Supporting Information

The Design and Synthesis of Alanine-Rich α-Helical Peptides Constrained by an *S*,*S*-Tetrazine Photochemical Trigger: A Fragment Union Approach

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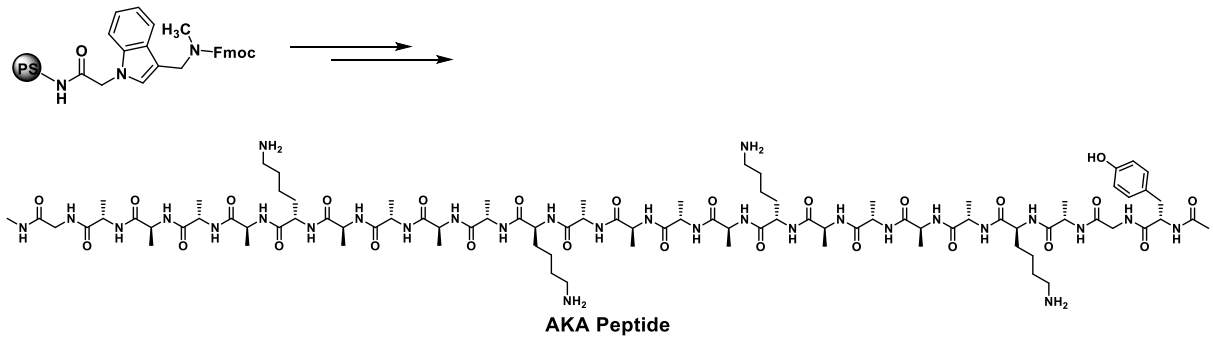
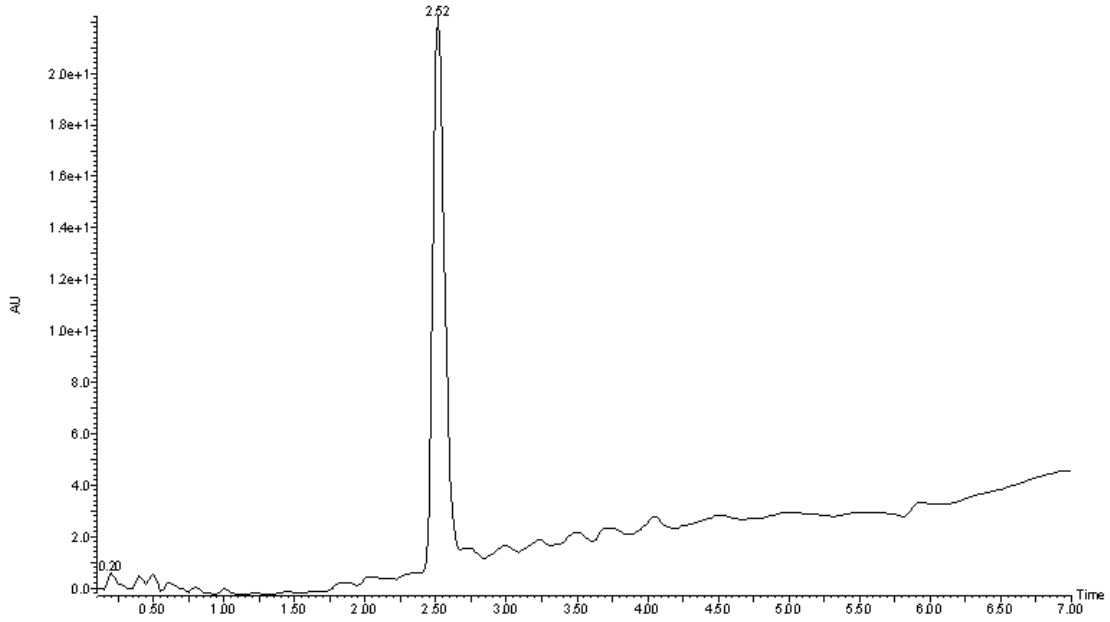
HPLC and HRMS Characterization of Peptides



LC-MS chromatogram of **AKA Peptide**:



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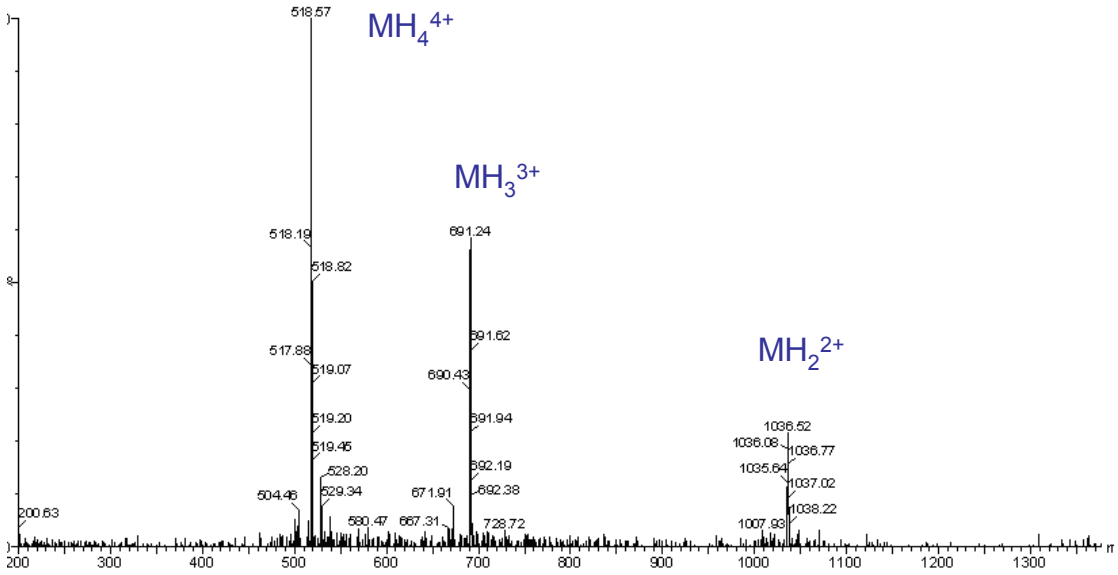

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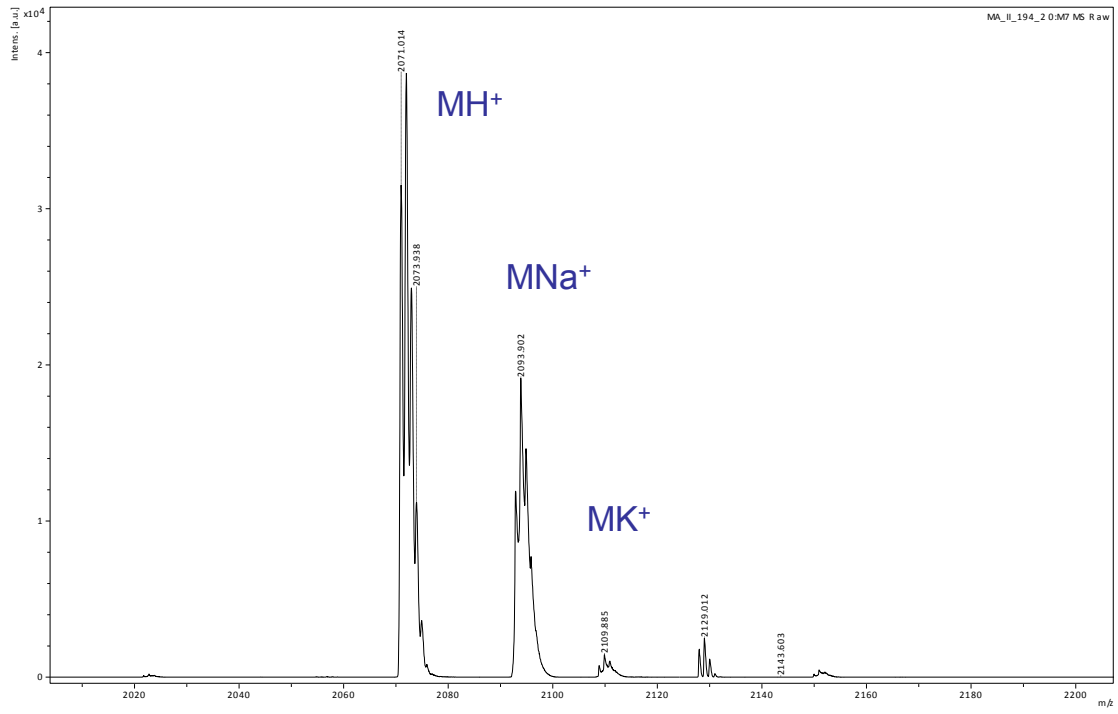

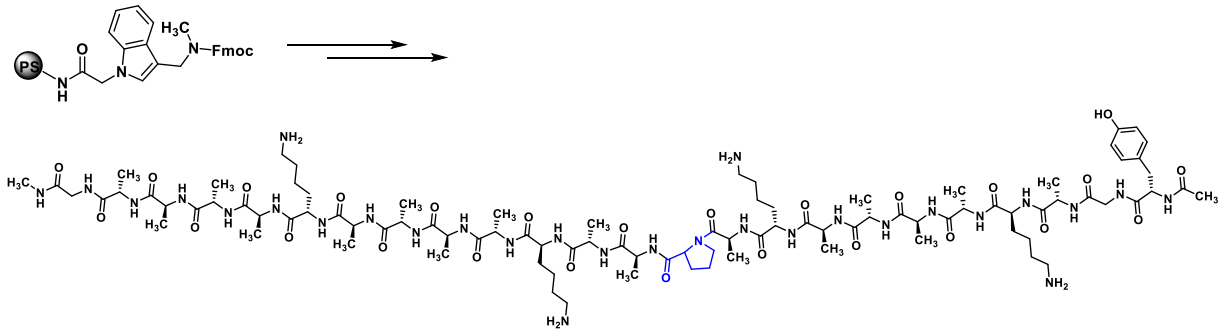

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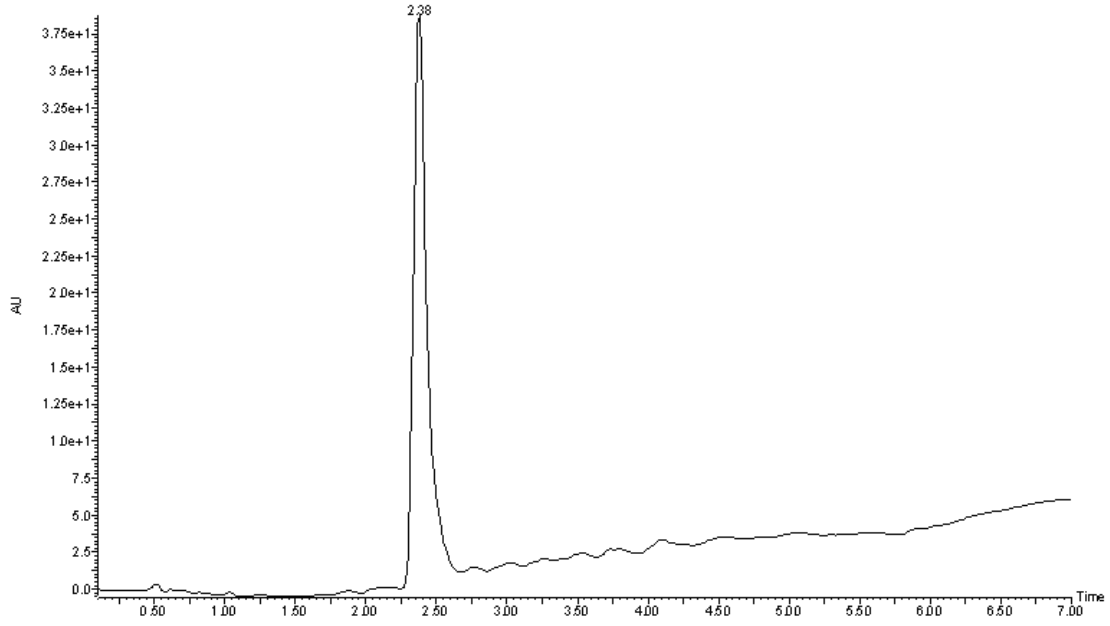

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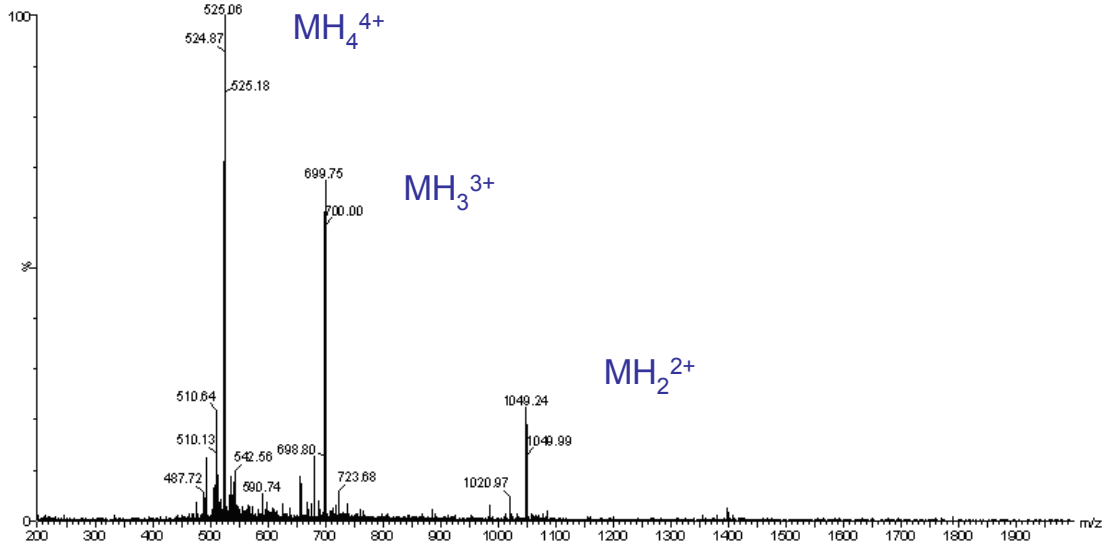

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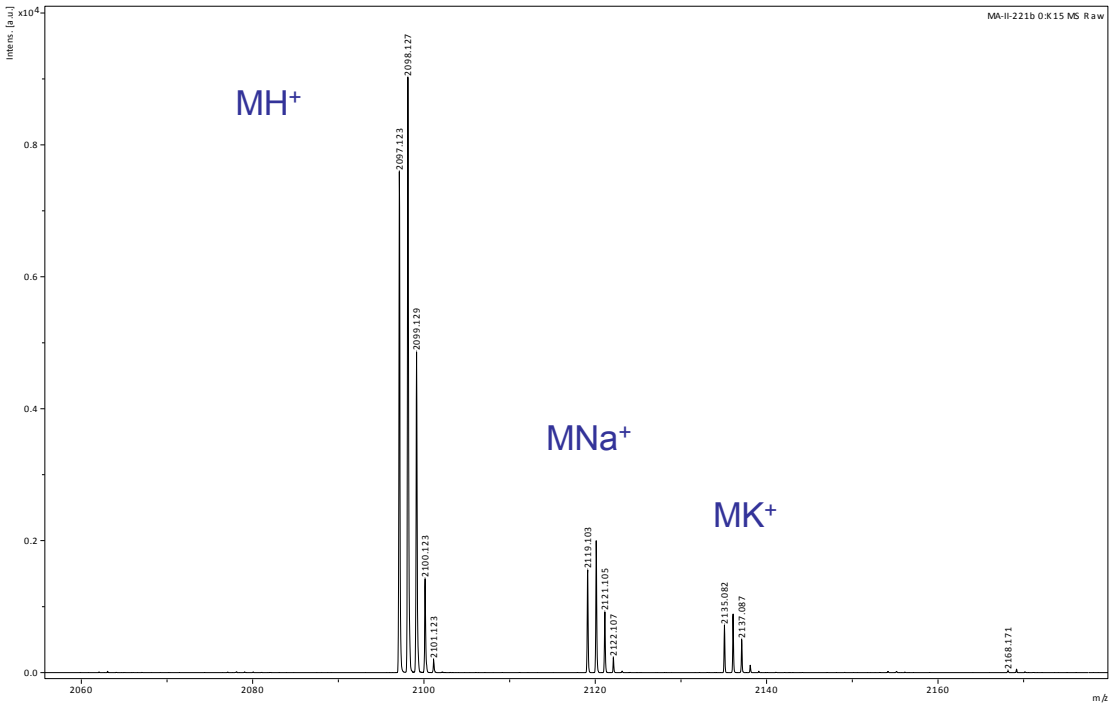
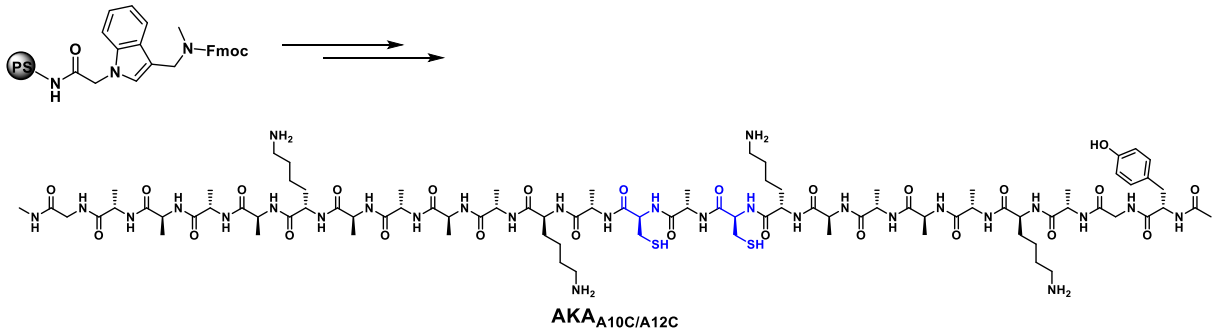
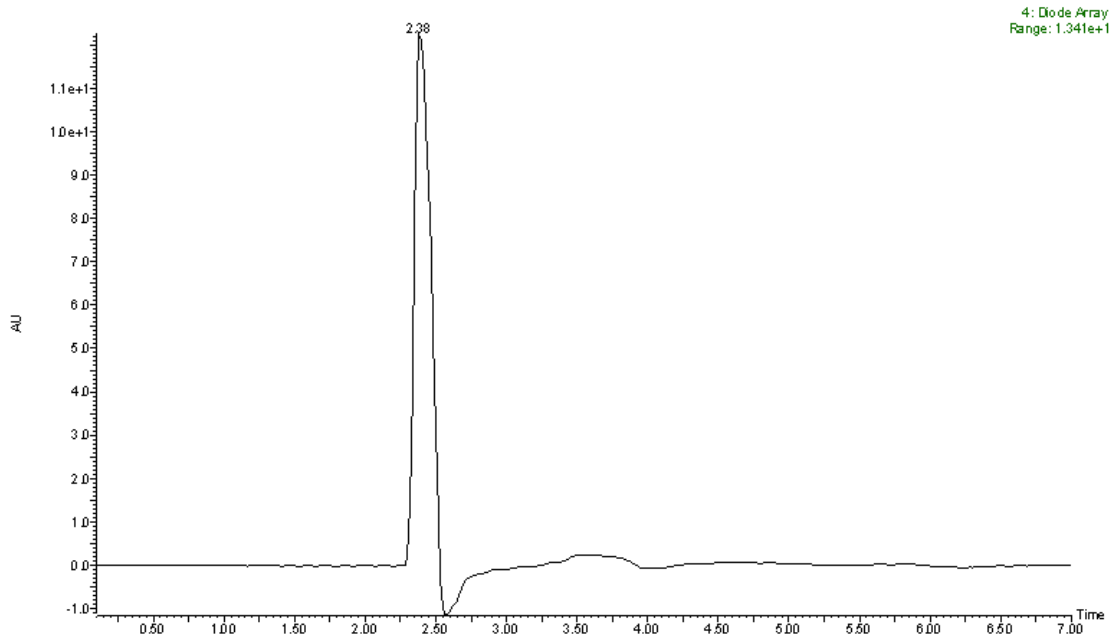




$AKA_{A10C/A12C}$

LC-MS chromatogram of **$AKA_{A10C/A12C}$:**



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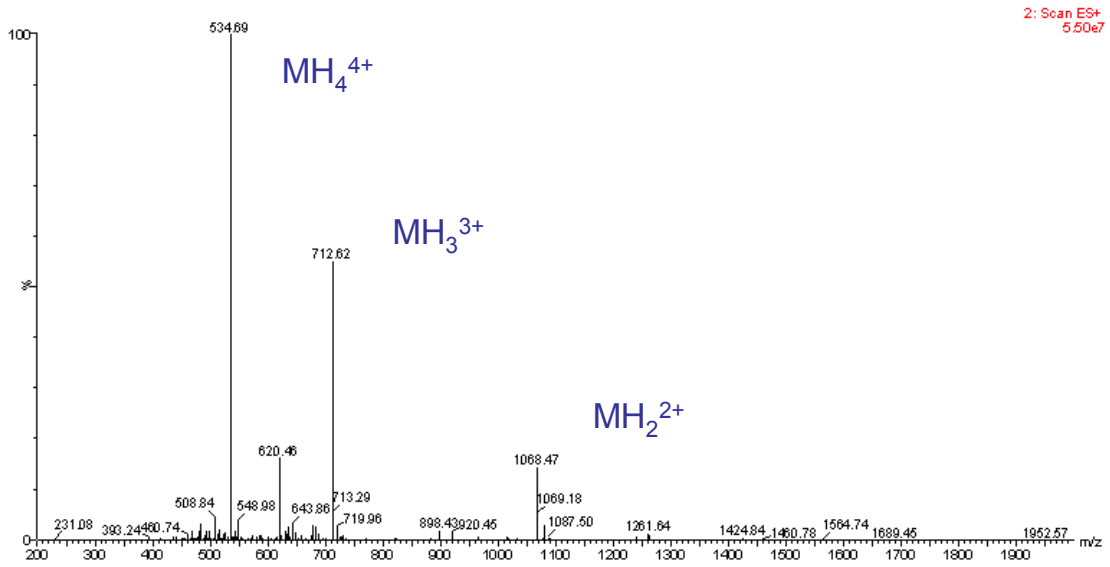

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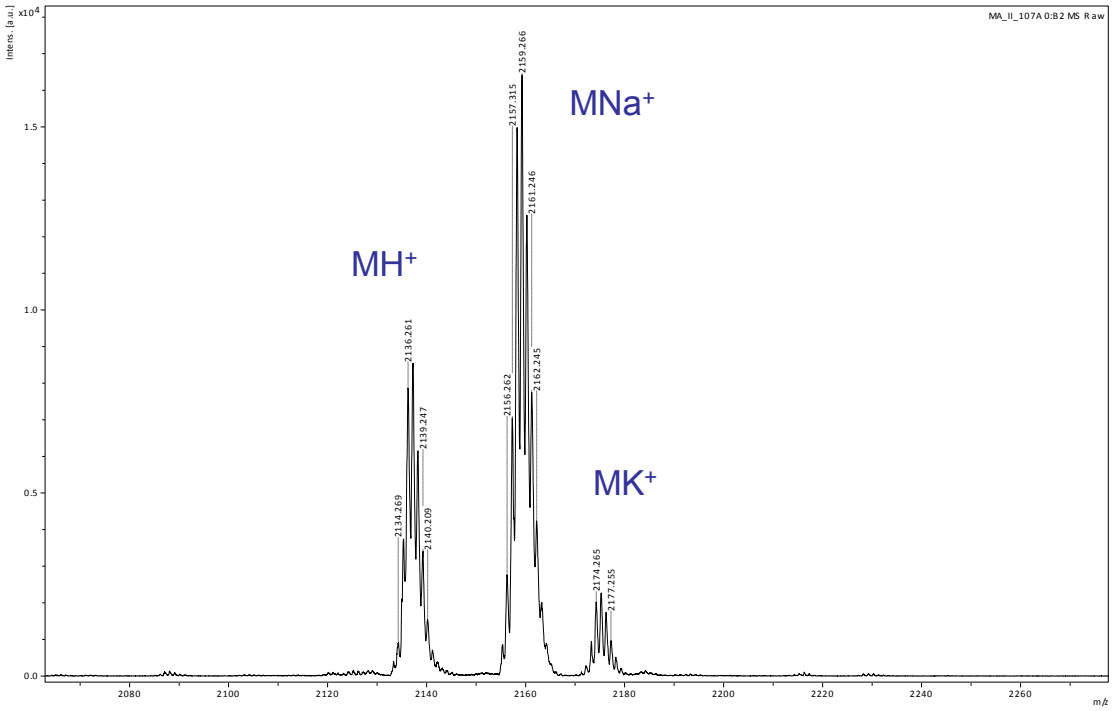

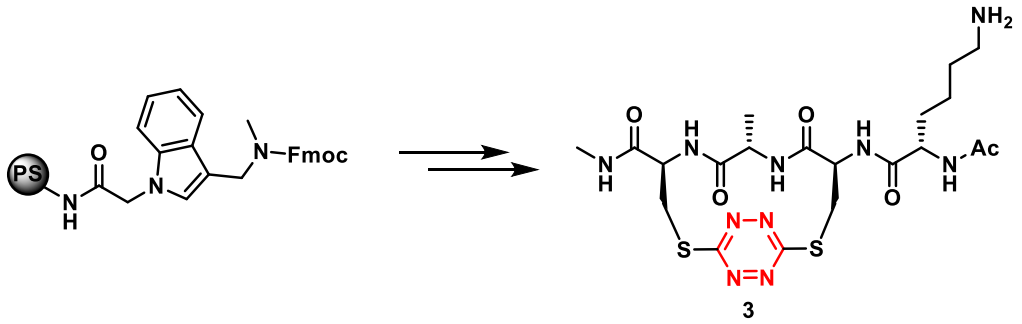

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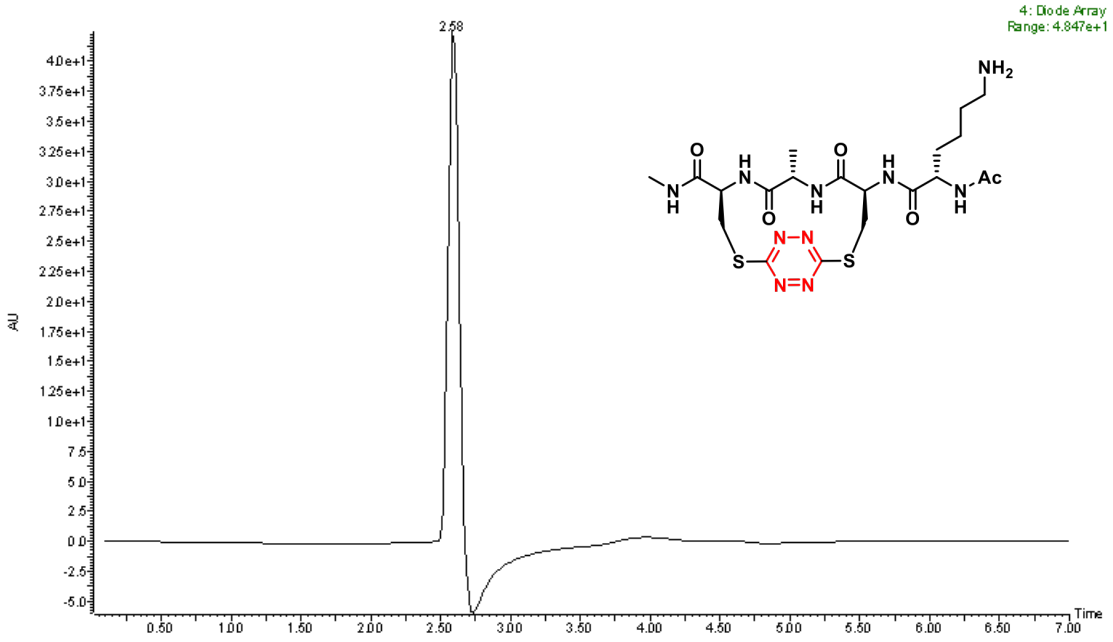

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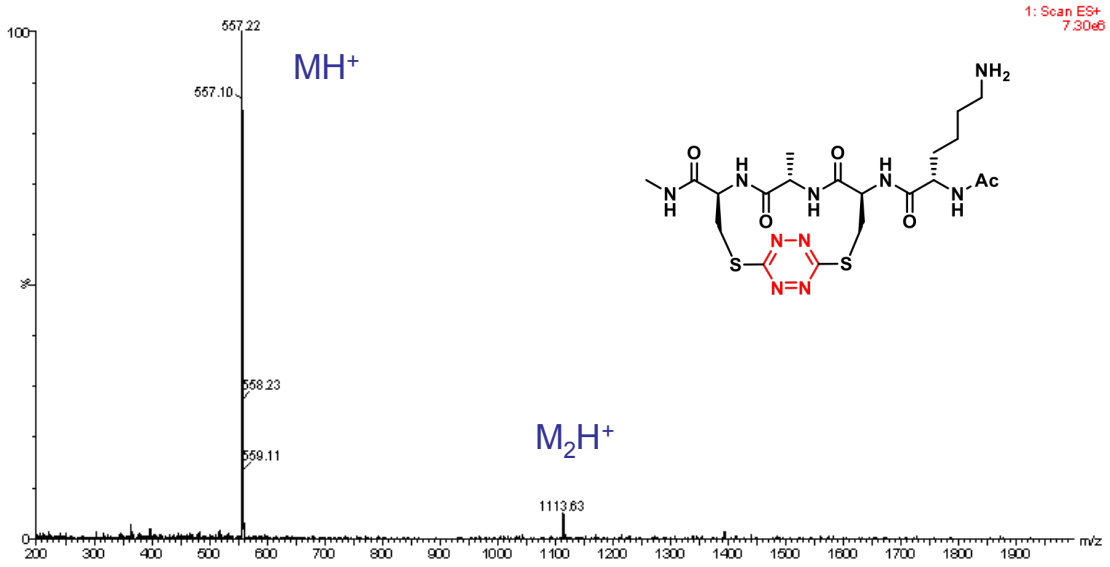

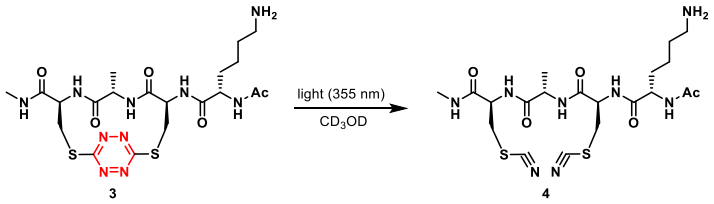

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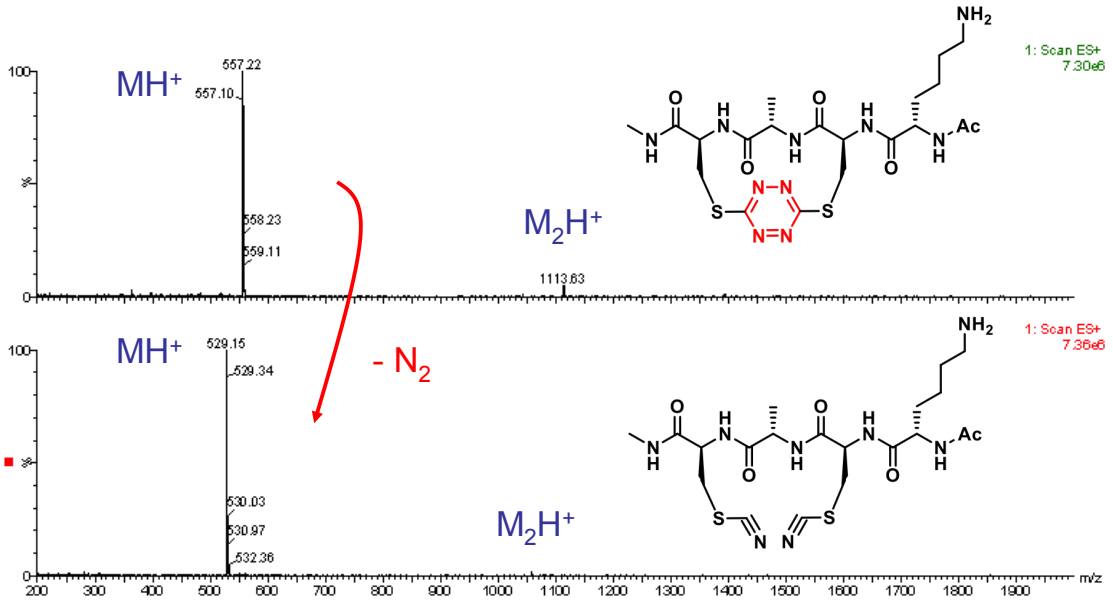

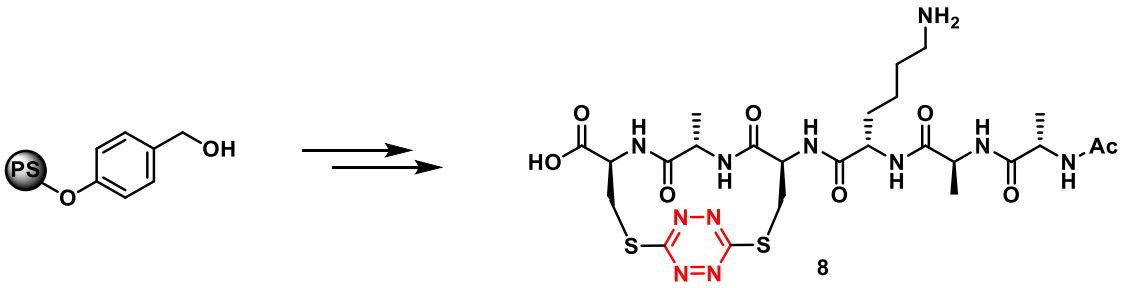

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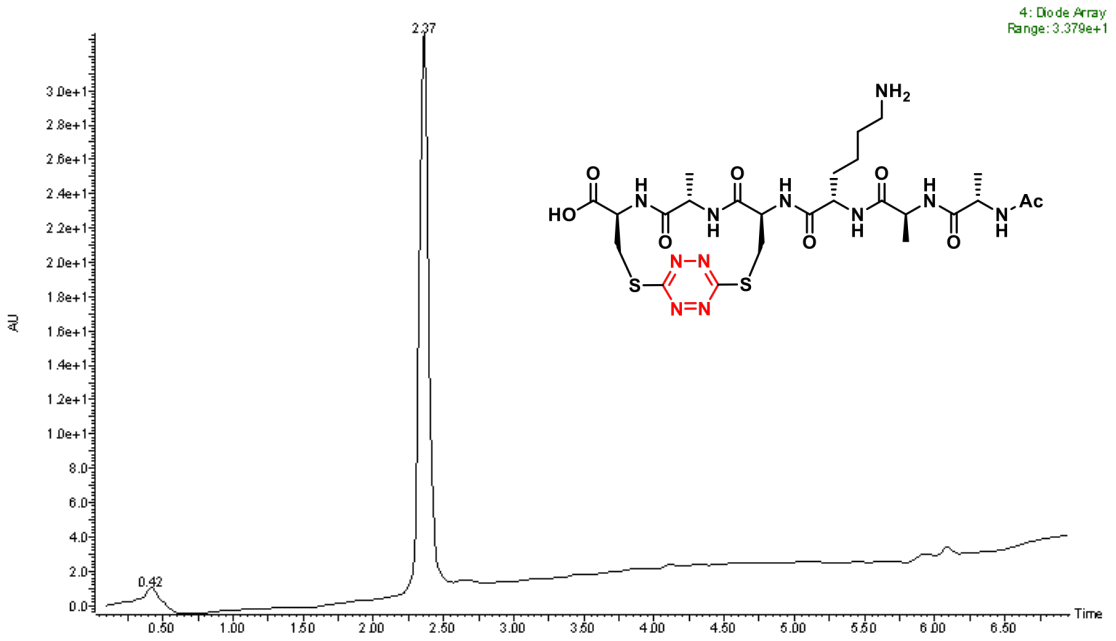

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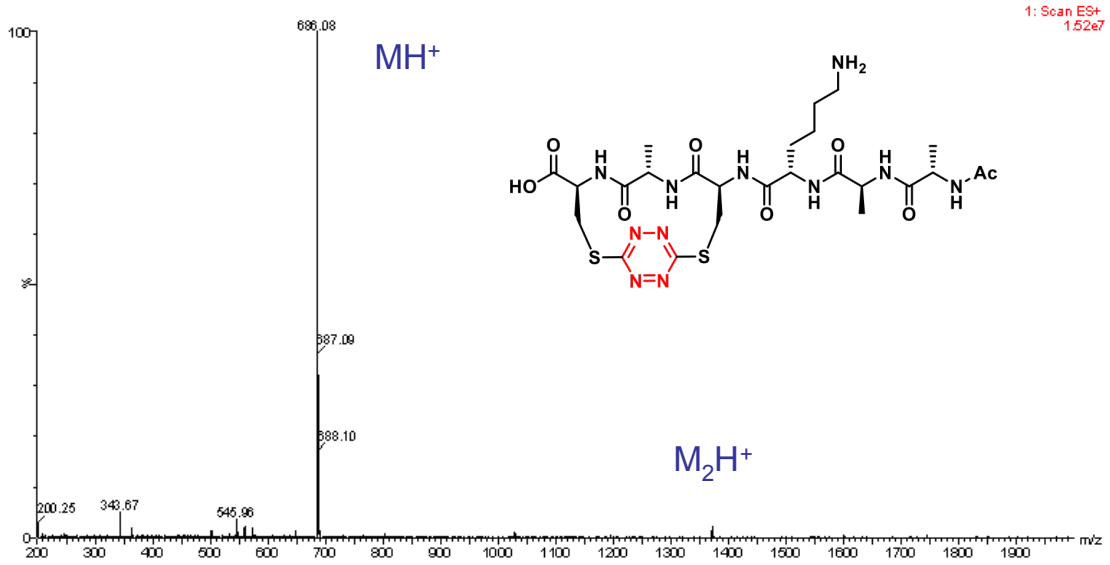

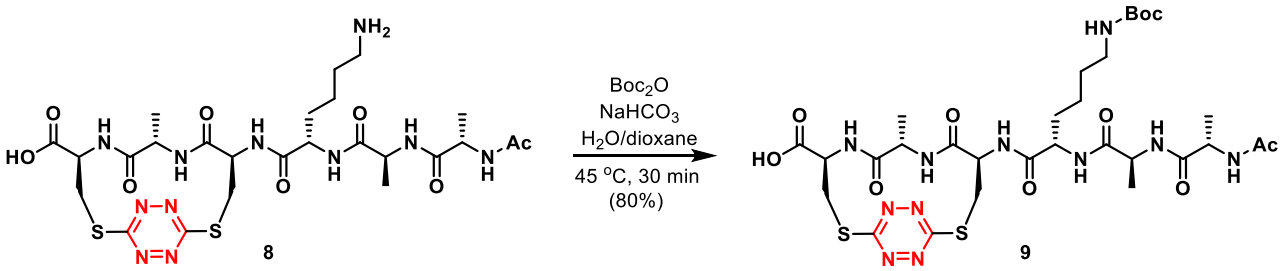

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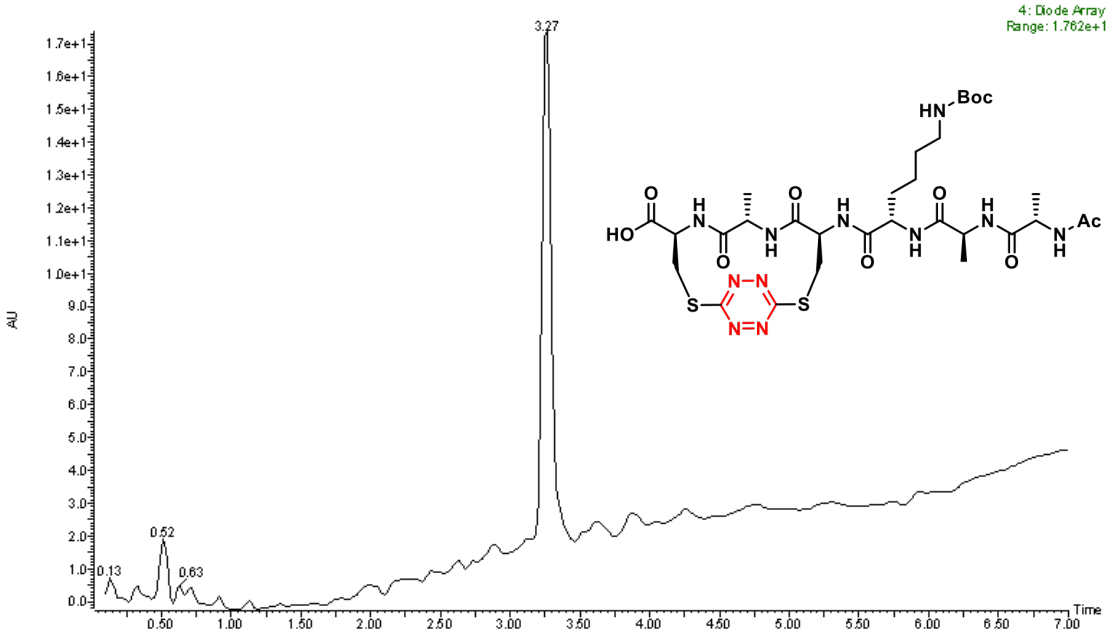

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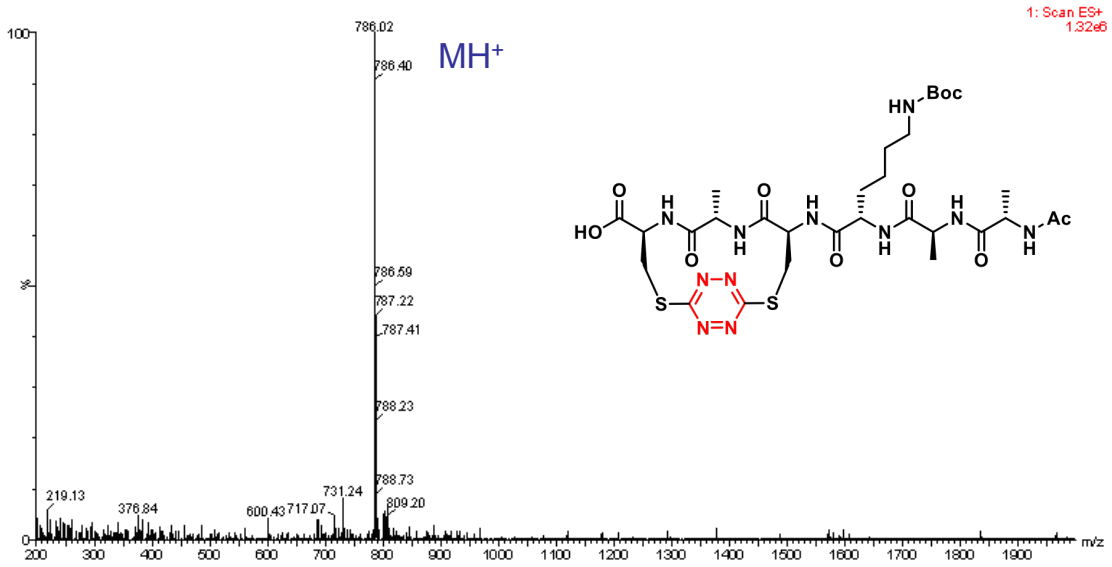
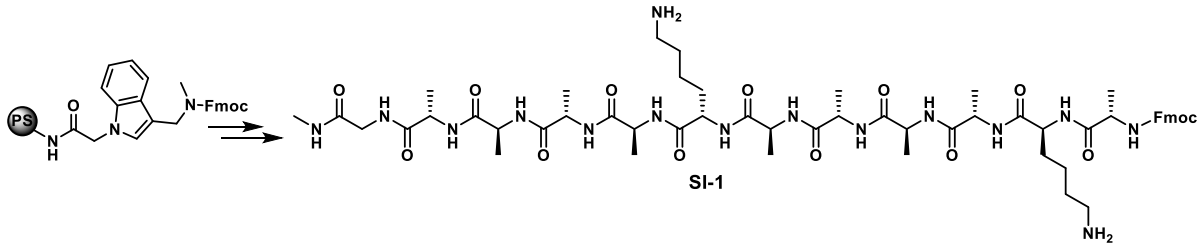
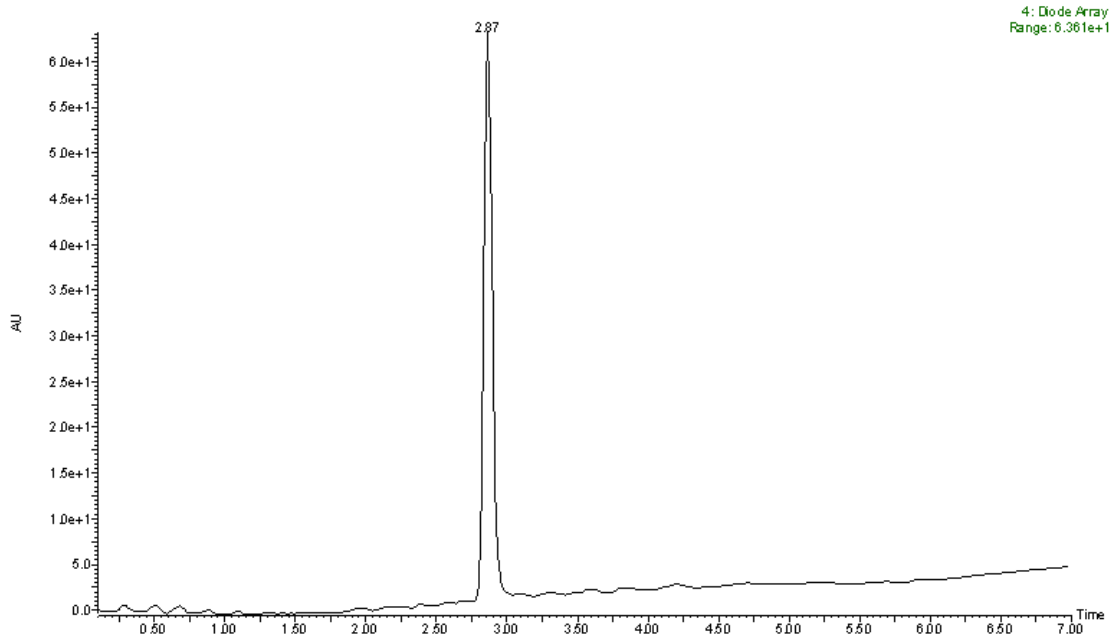


