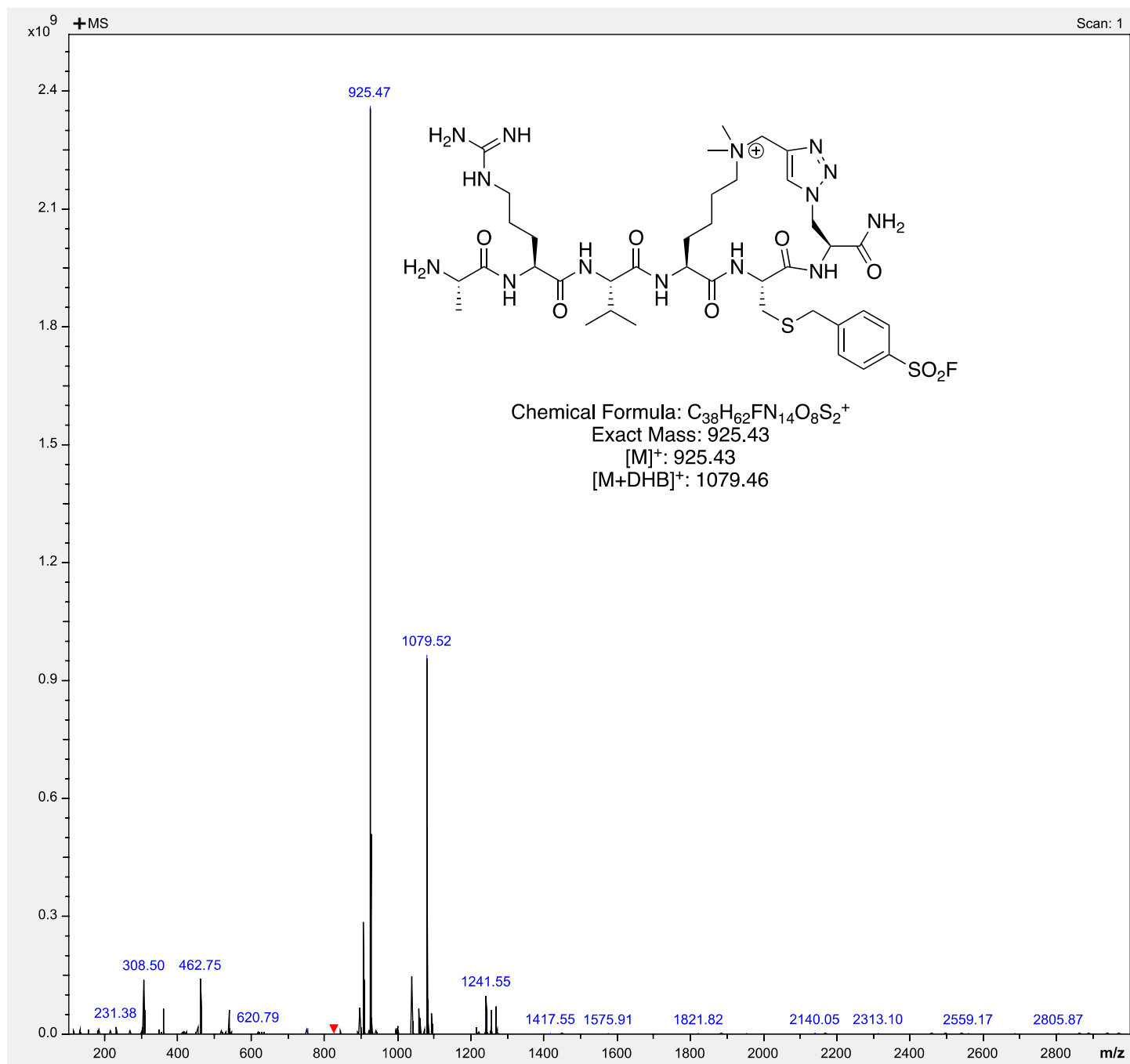
11/15/21

MYZ0128\_triazole\_T3V\_Q5CBnpSF\_QC 723 (1.698) Cm (707:741)

