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SUPPLEMENTARY MATERIAL

Propargyloxyproline regio- and stereoisomers for click-conjugation of peptides: Synthesis and application in linear and cyclic peptides.

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Contents:

Figure S1 Dose-response curves for BVD15 analogues 1 - 4, 10 - 15 in competition binding assays.

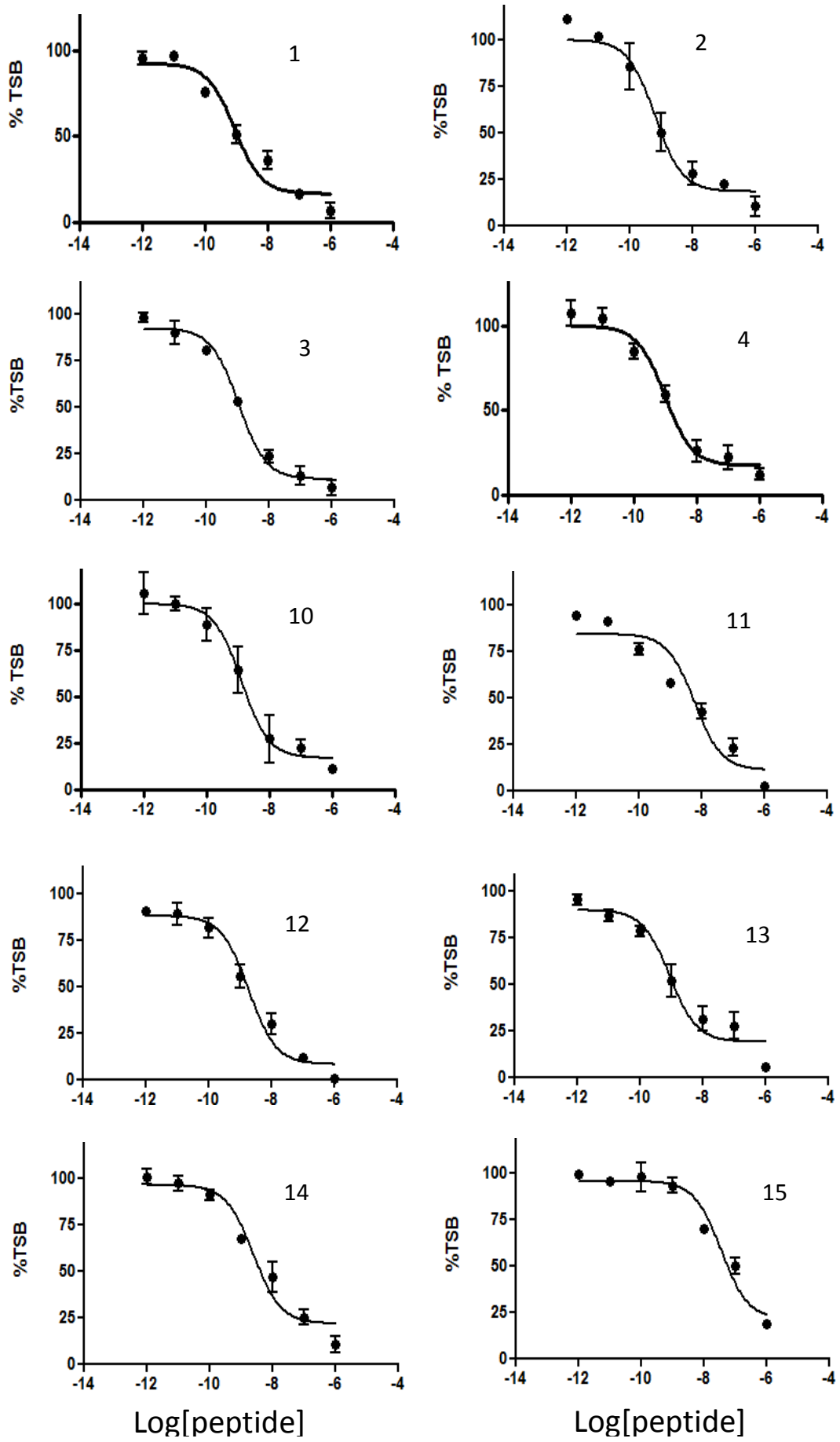


Figure S2 Dose-response curves for BVD15 analogues 1, 16 and 17 in competition binding assays.