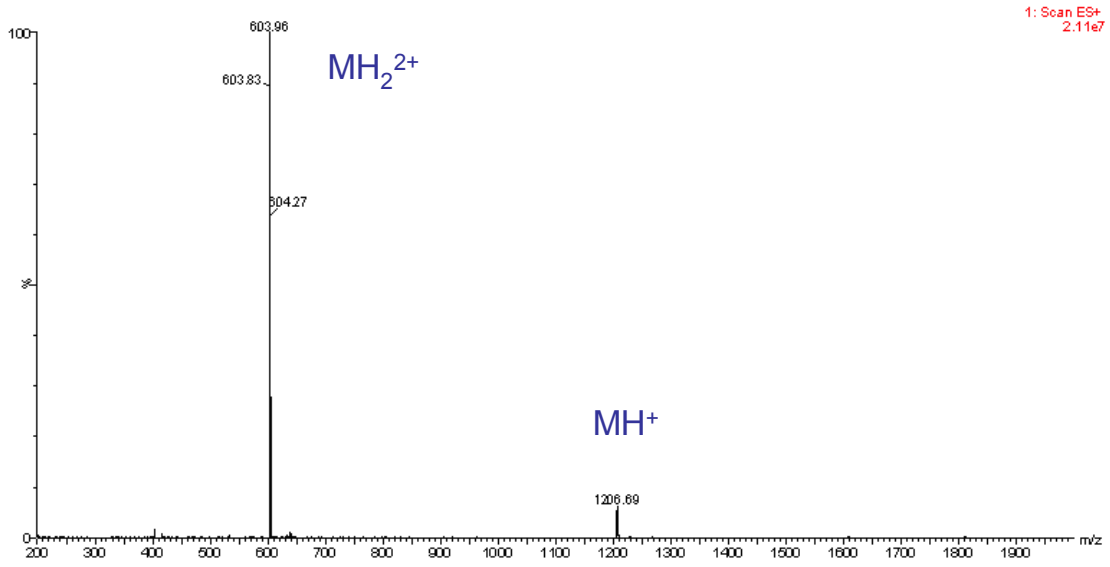


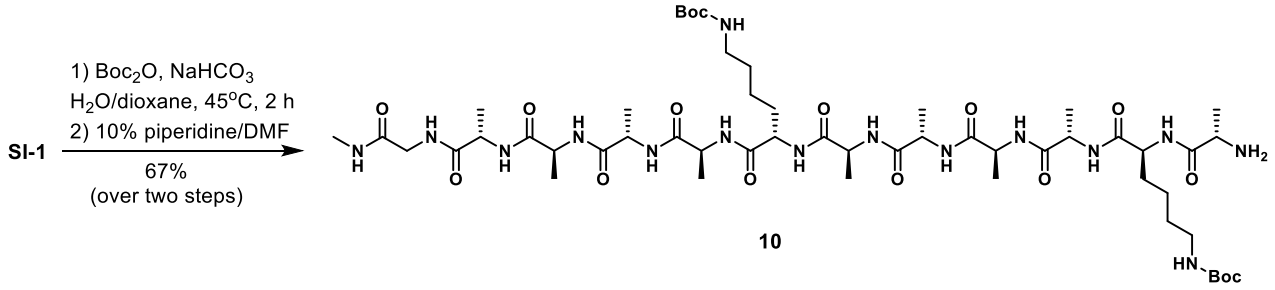
SI-1

LC-MS chromatogram of peptide **SI-1**:



LC-MS mass spectrum of peptide **SI-1**:





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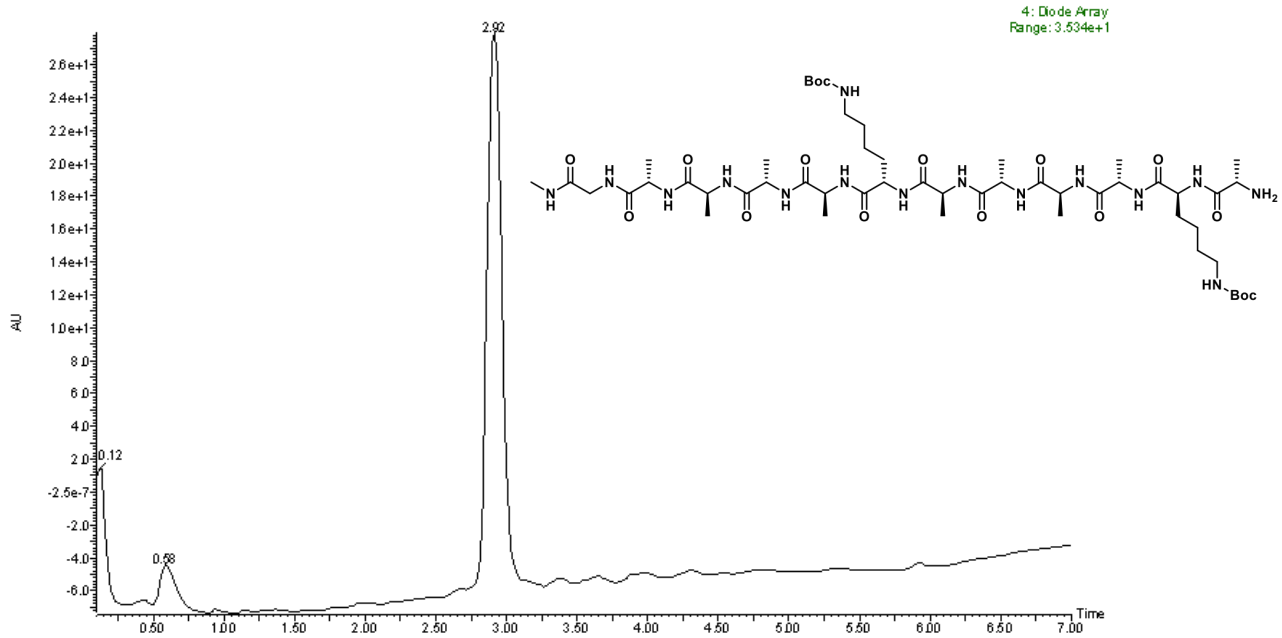

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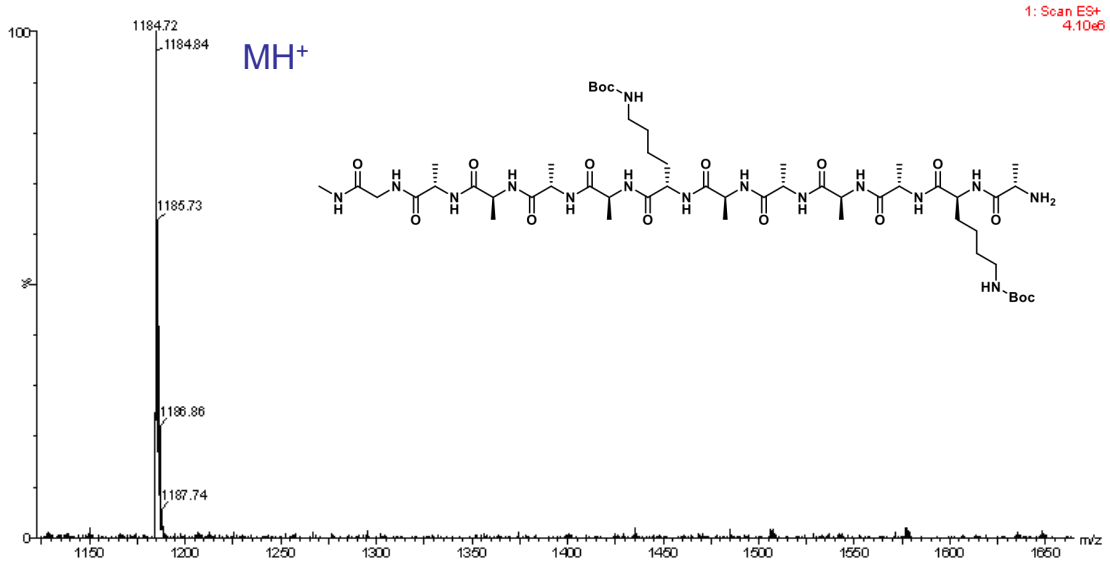

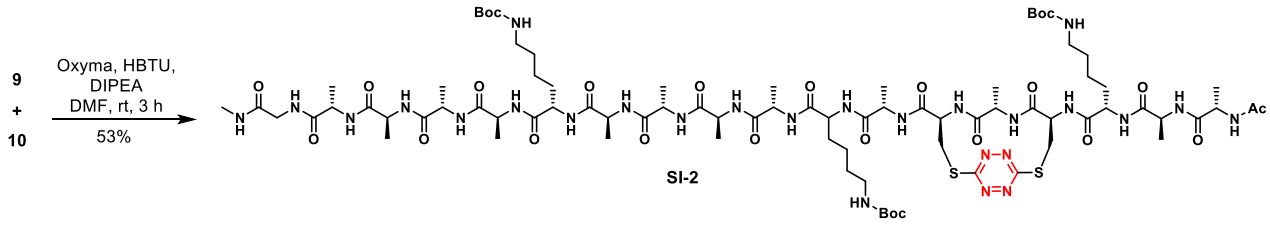

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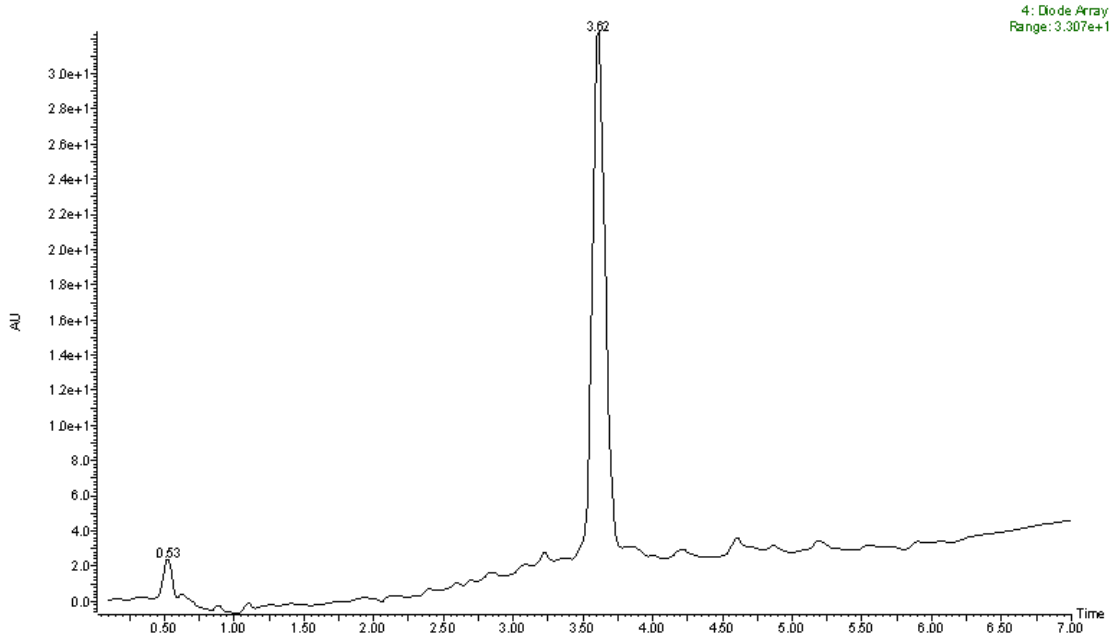
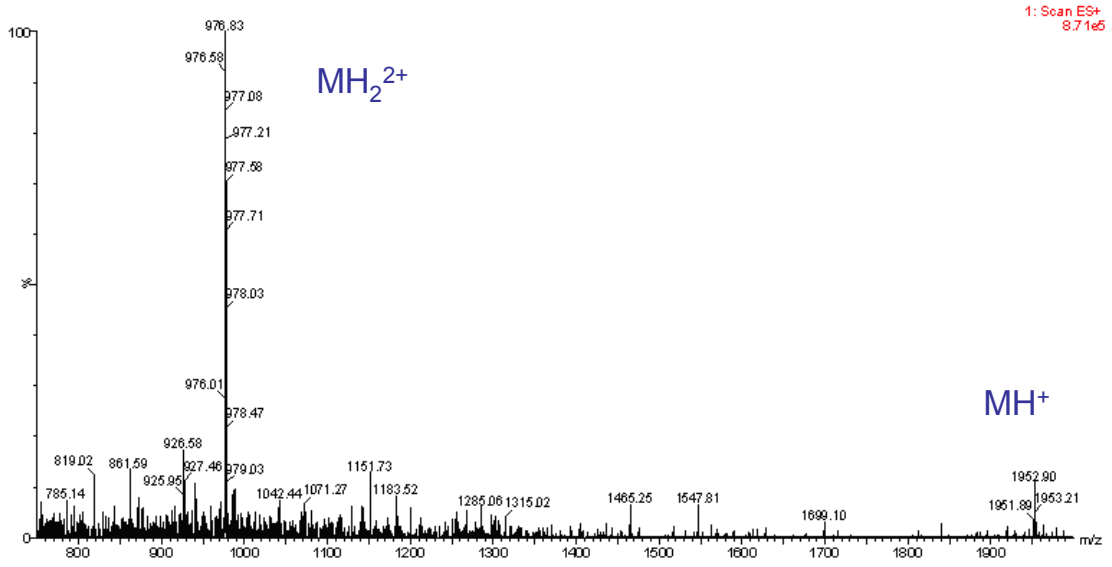


LC-MS mass spectrum of peptide **SI-2**:



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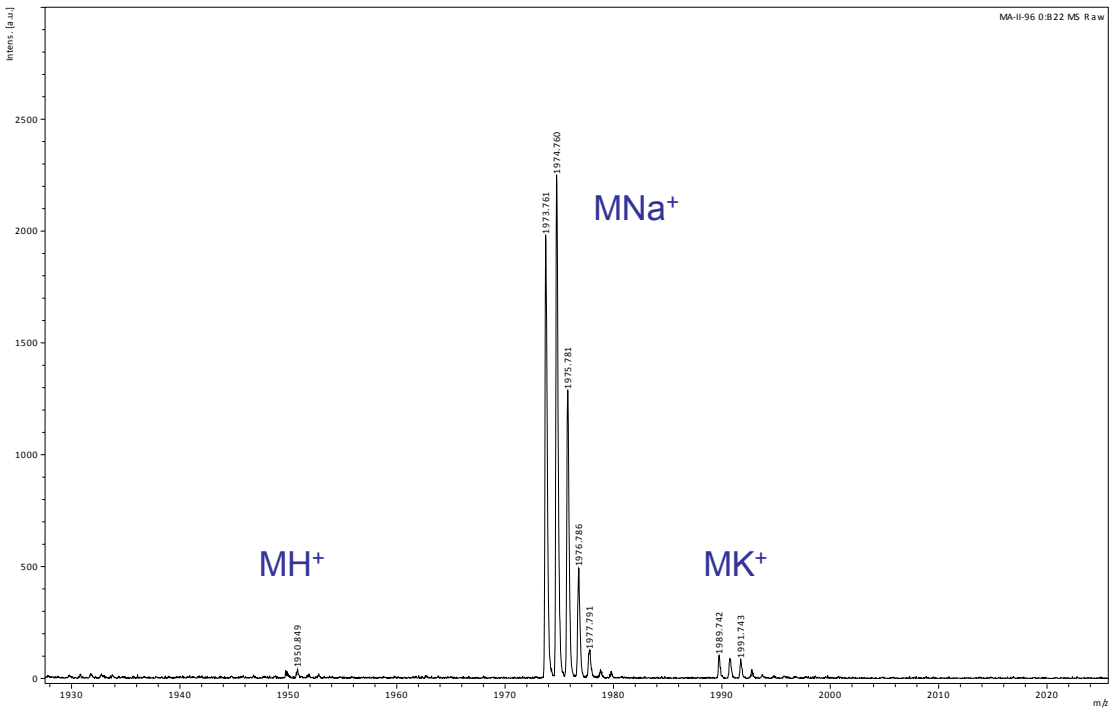

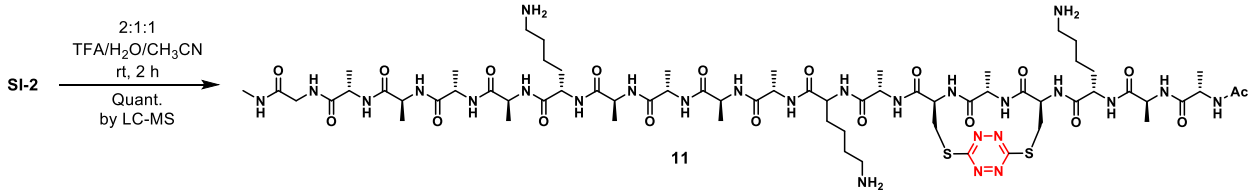

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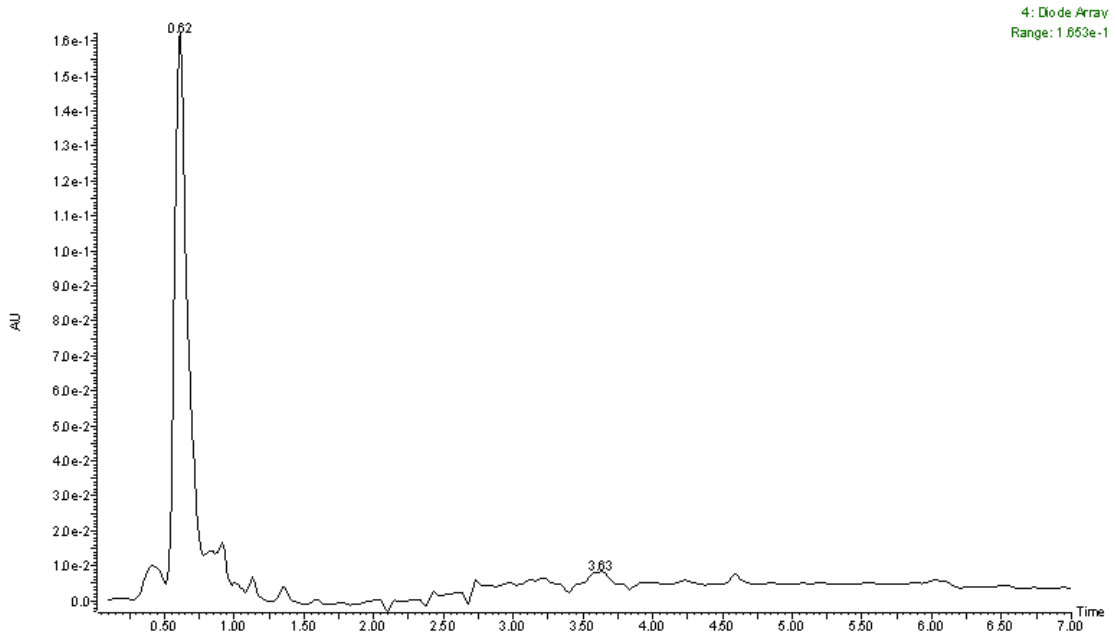

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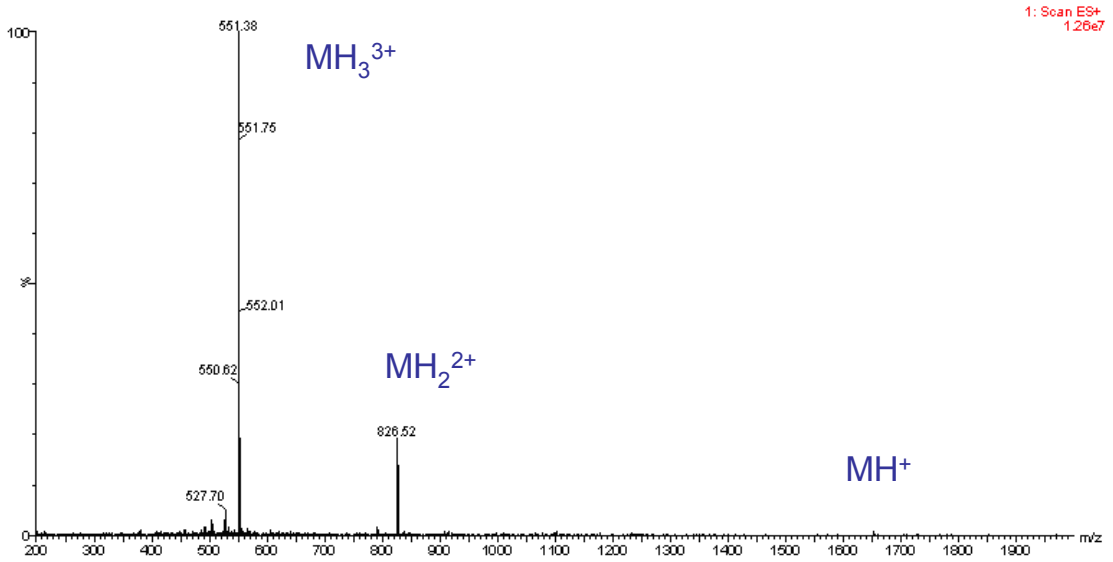
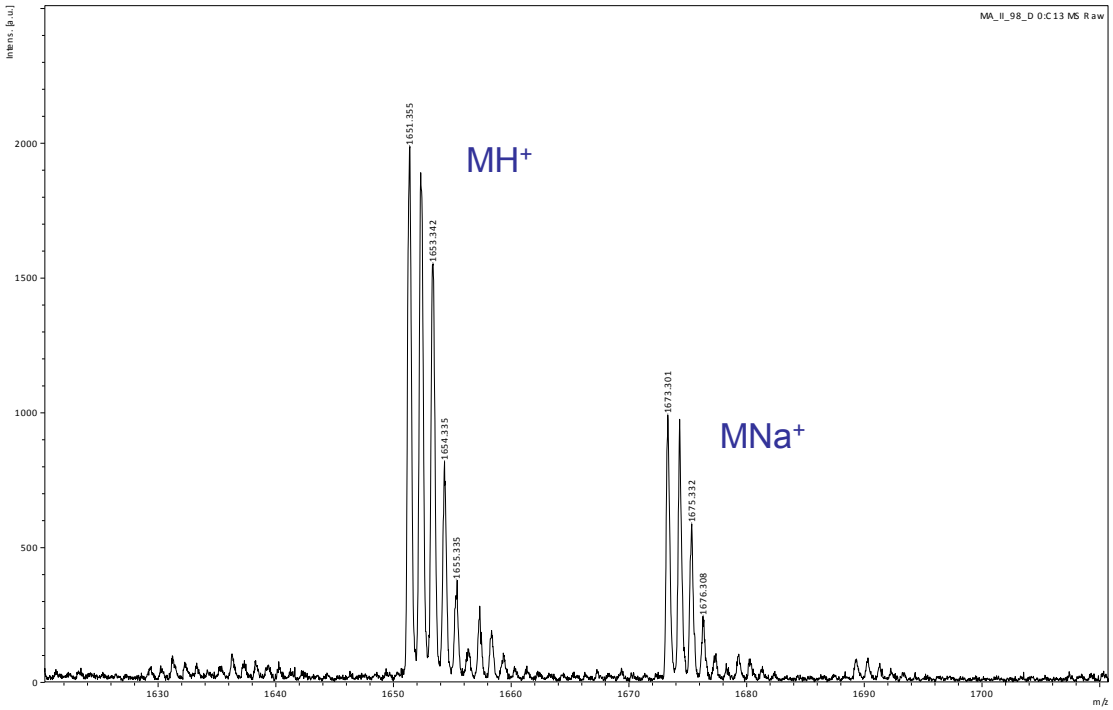
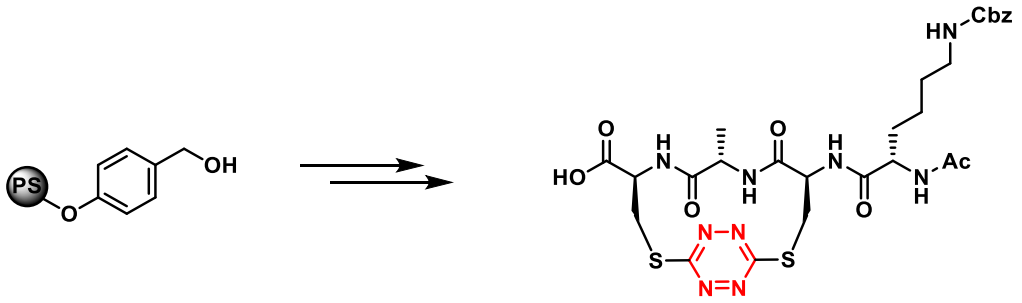


MALDI-TOF mass spectrum of peptide **11**:





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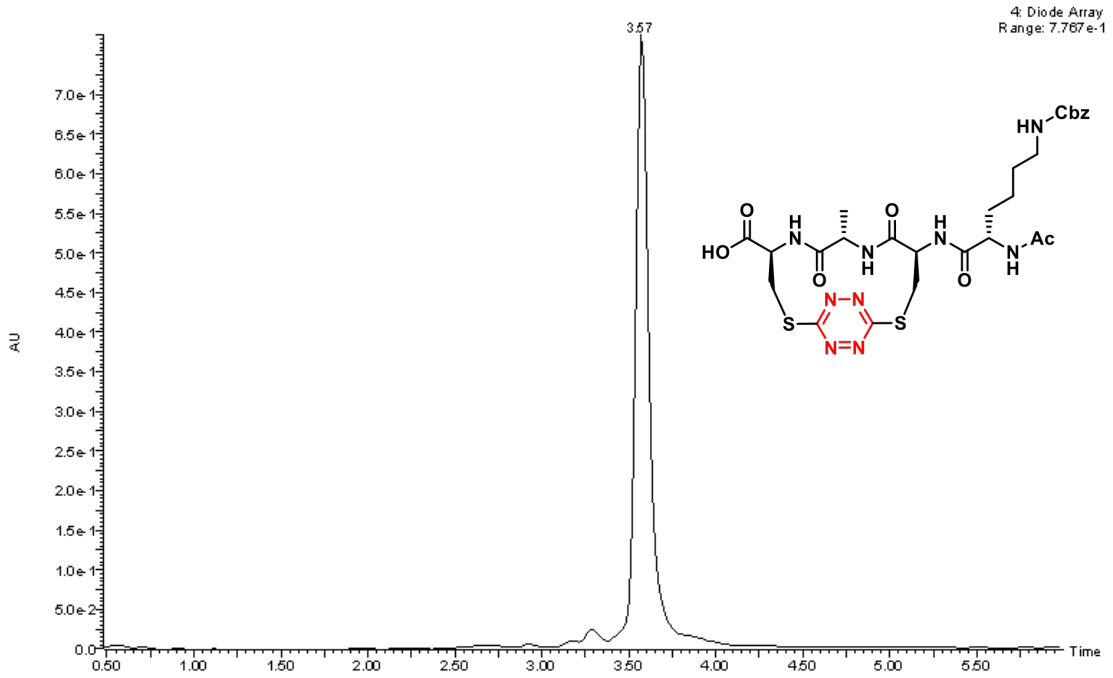

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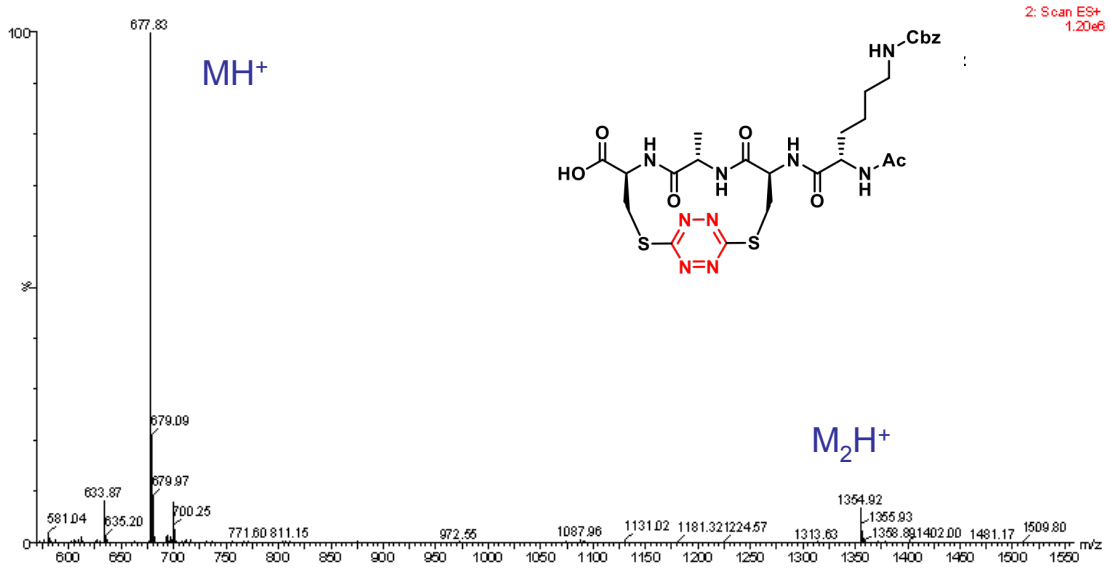

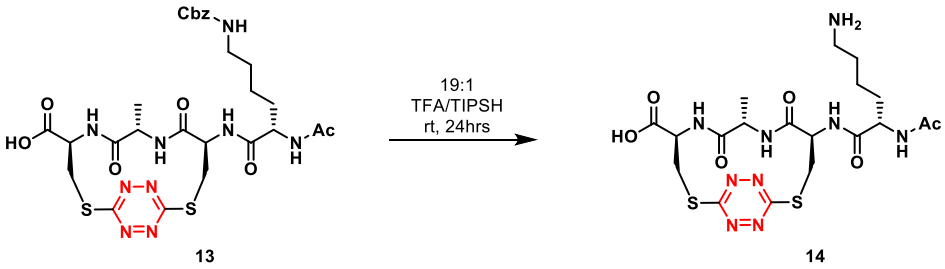

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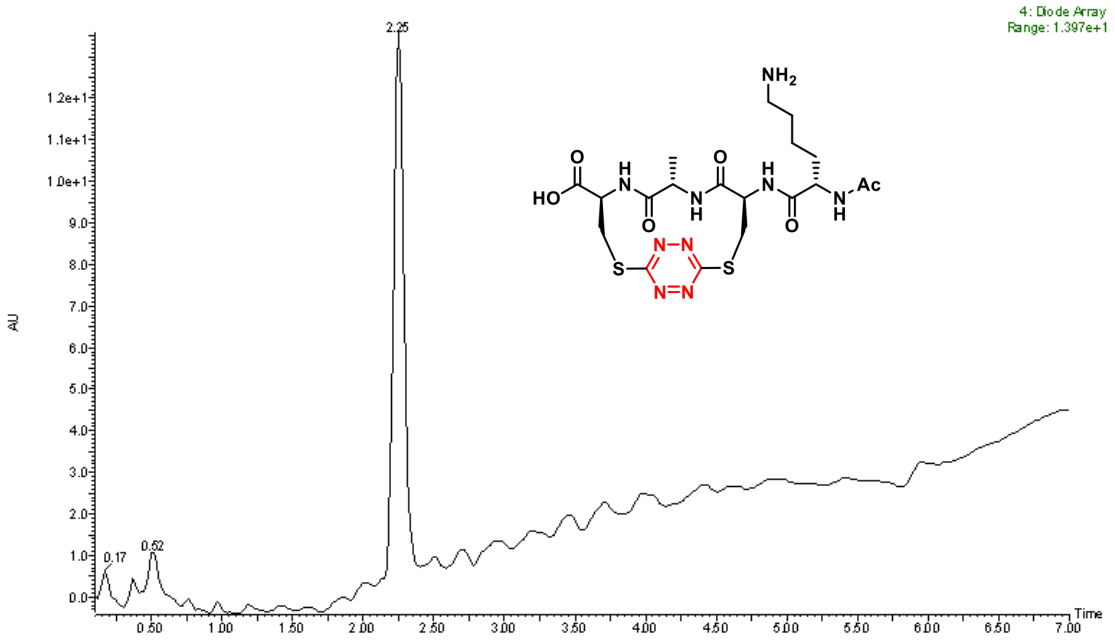

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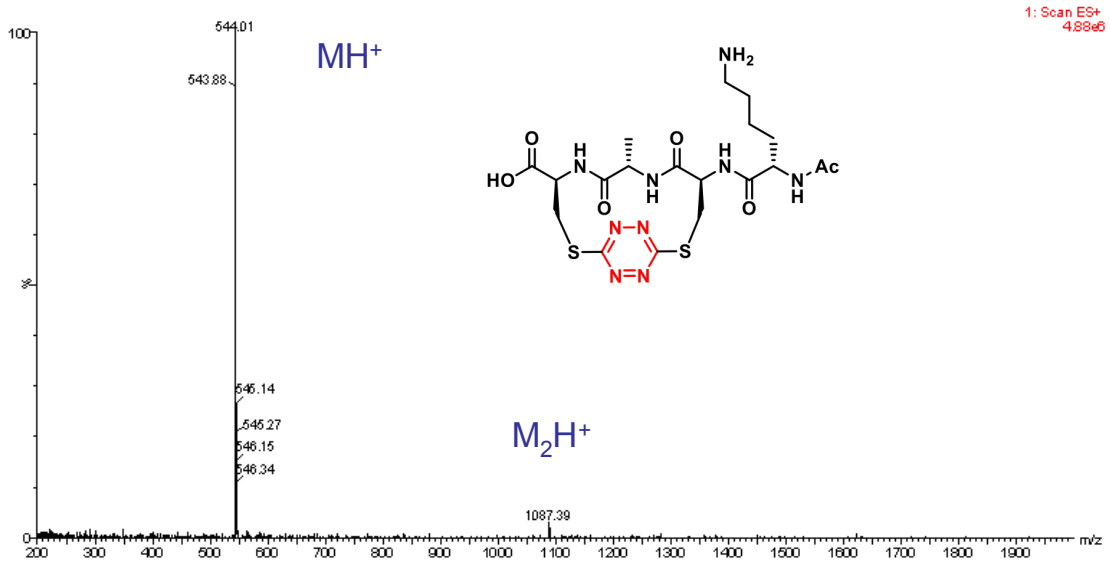

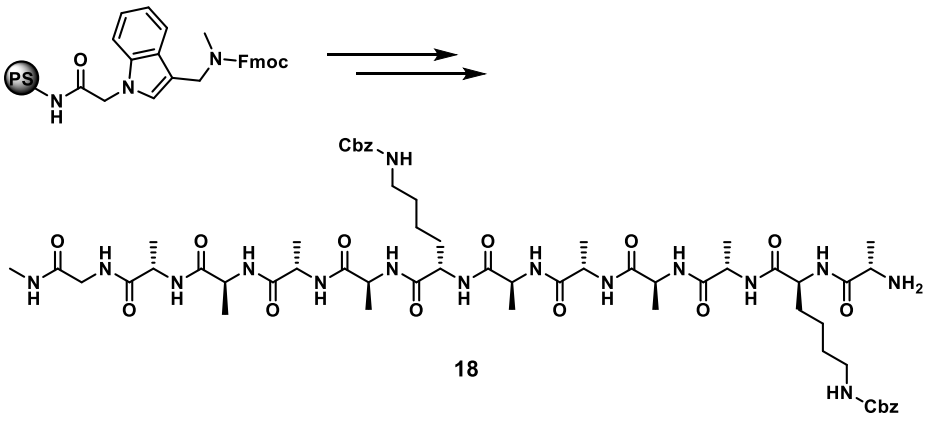

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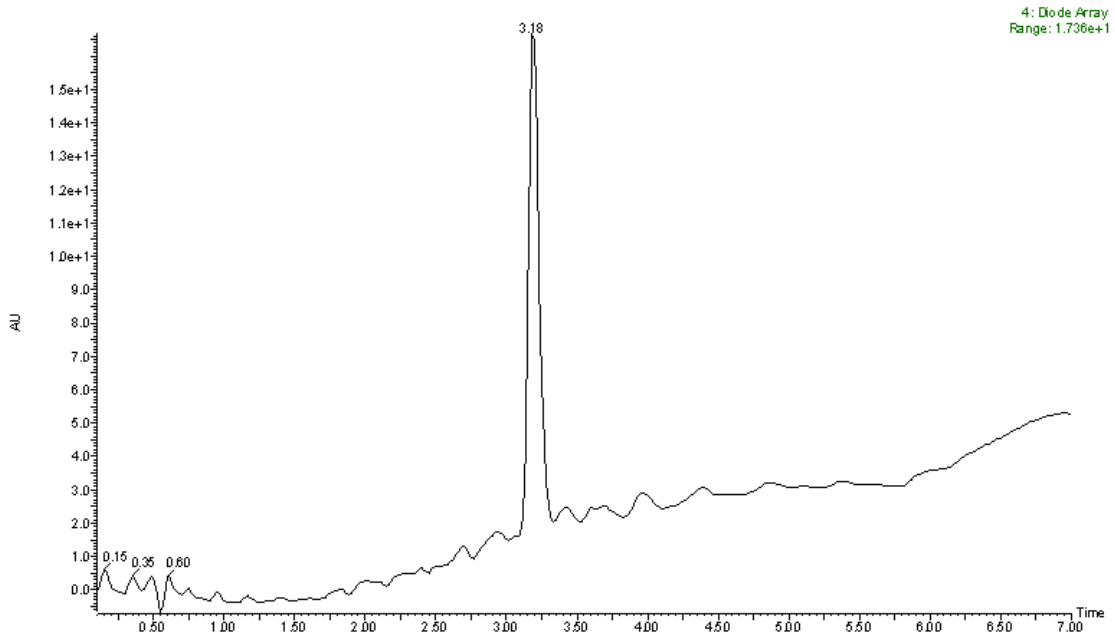

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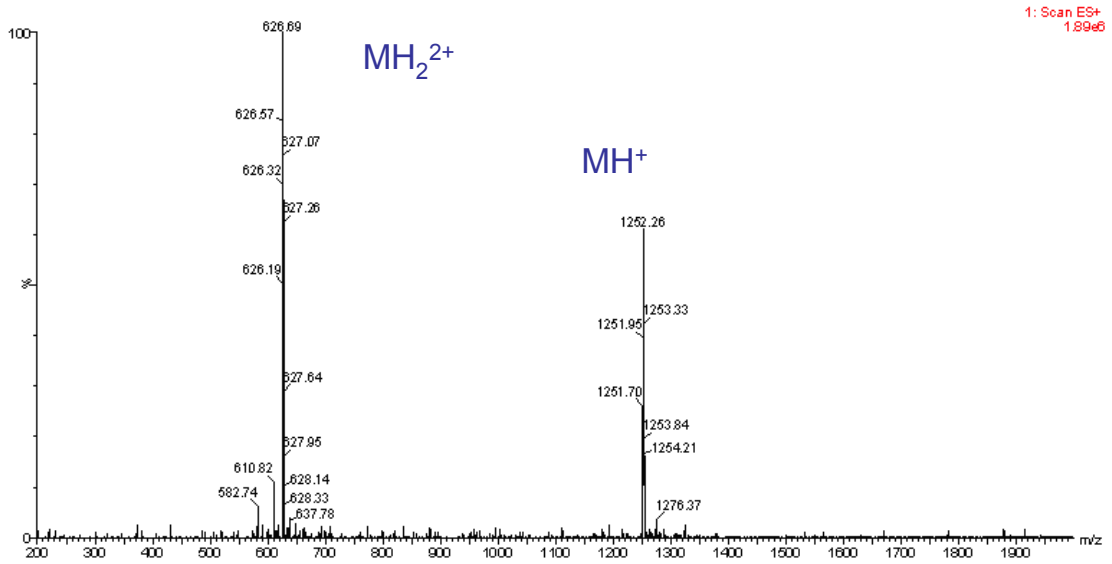

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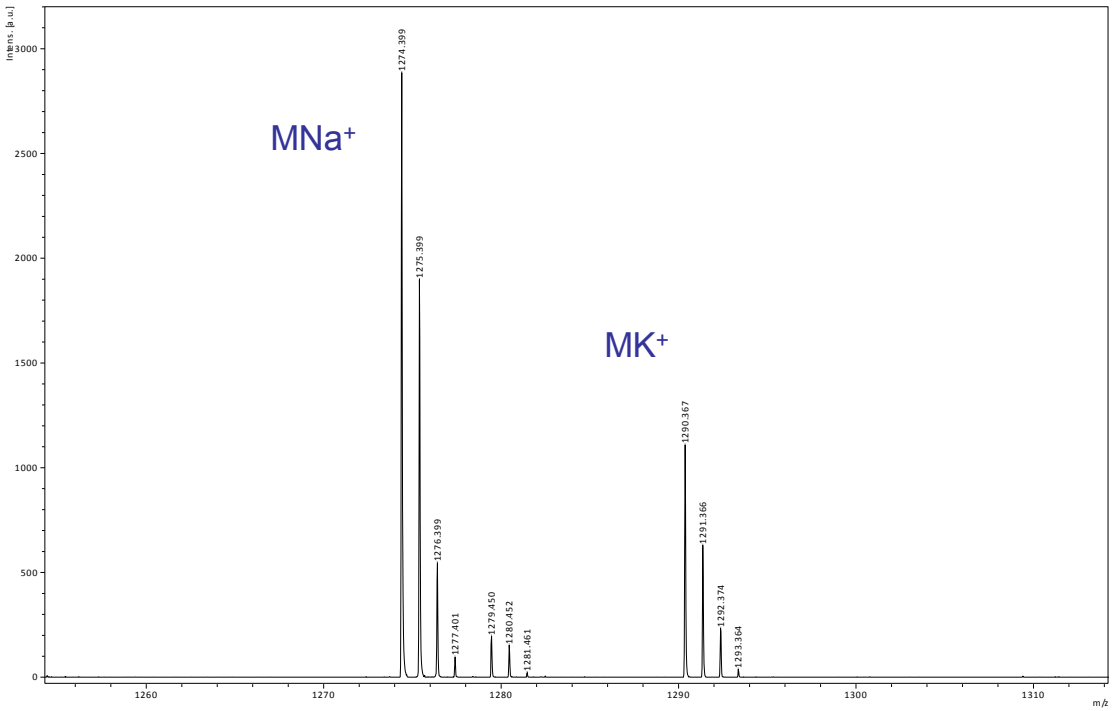