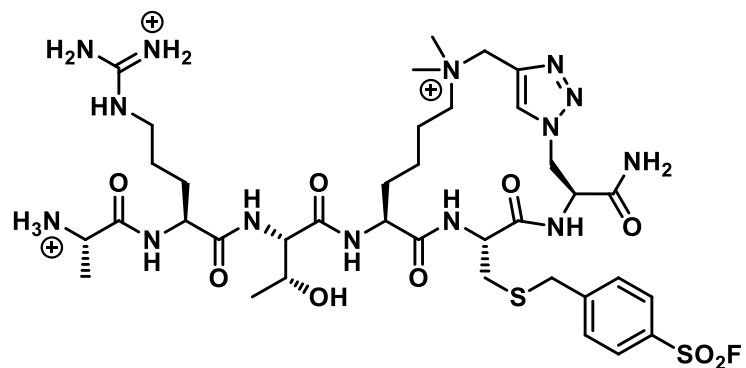
1: Scan ES+  
7.15e7



## MALDI



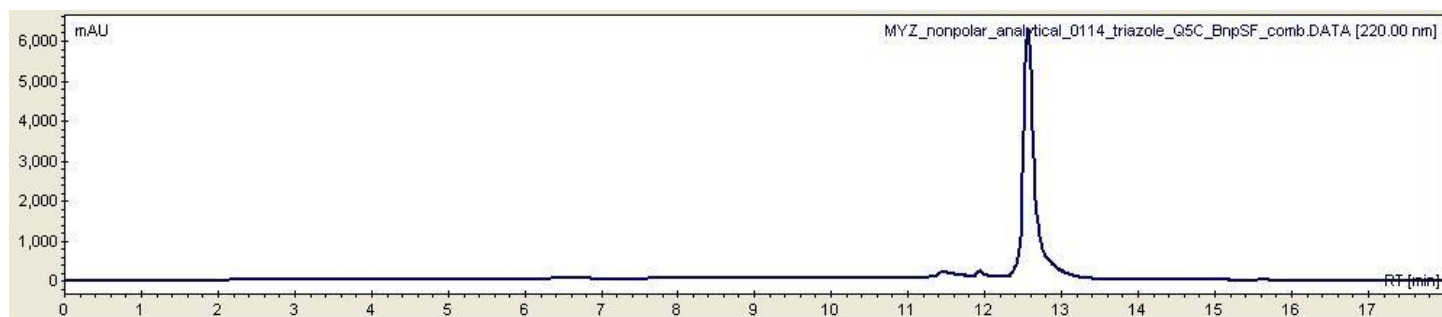
## Compound D-35



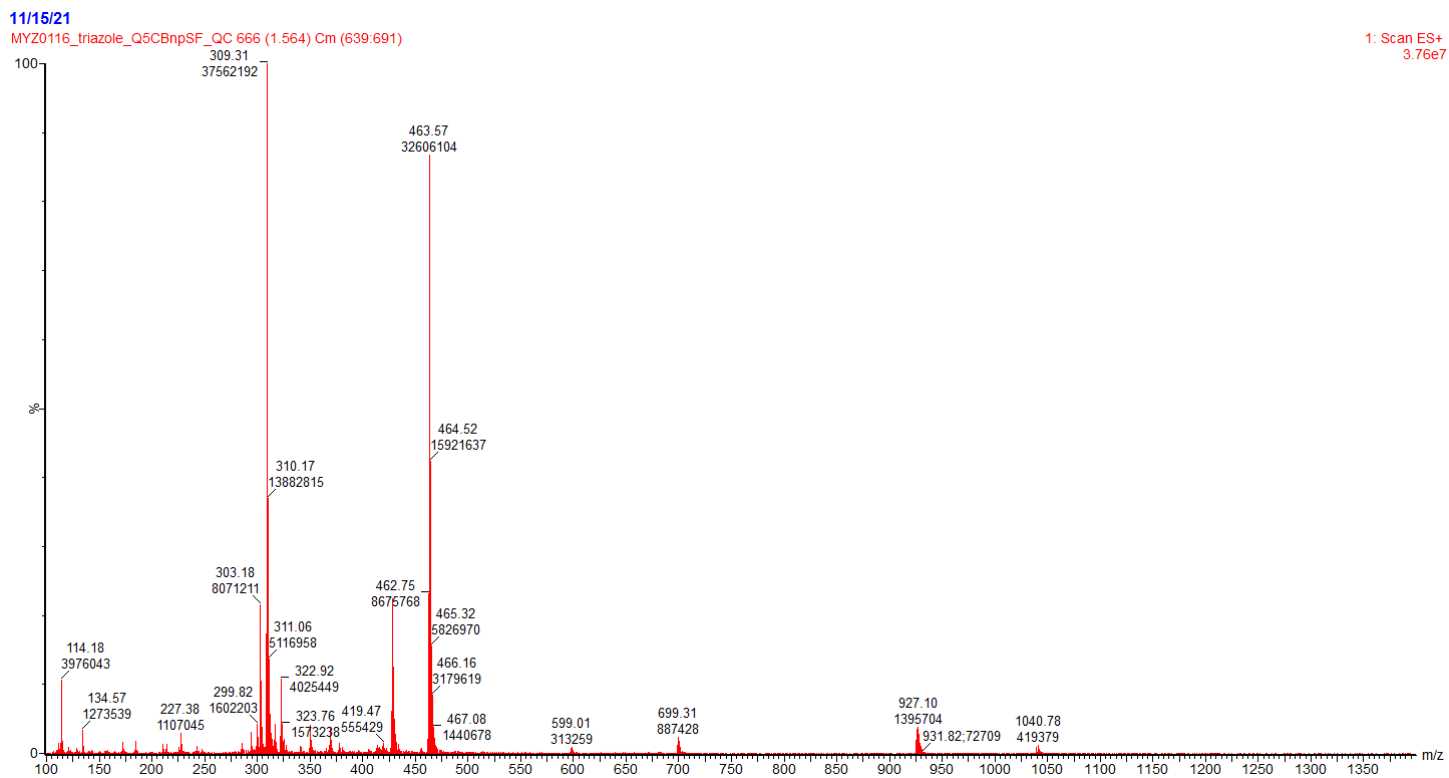
Yield: 4%

Exact mass: 929.42 [C<sub>37</sub>H<sub>62</sub>FN<sub>14</sub>O<sub>9</sub>S<sub>2</sub>]<sup>3+</sup>