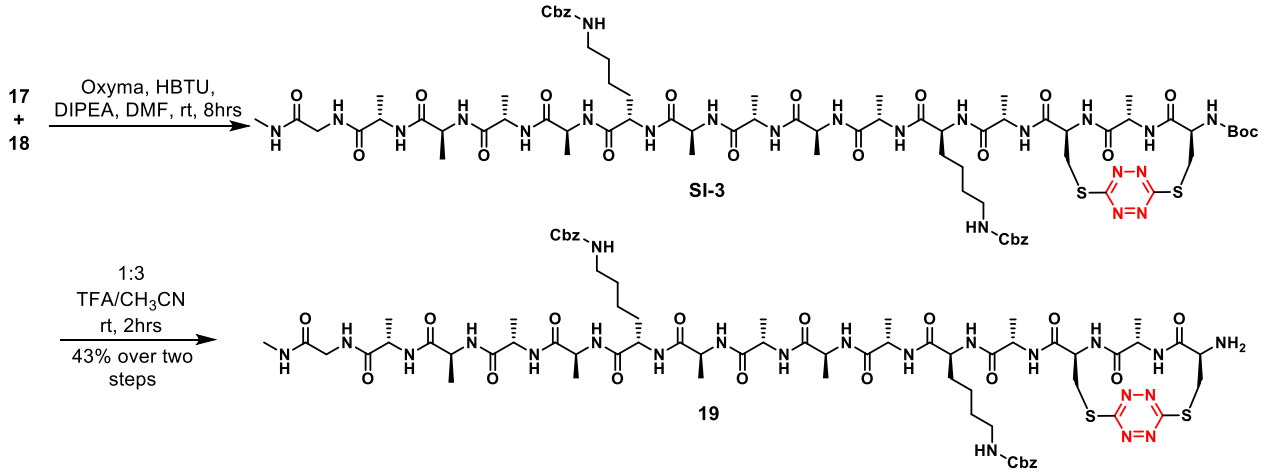

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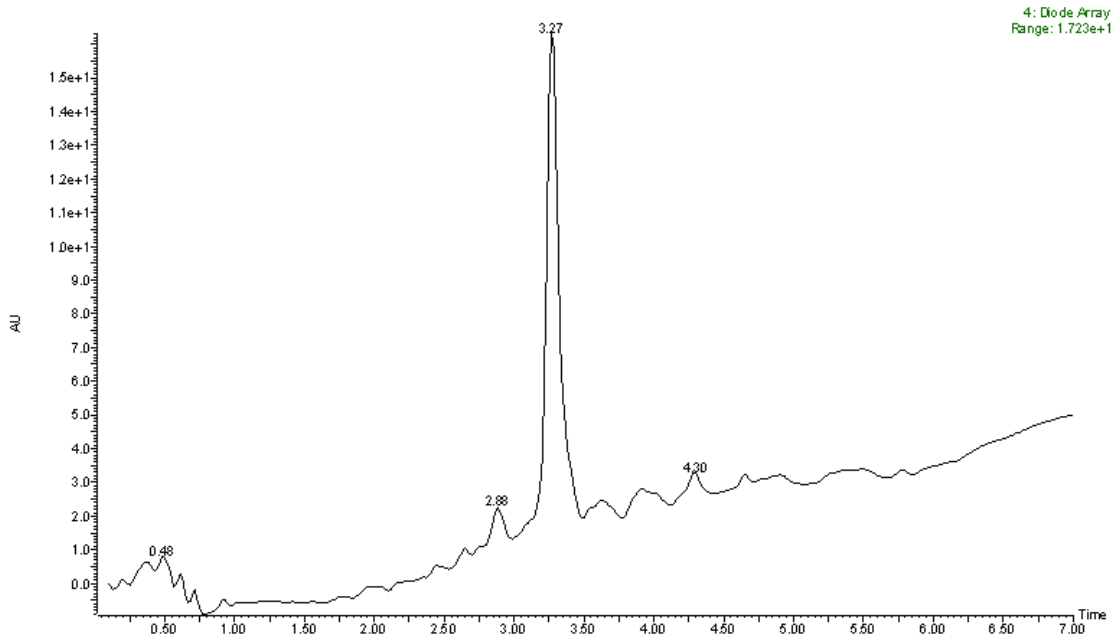
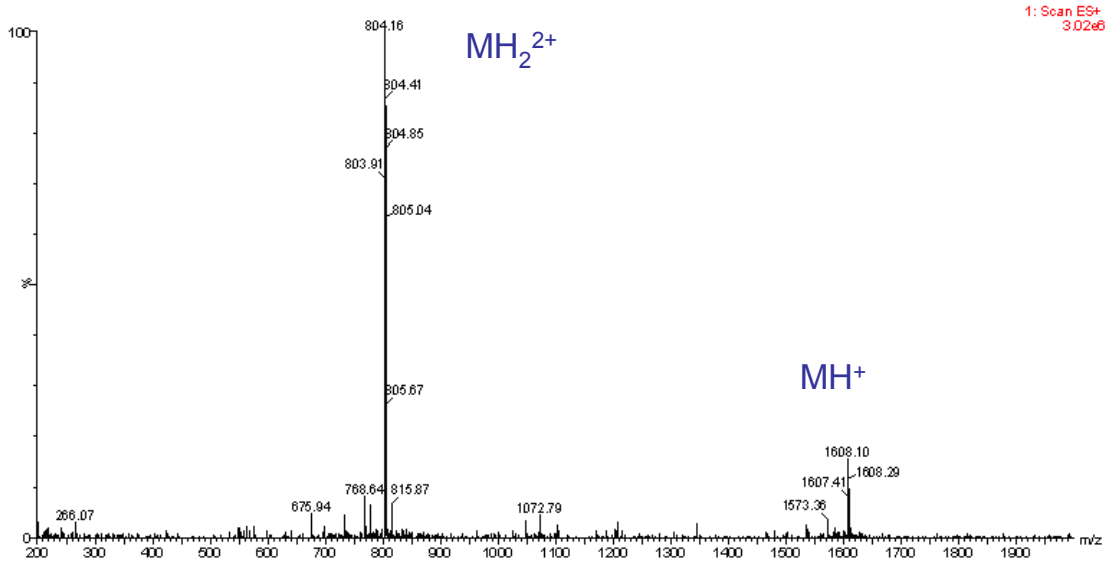
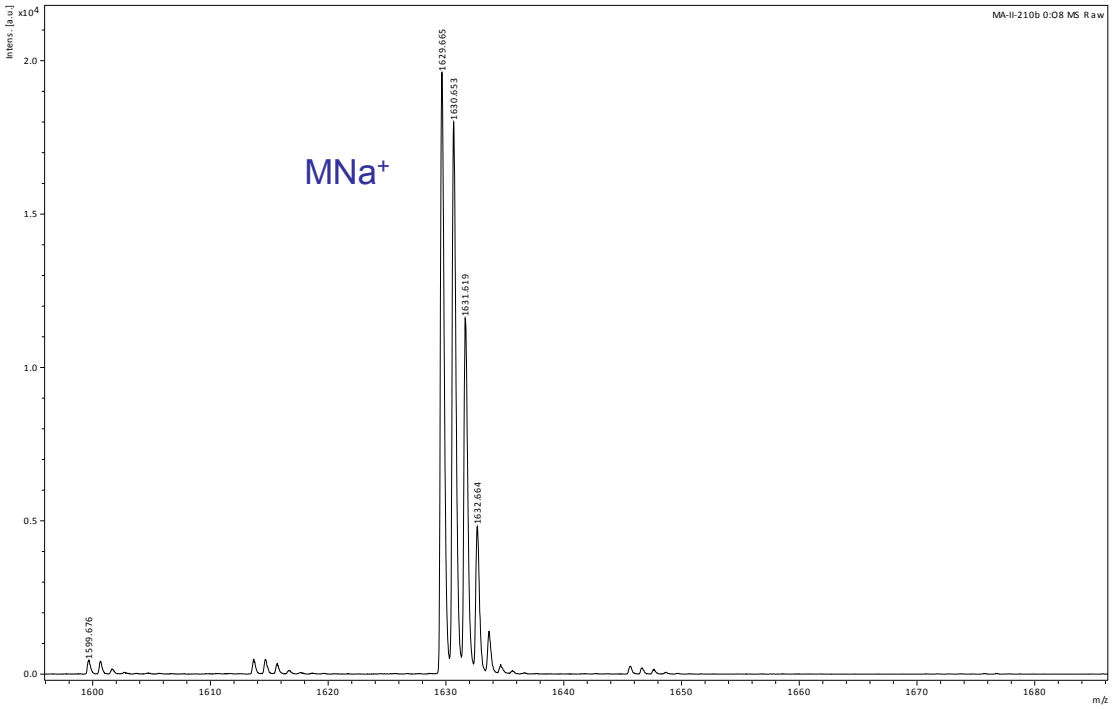


LC-MS mass spectrum of peptide **19**:



MALDI-TOF mass spectrum of peptide **19**:



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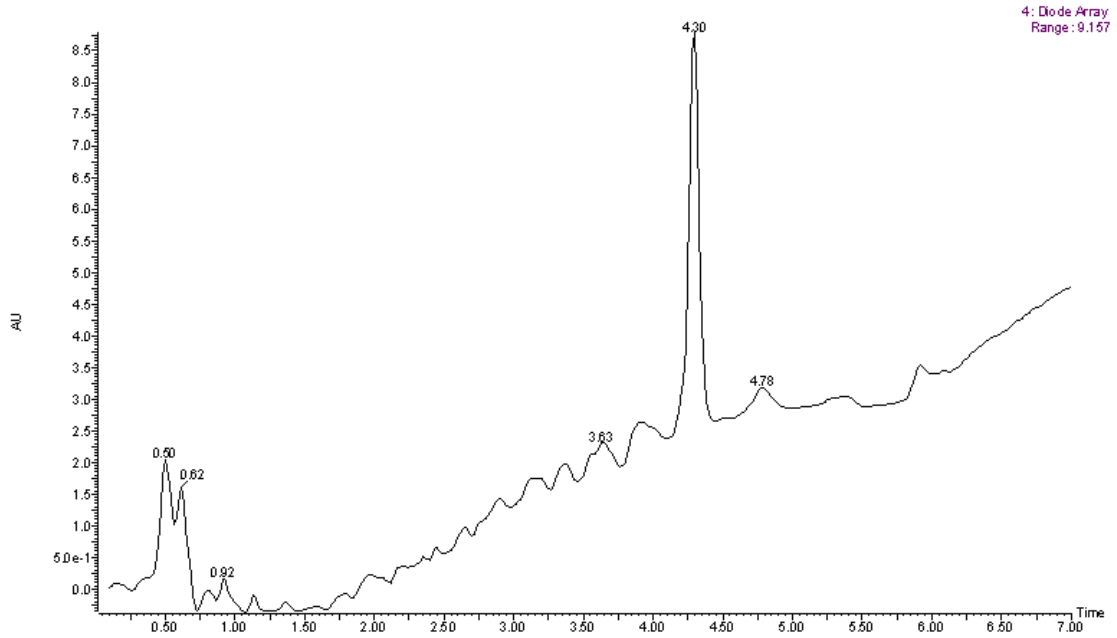
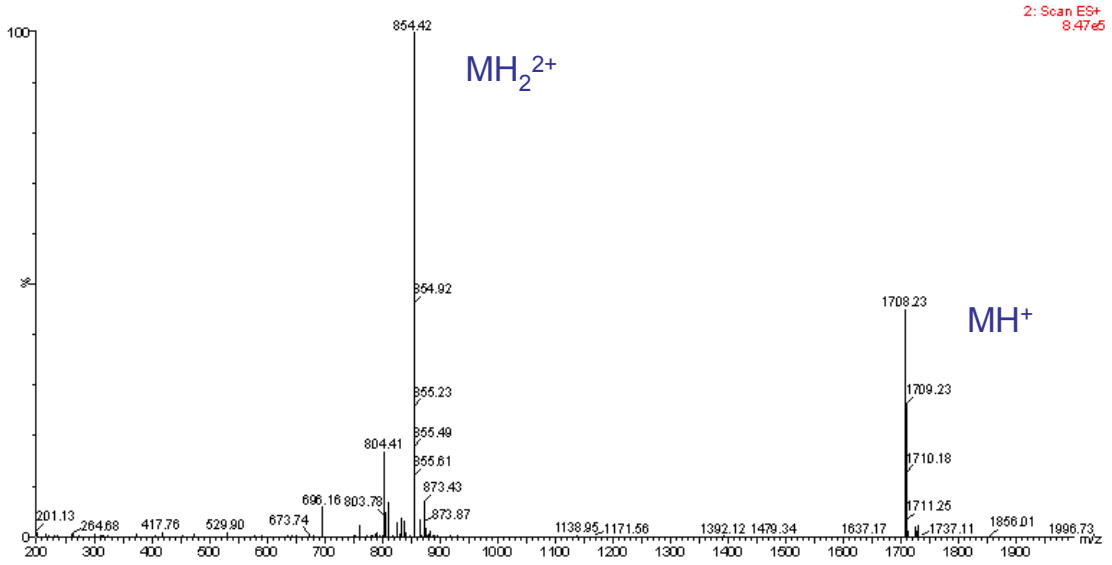
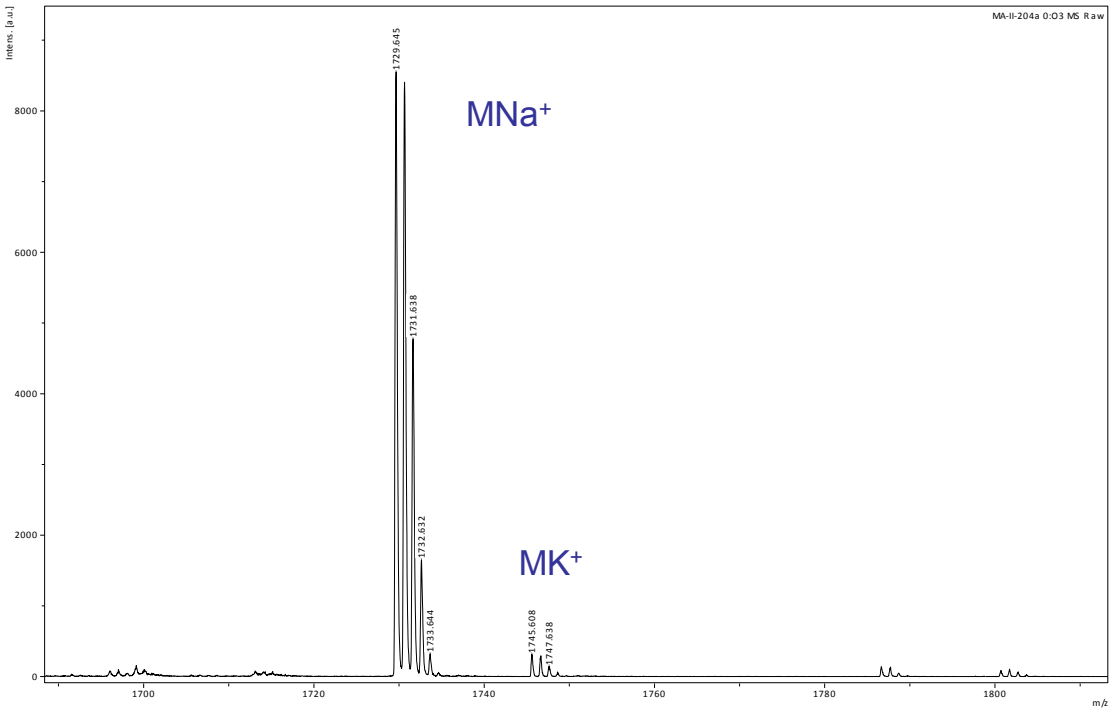
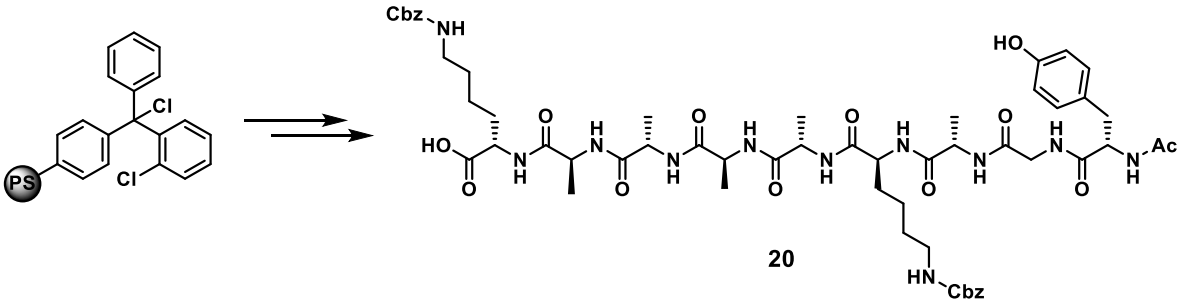


LC-MS mass spectrum of peptide **SI-3**:



MALDI-TOF mass spectrum of peptide **SI-3**:





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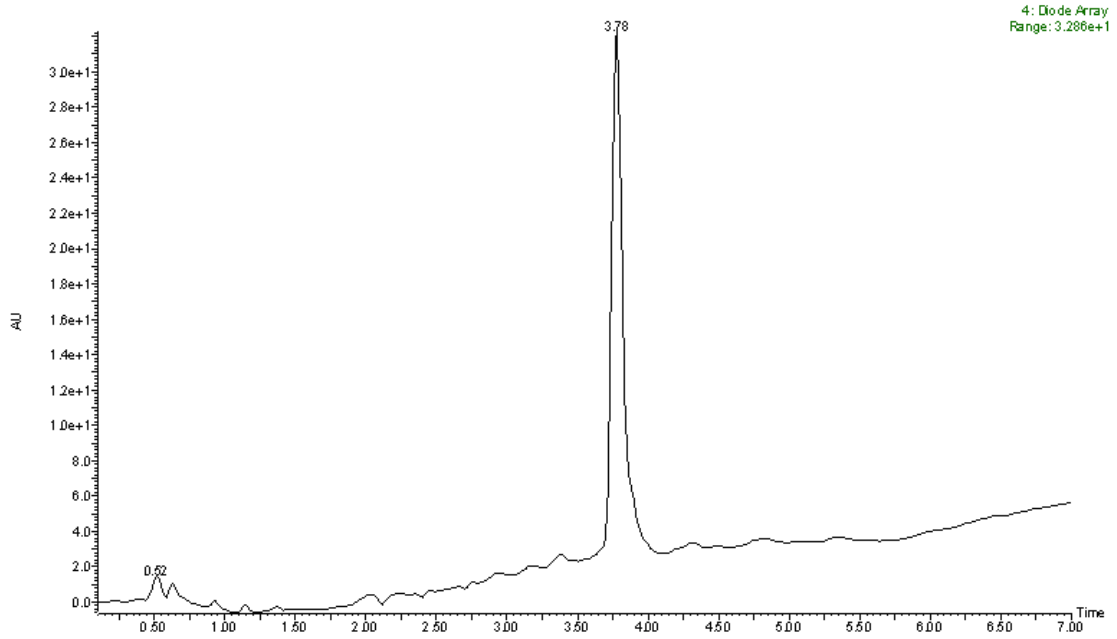

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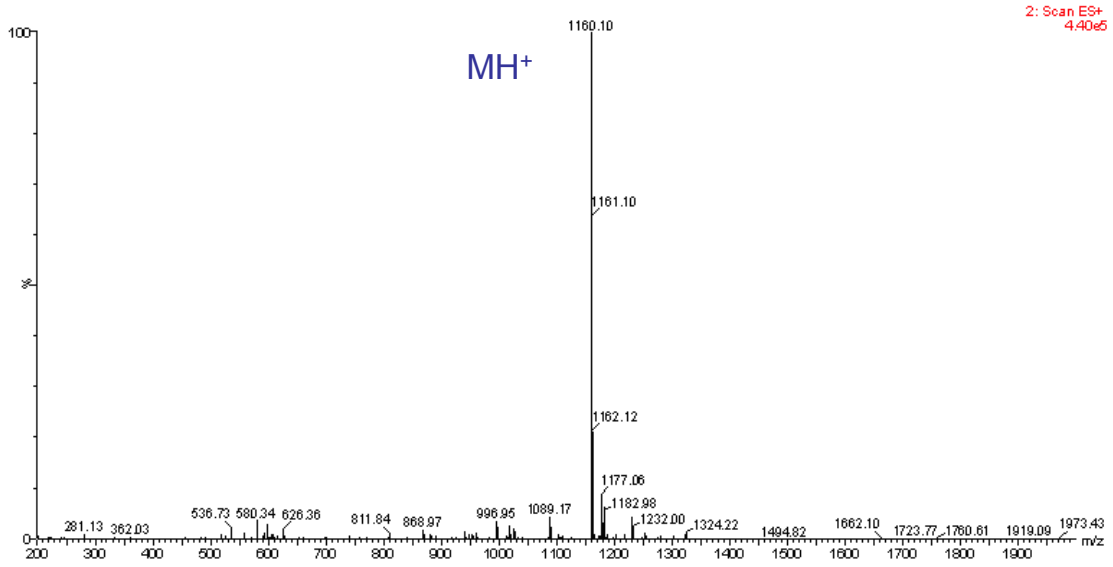

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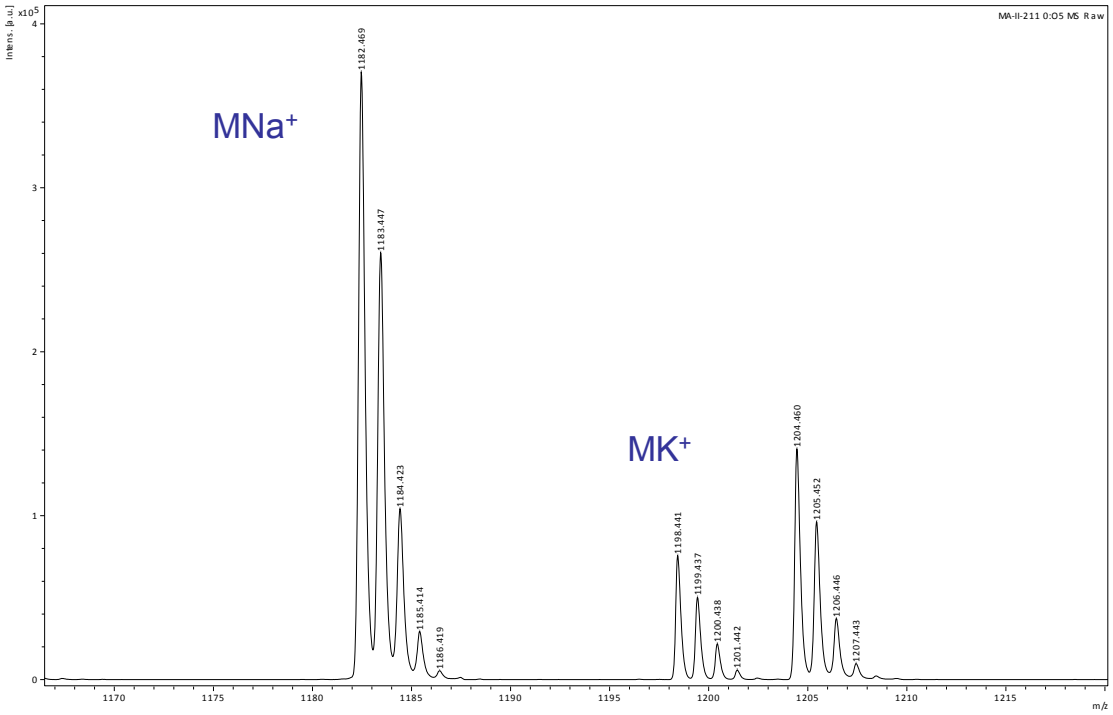
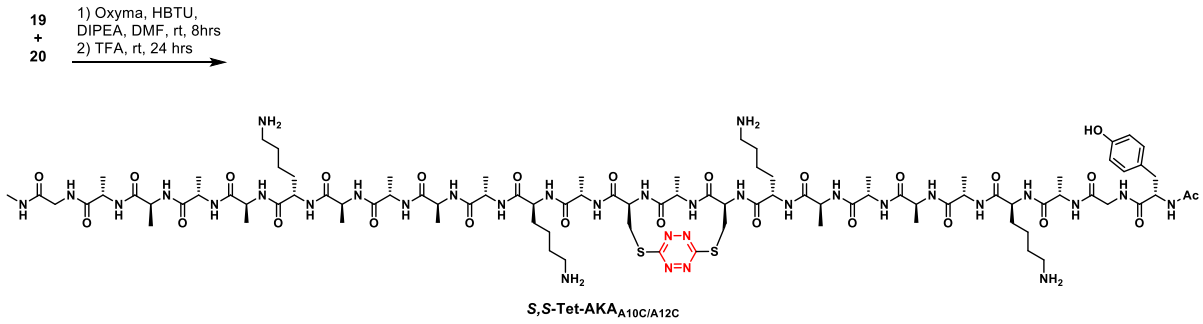
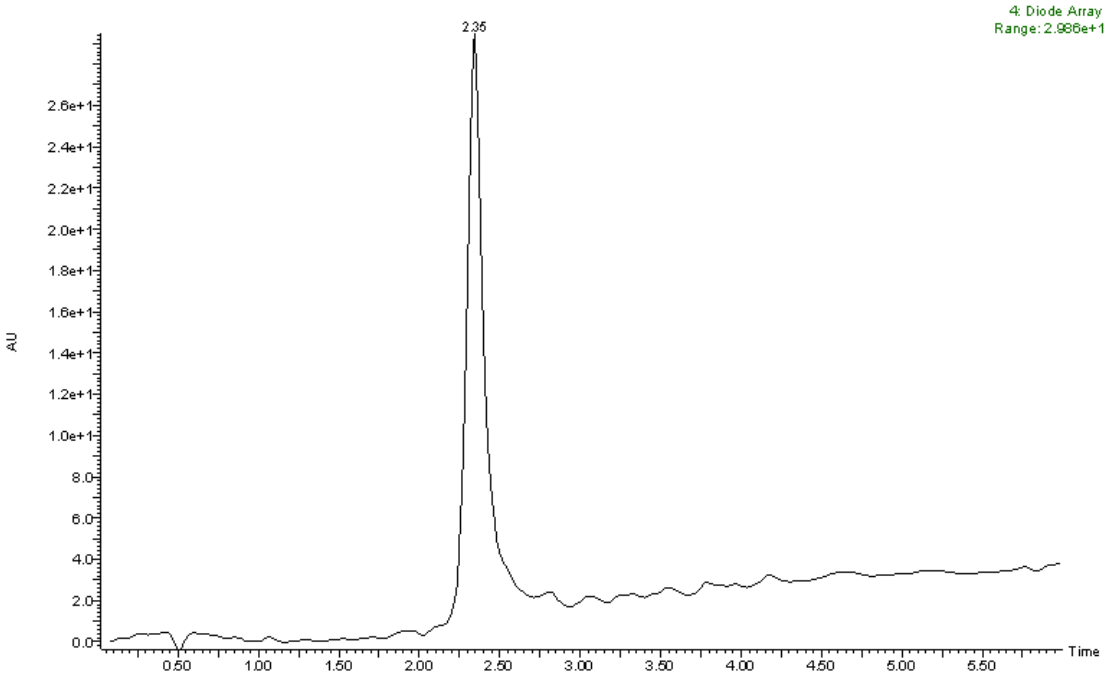


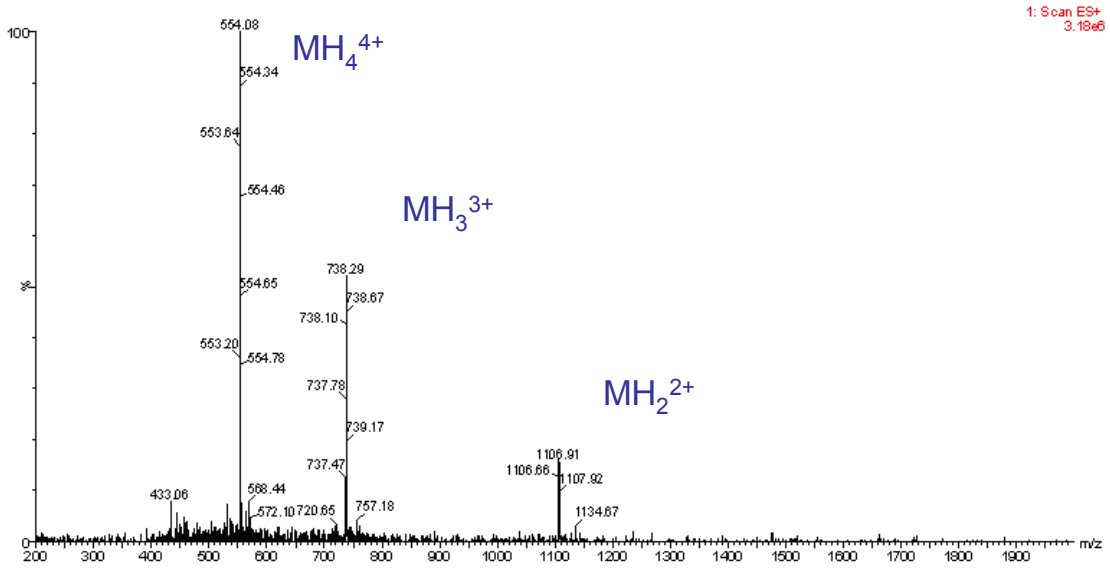


***S,S*-Tet-AKA$_{A10C/A12C}$**

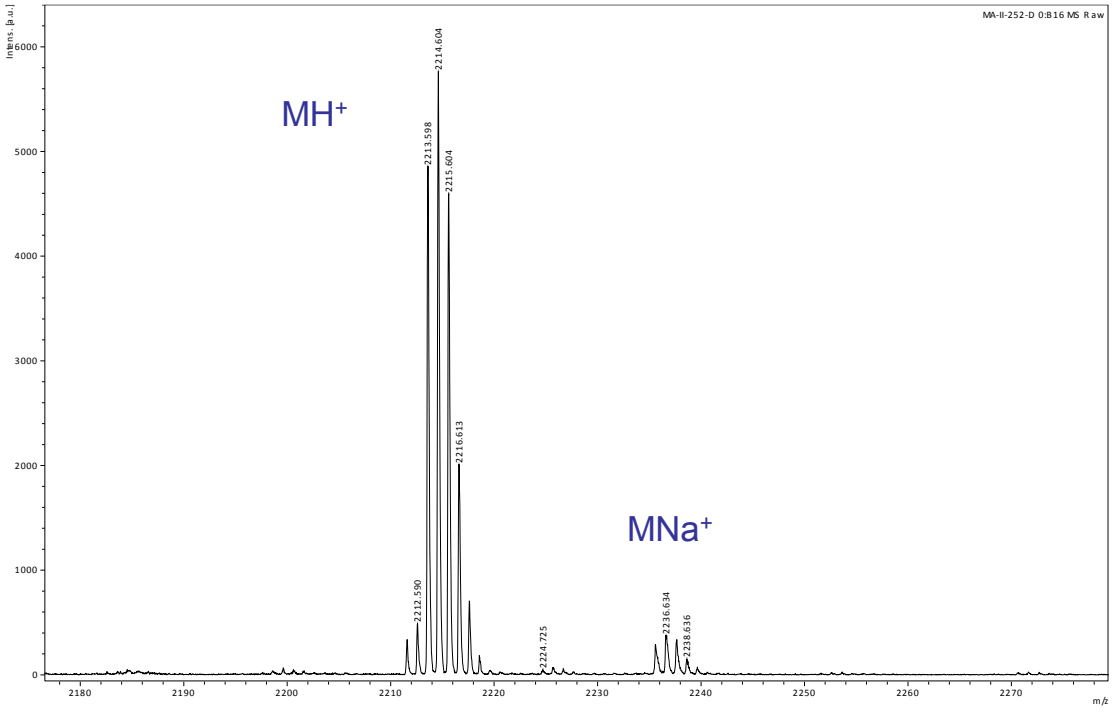
LC-MS chromatogram of peptide ***S,S*-Tet-AKA$_{A10C/A12C}$**:



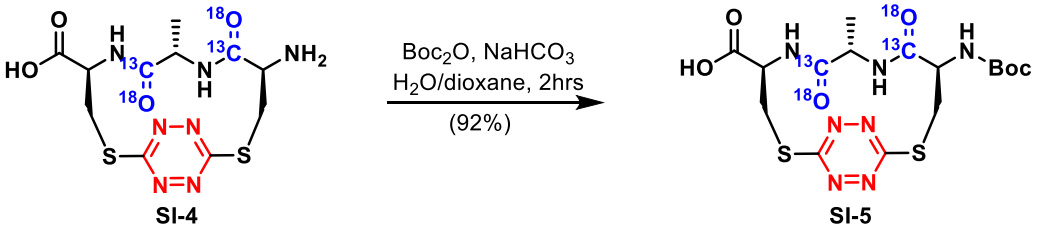
LC-MS mass spectrum of peptide ***S,S*-Tet-AKA$_{A10C/A12C}$**:



MALDI-TOF mass spectrum of peptide ***S,S*-Tet-AKA$_{A10C/A12C}$**:

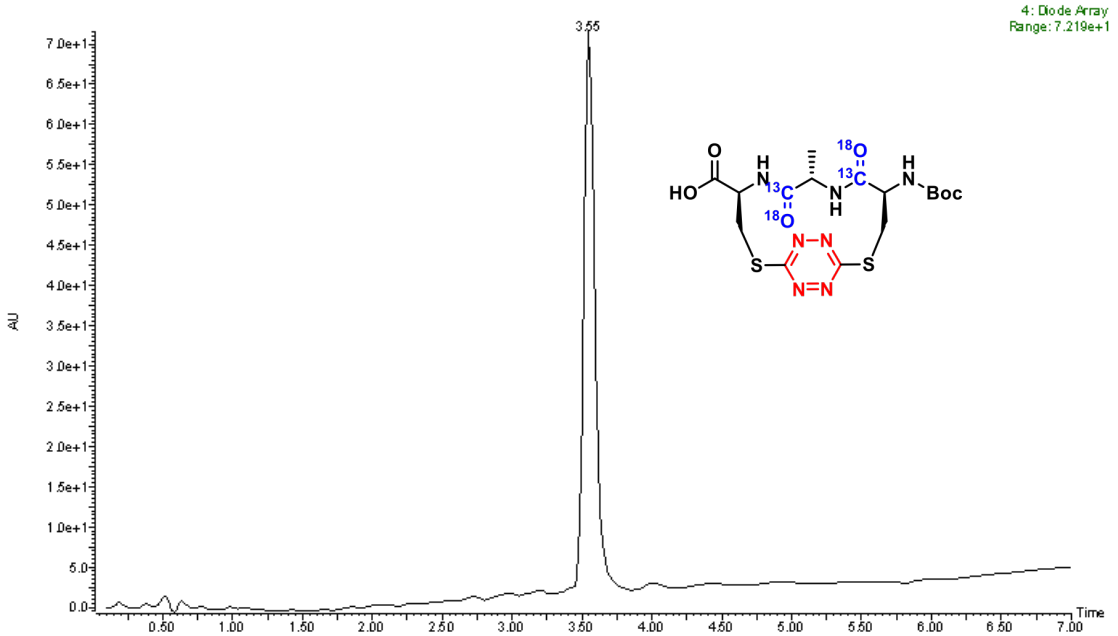


Enriched $^{13}C/^{18}O$ Amide Containing Peptides

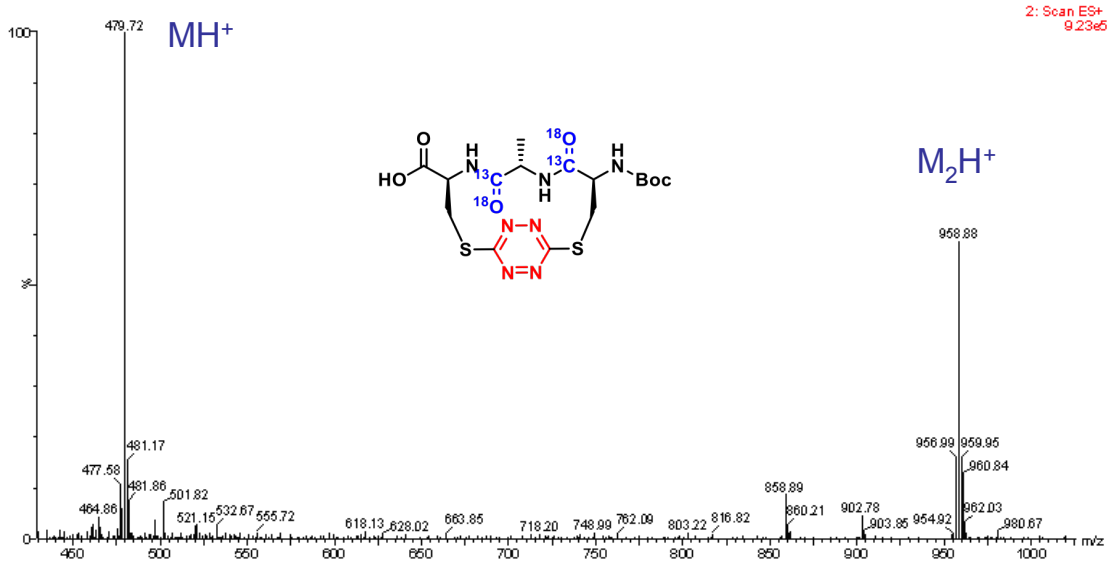


Peptide SI-5. A 25 mL round bottom flask was charged with **SI-4** (19.0 mg, 0.050 mmol) and $NaHCO_3$ (10.5 mg, 0.125 mmol, 2.5 equiv) then dissolved in water (2.0 mL). A pre-mixed solution of Boc_2O (16.4 mg, 0.0.75 mmol, 1.5 equiv) dissolved in 1,4-dioxane (2.0 mL) was added dropwise over 15 min with stirring. After 2 h, completion of the reaction was observed by LC-MS and the reaction solution was directly purified by reverse-phase chromatography (gradient 10 – 80% organic over 10 min) to give 22.1 mg (92%) of **SI-5** as a red/orange amorphous powder after lyophilization: HRMS (ES) *m/z* 478.1216 [$(M-H)^-$; calcd for $^{12}C_{14}{}^{13}C_2H_{22}N_7{}^{16}O_4{}^{18}O_2S_2$: 478.1225].

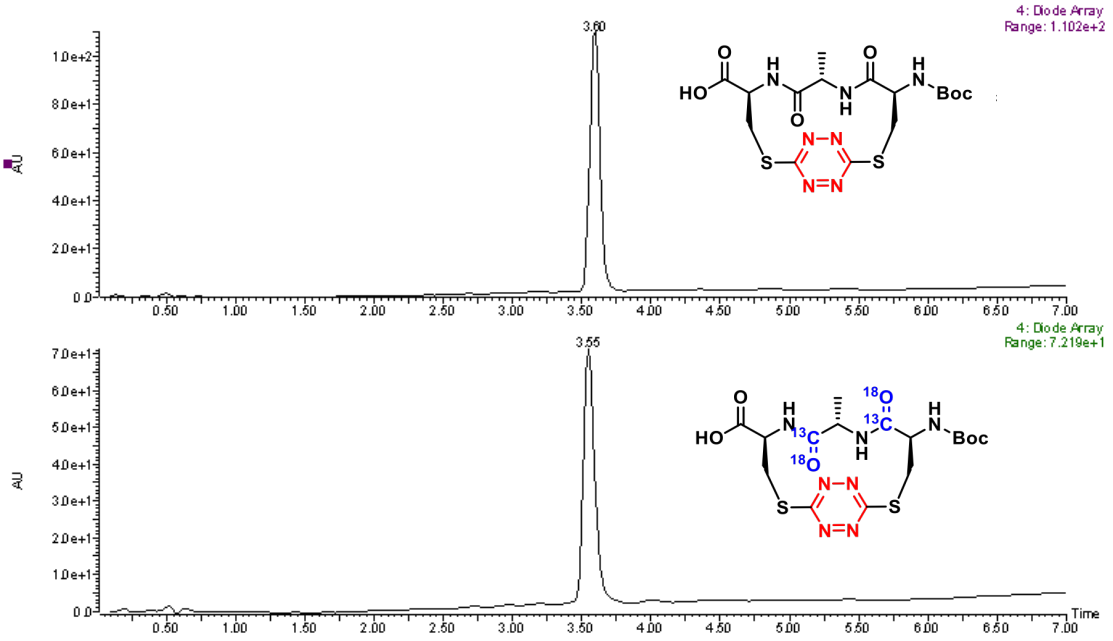
LC-MS chromatogram of peptide **SI-5**:



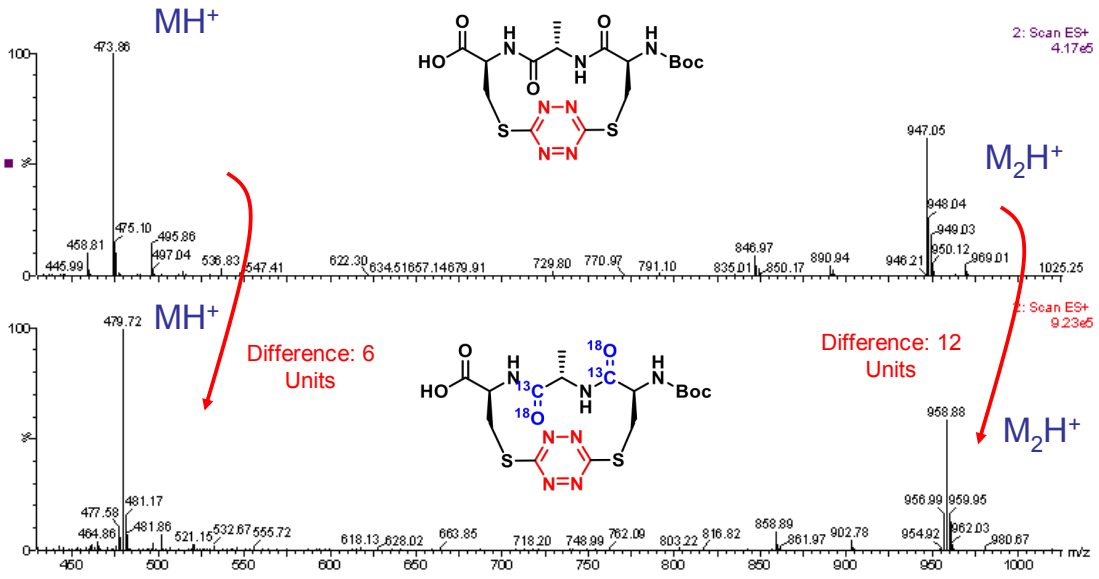
LC-MS mass spectrum of peptide **SI-5**:

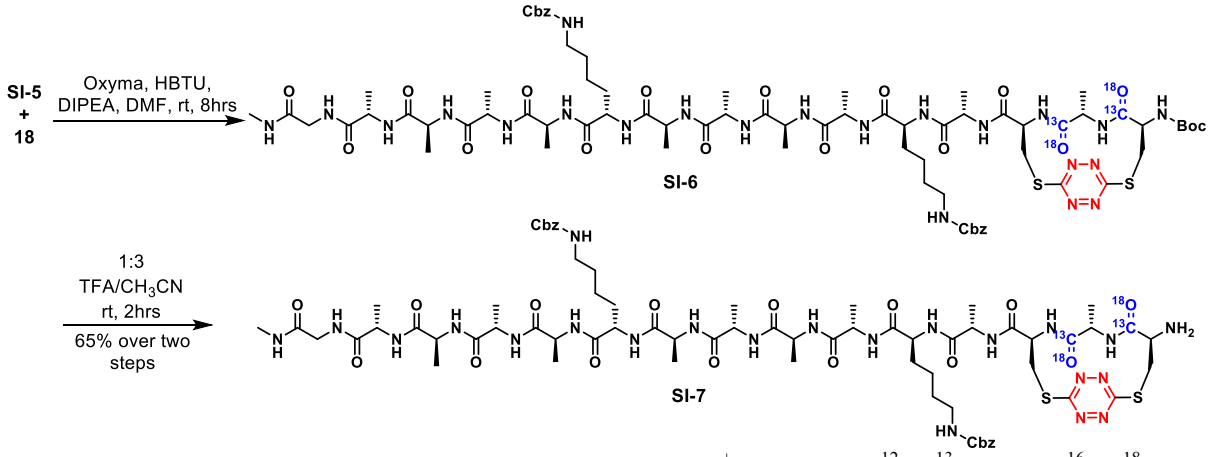


LC-MS chromatograms comparing peptide **17** to enriched peptide **SI-5**:



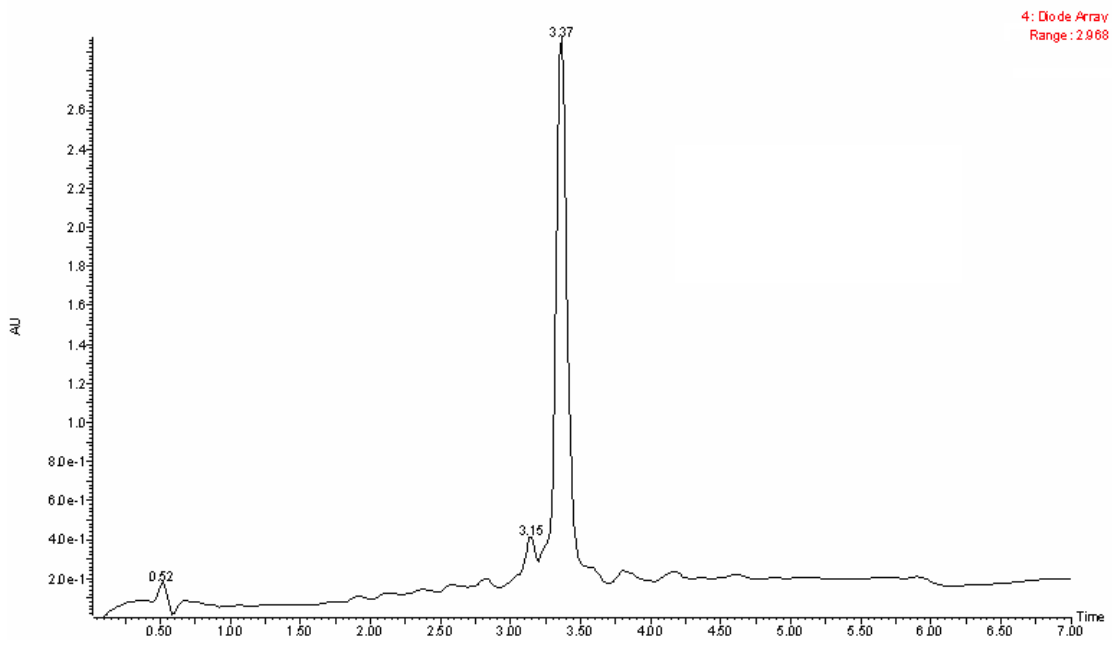
LC-MS mass spectra comparing peptide **17** to enriched peptide **SI-5**:





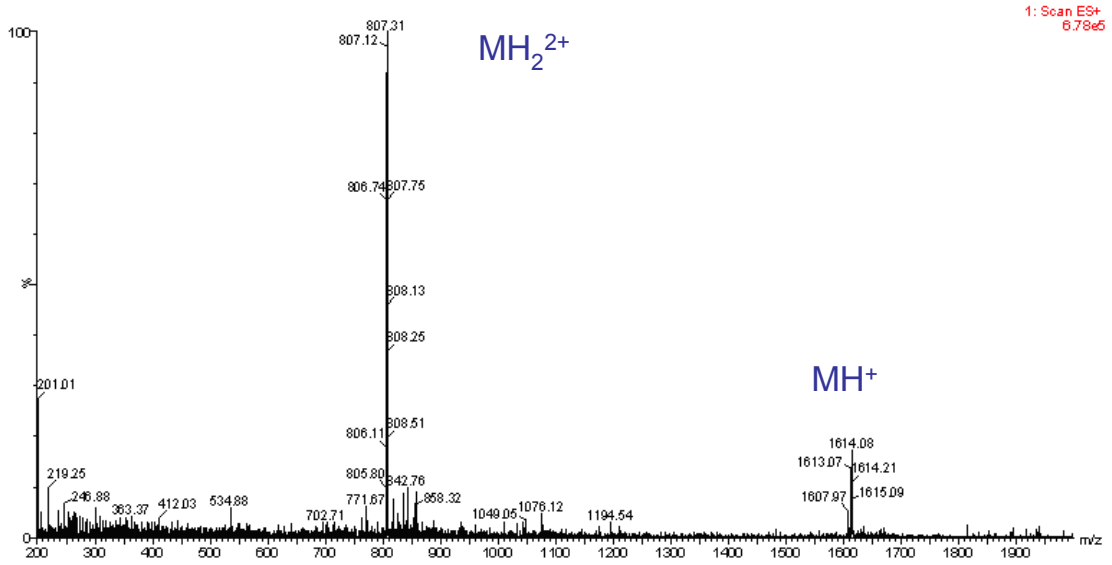
Peptide SI-7. HRMS (ES) *m/z* 1613.7458 [(M+H)$^+$; calcd for $^{12}C_{67}{}^{13}C_2H_{103}N_{22}{}^{16}O_{17}{}^{18}O_2S_2$: 1613.7363]; MALDI-TOF *m/z* 1636.069 [(M+Na)$^+$; calcd for $^{12}C_{67}{}^{13}C_2H_{102}N_{22}{}^{16}O_{17}{}^{18}O_2NaS_2$: 1635.7183].