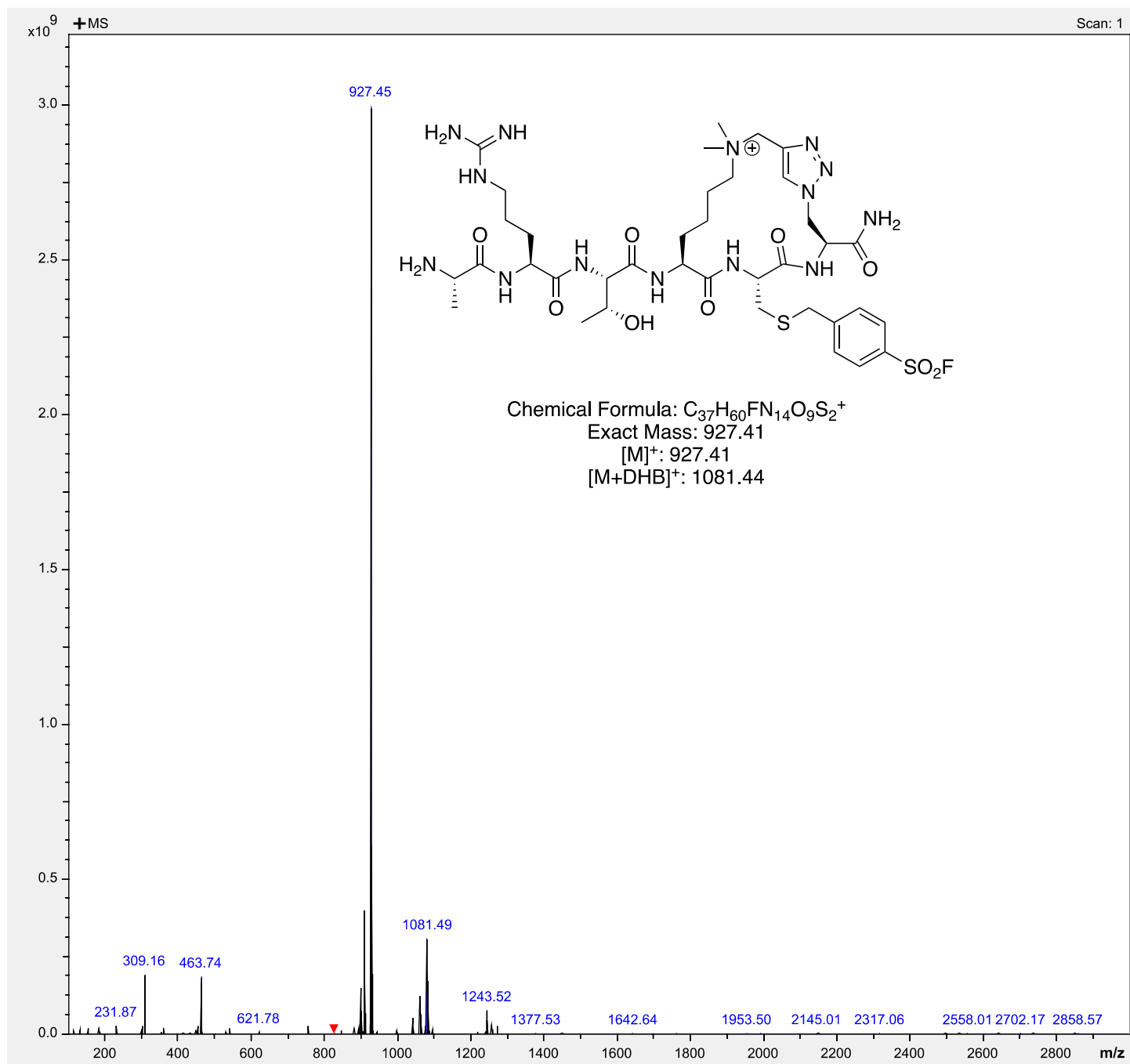
HPLC trace (Method 2):