LC-MS chromatogram of peptide **SI-7**:

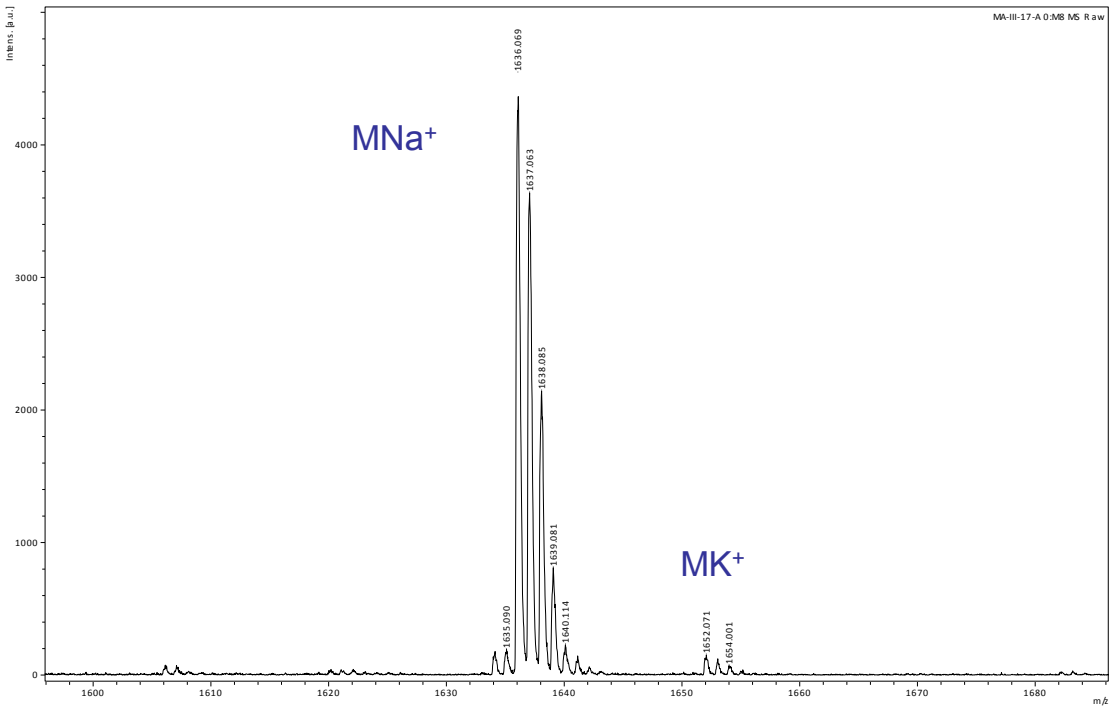


LCMS

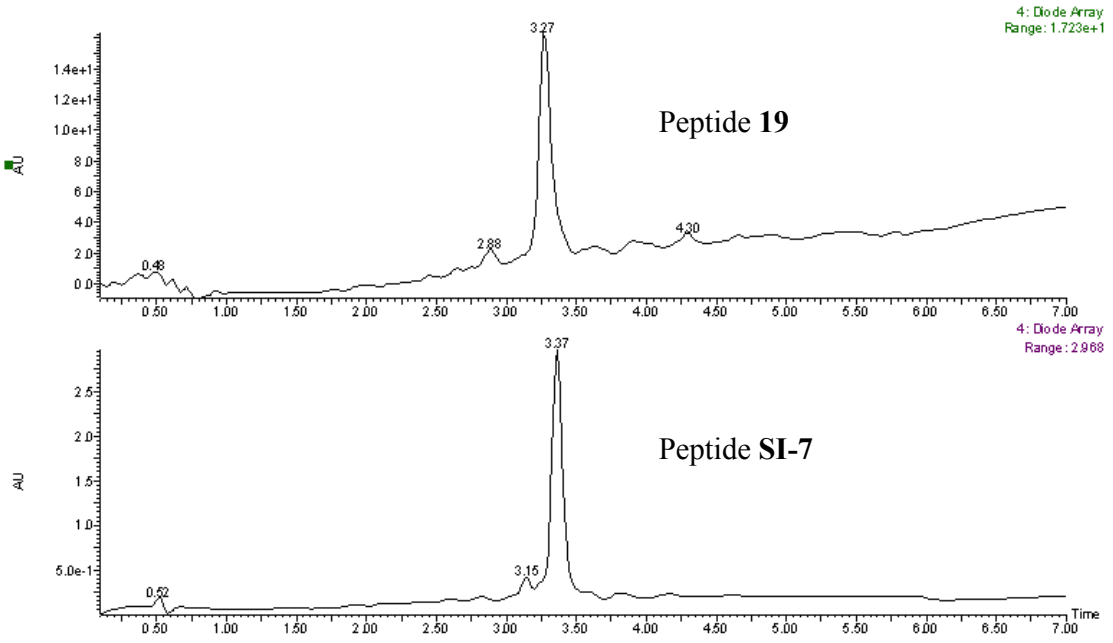
LC-MS mass spectrum of peptide **SI-7**:



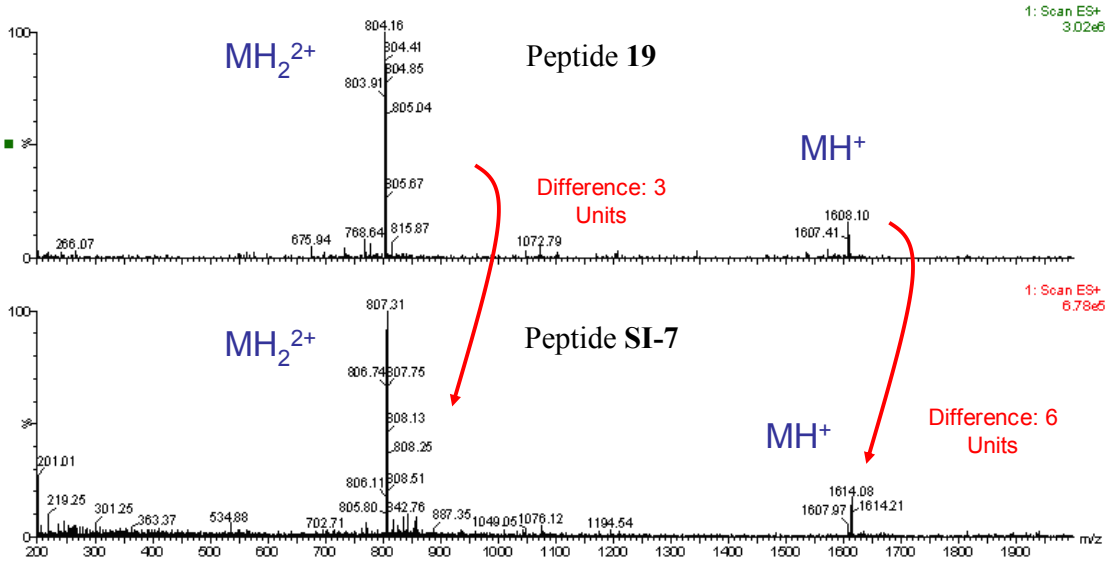
MALDI-TOF mass spectrum of peptide **SI-7**:



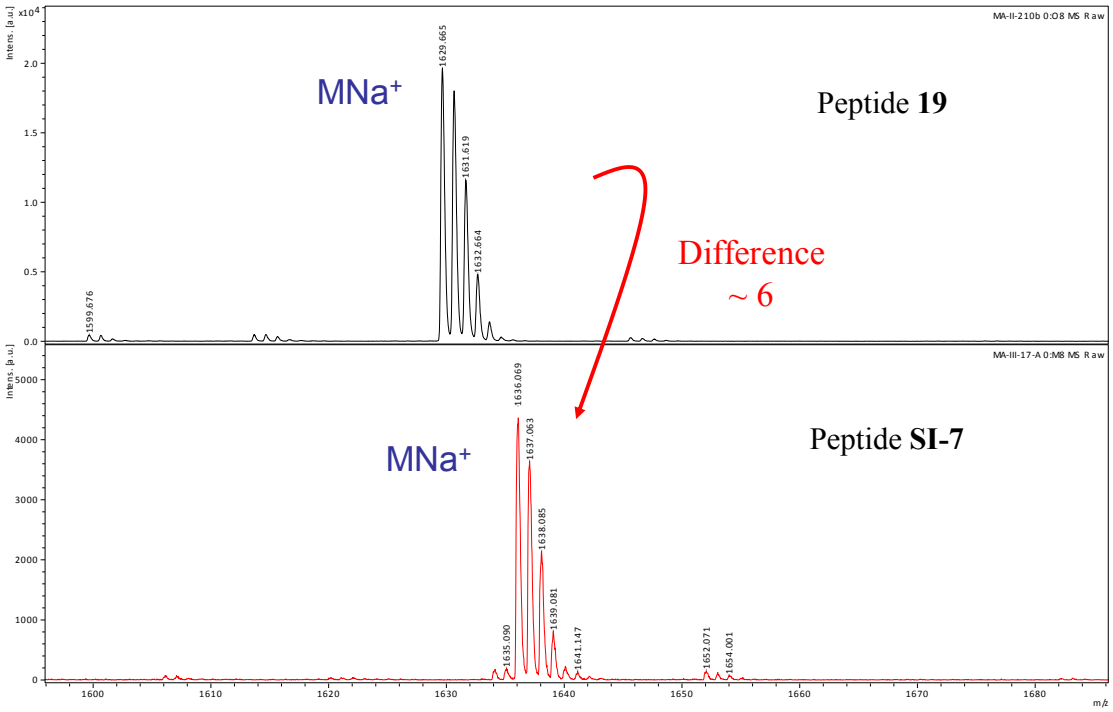
LC-MS chromatograms comparing peptide **19** to enriched peptide **SI-7**:

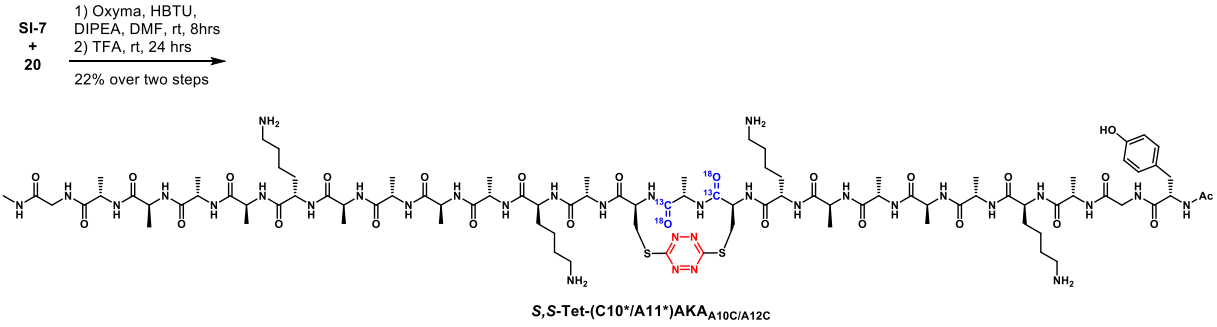


LC-MS mass spectra comparing peptide **19** to enriched peptide **SI-7**:



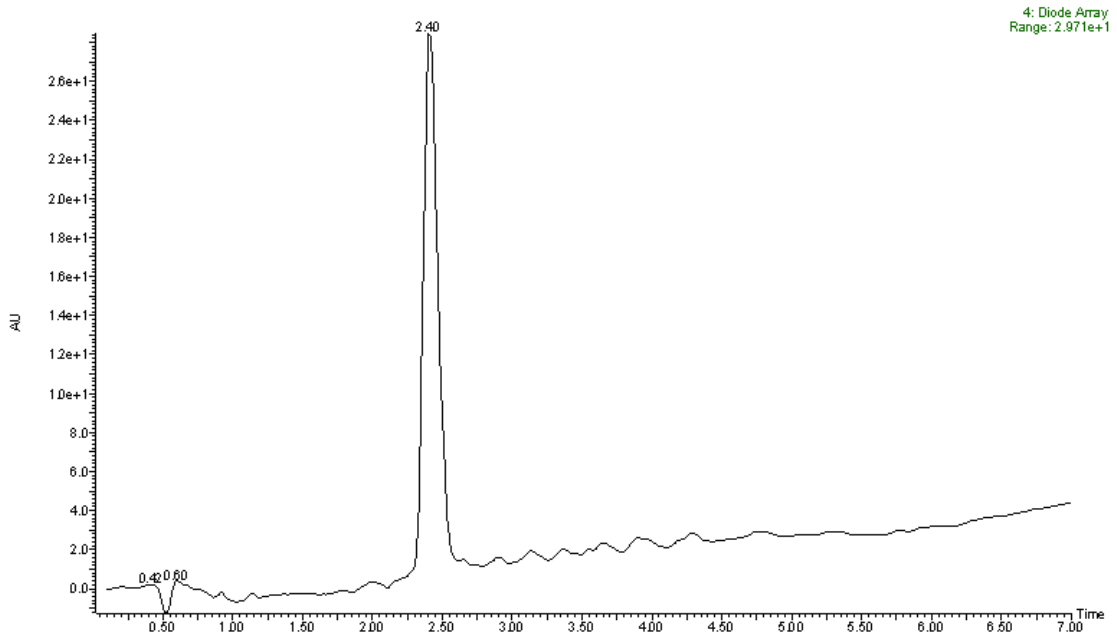
MALDI-TOF mass spectra comparing peptide **19** to enriched peptide **SI-7**:



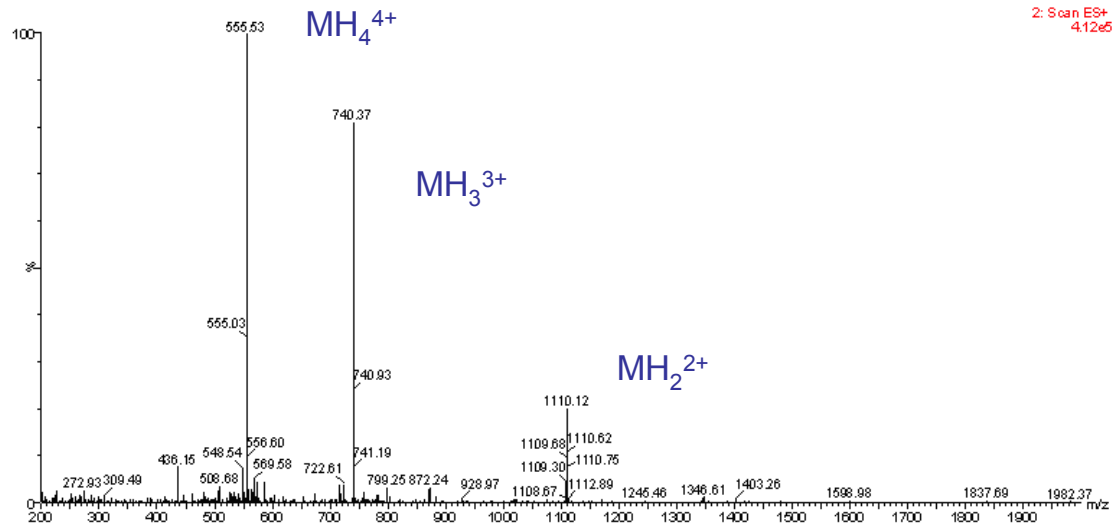


***S,S*-Tet-(C10*/A11*)AKA$_{A10C/A12C}$**

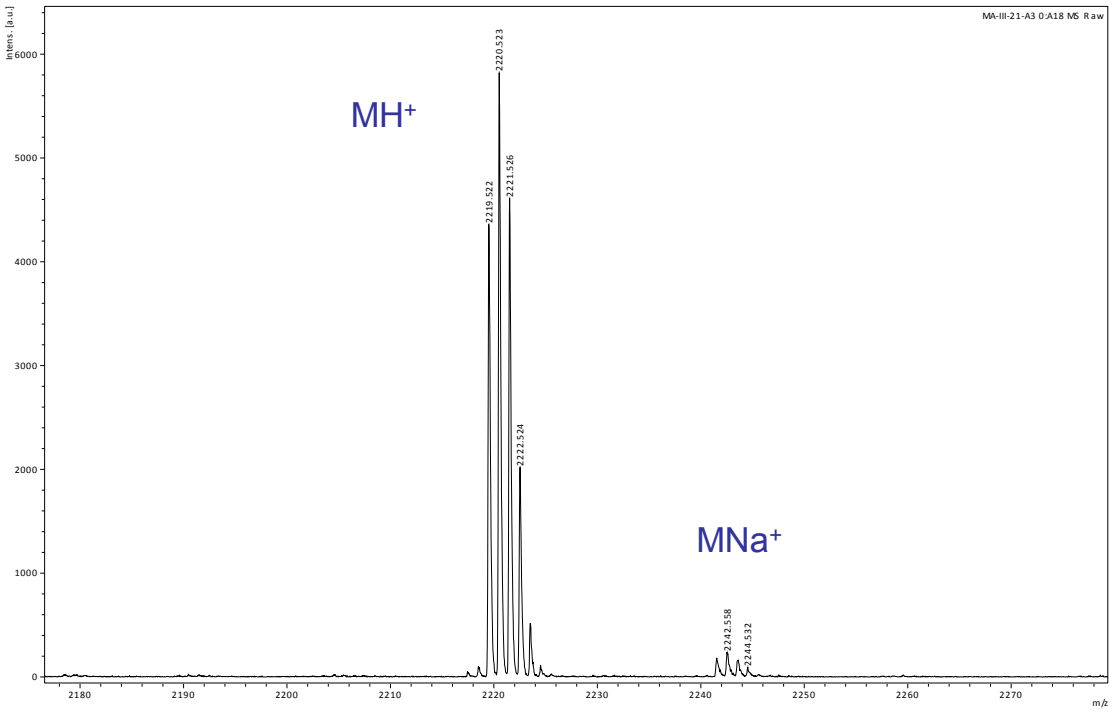
LC-MS chromatogram of peptide ***S,S*-Tet-(C10*/A11*)AKA$_{A10C/A12C}$**:



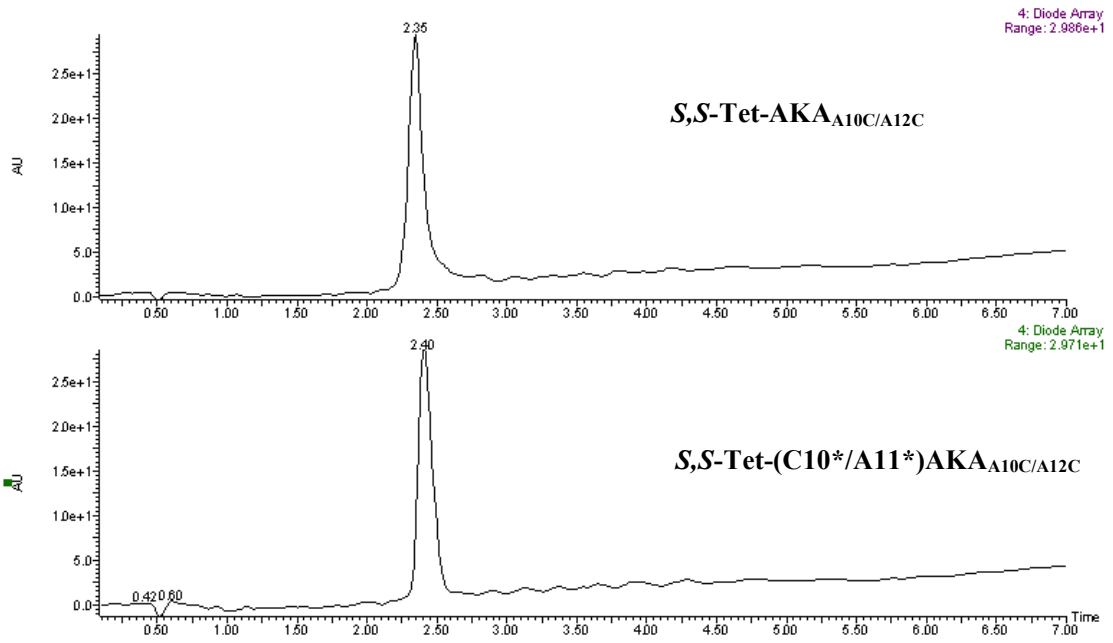
LC-MS mass spectrum of peptide ***S,S*-Tet-(C10*/A11*)AKA$_{A10C/A12C}$**:



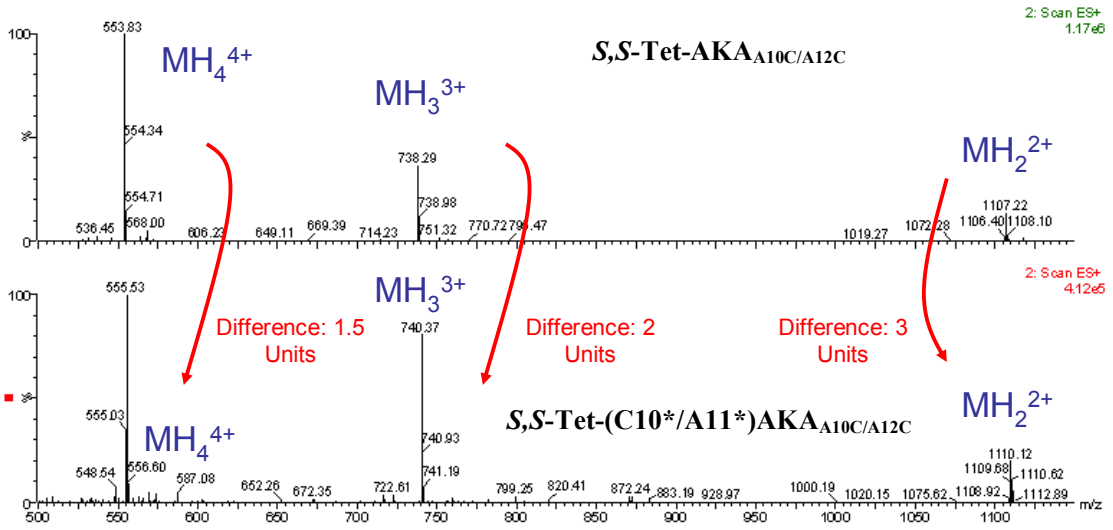
MALDI-TOF mass spectrum of peptide ***S,S*-Tet-(C10*/A11*)AKA$_{A10C/A12C}$**:



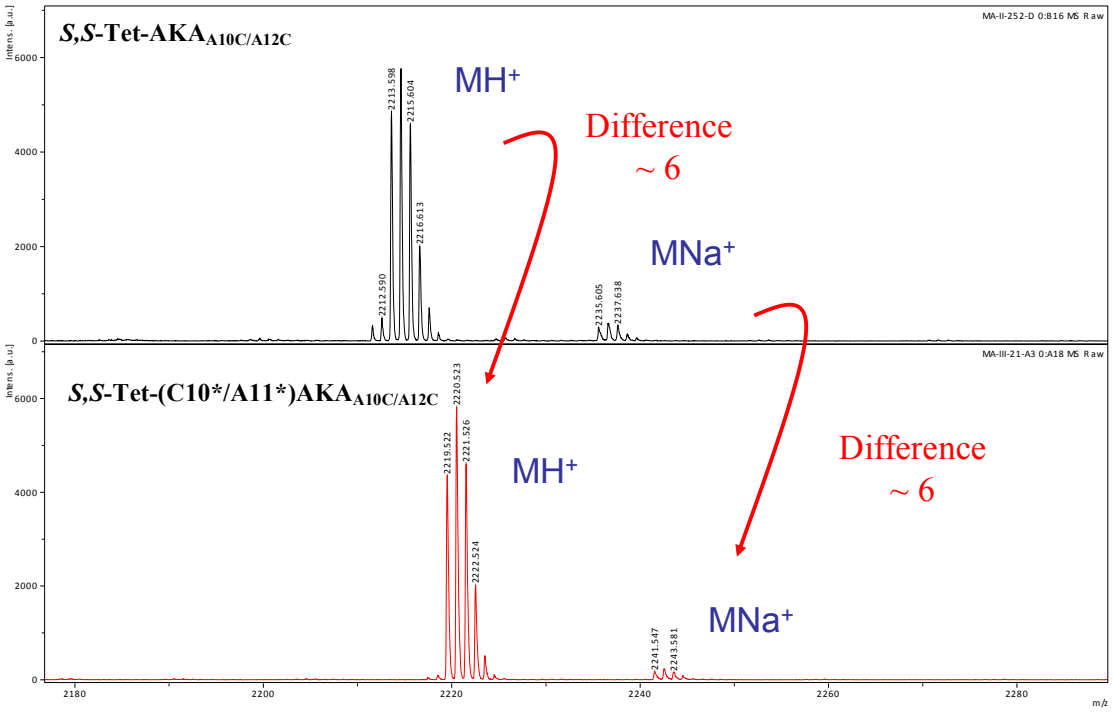
LC-MS chromatograms comparing peptide ***S,S*-Tet-AKA$_{A10C/A12C}$** to enriched peptide ***S,S*-Tet-(C10*/A11*)AKA$_{A10C/A12C}$**:

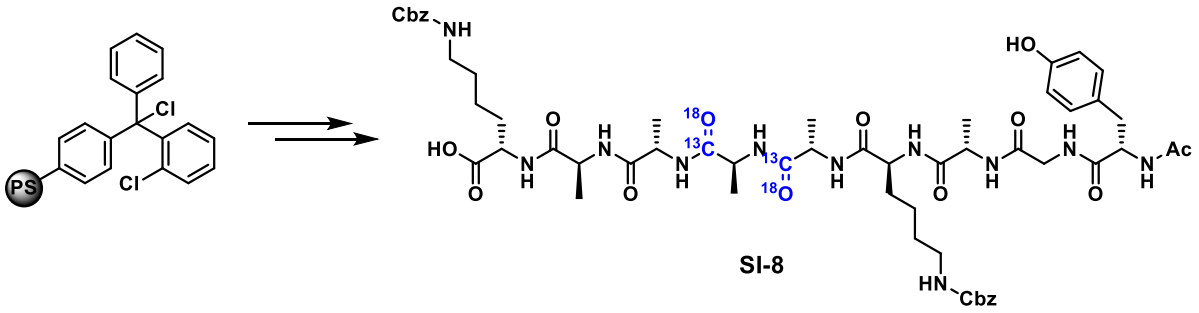


LC-MS mass spectra comparing peptide ***S,S*-Tet-AKA$_{A10C/A12C}$** to enriched peptide ***S,S*-Tet-(C10*/A11*)AKA$_{A10C/A12C}$**:



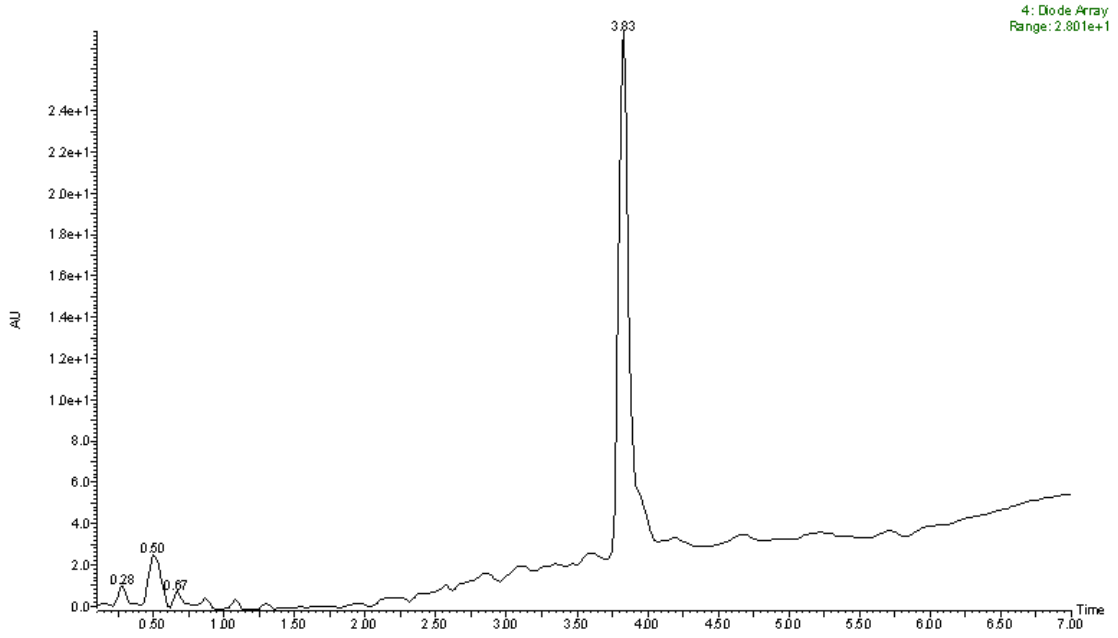
MALDI-TOF comparing peptide ***S,S*-Tet-AKA$_{A10C/A12C}$** to enriched peptide ***S,S*-Tet-(C10*/A11*)AKA$_{A10C/A12C}$**:



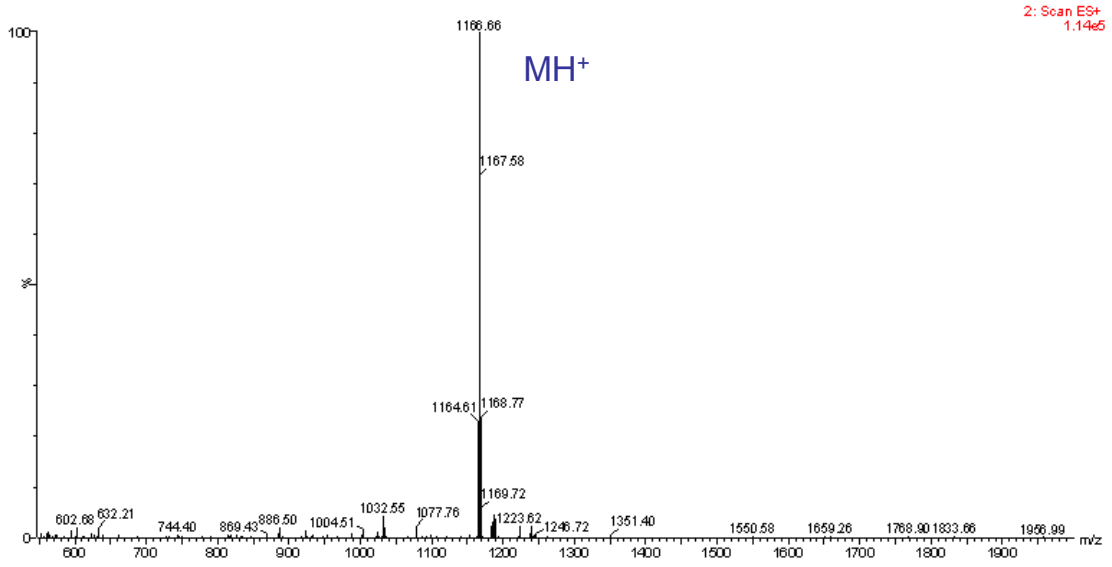


Peptide SI-8. HRMS (ES) *m/z* 1166.5781 [$(M+H)^+$; calcd for $C_{54}{}^{13}C_2H_{78}N_{11}O_{14}{}^{18}O_2$: 1166.5780]; MALDI-TOF *m/z* 1188.919 [$(M+Na)^+$; calcd for $C_{54}{}^{13}C_2H_{77}N_{11}O_{14}{}^{18}O_2Na$: 1188.5560].

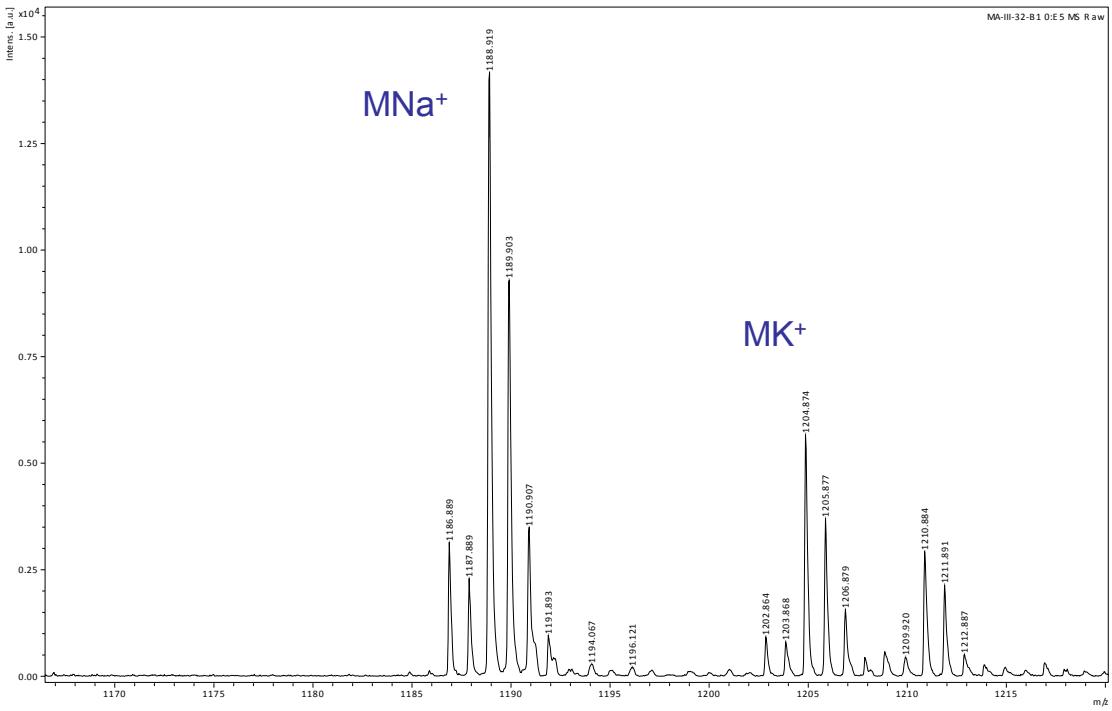
LC-MS chromatogram of peptide **SI-8**:



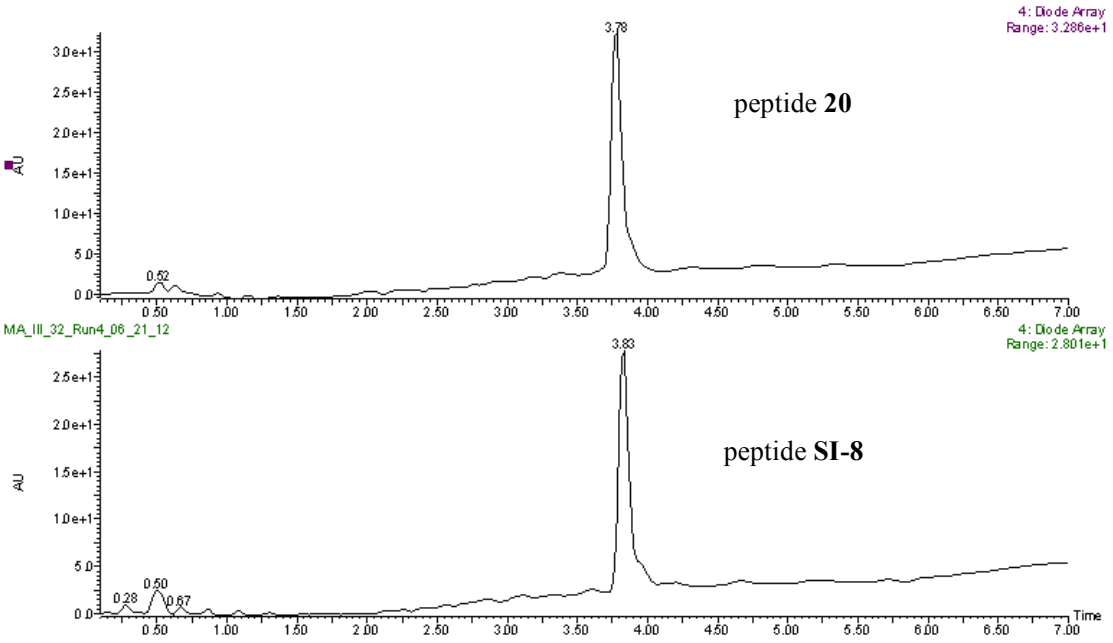
LC-MS mass spectrum of peptide **SI-8**:



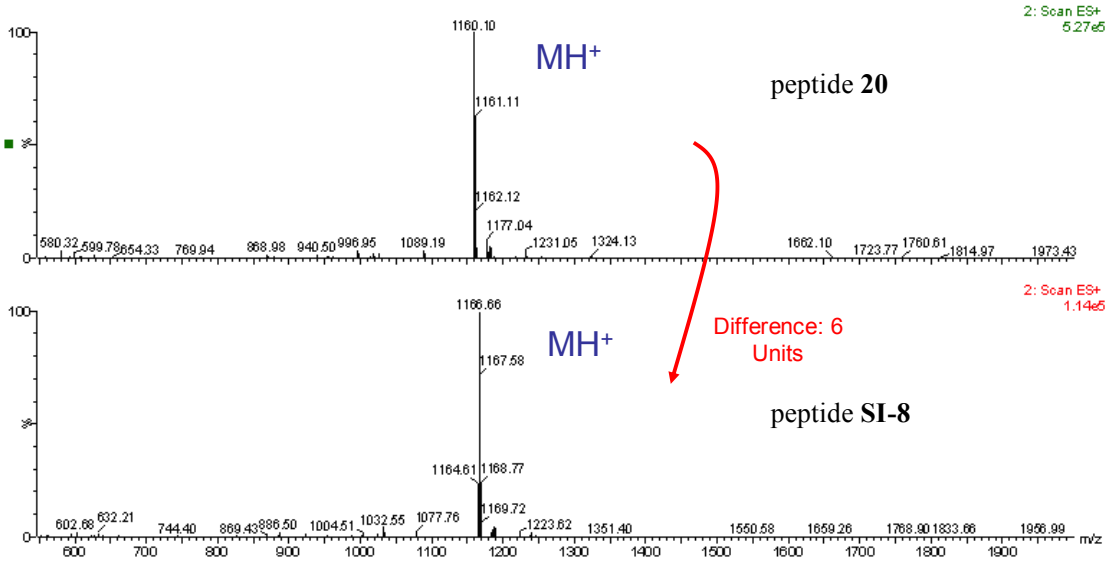
MALDI-TOF mass spectrum of peptide **SI-8**:



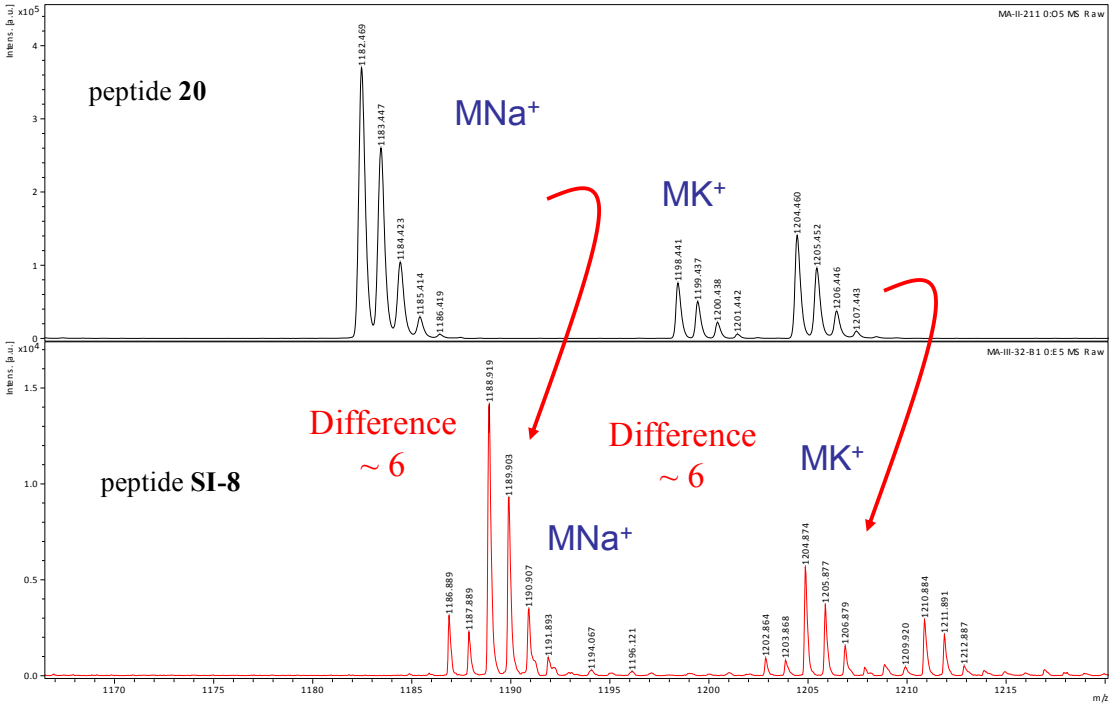
LC-MS chromatograms comparing peptide **20** to enriched peptide **SI-8**:

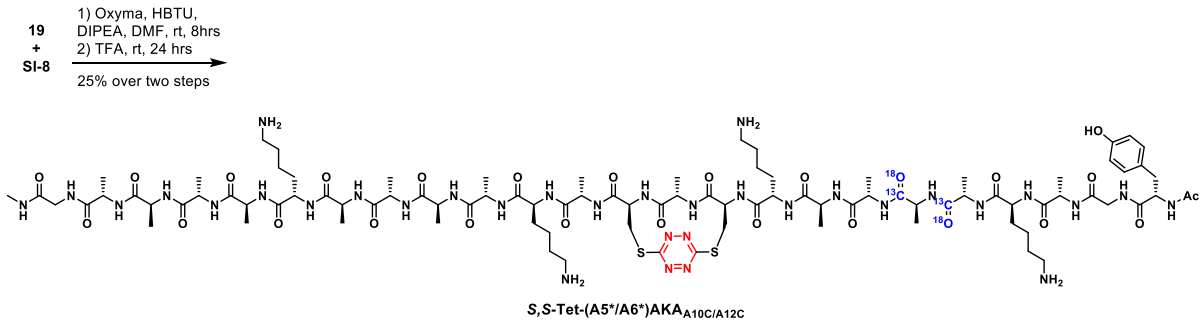


LC-MS mass spectra comparing peptide **20** to enriched peptide **SI-8**:



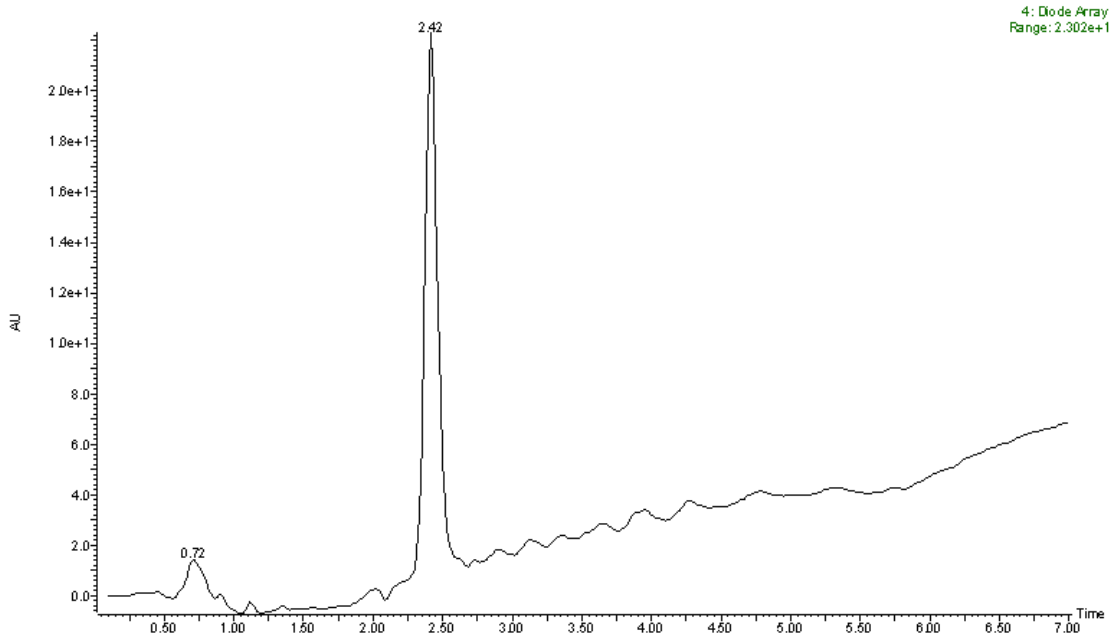
MALDI-TOF mass spectra comparing peptide **20** to enriched peptide **SI-8**:



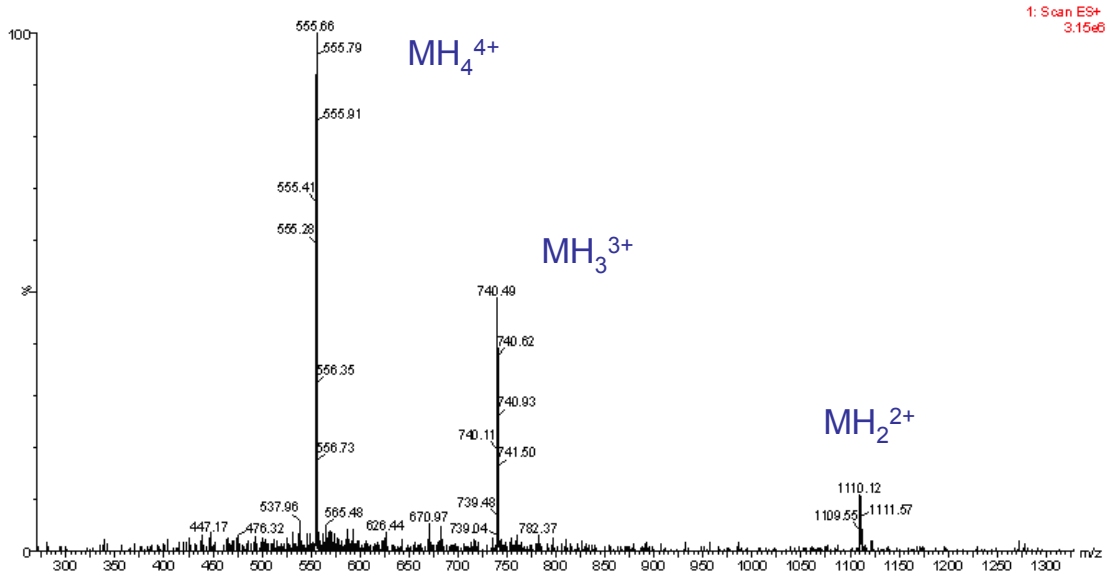


***S,S*-Tet-(A5*/A6*)AKA$_{A10C/A12C}$**

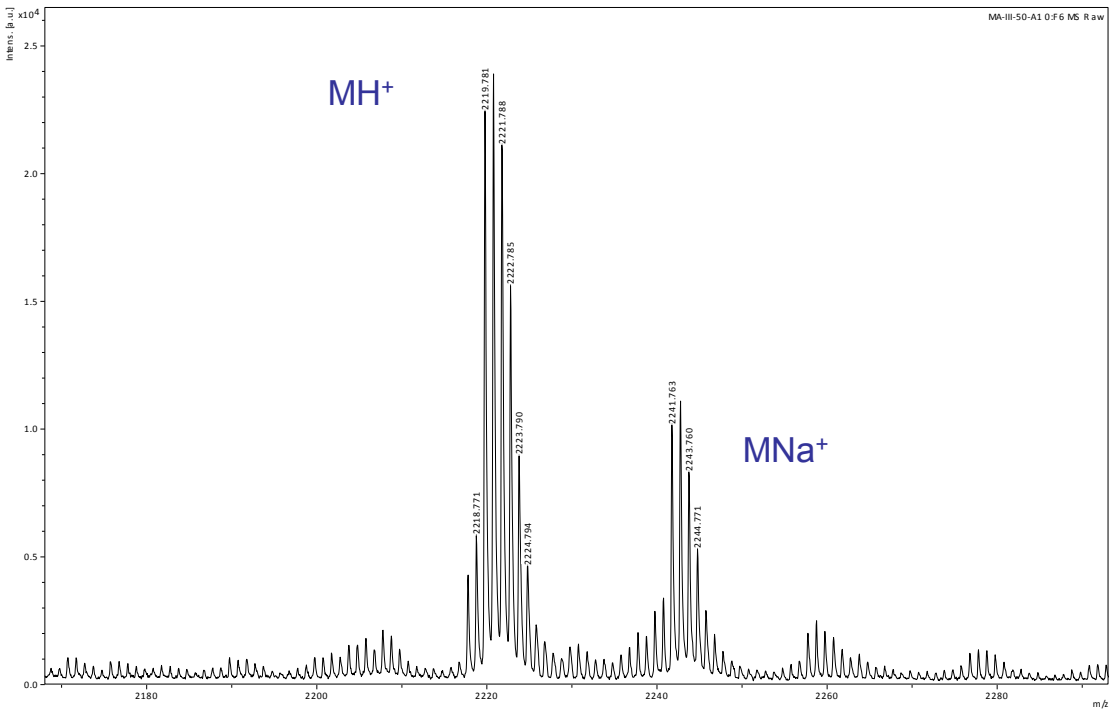
LC-MS chromatogram of peptide ***S,S*-Tet-(A5*/A6*)AKA$_{A10C/A12C}$**:



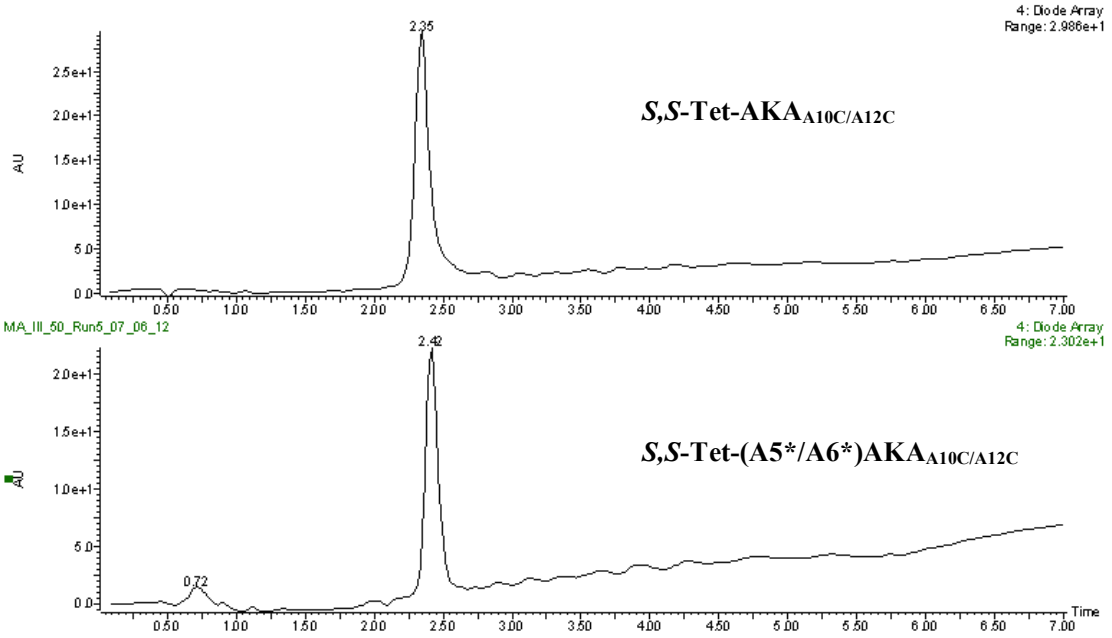
LC-MS mass spectrum of peptide ***S,S*-Tet-(A5*/A6*)AKA$_{A10C/A12C}$**:



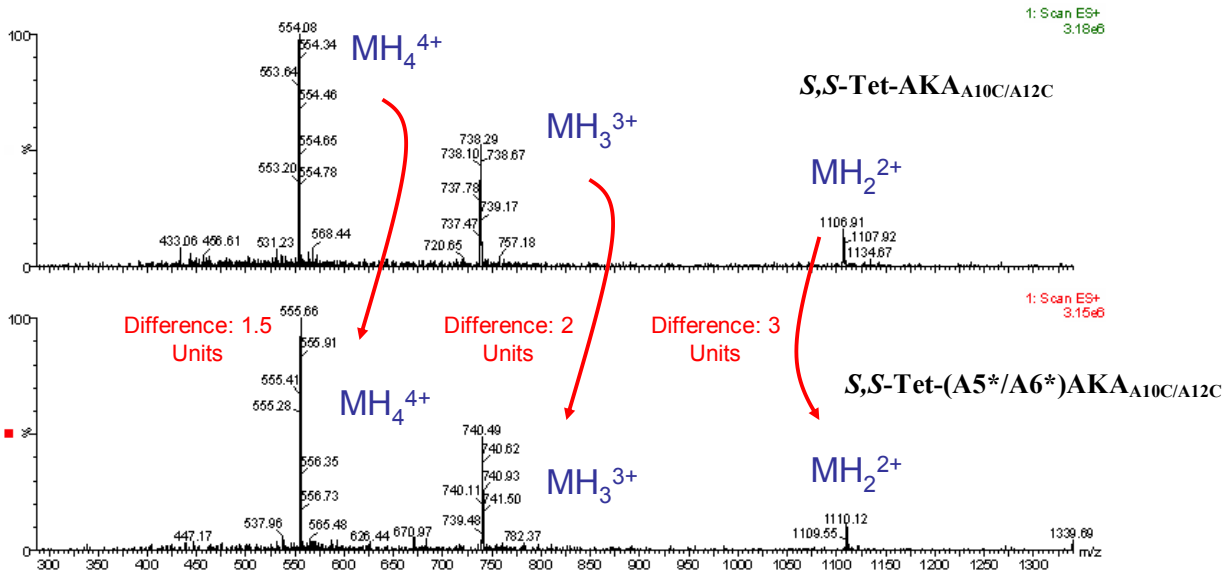
MALDI-TOF mass spectrum of peptide ***S,S*-Tet-(A5*/A6*)AKA$_{A10C/A12C}$**:



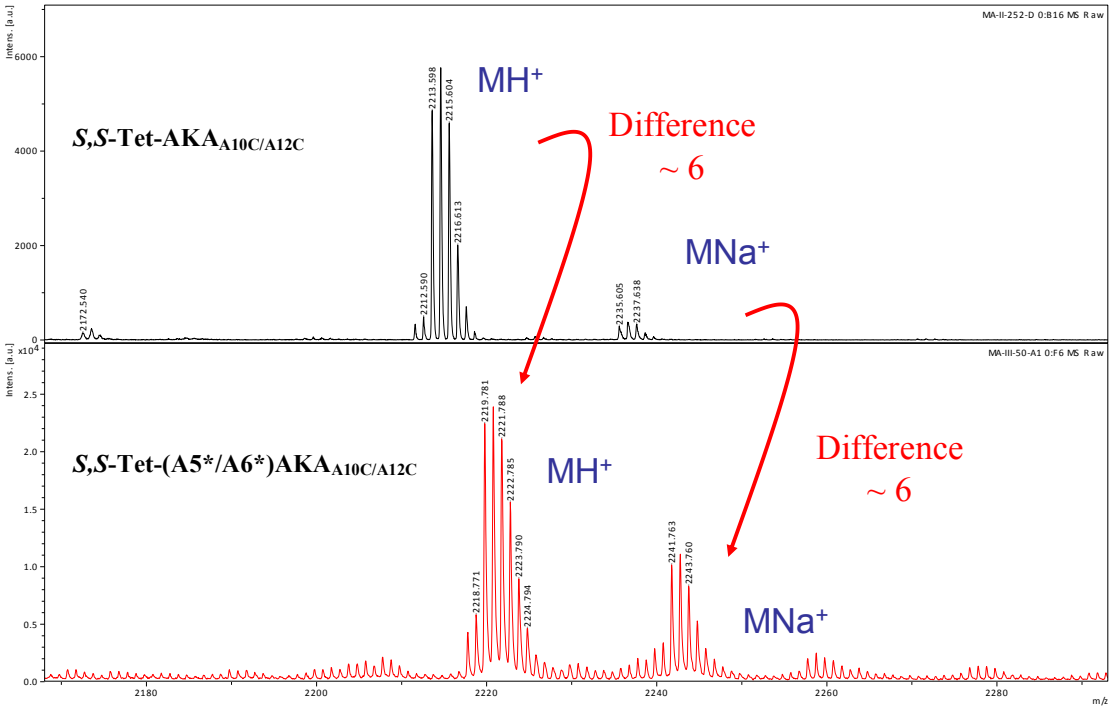
LC-MS chromatograms comparing peptide ***S,S*-Tet-AKA$_{A10C/A12C}$** to enriched peptide ***S,S*-Tet-(A5*/A6*)AKA$_{A10C/A12C}$**:

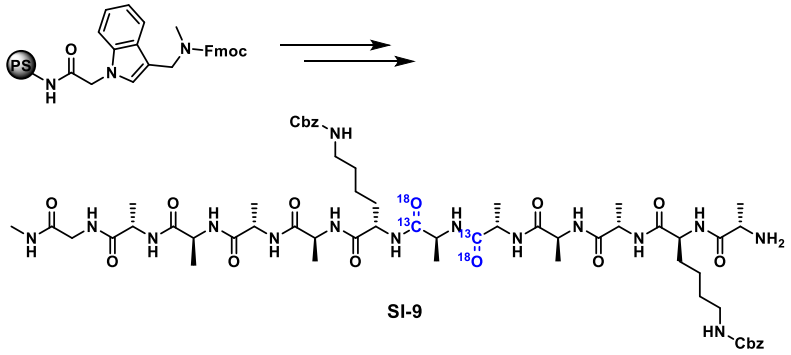


LC-MS mass spectra comparing peptide ***S,S*-Tet-AKA$_{A10C/A12C}$** to enriched peptide ***S,S*-Tet-(A5*/A6*)AKA$_{A10C/A12C}$**:



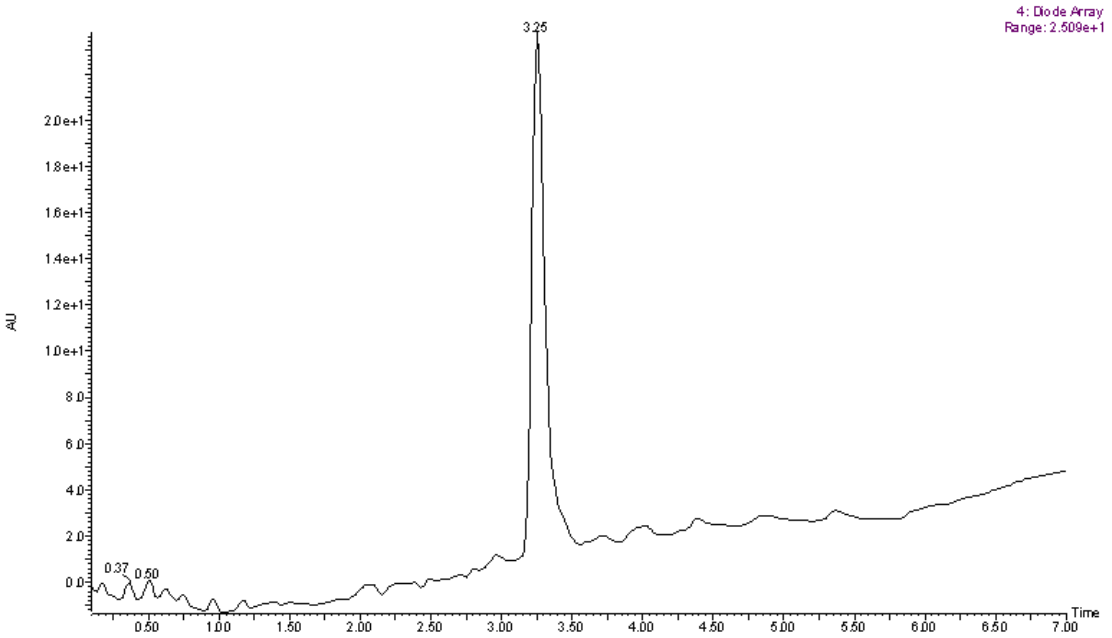
MALDI-TOF comparing peptide ***S,S*-Tet-AKA$_{A10C/A12C}$** to enriched peptide ***S,S*-Tet-(A5*/A6*)AKA$_{A10C/A12C}$**:



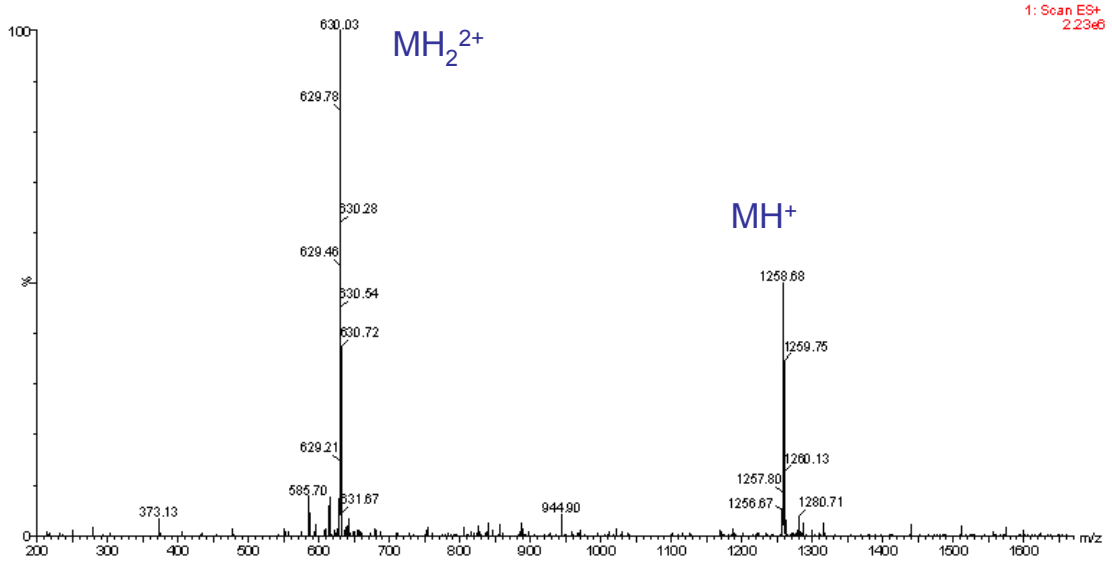


Peptide SI-9. HRMS (ES) *m/z* 1258.6870 [(M+H)$^+$; calcd for $C_{56}{}^{13}C_2H_{90}N_{15}O_{14}{}^{18}O_2$: 1258.6842]; MALDI-TOF *m/z* 1280.799 [(M+Na)$^+$; calcd for $C_{56}{}^{13}C_2H_{89}N_{15}O_{14}{}^{18}O_2Na$: 1280.6612].

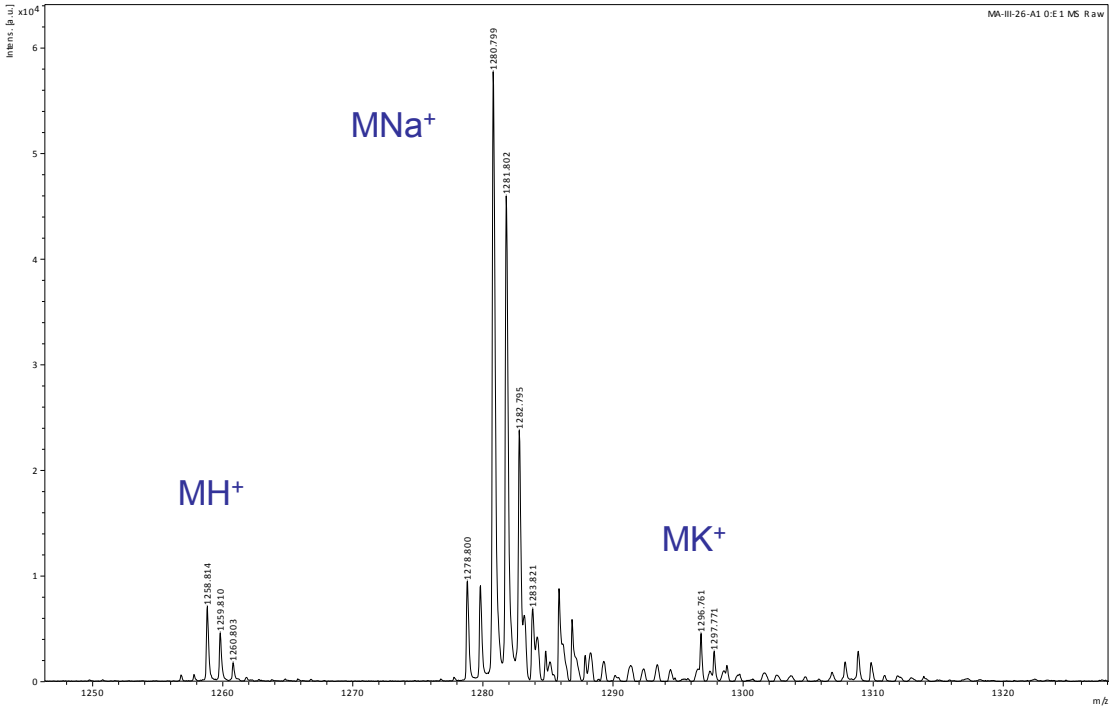
LC-MS chromatogram of peptide **SI-9**:



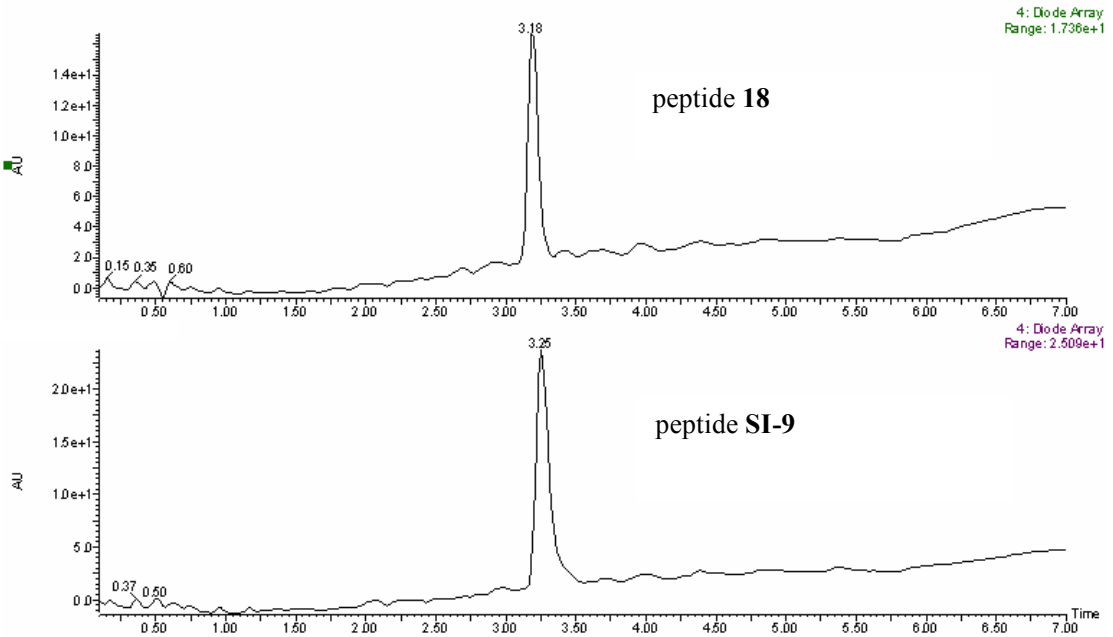
LC-MS mass spectrum of peptide **SI-9**:



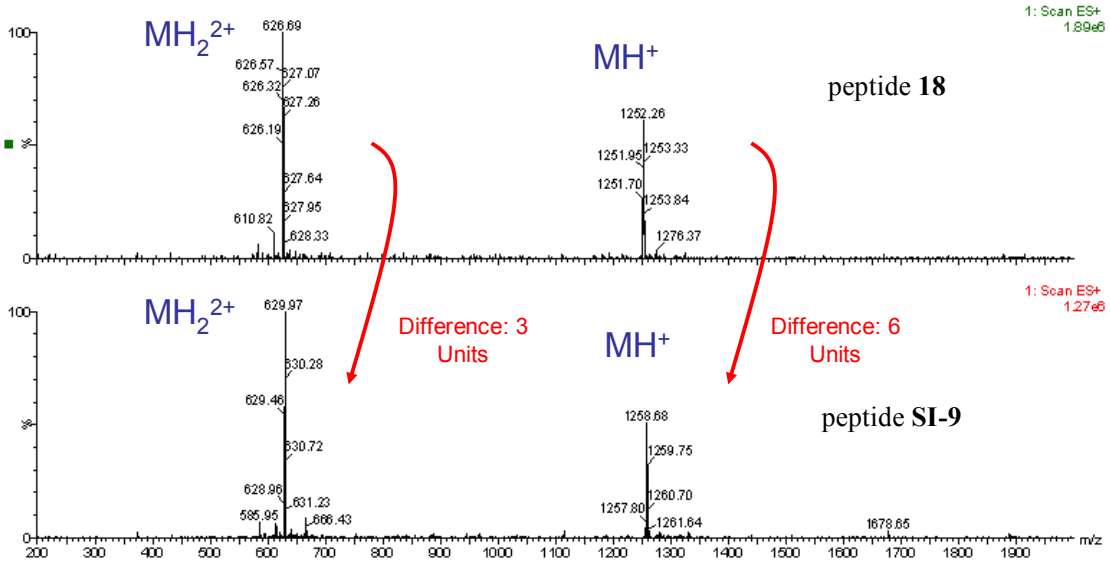
MALDI-TOF mass spectrum of peptide **SI-9**:



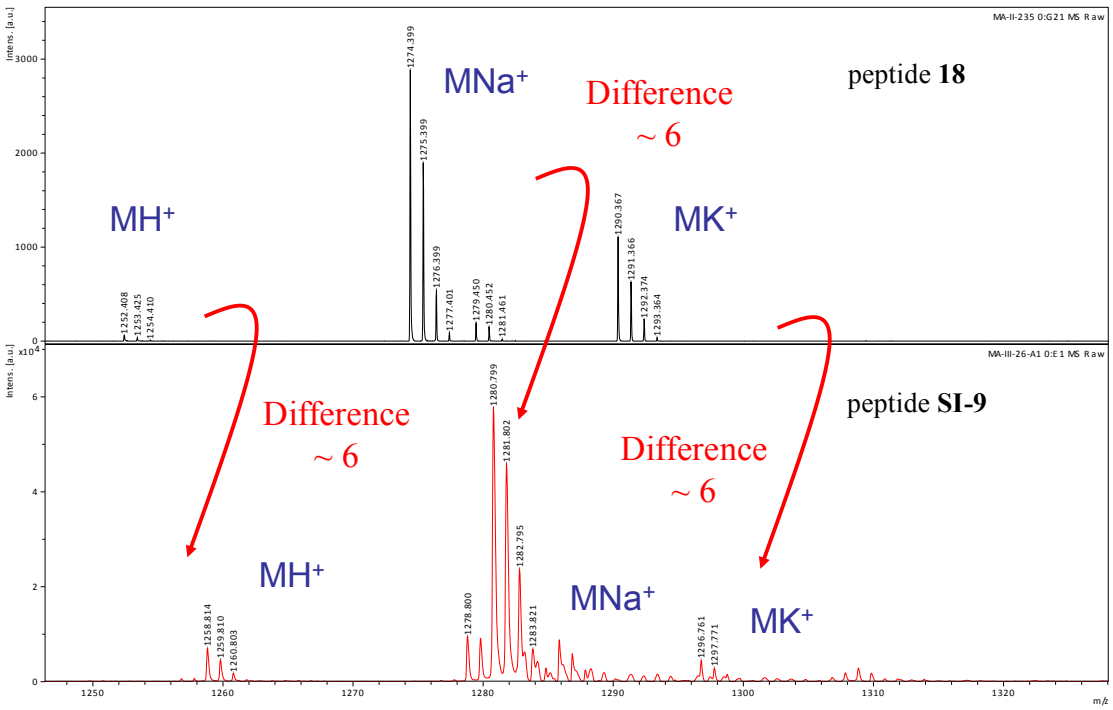
LC-MS chromatograms comparing peptide **18** to enriched peptide **SI-9**:

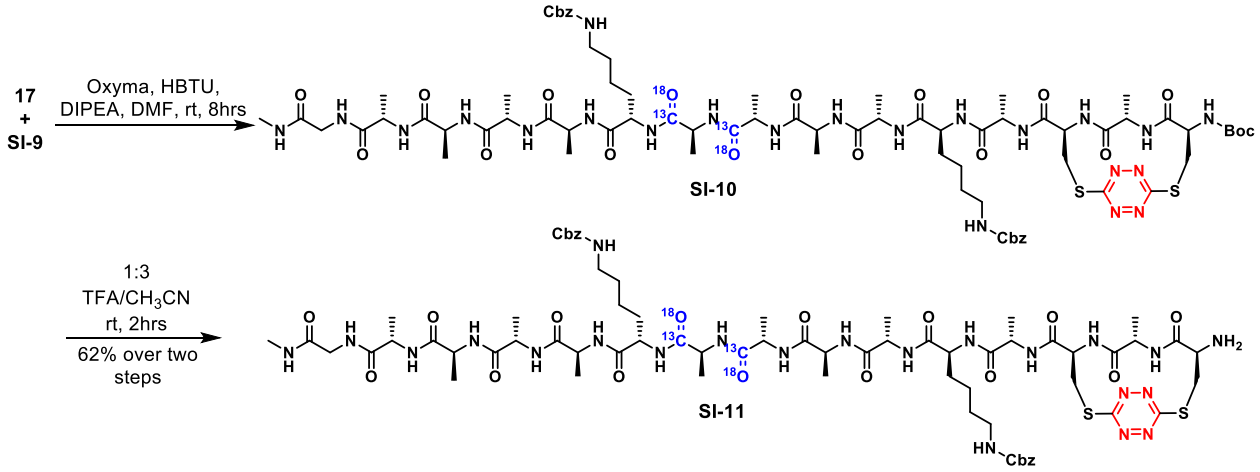


LC-MS mass spectra comparing peptide **18** to enriched peptide **SI-9**:



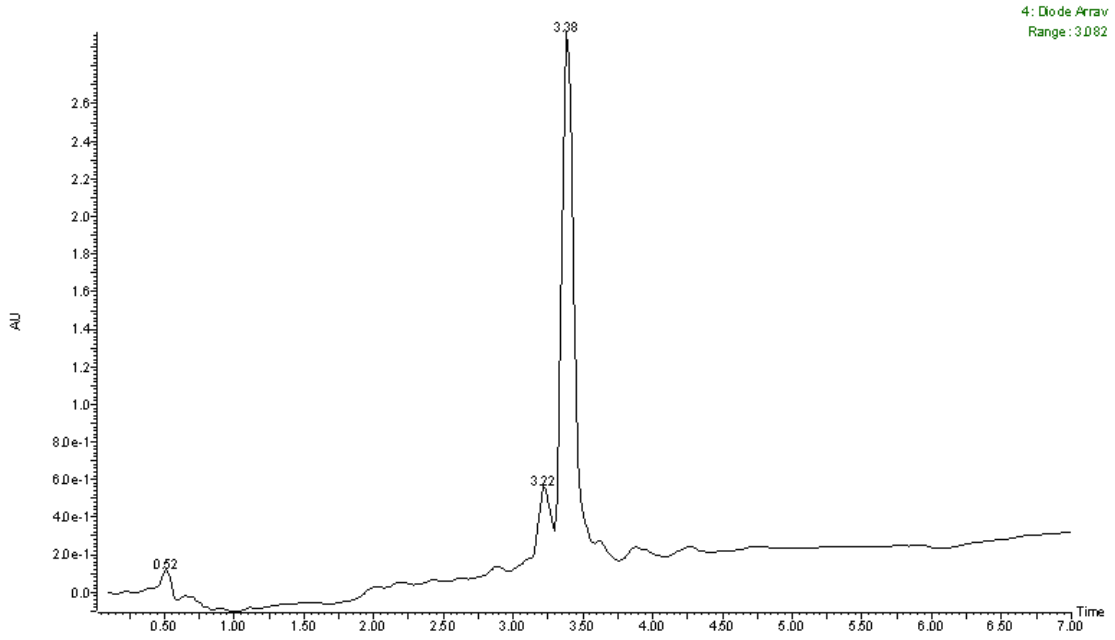
MALDI-TOF mass spectra comparing peptide **18** to enriched peptide **SI-9**:



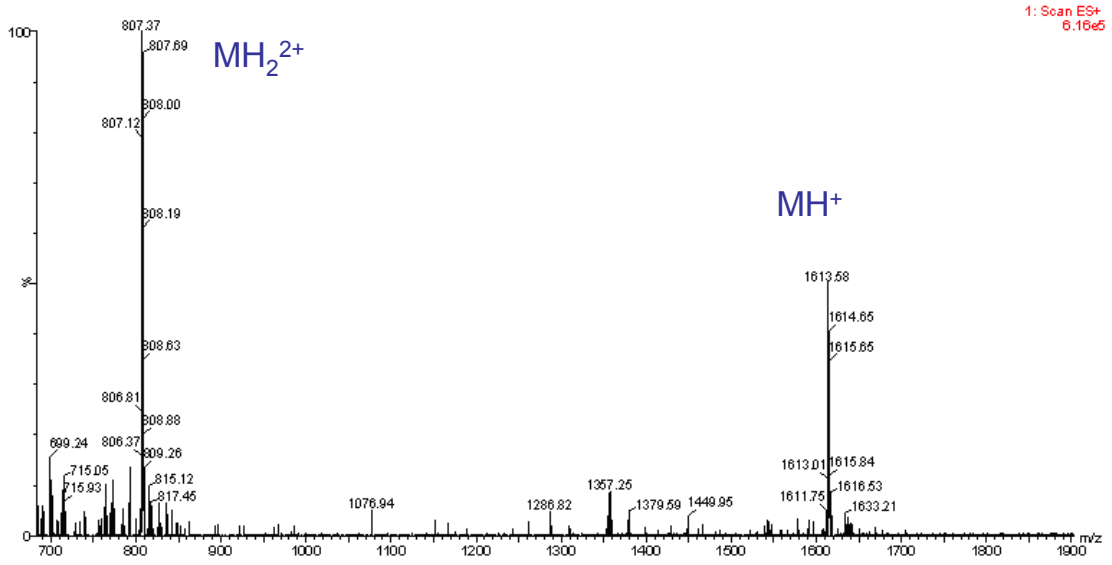


Peptide SI-11. HRMS (ES) *m/z* 1635.7179 [(M+Na)$^+$; calcd for $^{12}C_{67}{}^{13}C_2H_{102}N_{22}{}^{16}O_{17}{}^{18}O_2NaS_2$: 1635.7183]; MALDI-TOF *m/z* 1636.208 [(M+Na)$^+$; calcd for $^{12}C_{67}{}^{13}C_2H_{102}N_{22}{}^{16}O_{17}{}^{18}O_2NaS_2$: 1635.7183].