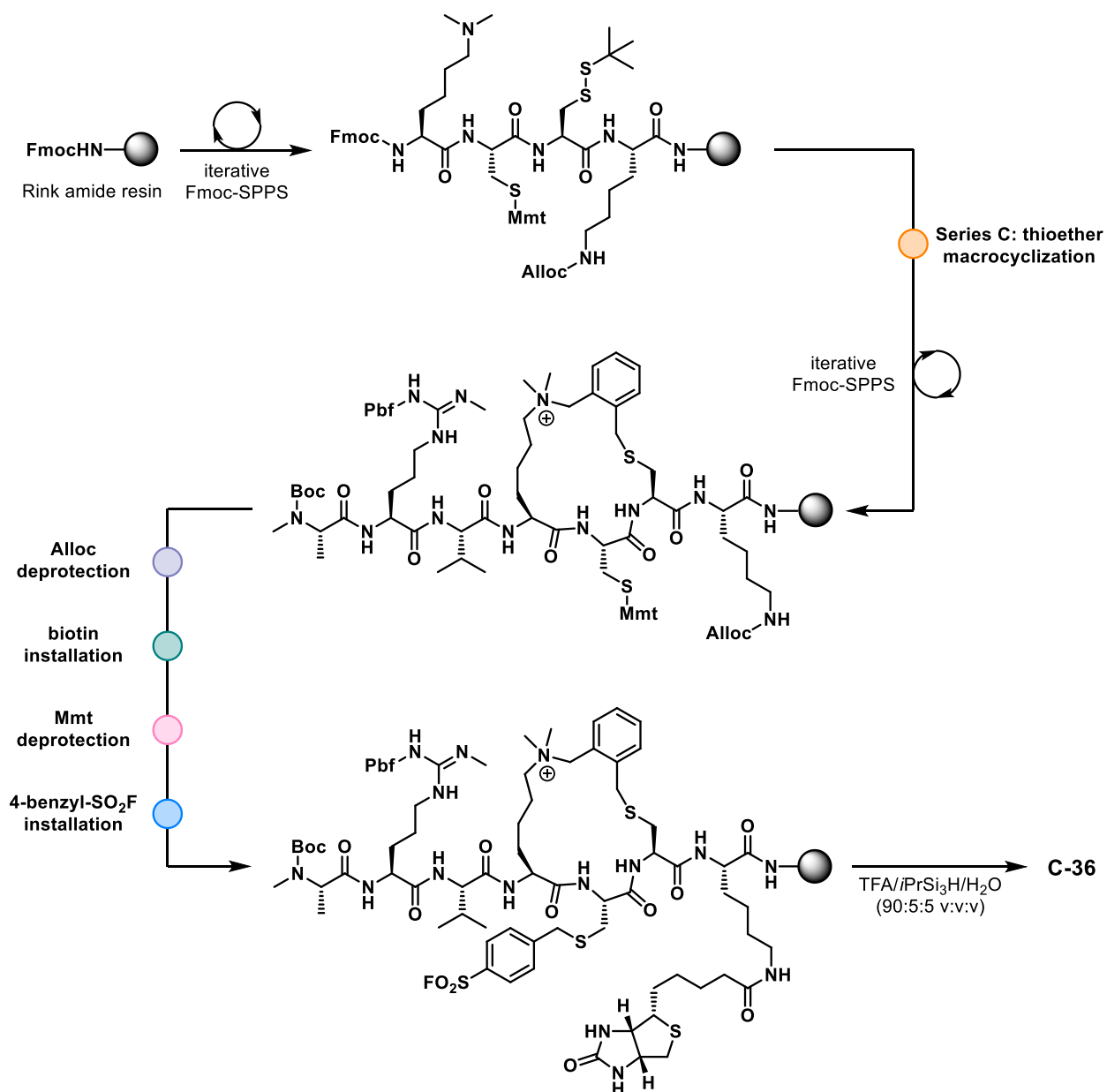
## LCMS



# MALDI

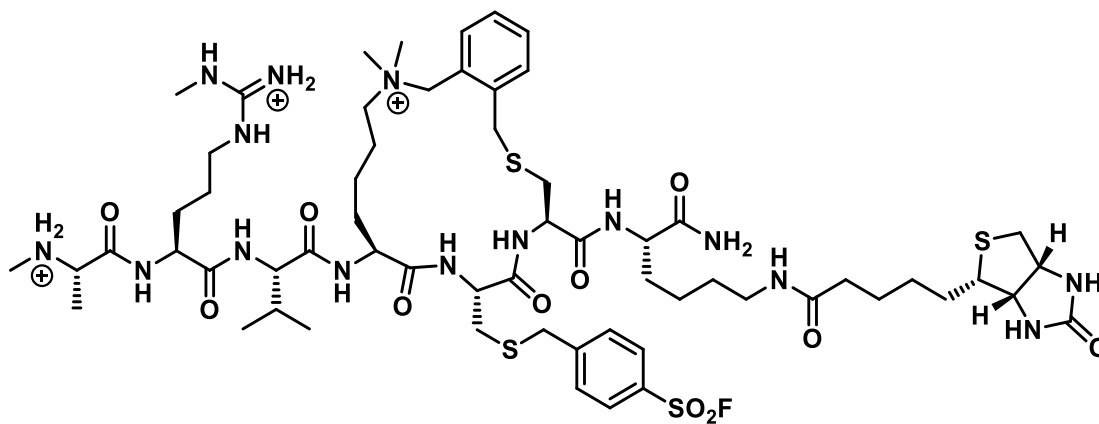


## Synthesis of biotinylated covalent thioether cyclopeptide C-36



Tetrapeptide Fmoc-Lys(me<sub>2</sub>)-Cys(Mmt)-Cys(S*t*Bu)-Lys(Alloc)-CONH-Rink amide resin (1 equiv) was prepared on Rink amide resin according to the procedures outlined in the **Iterative Fmoc-SPPS workflow**. The tetrapeptide intermediate was subjected to **Series C: thioether macrocyclization** conditions and elongated using the procedures outlined in the **Iterative Fmoc-SPPS workflow**. The Alloc group was removed using the procedures outlined in **Deprotection procedures**. The resin was treated with a solution of biotin (4 equiv), PyBOP (4 equiv) and DIPEA (8 equiv) in DMF and agitated at room temperature for one hour. The liquid was