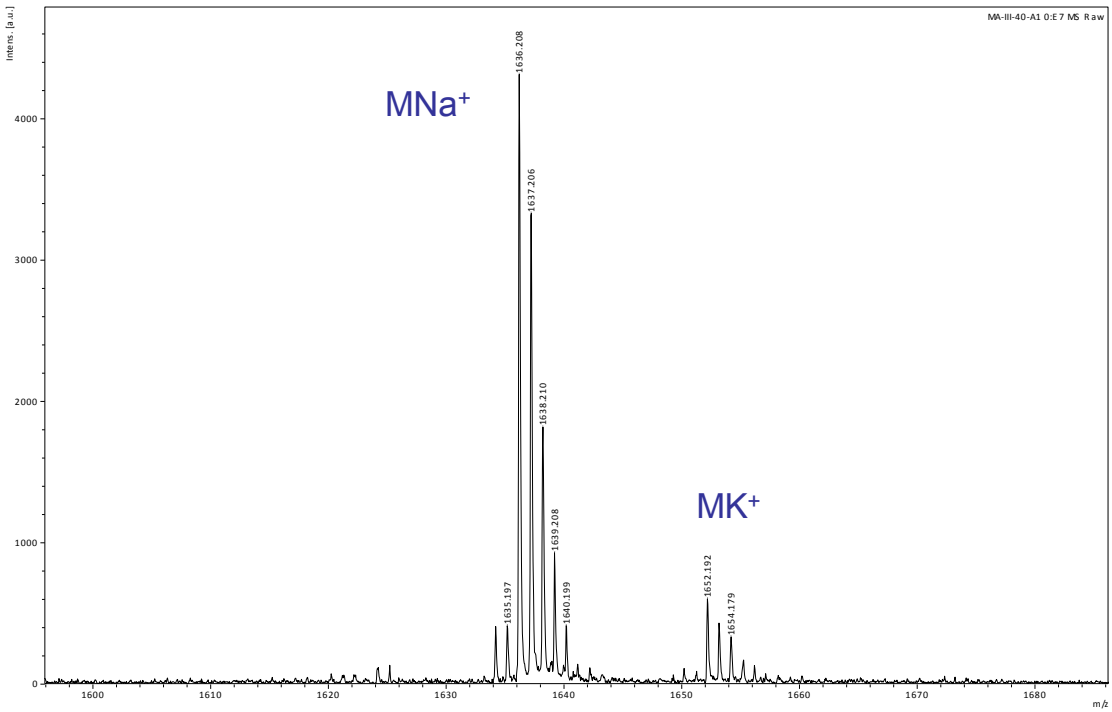
LC-MS chromatogram of peptide **SI-11**:



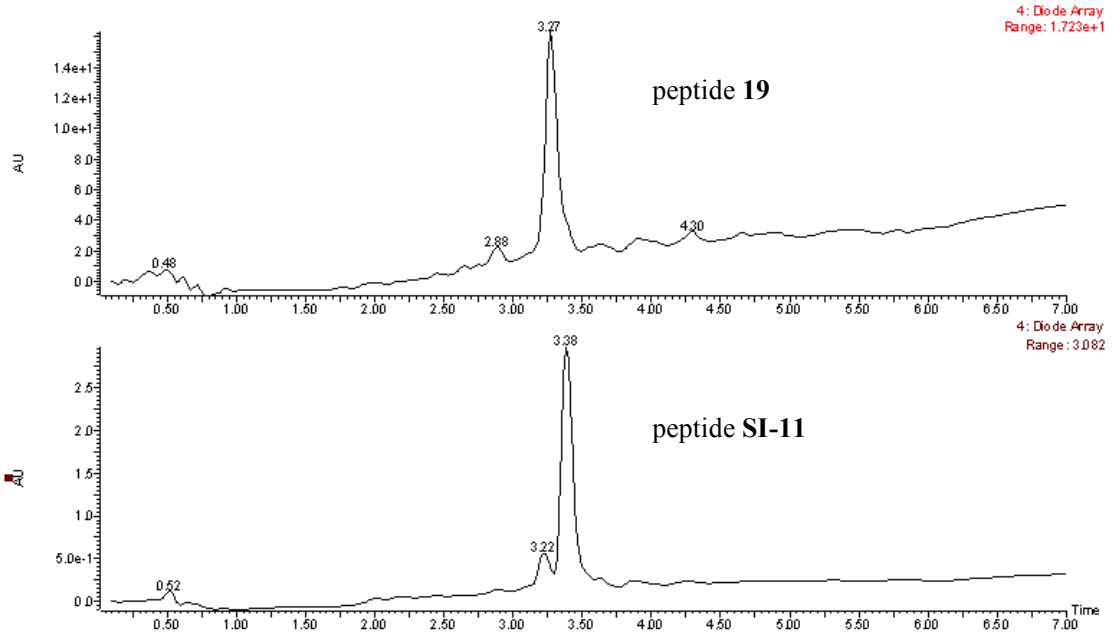
LC-MS mass spectrum of peptide **SI-11**:



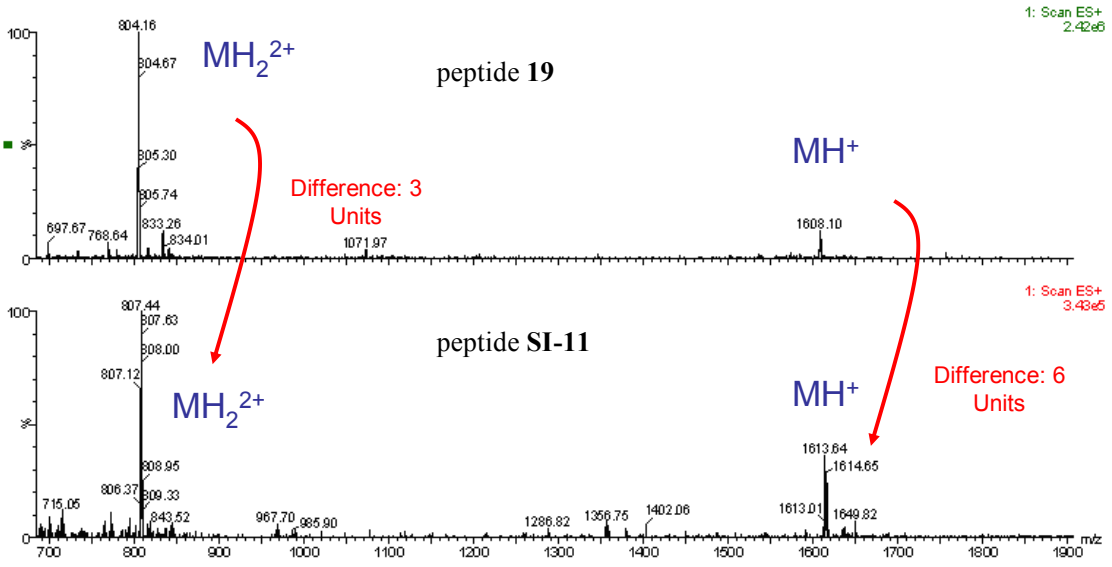
MALDI-TOF mass spectrum of peptide **SI-11**:



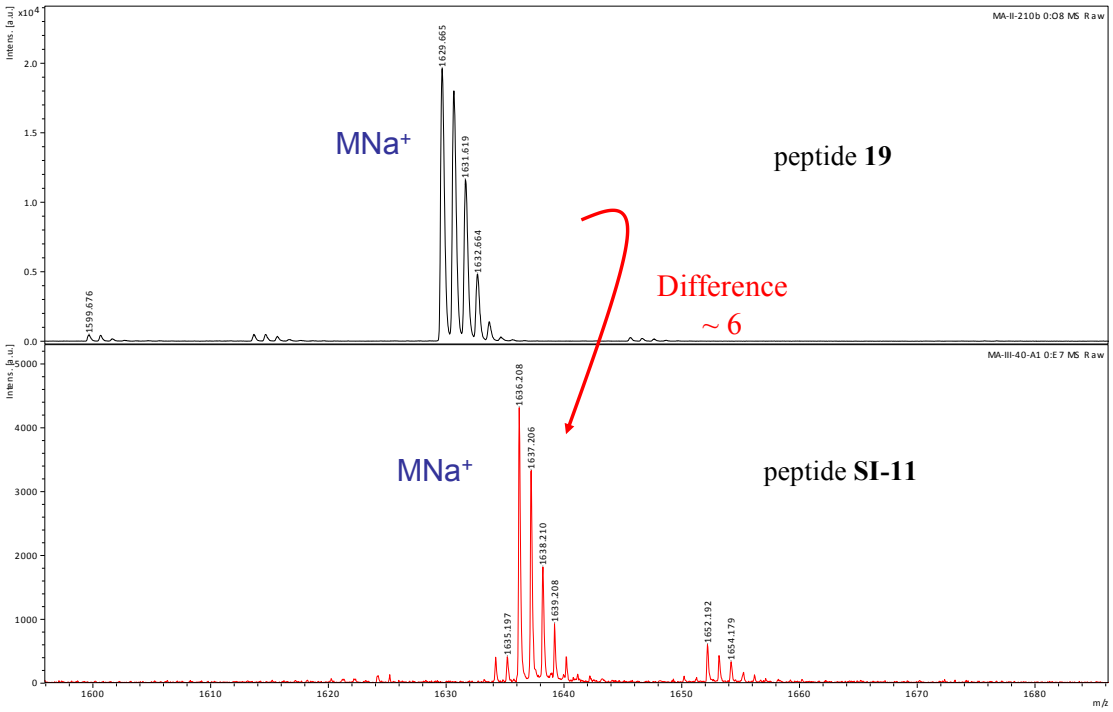
LC-MS chromatograms comparing peptide **19** to enriched peptide **SI-11**:

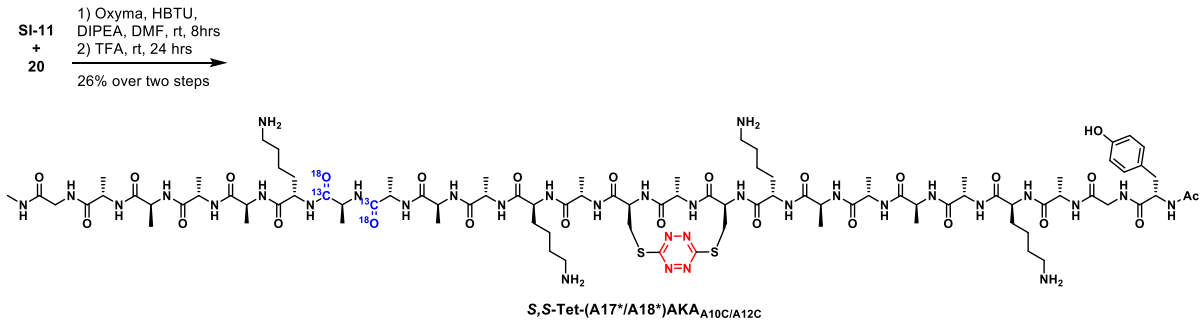


LC-MS mass spectra comparing peptide **19** to enriched peptide **SI-11**:



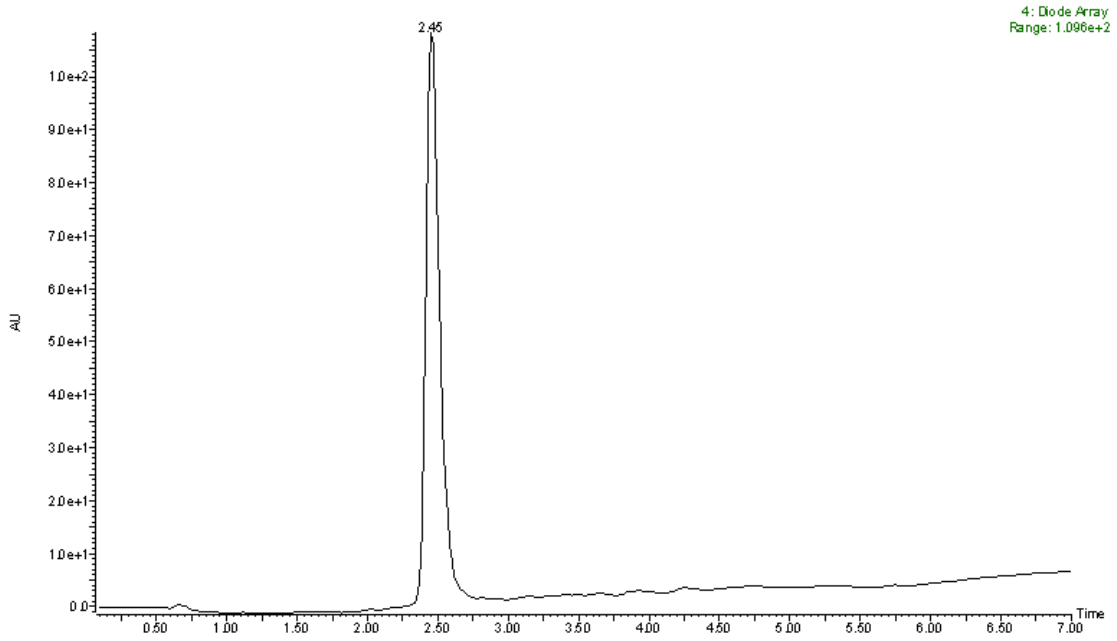
MALDI-TOF mass spectra comparing peptide **19** to enriched peptide **SI-11**:



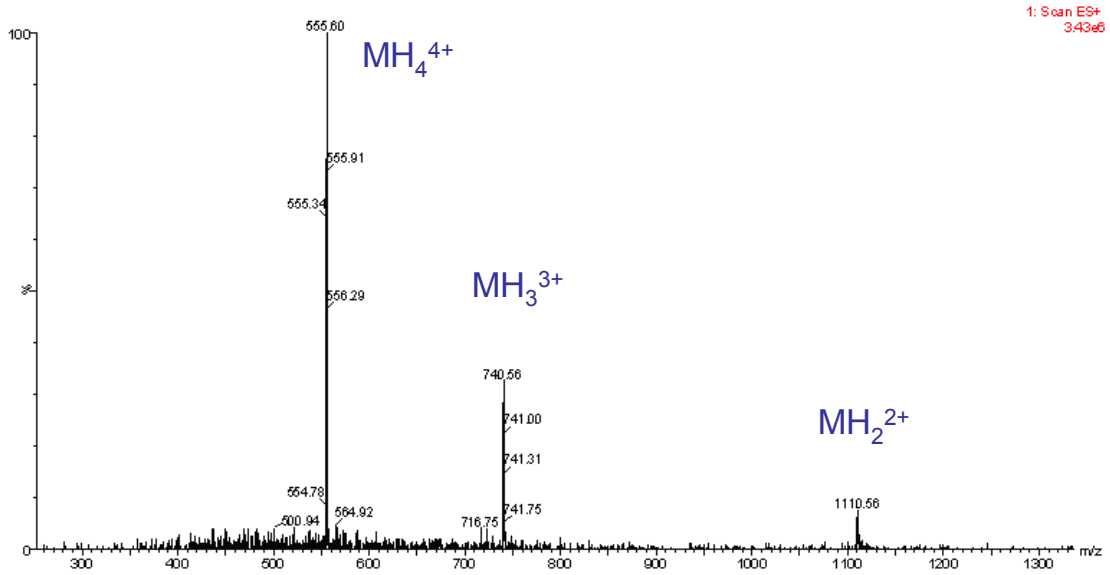


***S,S*-Tet-(A17*/A18*)AKA$_{A10C/A12C}$**

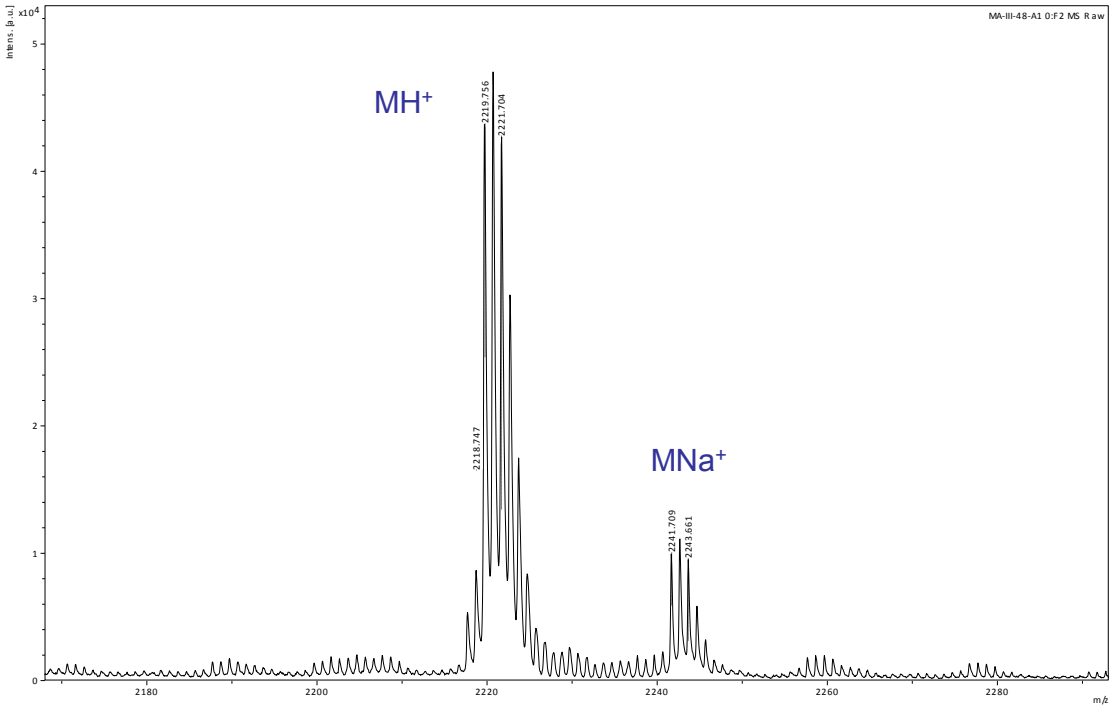
LC-MS chromatogram of peptide ***S,S*-Tet-(A17*/A18*)AKA$_{A10C/A12C}$**:



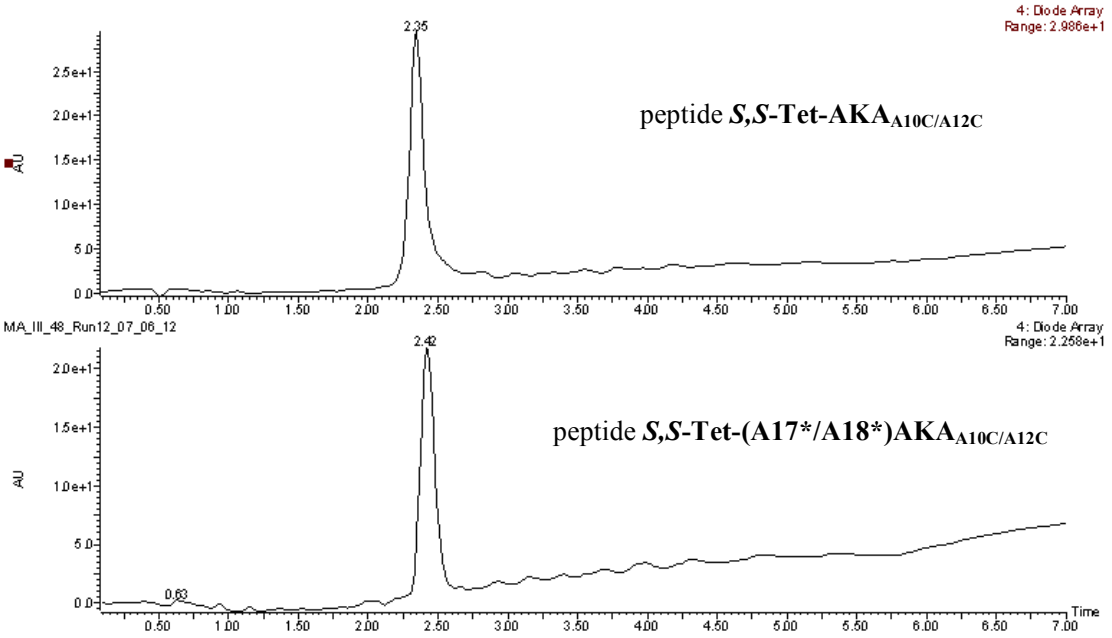
LC-MS mass spectrum of peptide ***S,S*-Tet-(A17*/A18*)AKA$_{A10C/A12C}$**:



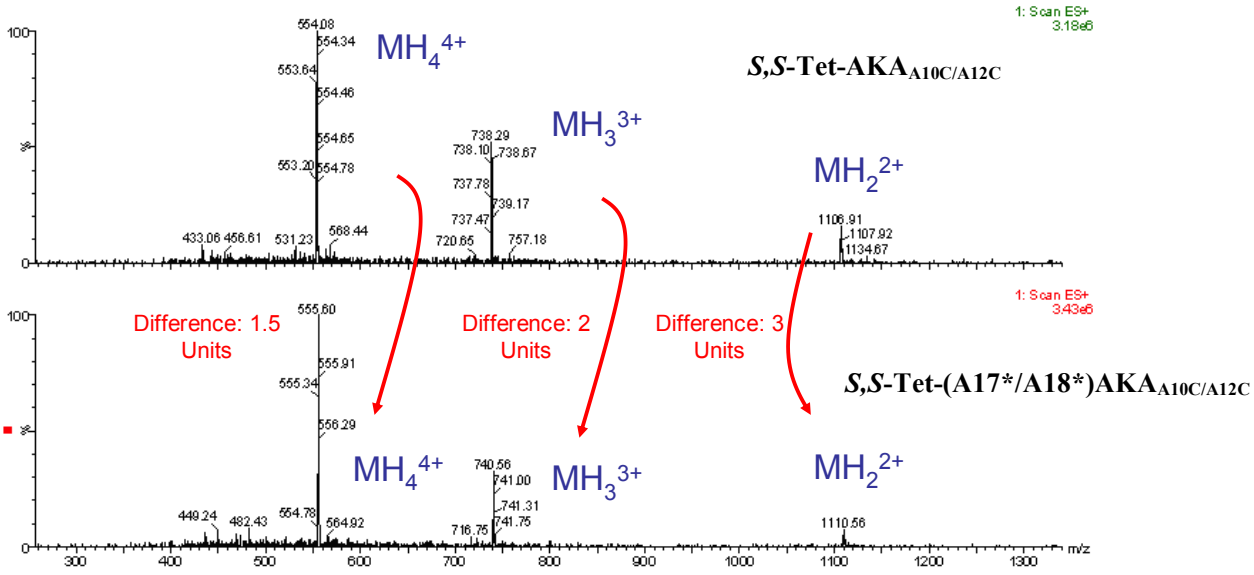
MALDI-TOF mass spectrum of peptide ***S,S*-Tet-(A17*/A18*)AKA$_{A10C/A12C}$**:



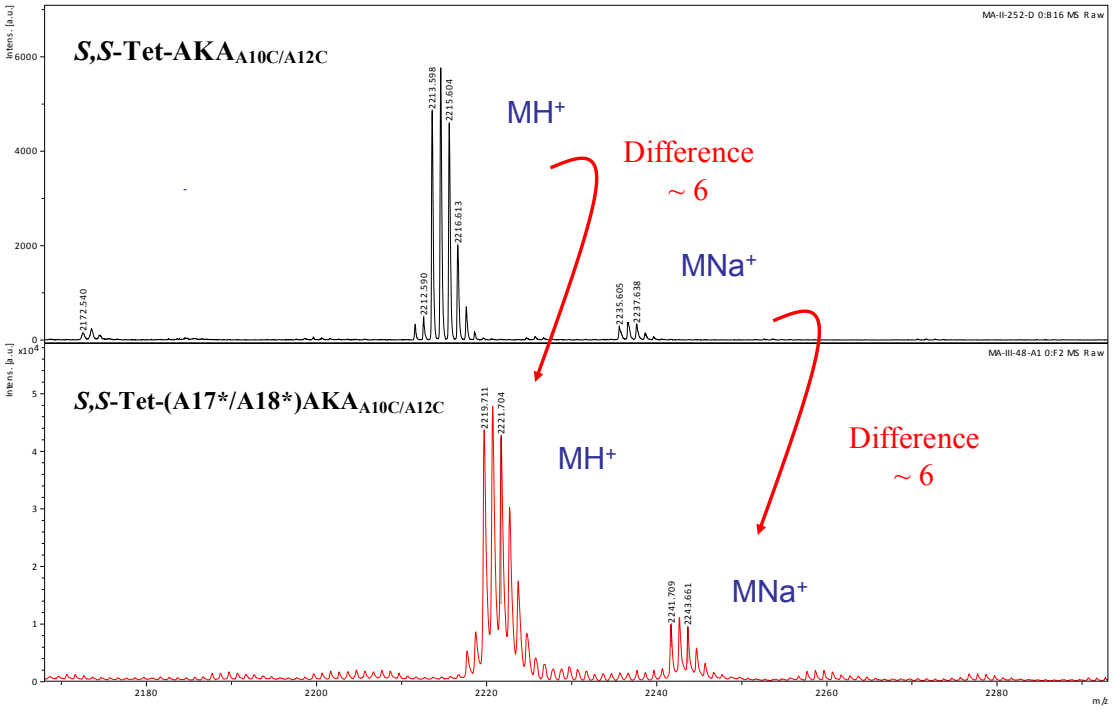
LC-MS chromatograms comparing peptide peptide ***S,S*-Tet-AKA$_{A10C/A12C}$** to enriched peptide ***S,S*-Tet-(A17*/A18*)AKA$_{A10C/A12C}$**:



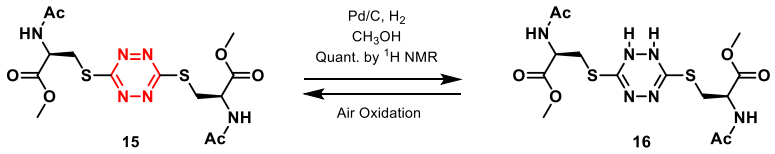
LC-MS mass spectra comparing peptide ***S,S*-Tet-AKA$_{A10C/A12C}$** to enriched peptide ***S,S*-Tet-(A17*/A18*)AKA$_{A10C/A12C}$**:



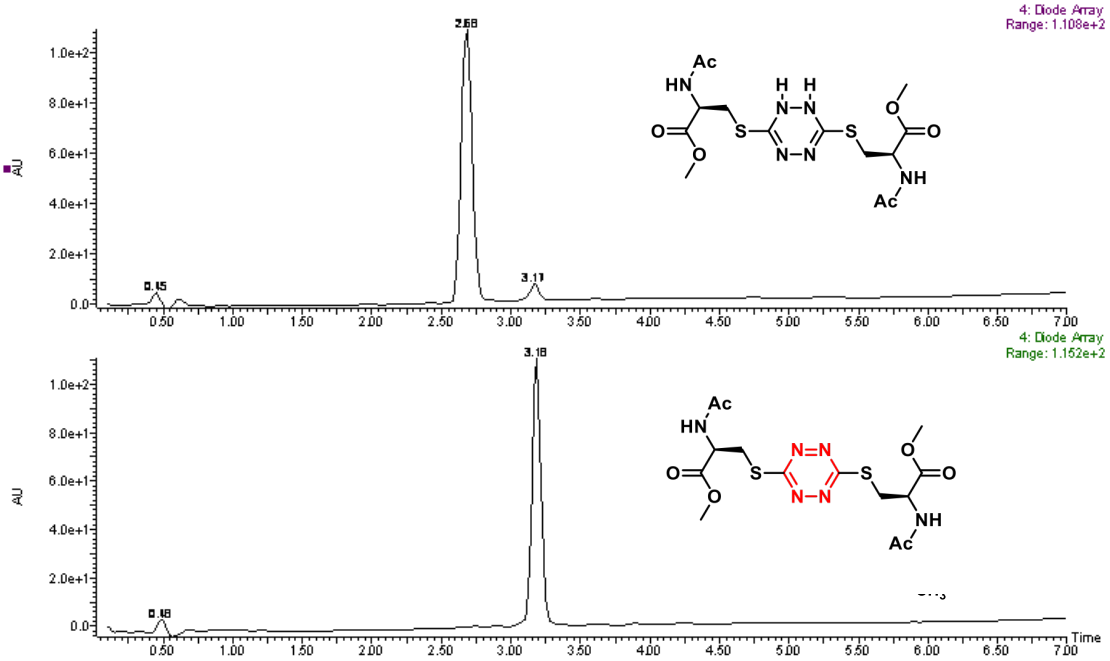
MALDI-TOF comparing peptide ***S,S*-Tet-AKA$_{A10C/A12C}$** to enriched peptide ***S,S*-Tet-(A17*/A18*)AKA$_{A10C/A12C}$**:



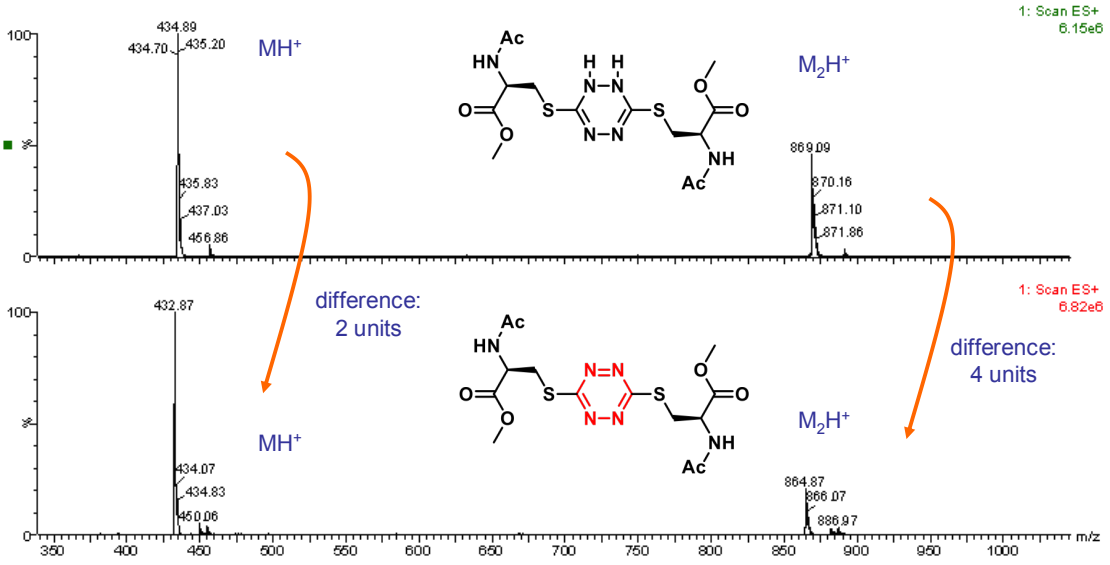
S,S-Tetrazine Reduction and Re-Oxidation



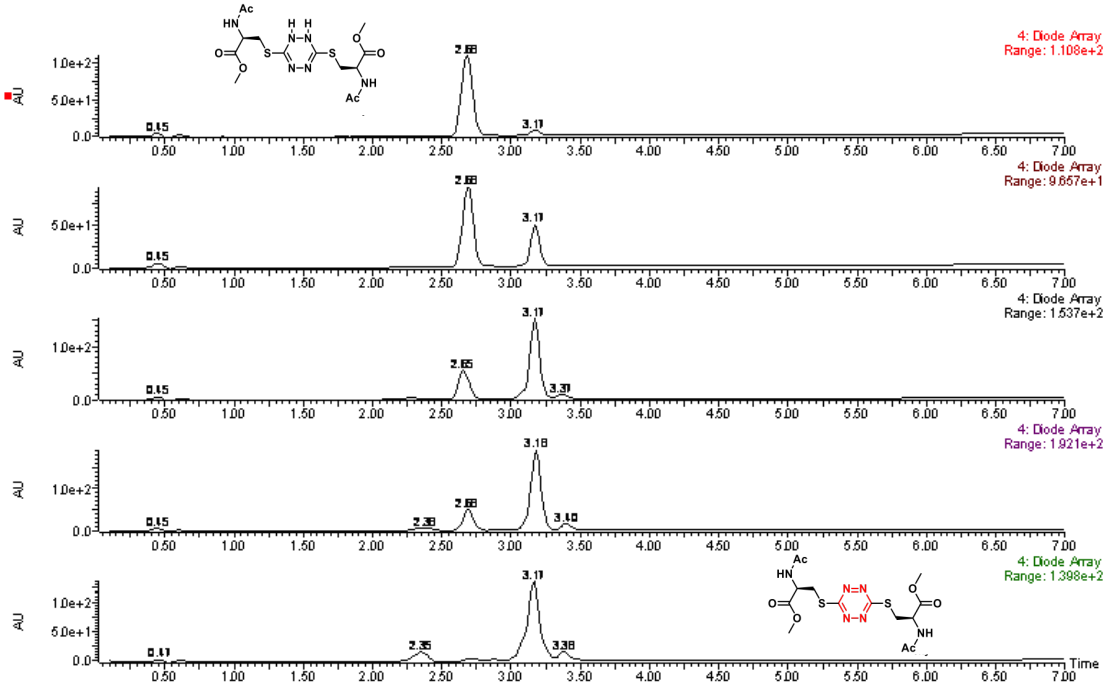
LC-MS chromatograms comparing compound **15** to compound **16**:



LC-MS mass spectra comparing compound **15** to compound **16**:



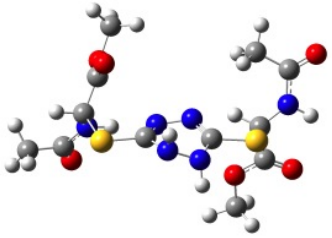
LC-MS chromatograms depicting air oxidation of compound **16** to compound **15**:



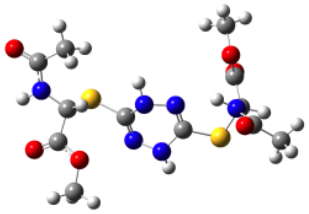
Experimental Evidence for the 1,2-dihydro-*S,S*-tetrazine

Simulated structures of compound **16** as 1,2- vs 1,4-dihydrotetrazine:

Simulated Structure of 1,2-dihydrotetrazine

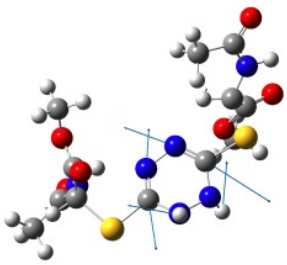


Simulated Structure of 1,4-dihydrotetrazine

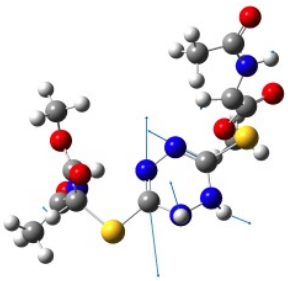


Based on the simulations performed, the ring mode frequencies for the 1,2- and 1,4-dihydrotetrazine systems were calculated and their IR Spectrum generated. Although 1,4-dihydrotetrazine is energetically more favorable by ~ 7 kcal/mol, the ring mode frequencies from the experimental IR of **16** matched those of the 1,2-dihydrotetrazine.

A diagram representing the ring modes for 1,2-dihydrotetrazine:



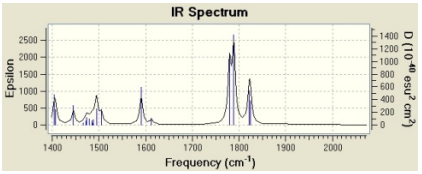
Ring mode 1: higher frequency



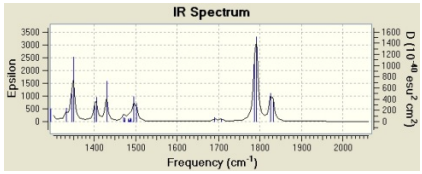
Ring mode 2: lower frequency

Calculated and experimental IR of compound **16**:

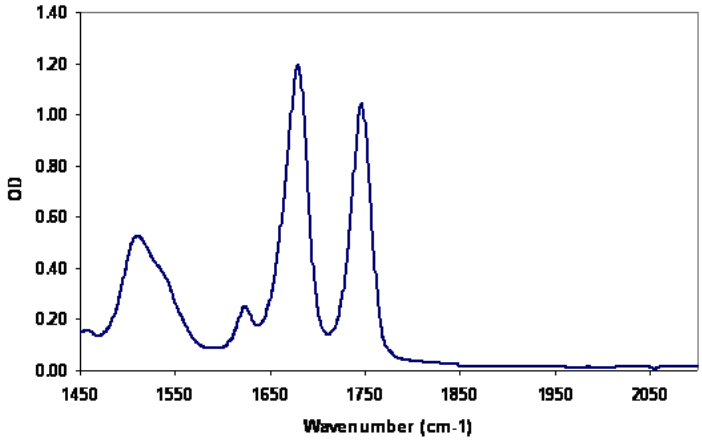
Calculated IR Spectrum of 1,2-dihydrotetrazine



Calculated IR Spectrum of 1,4-dihydrotetrazine



Collected IR Spectrum of 16



The ester and the acetate C=O frequency is used as a reference:

Experiment	Calculated average	RatioExperiment/Calculated
1679 cm^{-1} amide	1789 cm^{-1} amide	0.938
1745 cm^{-1} ester	1823 cm^{-1} ester	0.955

Average of the ratio = 0.947

From 1,2 - dihydrotetrazine calculation :

1590 cm^{-1} x 0.947 = 1506 cm^{-1} 1691 cm^{-1} x 0.947 = 1601 cm^{-1}

1612 cm^{-1} x 0.947 = 1527 cm^{-1} 1701 cm^{-1} x 0.947 = 1610 cm^{-1}

From 1,4 - dihydrotetrazine calculation :

1691 cm^{-1} x 0.947 = 1601 cm^{-1}

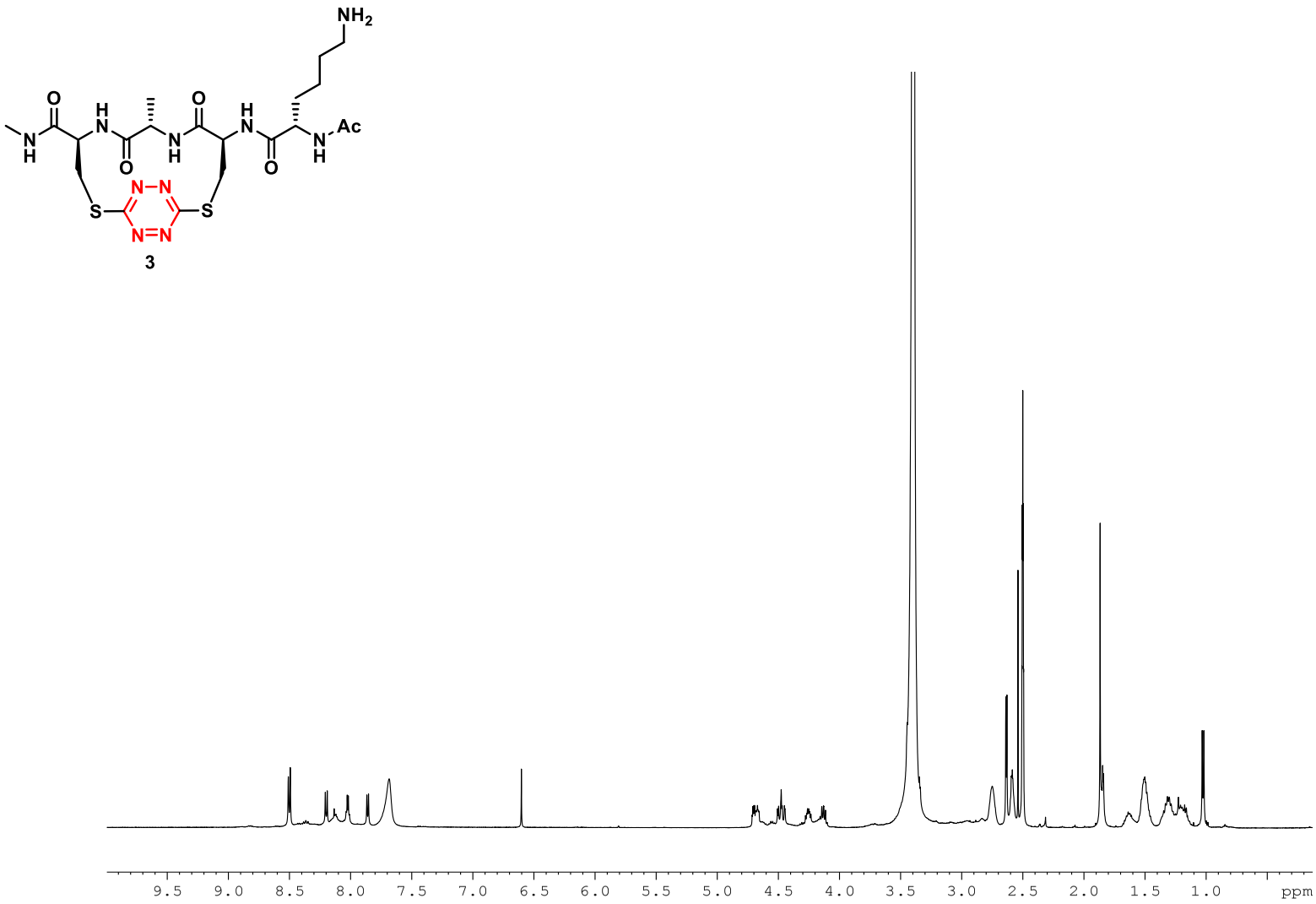
1701 cm^{-1} x 0.947 = 1610 cm^{-1}

From Experiment:

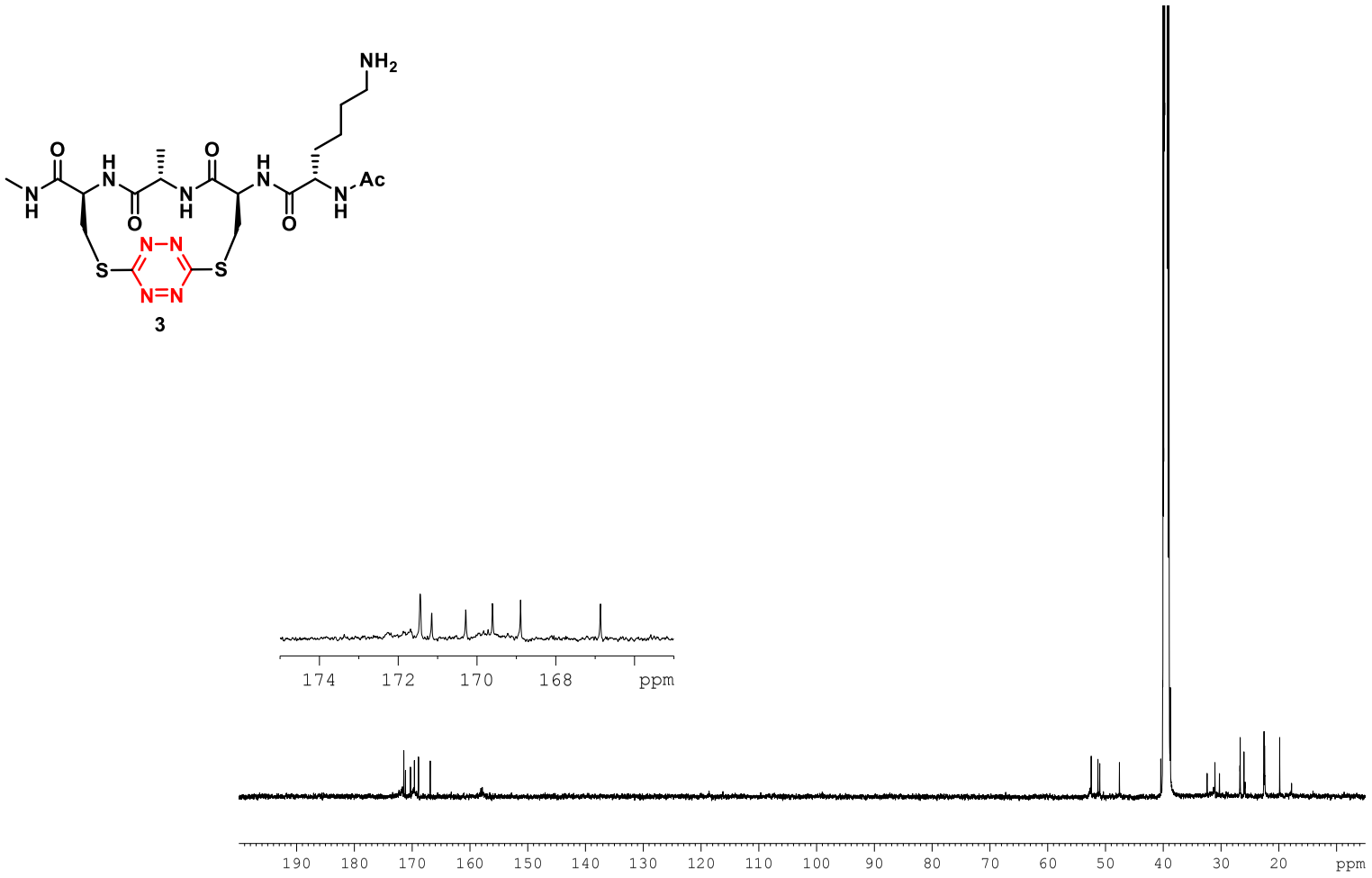
1510 cm^{-1}

1536 cm^{-1}

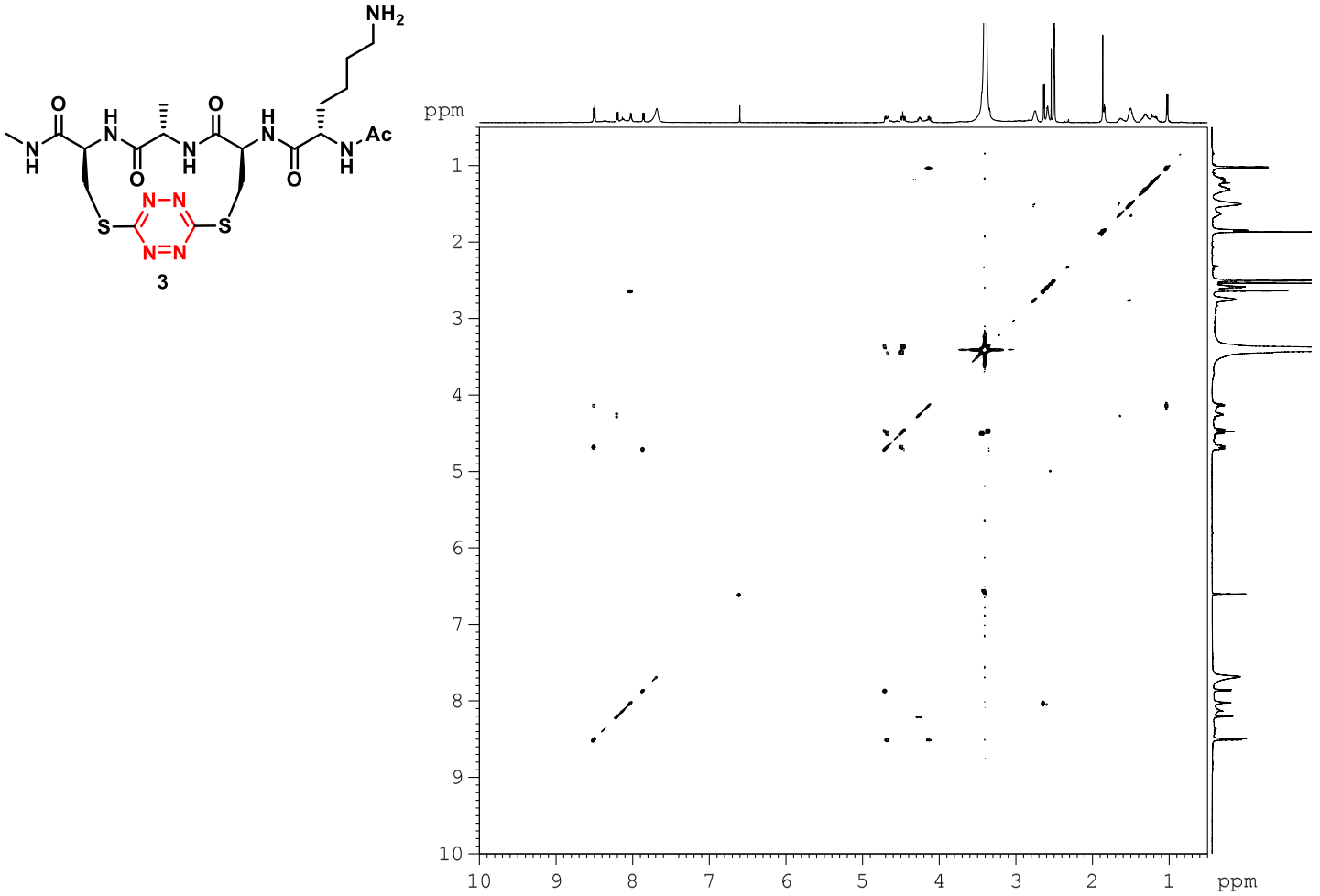
The ring mode frequencies from the experimental IR match those of the calculated frequencies of the 1,2 – dihydrotetrazine. Furthermore, the correlation in the peak shape was the same; higher ring mode frequency was less intense.



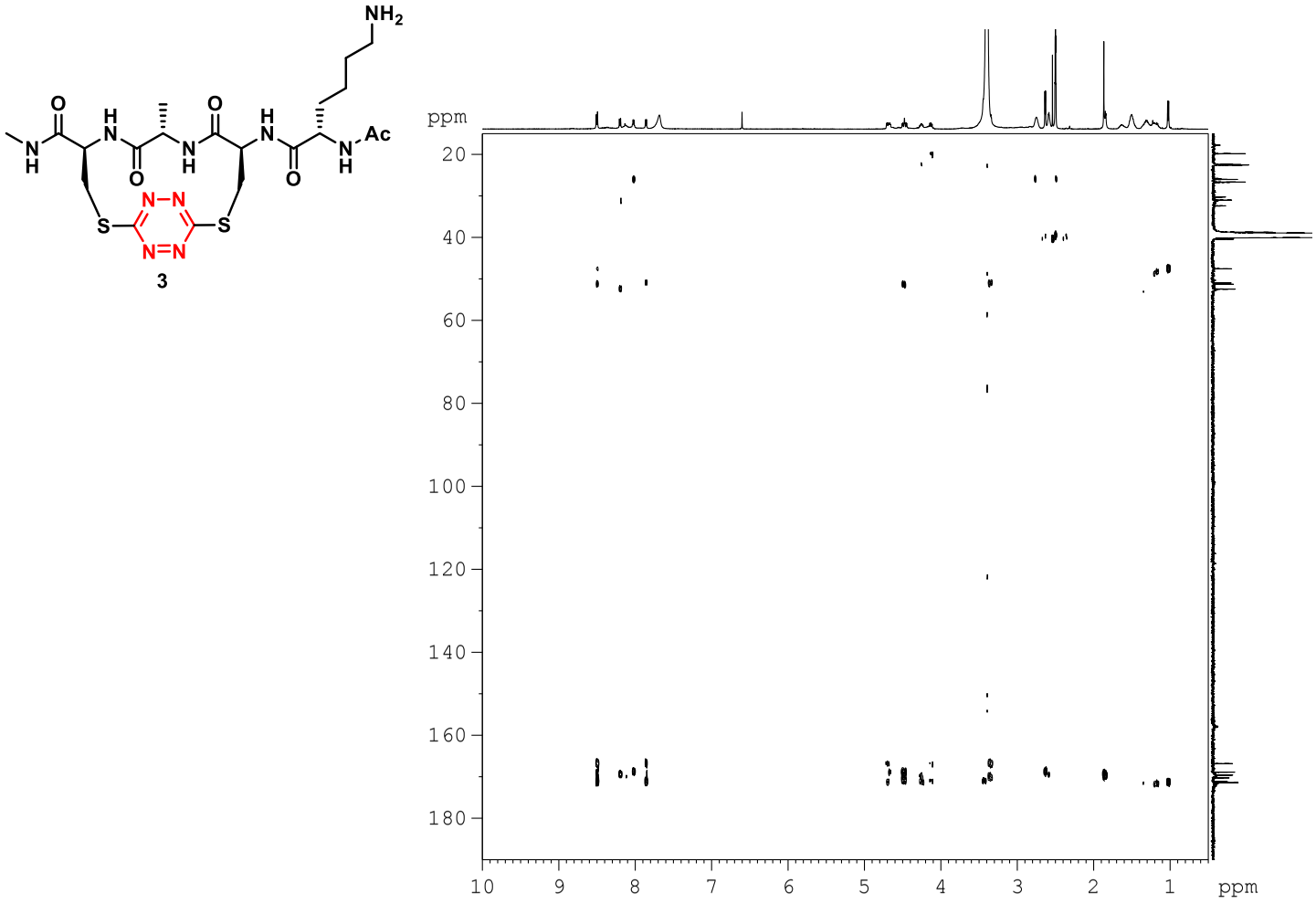
3 (^{1}H NMR, DMSO-d_6, 500 MHz)



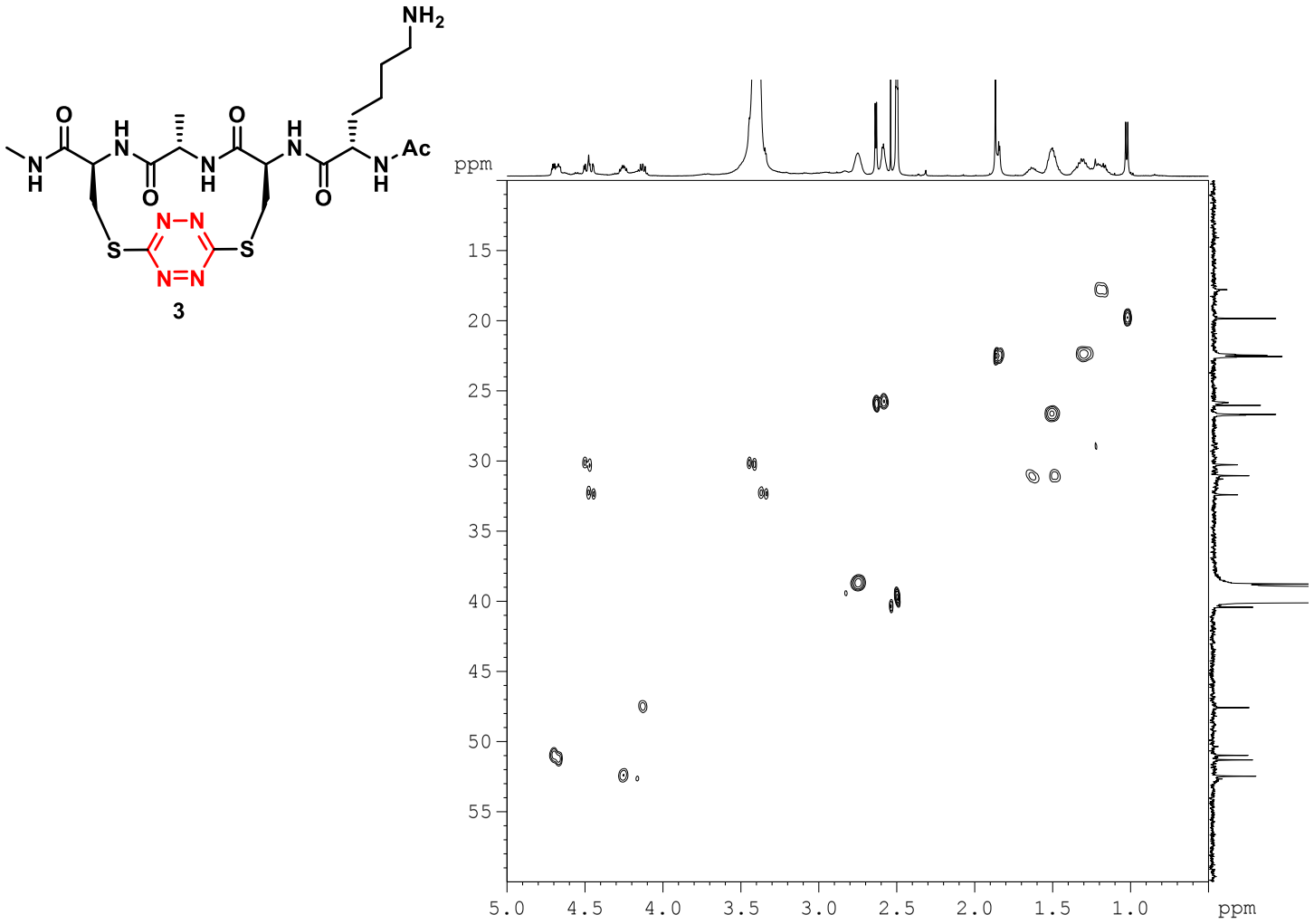
3 (^{13}C NMR, DMSO-d_6, 125 MHz)



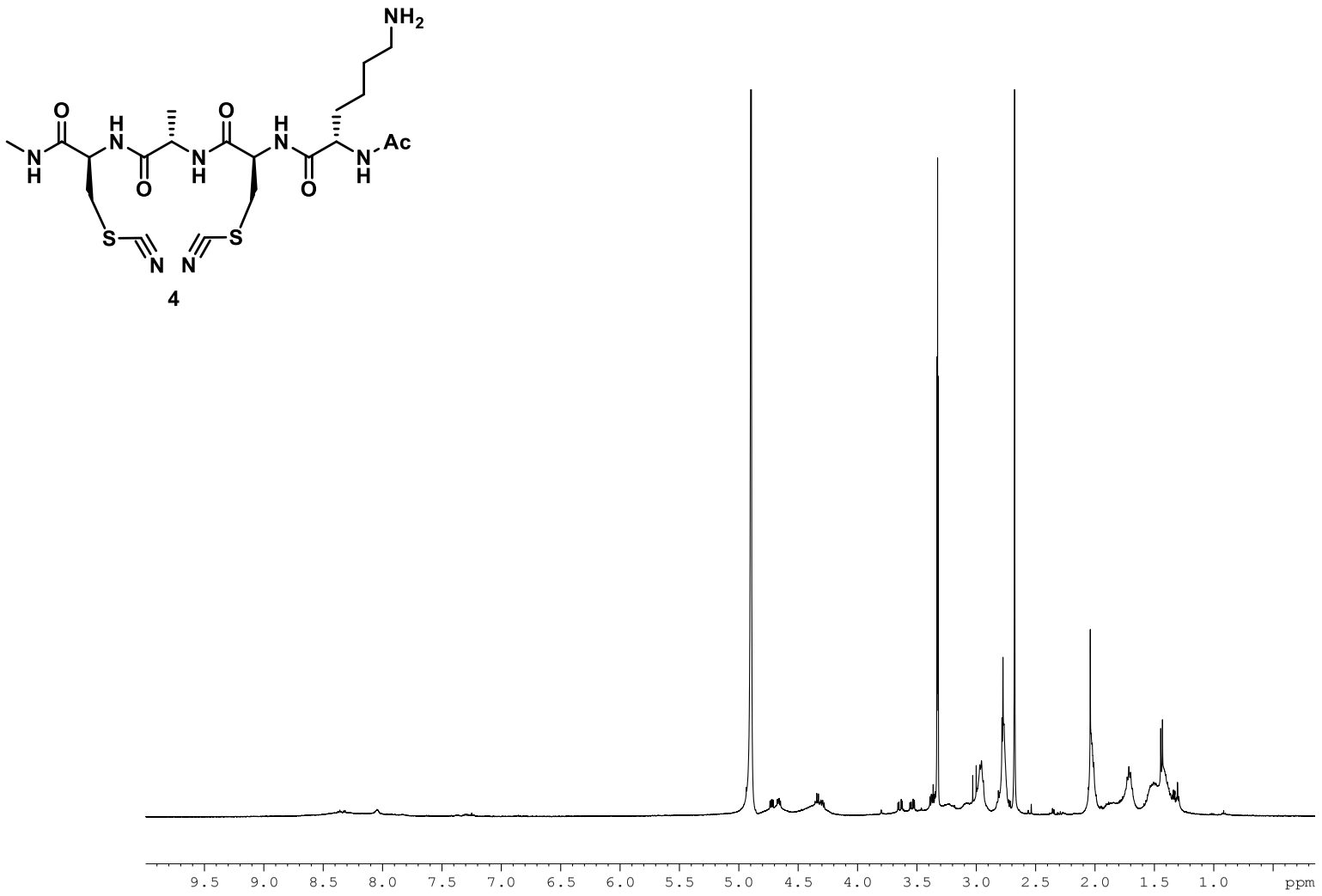
3 (COSY, DMSO-d_6)



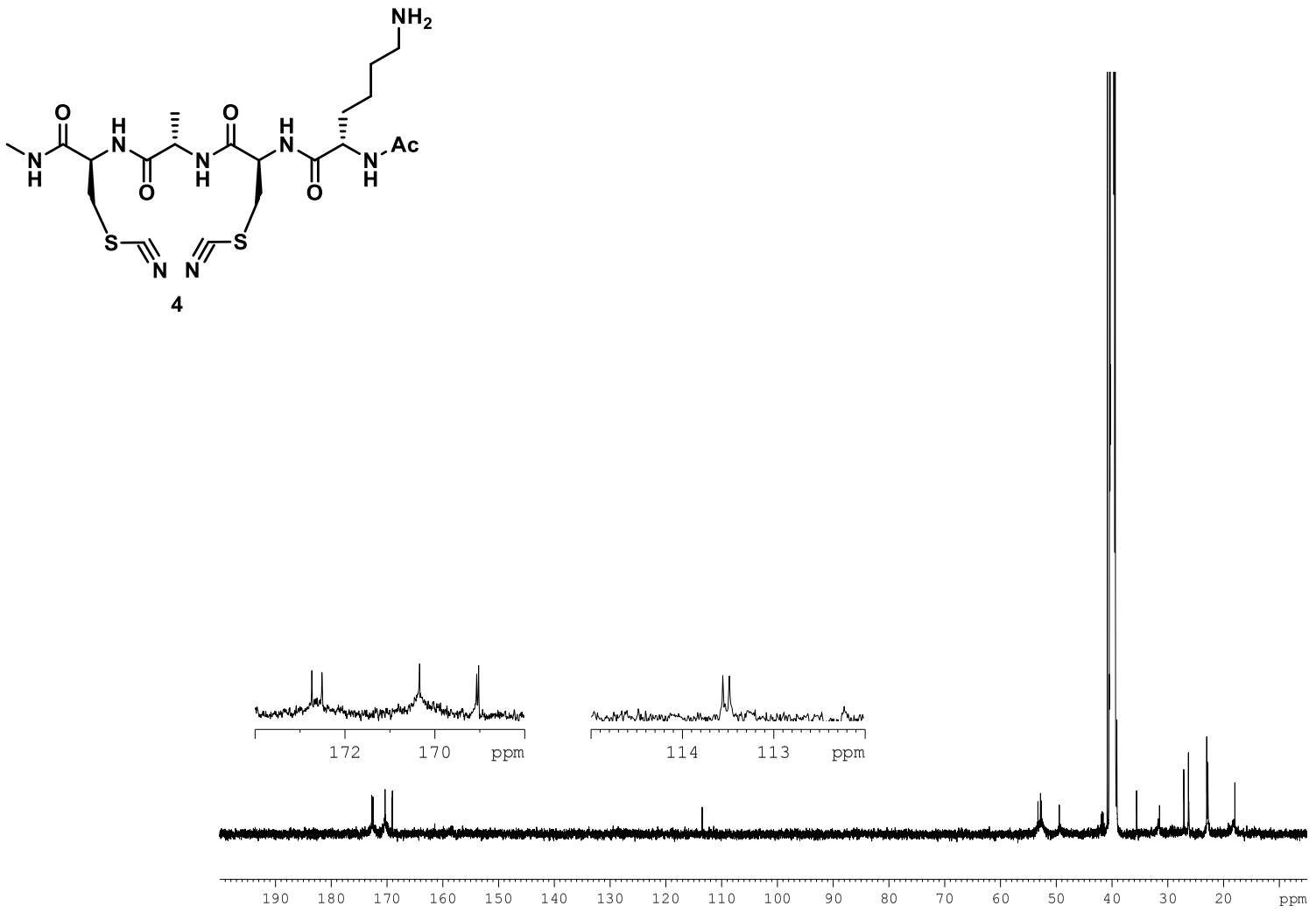
3 (HMBC, DMSO-d_6)



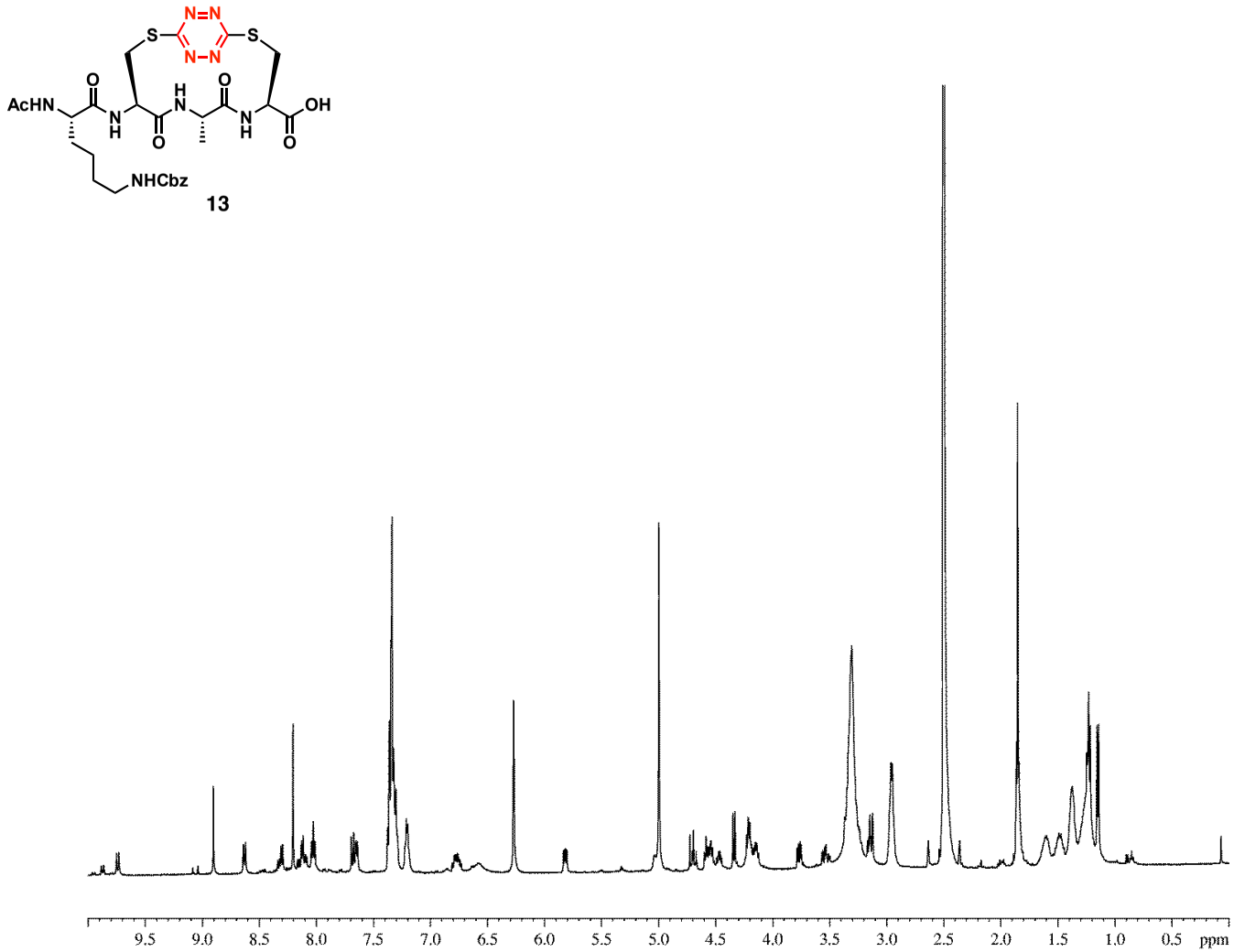
3 (HSQC, DMSO-d_6)



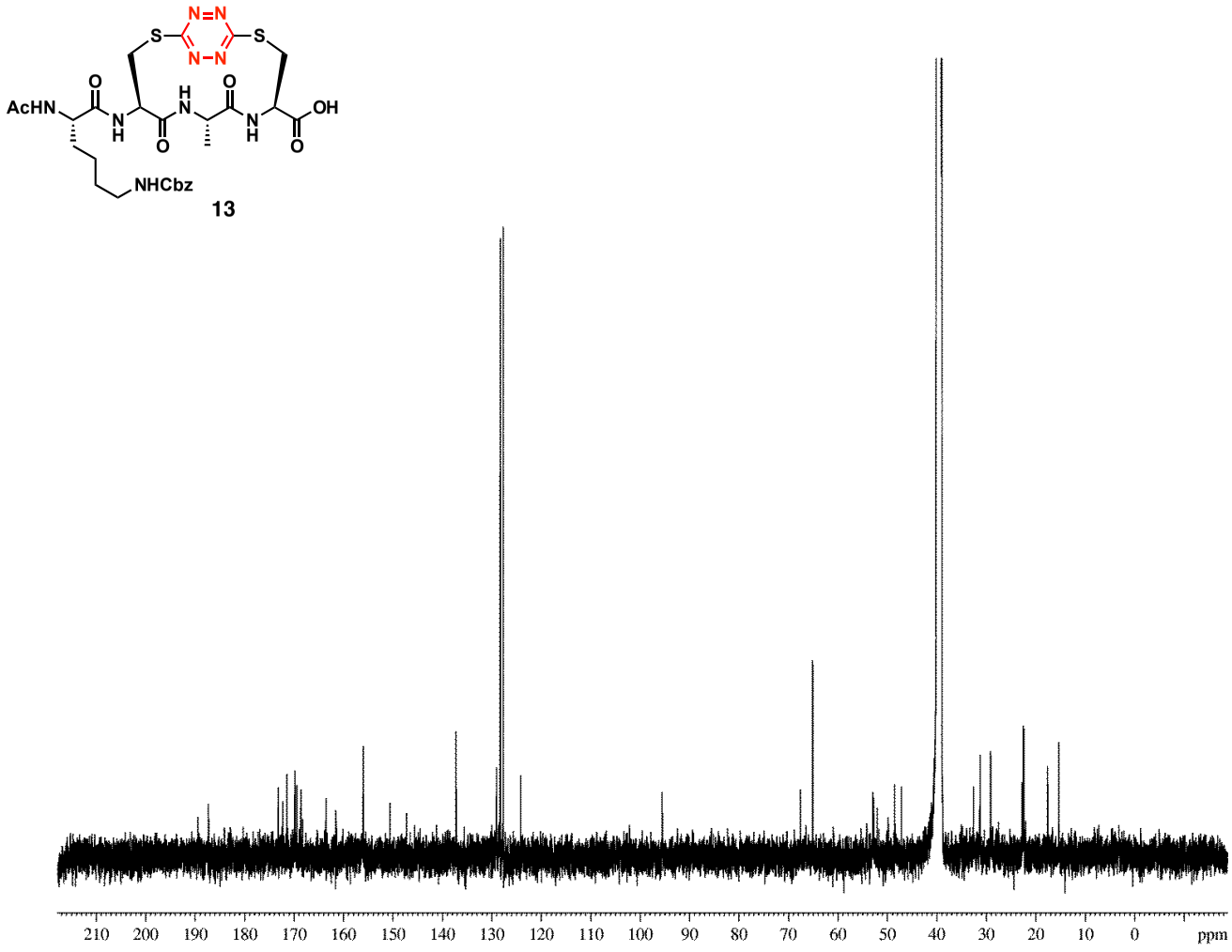
4 (^{1}H NMR, CD_3OD, 500 MHz)



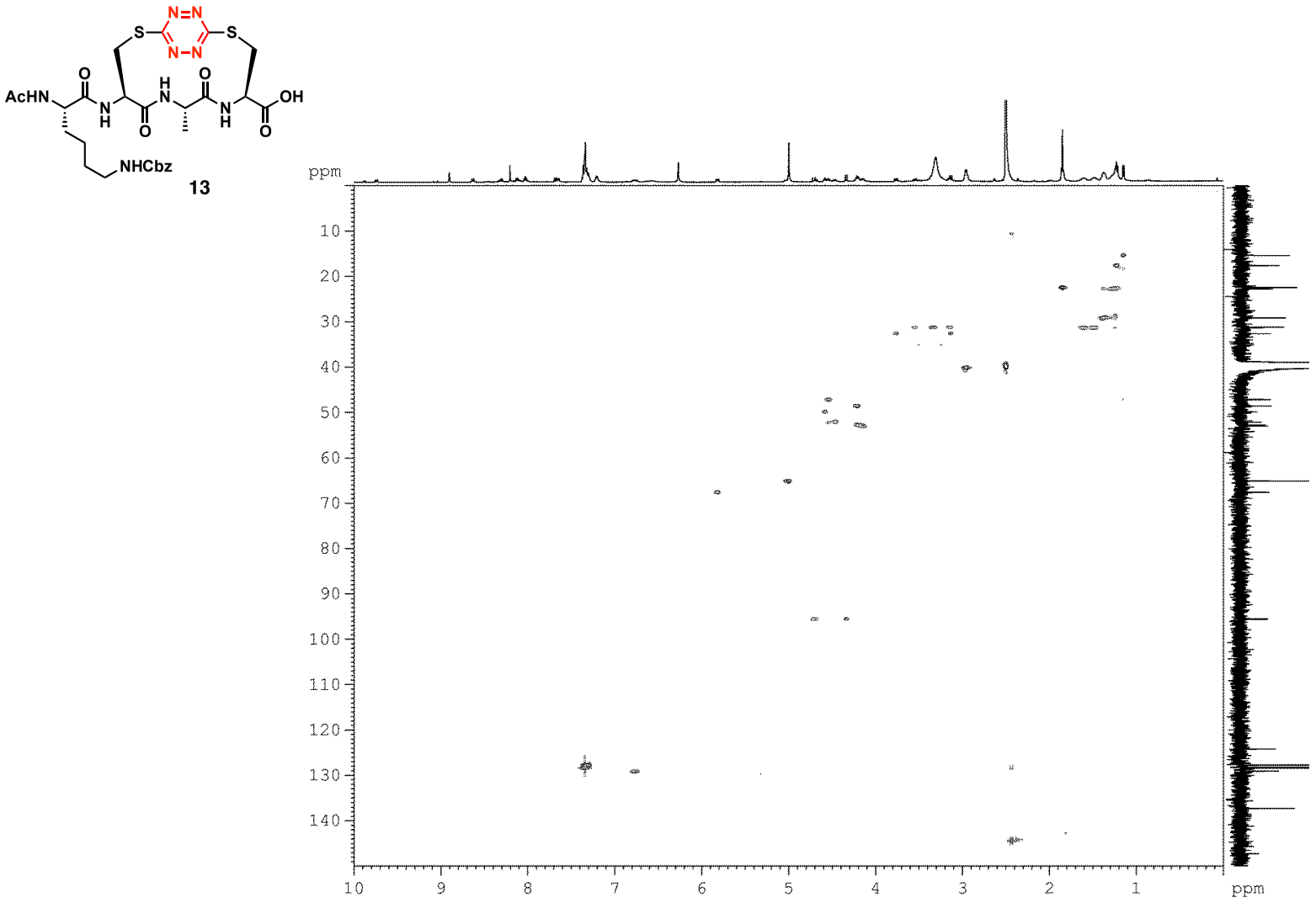
4 (^{13}C NMR, DMSO-d_6, 125 MHz)



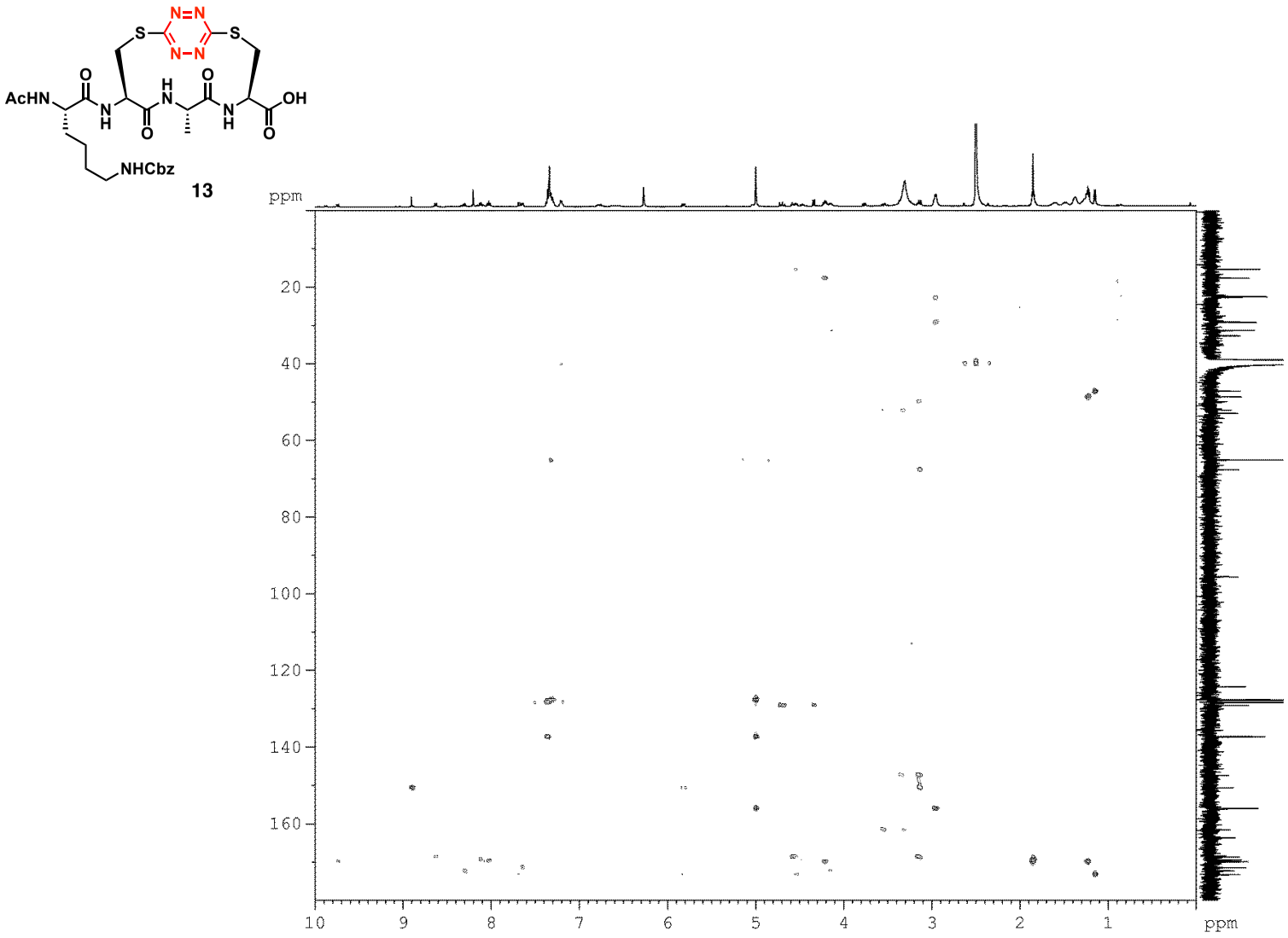
13 (^{1}H NMR, DMSO-d_6, 500 MHz)



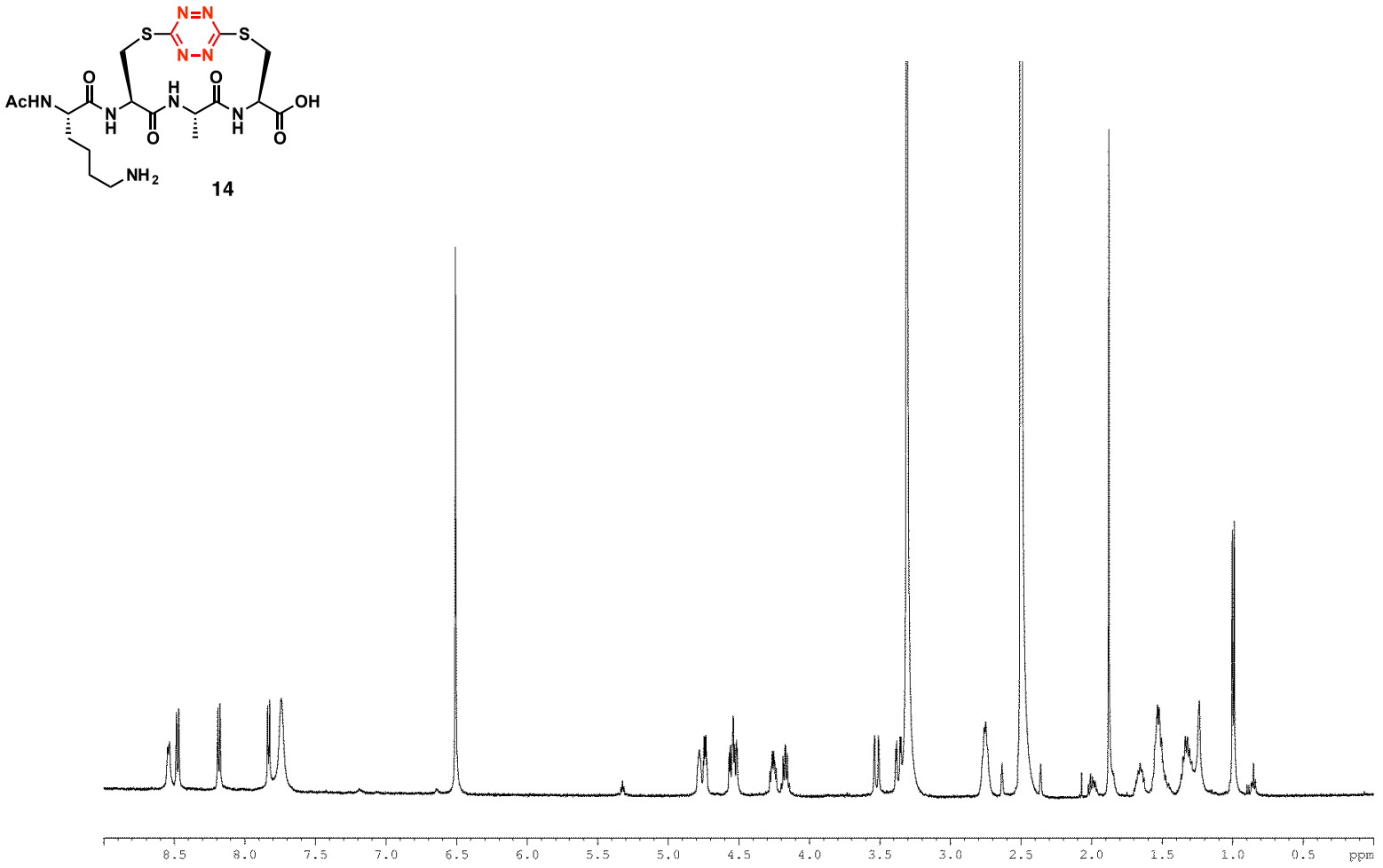
13 (^{13}C NMR, DMSO-d_6, 125 MHz)



13 (HSQC, DMSO-d_6)

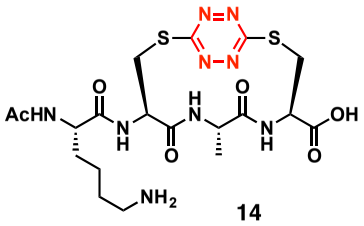


13 (HMBC, DMSO-d_6)

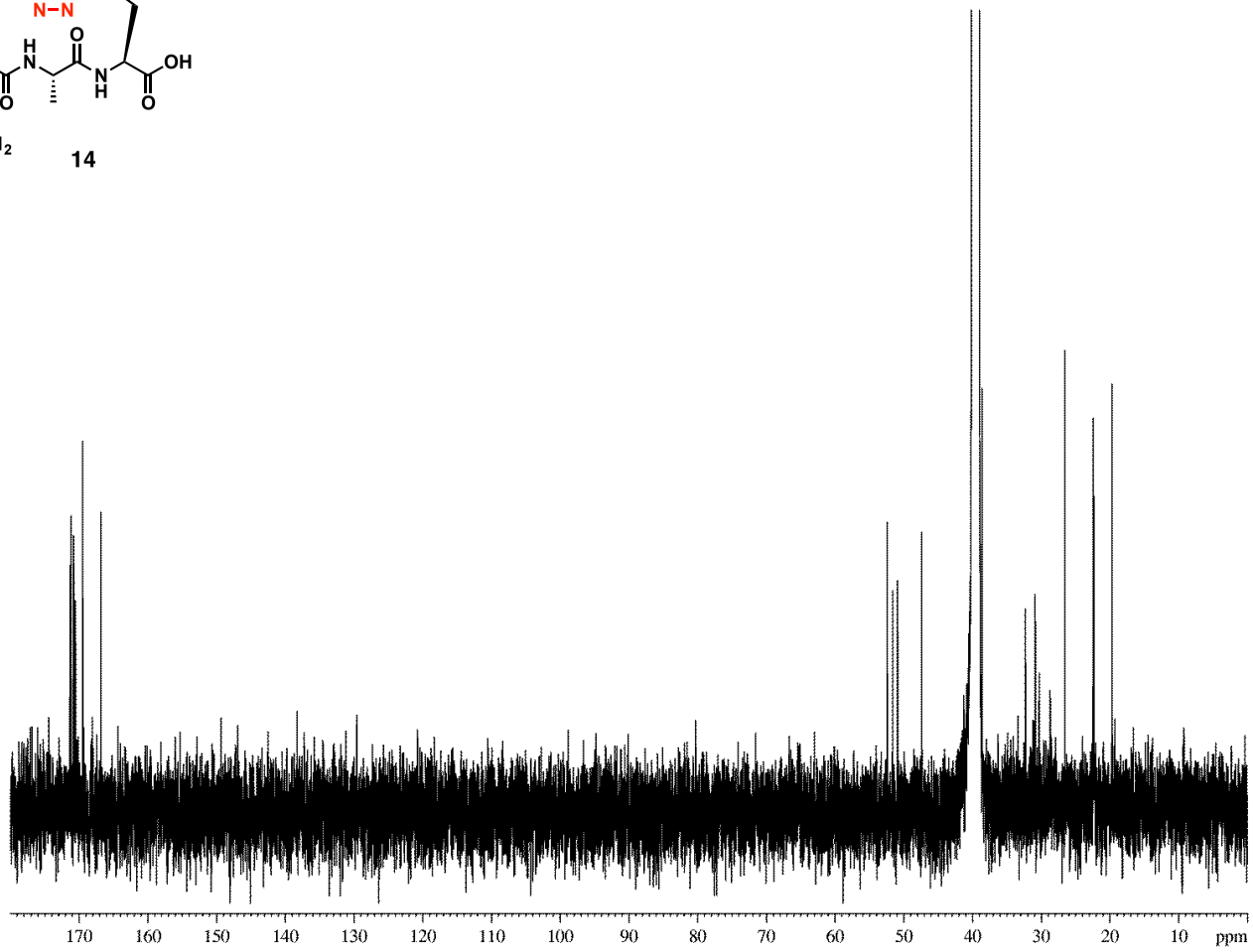


14

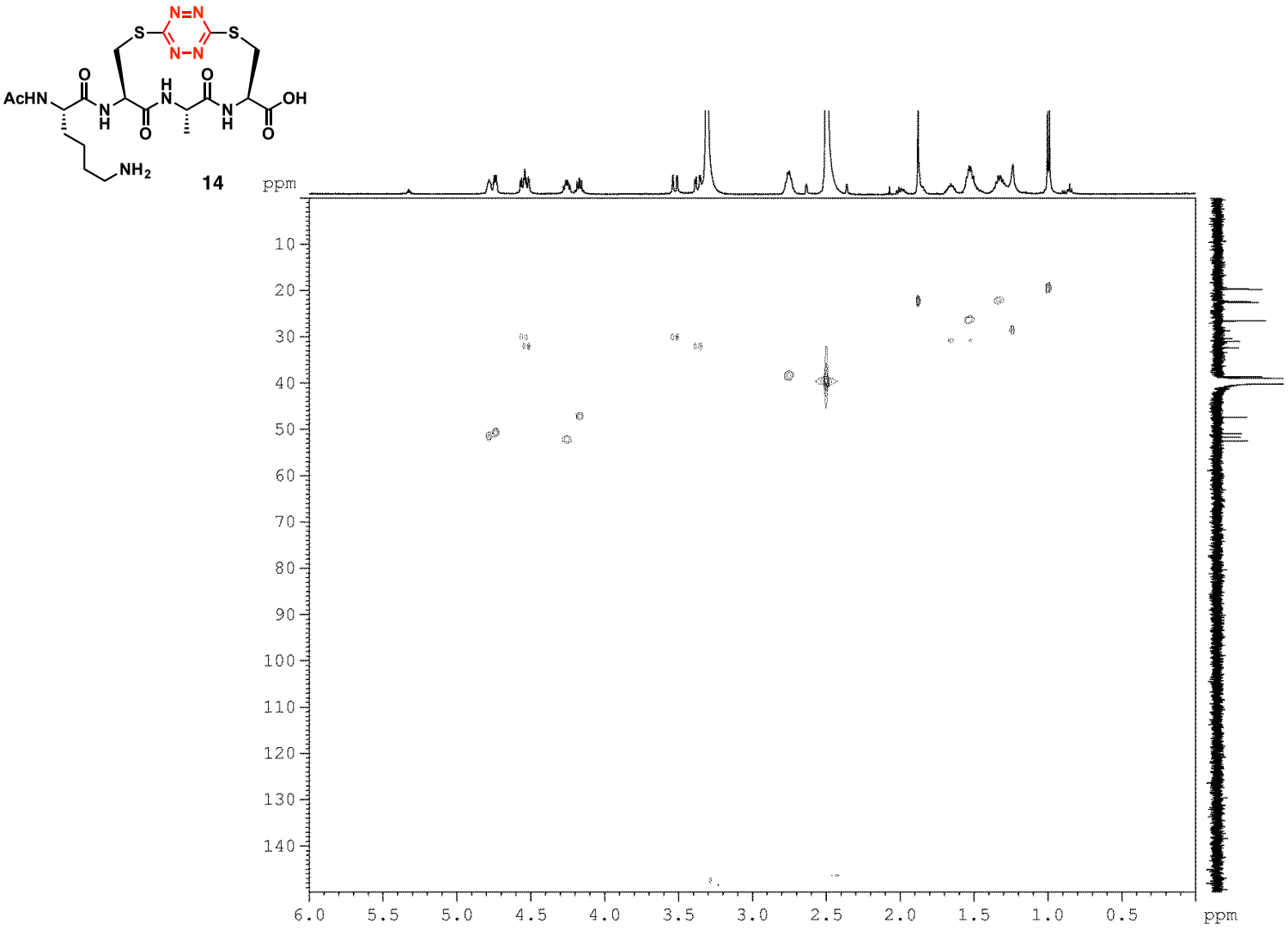
14 (^{1}H NMR, DMSO-d_6, 500 MHz)