expelled from the reaction vessel and the resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL), and DMF (5 × 3 mL). The Mmt group was cleaved using the procedures described in **Deprotection procedures**, and the 4-benzylsulfonyl fluoride functionality was introduced according to the protocols outlined in **Installation of the 4-benzylsulfonyl fluoride covalent warhead**. The resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL), DMF (5 × 3 mL) and DCM (5 × 3 mL). The peptide was cleaved from resin and purified according to the procedures outlined in the **Iterative Fmoc-SPPS workflow** to afford **C-36**.

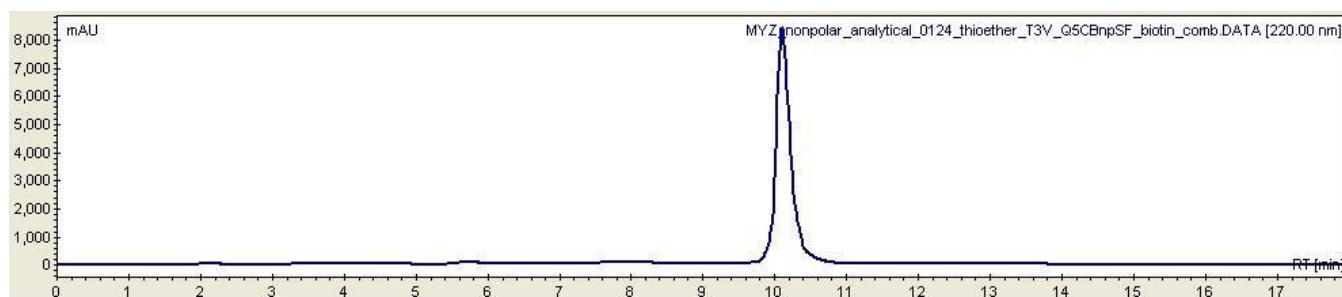
# Compound C-36



Yield: 9%

Exact mass: 1364.65 [C<sub>61</sub>H<sub>99</sub>FN<sub>15</sub>O<sub>11</sub>S<sub>4</sub>]<sup>3+</sup>

HPLC trace (Method 2):

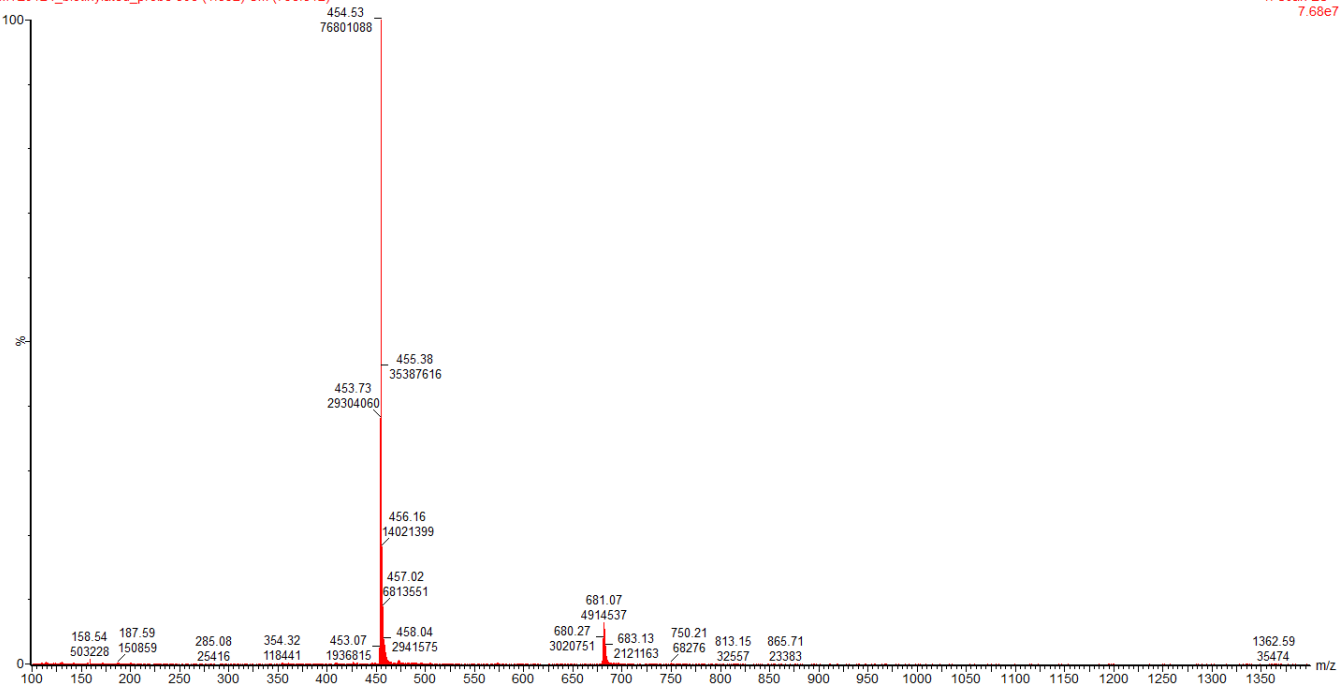


## LCMS

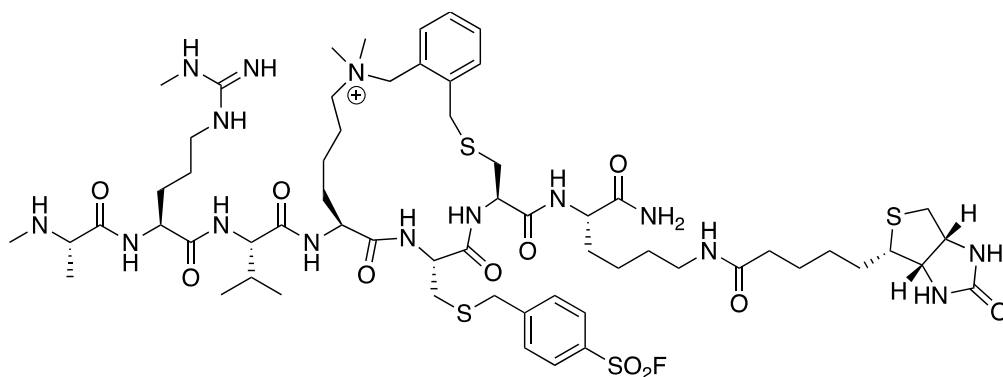
11/10/21

MYZ0124\_biotinylated\_probe 806 (1.892) Cm (796.812)