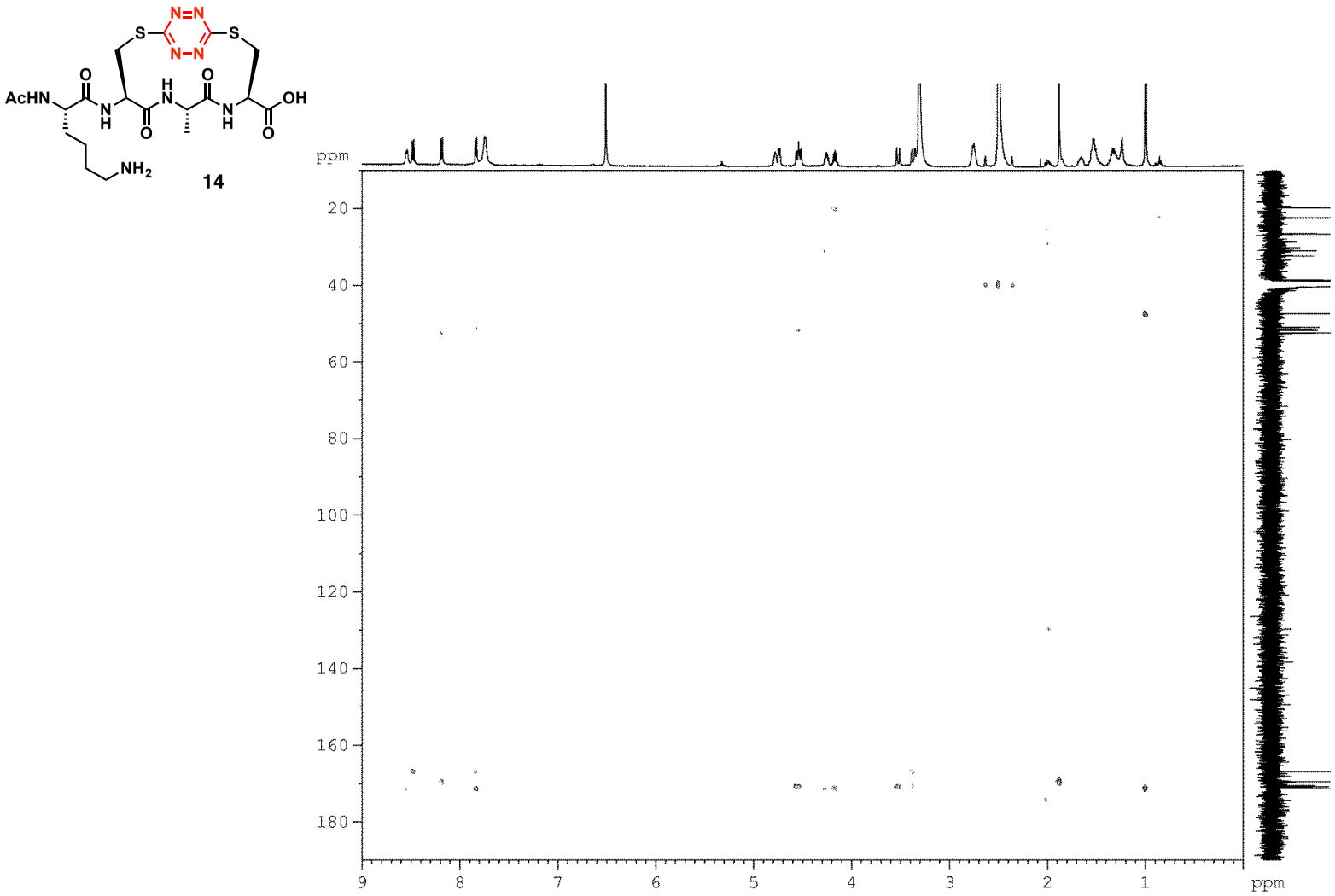
14



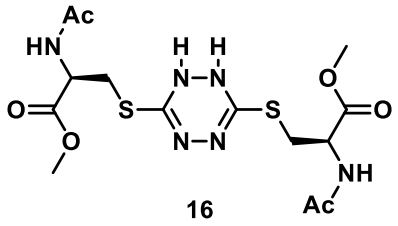
14 (^{13}C NMR, DMSO-d_6, 125 MHz)



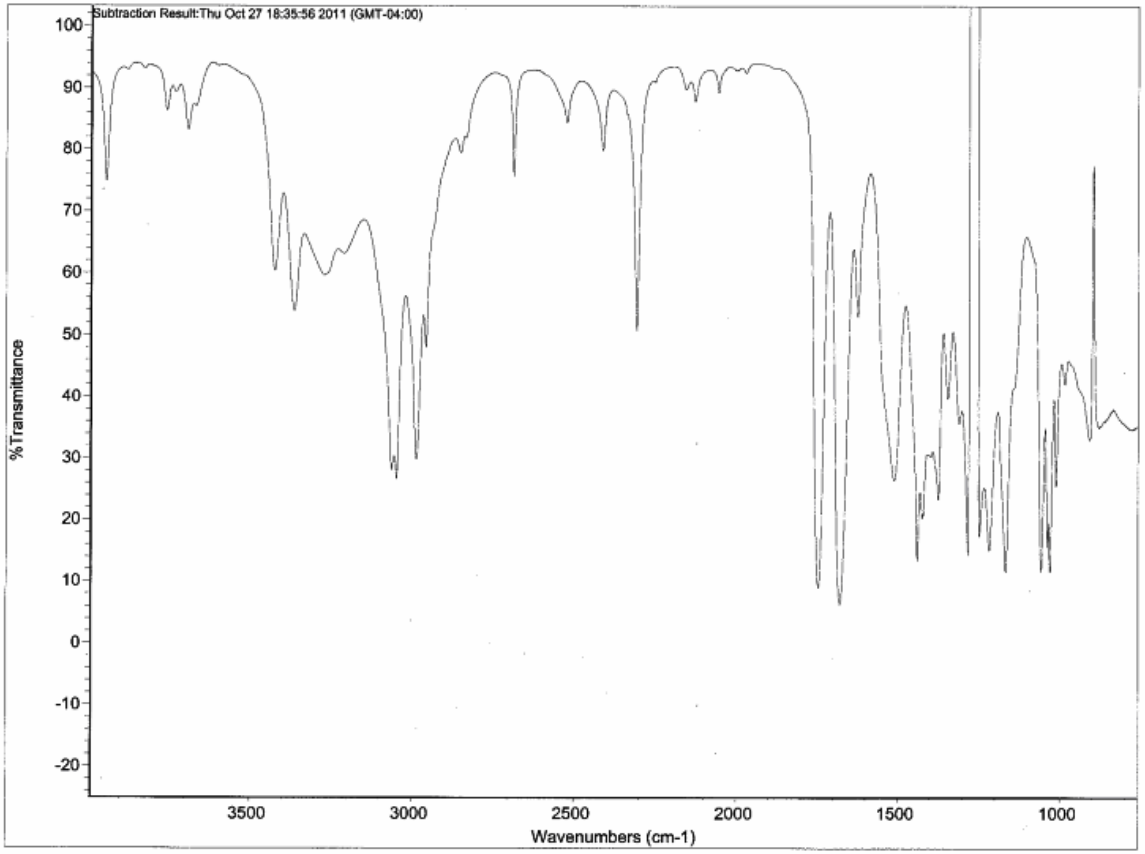
14 (HSQC, DMSO-d_6)



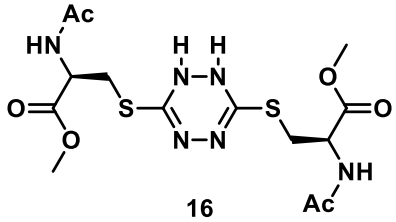
14 (HMBC, DMSO-d_6)



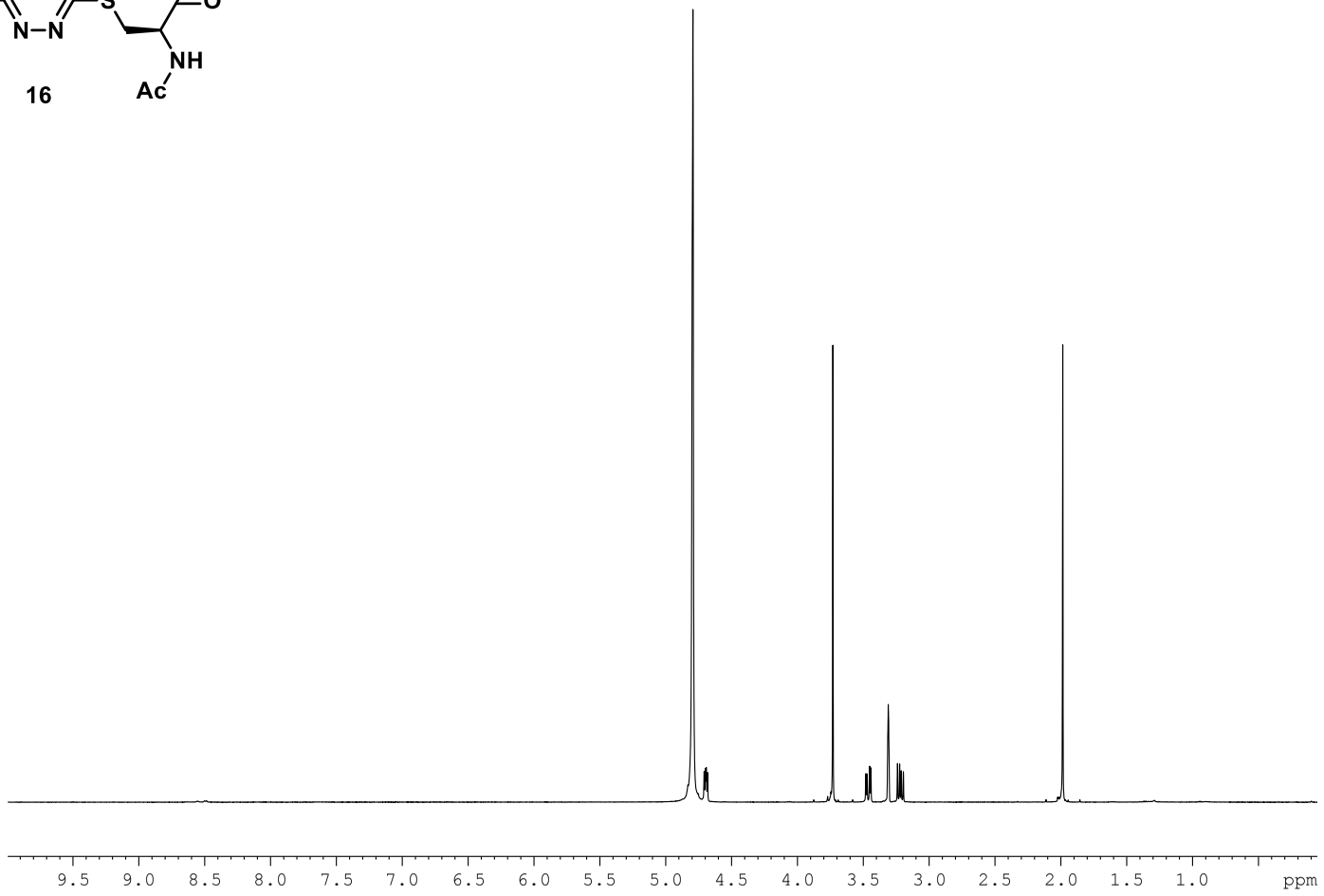
16



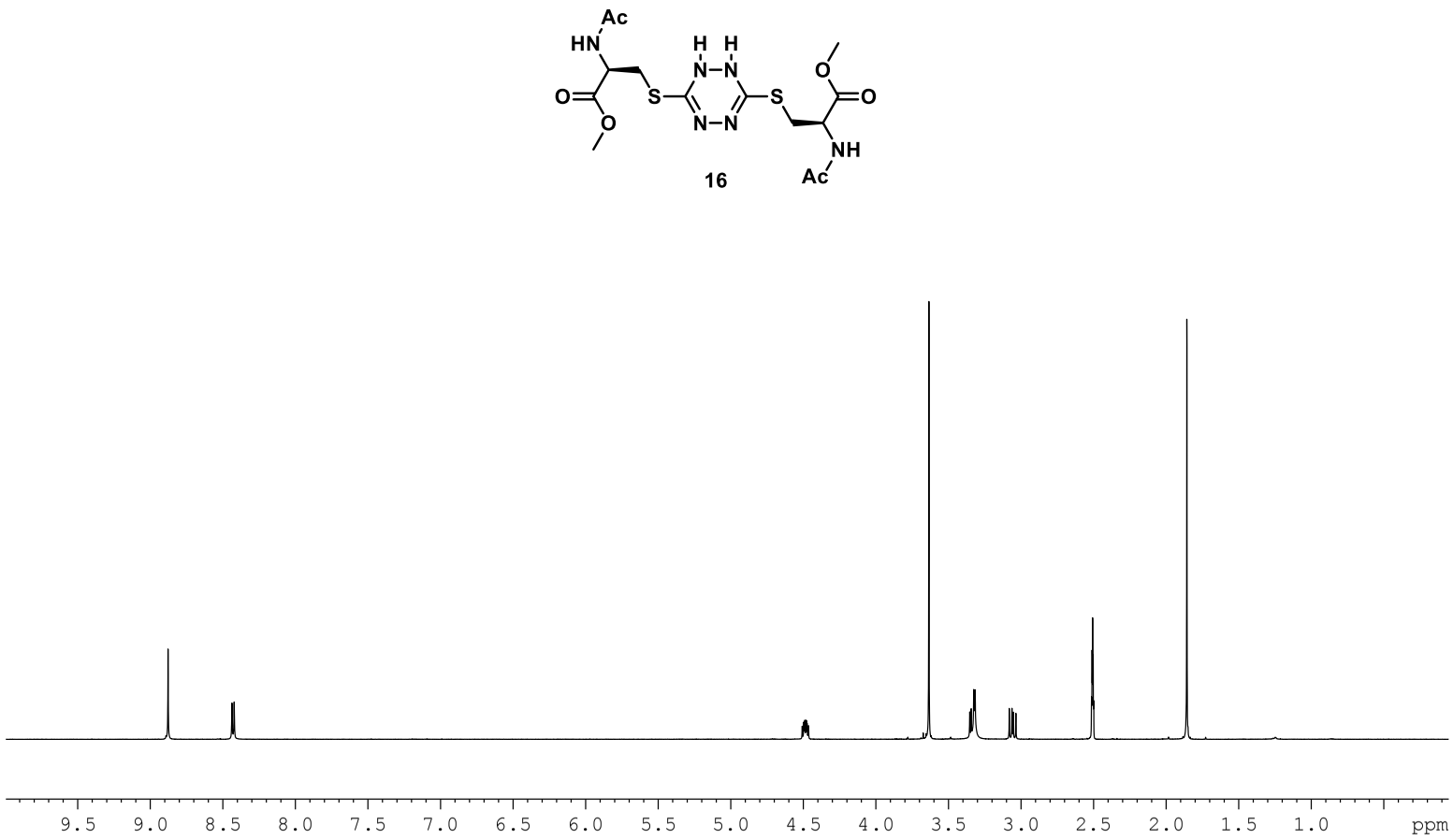
16 (IR, CH_2Cl_2)



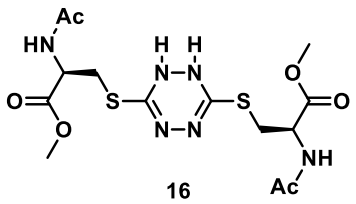
16



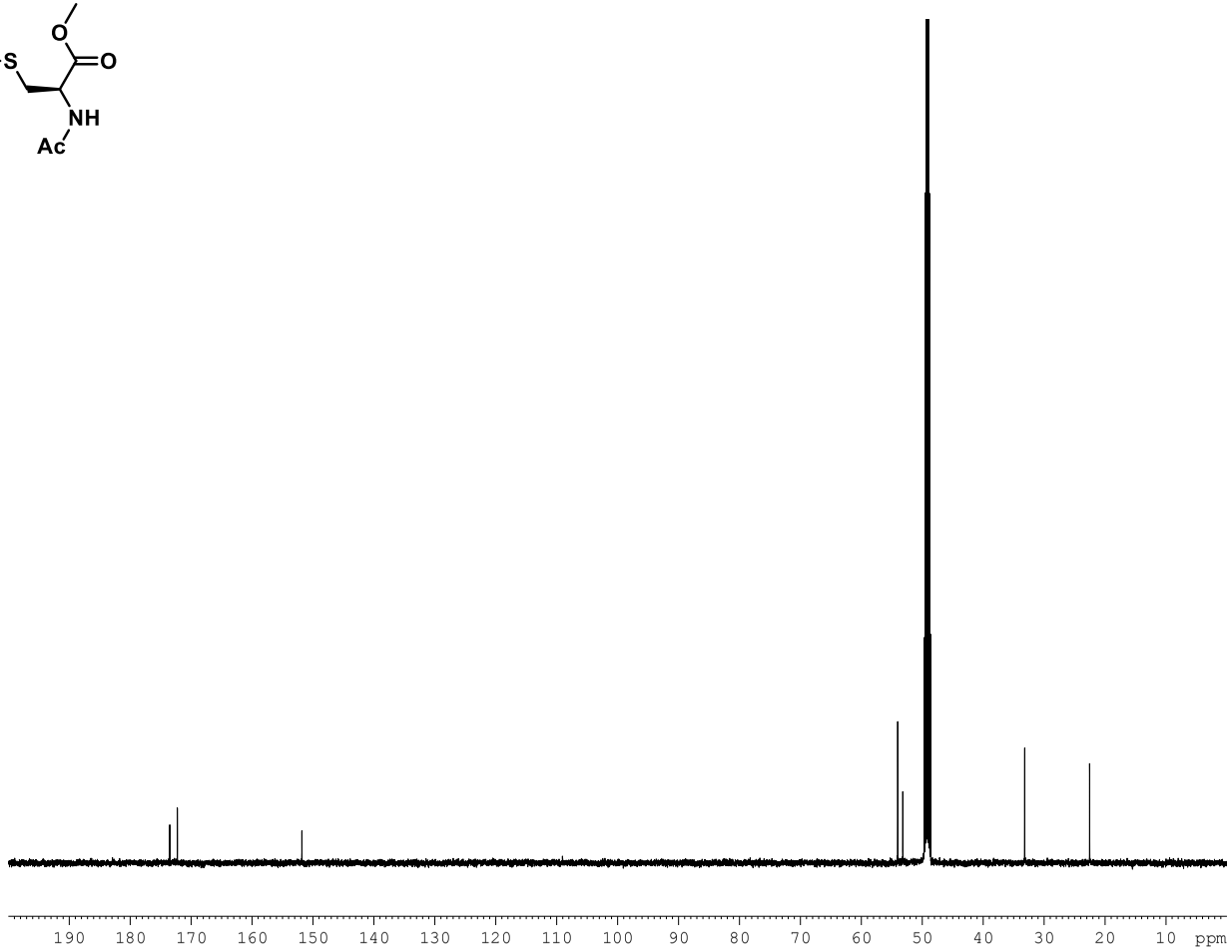
16 (^{1}H NMR, CD_3OD, 500 MHz)



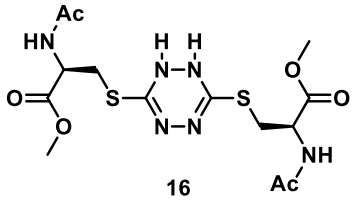
16 (^{1}H NMR, DMSO-d_6, 500 MHz)



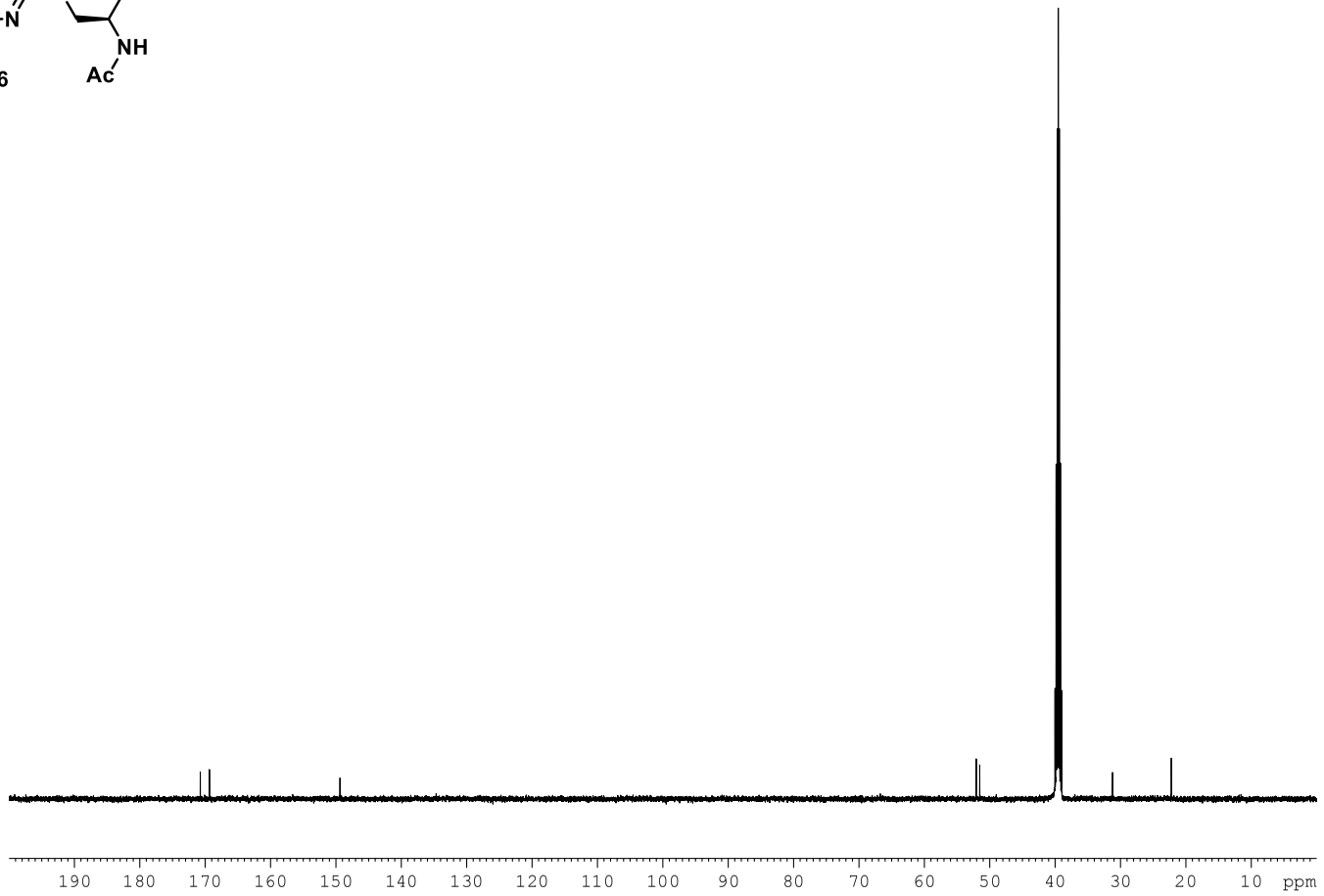
16



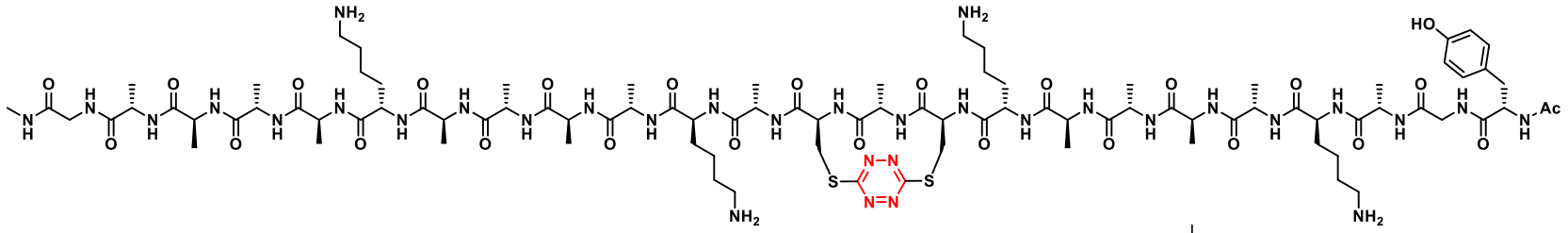
16 (^{13}C NMR, CD_3OD, 125 MHz)



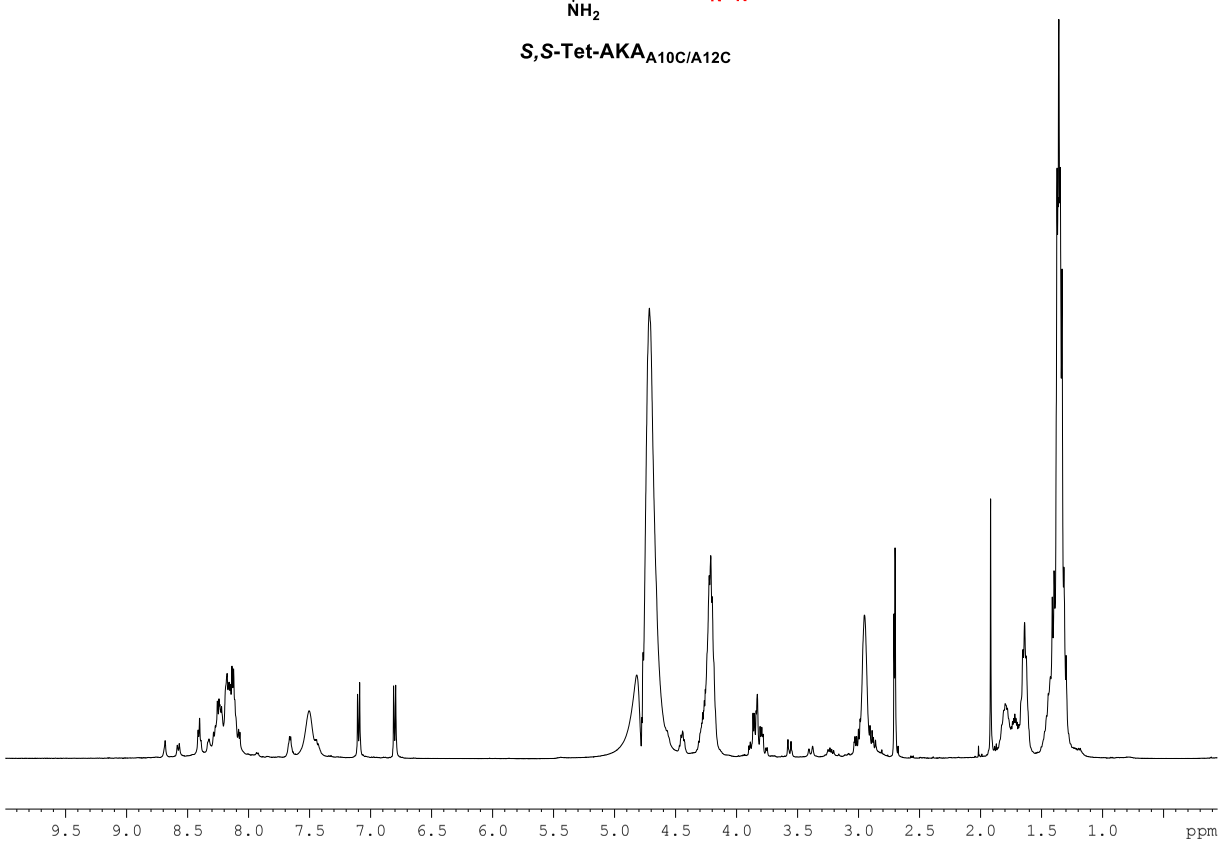
16



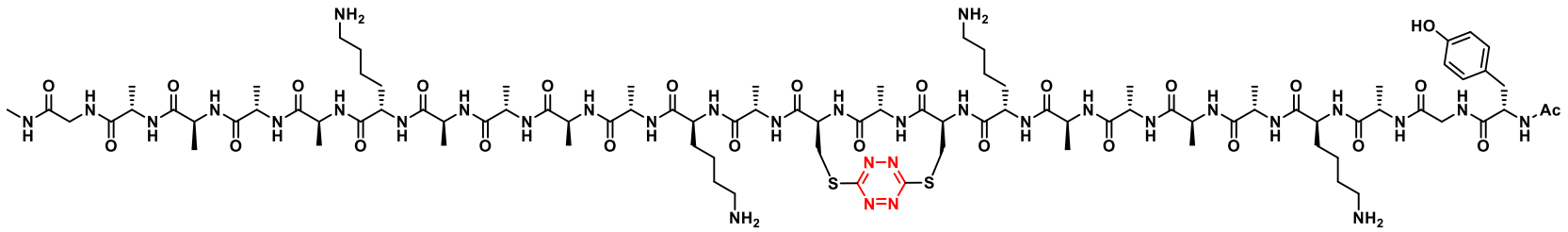
16 (^{13}C NMR, DMSO-d_6, 125 MHz)



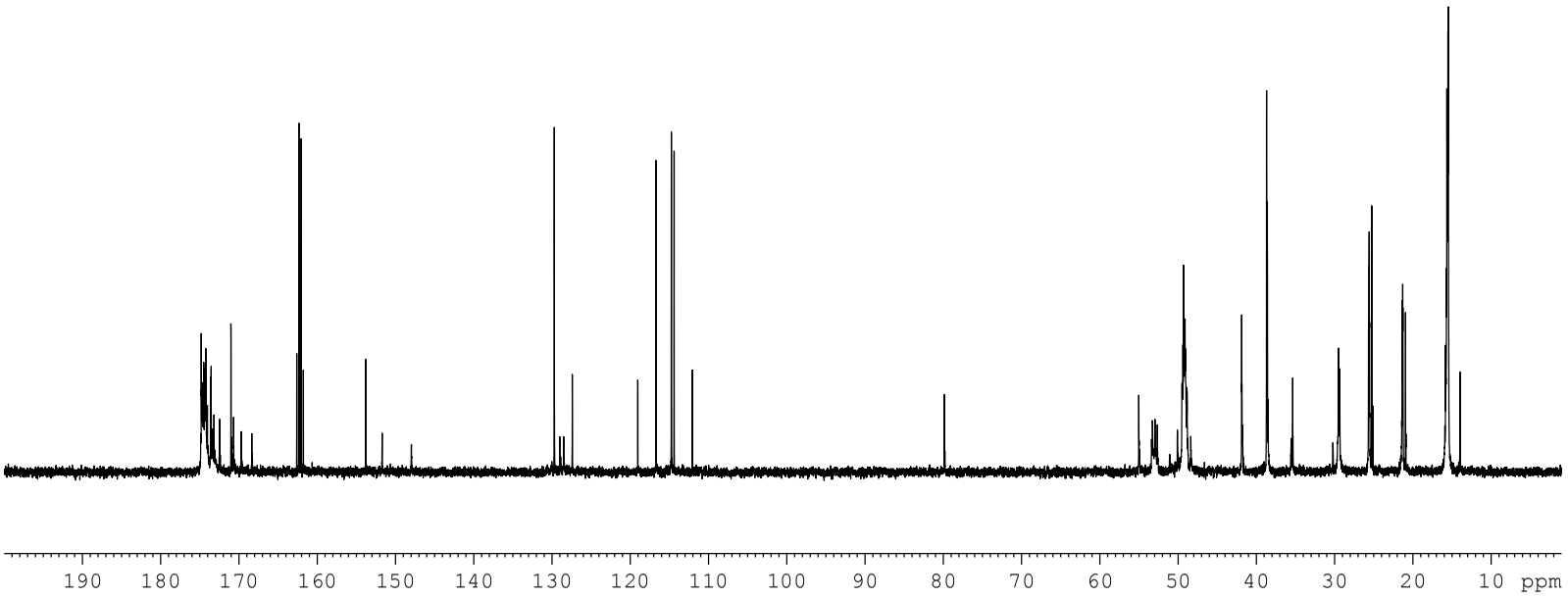
***S,S*-Tet-AKA$_{A10C/A12C}$**



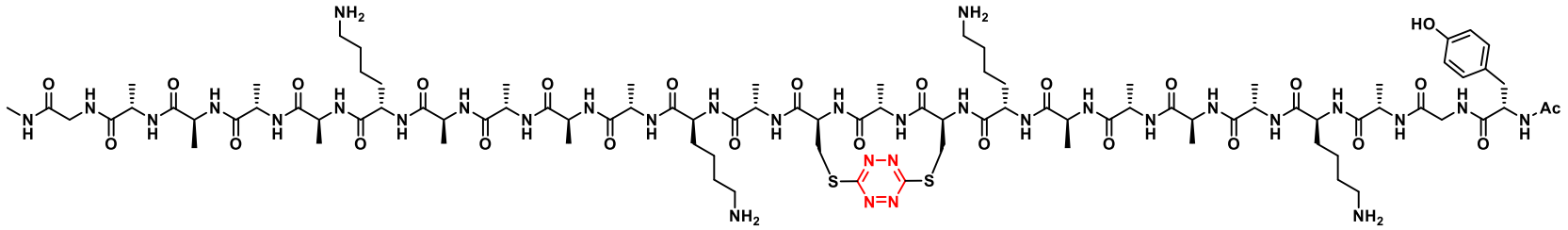
***S,S*-Tet-AKA$_{A10C/A12C}$ (^{1}H NMR, 1:9 D_2O/H_2O, 500 MHz)**



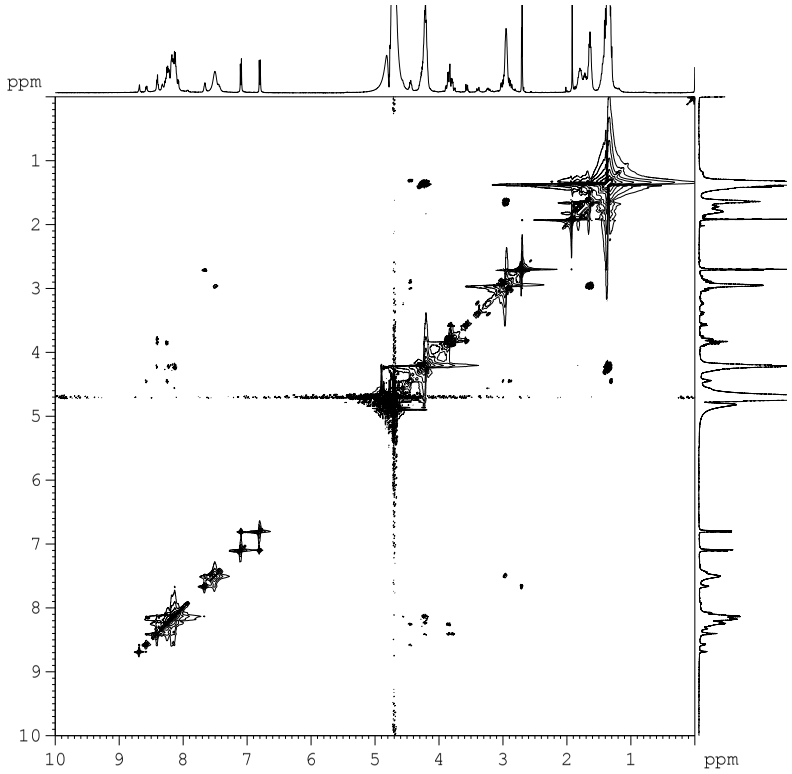
***S,S*-Tet-AKA$_{A10C/A12C}$**



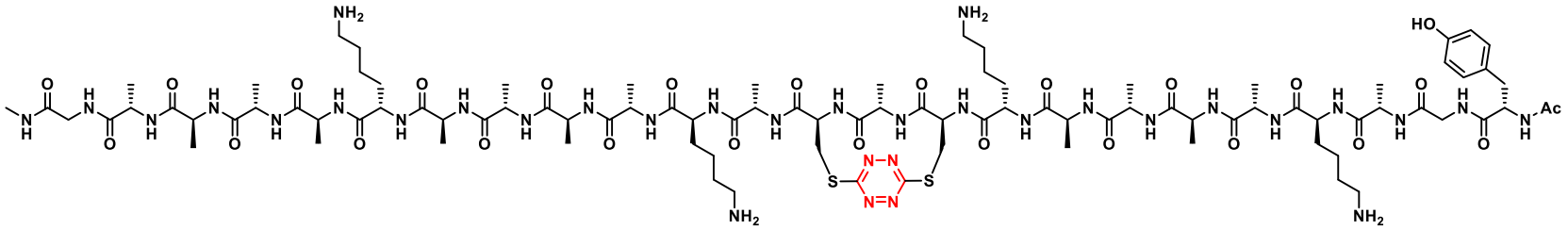
***S,S*-Tet-AKA$_{A10C/A12C}$** (^{13}C NMR, 1:9 D_2O/H_2O, 125 MHz)



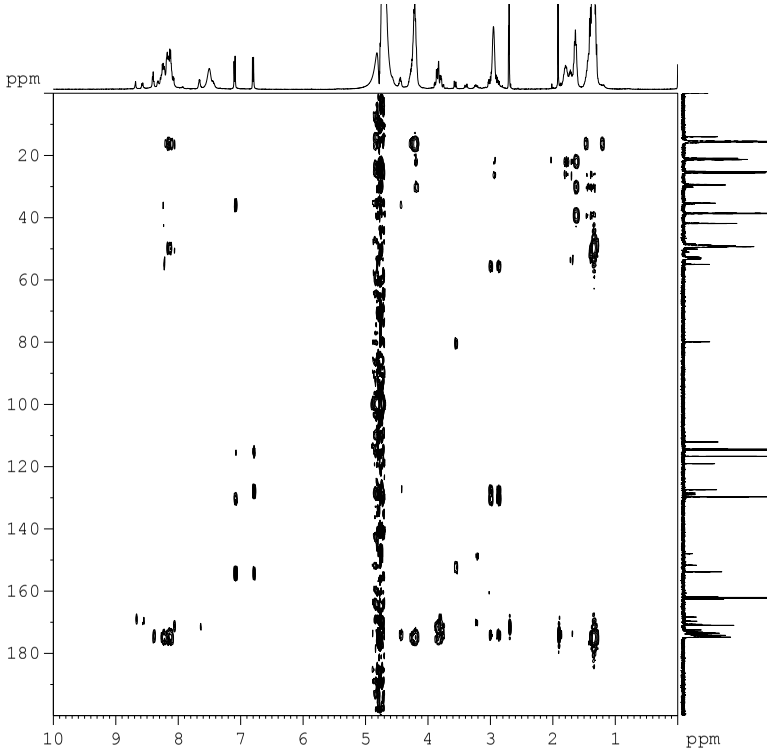
***S,S*-Tet-AKA$_{A10C/A12C}$**



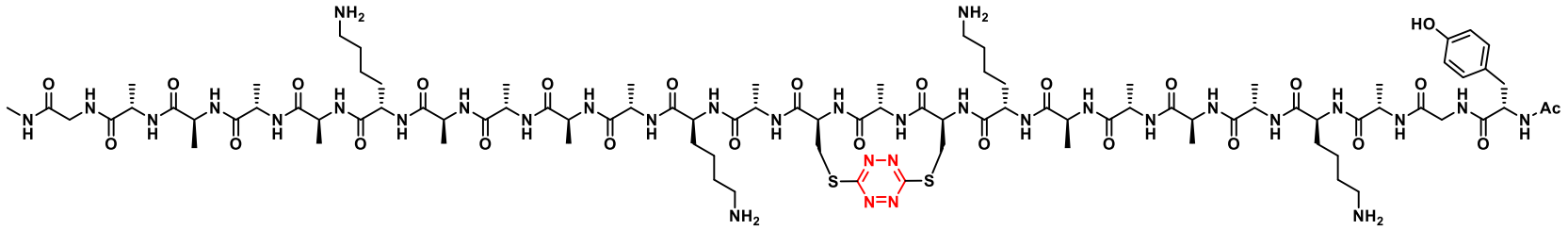
***S,S*-Tet-AKA$_{A10C/A12C}$** (COSY, 1:9 D_2O/H_2O)



***S,S*-Tet-AKA$_{A10C/A12C}$**



***S,S*-Tet-AKA$_{A10C/A12C}$** (HMBC, 1:9 D_2O/H_2O)



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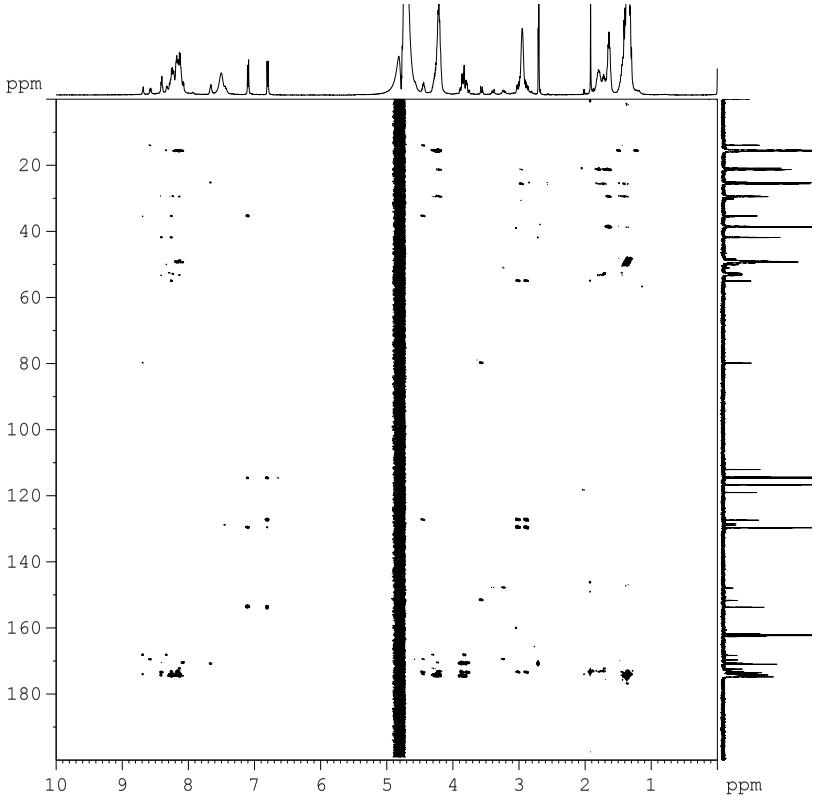
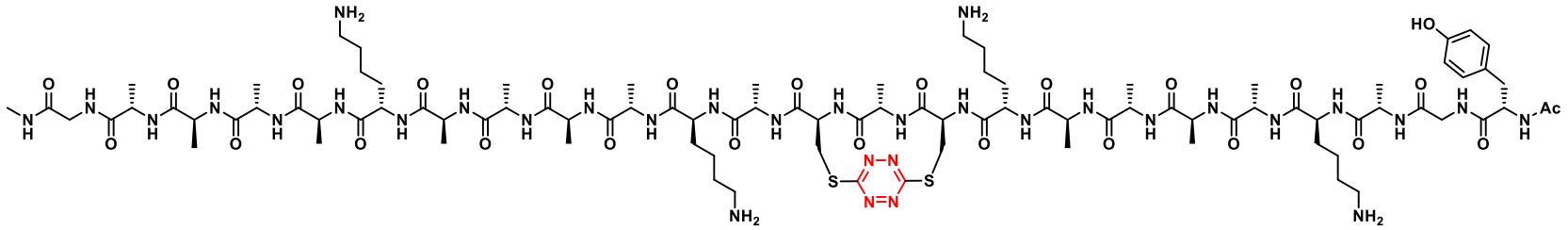
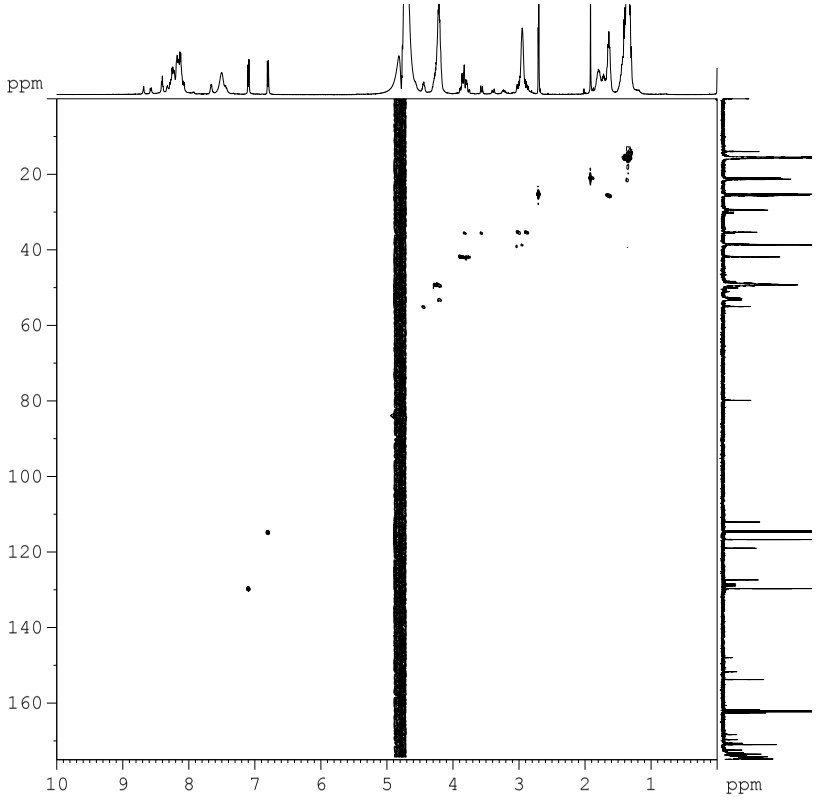


***S,S*-Tet-AKA$_{A10C/A12C}$** (Long Range HMBC, 1:9 D_2O/H_2O)



***S,S*-Tet-AKA$_{A10C/A12C}$**



***S,S*-Tet-AKA$_{A10C/A12C}$** (HMQC, 1:9 D_2O/H_2O)