1: Scan ES+  
7.68e7



# MALDI

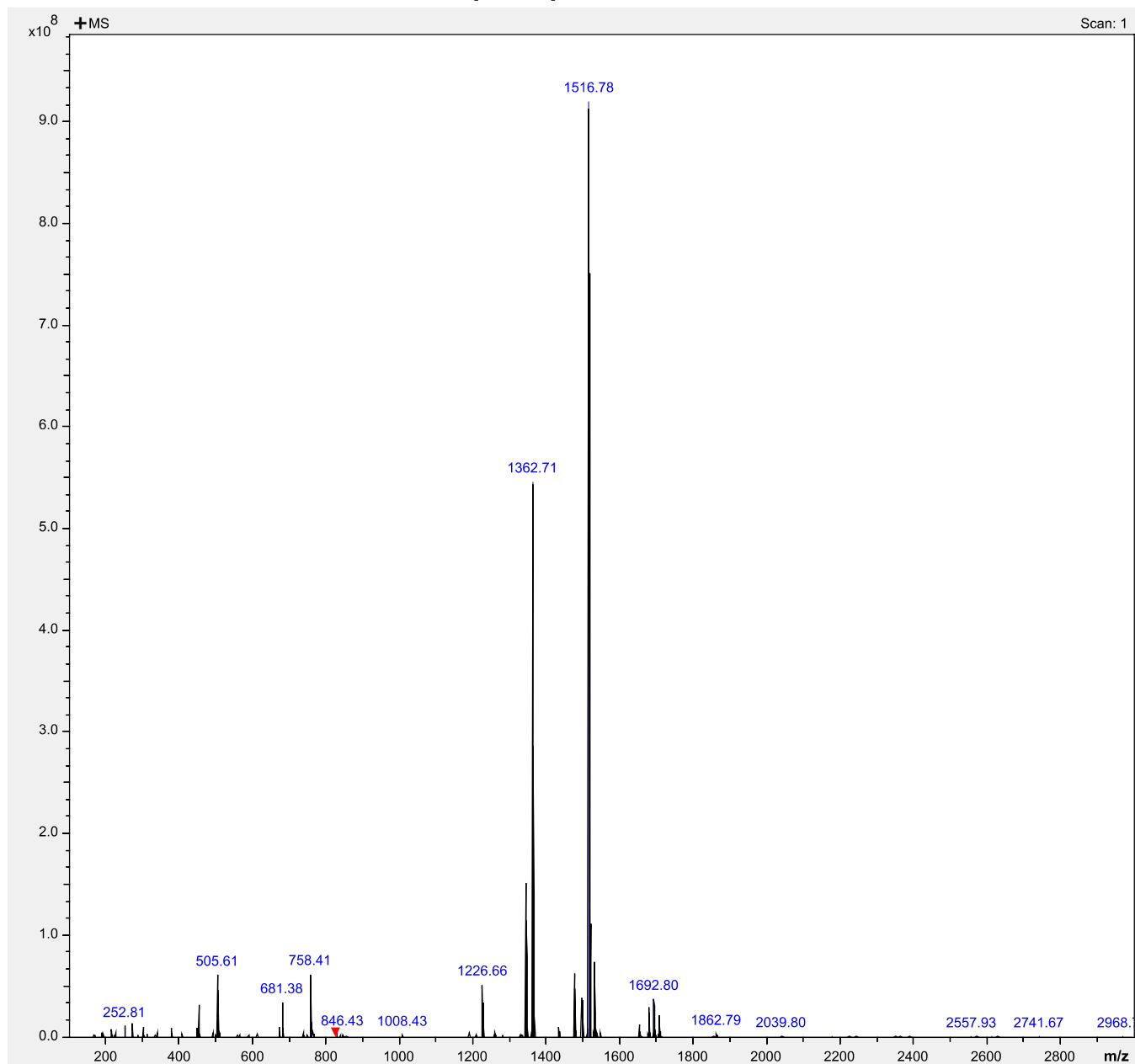


Chemical Formula: C<sub>61</sub>H<sub>97</sub>FN<sub>15</sub>O<sub>11</sub>S<sub>4</sub><sup>+</sup>

Exact Mass: 1362.64

[M]<sup>+</sup>: 1362.64

[M+DHB]<sup>+</sup>: 1516.67



## **Expression and purification of His<sub>6</sub>-MBP-PHD3, WT and mutant PHD3**

Wild-type His<sub>6</sub>-MBP-PHD3 and mutant PHD3 constructs (K1620A, K1622A and K1620A/K1622A) were cloned into a pET His<sub>6</sub> MBP TEV vector and expressed in BL21(DE3) *E. coli* cells. Expression and purification of WT and mutant PHD3 followed the same protocol. Cells were induced with 0.3 mM IPTG and grown at 18 °C overnight. Collected pellets were flash-frozen in liquid nitrogen and stored at -80 °C until further use. The pellets were suspended in lysis buffer (20 mM Tris pH 8.0, 0.5 M NaCl, 10% glycerol, 2.5 mM BME, 50 μM ZnCl<sub>2</sub>, 1x Pierce™ Protease Inhibitor Tablet, EDTA-free), lysed by sonication and centrifuged. The clarified lysate was purified using immobilized metal ion affinity chromatography (HisTrap™ Fast Flow 5 mL), washed with high salt buffer (20 mM Tris pH 8.0, 1 M NaCl, 2.5 mM BME, 50 μM, 20 mM imidazole), followed by low salt buffer (20 mM Tris pH 8.0, 50 mM NaCl, 2.5 mM BME, 50 μM, 20 mM imidazole), and His-MBP-PHD3 was eluted with low salt buffer with supplemented with increasing imidazole concentration (20 → 200 mM imidazole concentration). Collected protein fractions were combined and dialyzed at 4 °C overnight into storage buffer (25 mM HEPES pH 7.5, 50 mM KCl, 5 mM BME, 50 μM ZnCl<sub>2</sub>). The sample was further purified by size-exclusion chromatography using a HiLoad 26/60 Superdex 75 gel filtration column in storage buffer. Eluted protein fractions were collected, concentrated, aliquoted and flash-frozen in liquid nitrogen.

During the dialysis step, cleavage of the MBP tag with TEV protease afforded the WT PHD3 construct and other related PHD3 mutants.



## Fluorescence polarization assays

The binding of His<sub>6</sub>-MBP-PHD3 to H3K4me3 WT and mutant peptides, as well as the binding of recombinant His<sub>6</sub>-MBP-PHD3 lysine mutant proteins to H3K4me3 WT peptide were measured using direct or competitive fluorescence-polarization (FP) assays.<sup>1</sup> For a direct FP binding assay, 10 nM of C-terminal 5FAM-labelled H3K4me3 10mer peptide was incubated at room temperature for 30 min with varying concentrations of recombinant His<sub>6</sub>-MBP-PHD3 protein. Results from direct FP assays were fitted to **equation 1** to deduce  $K_d$  values.

$$\text{equation 1: } FP_{\text{obs}} = \frac{FP_{\text{max}}[\text{PHD3}] + FP_{\text{min}}K_d}{K_d + [\text{PHD3}]}$$

For competitive FP binding assays, 100 nM of His<sub>6</sub>-MBP-PHD3 was incubated with 10 nM of C-terminal 5FAM-labelled H3K4me3 10mer peptide, where varying concentrations of mutant peptides were added as competitors. Results from competitive FP assays were fitted to **equation 2** to deduce  $K_i$  values for each mutant competitor peptide.

$$\text{equation 2: } FP_{\text{obs}} = \frac{K_i(FP_{\text{max}}[\text{PHD3}] + FP_{\text{min}}K_d) + FP_{\text{min}}K_d[I]}{K_i(K_d + [\text{PHD3}]) + K_d[I]}$$

$FP_{\text{obs}}$  is the observed fluorescence polarization,  $FP_{\text{max}}$  is the maximum FP value,  $FP_{\text{min}}$  is the minimum FP value,  $[\text{PHD3}]$  is the concentration of His<sub>6</sub>-MBP-PHD3,  $K_d$  is the dissociation constant,  $K_i$  is the inhibition constant of the competitor peptides and  $[I]$  is the concentration of the competitor peptides.

## **In vitro PHD3 labelling reactions**

All covalent labelling reactions were performed in 50 mM HEPES pH 7.5, 50 mM KCl. Wild type or mutant PHD3 (10  $\mu$ M) and covalent peptide (50  $\mu$ M) were incubated at 37 °C. At each time point, an aliquot was taken and analyzed by LC-MS. The ESI mass spectra were deconvoluted using MassEnt software.

## **Cell lysate labelling experiments**

Human embryonic kidney 293T cells were cultured in Dulbecco's modified eagle medium (DMEM), high glucose supplemented with 10% fetal bovine serum (FBS), 1% Pen-Strep at 37 °C in a humidified incubator with 5% CO<sub>2</sub> atmosphere. HEK293T cells were grown in a T150 flask to 30,000,000 cells, at which time cells were trypsinized, collected, washed with phosphate-buffered saline twice and flash-frozen in liquid nitrogen. Thawed cell pellets were treated with lysis buffer (50 mM HEPES pH 7.5, 50 mM KCl, 4x Pierce™ Protease Inhibitor EDTA-free). Protein concentration was normalized to 5 mgmL<sup>-1</sup> using the Bradford assay. Cell lysates were aliquoted and treated with biotinylated thioether probe **C-36** and/or recombinant His<sub>6</sub>-MBP-PHD3 protein ( $t = 4.5$  h at RT) at which time reactions were quenched by flash-freezing in liquid nitrogen. Thawed samples were treated with 4x SDS buffer and boiled for 10 minutes.

Samples were resolved by 4-20% SDS-PAGE gel (Bio-Rad) and were transferred to a 0.2  $\mu$ M nitrocellulose membrane using a Trans-Blot Turbo transfer system (Bio-Rad, 7-minute mixed MW protocol). The membrane was briefly stained with Ponceau S solution and washed with TBST buffer (3  $\times$  5 minutes). The membrane was blocked with 1x TBS 1% Casein Blocker (Bio-Rad, Cat. #1610782) for 1 h at RT and incubated with NeutrAvidin-HRP antibody (Thermo Scientific™ Pierce™ High Sensitivity NeutrAvidin™-HRP Prod # 31030, 1:10,000) for 1 h at RT. The membrane was subsequently washed 4  $\times$  10 minutes with TBST buffer and treated with chemiluminescent western blotting substrate (Amersham™ ECL™ Prime Western Blotting Detection System) for five minutes, then imaged with a Bio-Rad ChemiDoc Molecular Imager.

## **References**

- (1) Longbotham, J. E.; Chio, C. M.; Dharmarajan, V.; Trnka, M. J.; Torres, I. O.; Goswami, D.; Ruiz, K.; Burlingame, A. L.; Griffin, P. R.; Fujimori, D. G. Histone H3 Binding to the PHD1 Domain of Histone Demethylase KDM5A Enables Active Site Remodeling. *Nat. Commun.* **2019**, *10* (1), 94. <https://doi.org/10.1038/s41467-018-07829-z>.
- (2) Turner, R. A.; Oliver, A. G.; Lokey, R. S. Click Chemistry as a Macrocyclization Tool in the Solid-Phase Synthesis of Small Cyclic Peptides. *Org. Lett.* **2007**, *9* (24), 5011–5014. <https://doi.org/10.1021/ol702228u>.
- (3) Zhou, H.; Mukherjee, P.; Liu, R.; Evrard, E.; Wang, D.; Humphrey, J. M.; Butler, T. W.; Hoth, L. R.; Sperry, J. B.; Sakata, S. K.; Helal, C. J.; am Ende, C. W. Introduction of a Crystalline, Shelf-Stable Reagent for the Synthesis of Sulfur(VI) Fluorides. *Org. Lett.* **2018**, *20* (3), 812–815. <https://doi.org/10.1021/acs.orglett.7b03950>.