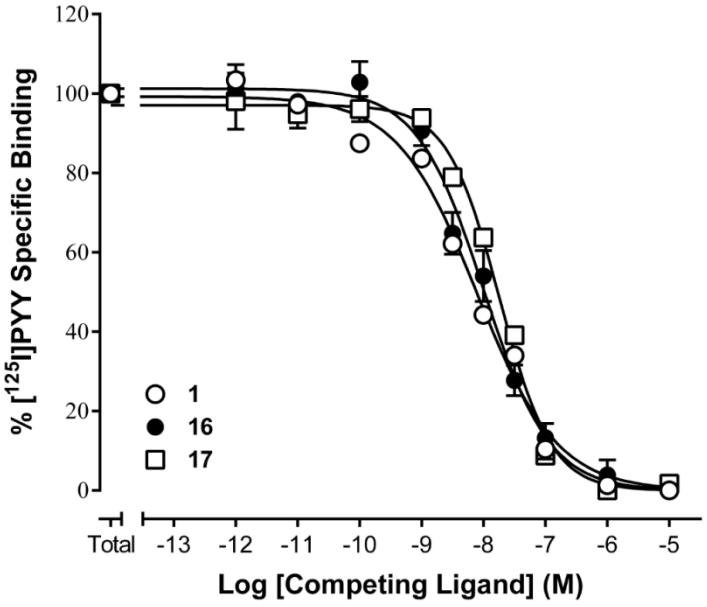
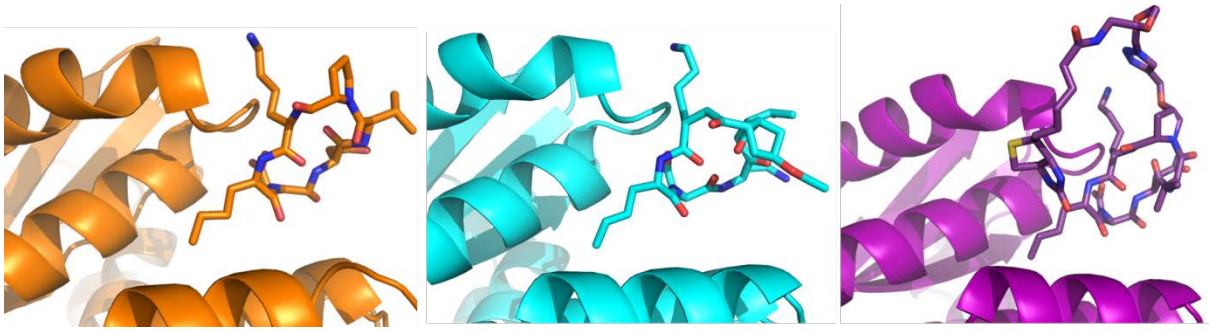


Figure S3 **Comparison of crystal structures of compounds 19, 28 and 22 (*left to right*) in complex with IN**



Amino acid synthesis

General Method A: Boc-protection of Hyp

To a stirred solution of the hydroxyproline (1 eq.) in MeOH (10-36 ml) was added Et_3N (1.9 eq.) and Boc anhydride (2 eq.). The reaction was heated to reflux for 3.5 hrs and then allowed to cool and stirred at room temperature for a further 20 hrs. The solvent was removed *in vacuo* and the residue cooled to 0˚C. Following the addition of NaH_2PO_4 (150 mg), the solution was acidified to pH 2 with 0.5 M HCl. The mixture was stirred at 0˚C for 30 min prior to extracting the product with EtOAc (4 x 20 ml). The combined organic layers were dried with $MgSO_4$, filtered and the solvent removed *in vacuo* to yield the crude product.

General Method B: Generating Boc-Propargyloxyproline

A solution of the Boc protected hydroxyproline (1 eq.) in dry DMF (1 ml) was added dropwise to a suspension of NaH (2.75 eq.) in dry DMF (5-15 ml) under nitrogen at 0˚C. After 15 min propargyl bromide (80% in toluene) (1.25 eq.) was added drop wise to the reaction. The reaction was stirred at 0˚C for 2 hrs then quenched with H_2O followed by ACN and lyophilised. The resulting residue was taken up in EtOAc and H_2O and adjusted to pH 2 with 10% citric acid. The phases were separated and the aqueous layer was extracted with EtOAc (2 x 20 ml). The combined organic layers were dried with $MgSO_4$ and filtered. The solvent was removed *in vacuo* to yield the crude product.

General Method C:Fmoc-Propargyloxyproline

The Boc protected propargyloxyproline (1 eq.) was treated with 1:1 TFA/DCM at room temperature for 45 min and then the solvent was removed *in vacuo*. The residue was dissolved in H_2O (5 ml) and adjusted to pH 9 with a solution of Na_2CO_3 (2.2 eq.). Fmoc-OSu (1 eq.) in dioxane (or acetone) (5-10 ml) was added to the reaction mixture at 0˚C and stirred for 1 hr. The reaction was then brought to room temperature and stirred overnight. Dioxane (or acetone) was removed *in vacuo* and the reaction mixture acidified to pH 2-3 with 1M HCl. The product was extracted with EtOAc (3 x 20 ml), washed with brine, dried ($MgSO_4$), filtered and solvent removed *in vacuo to* yield the crude product.

*Boc-trans-4-hydroxy-L-proline (**v**)*

The title compound was prepared according to Method A from *trans*-4-hydroxy-L-proline (**i** , 2.0 g, 15.3 mmol) to yield a white foam (3.23 g) which was reacted directly on for the next step. ^{1}H NMR: (CD_3OD, 400 MHz) δ 4.40 (dd, *J*=5.5, 3.4 Hz, CH, 1H), 4.32 (dt, *J*=12.9, 8.0 Hz, CH, 1H), 3.54 (dt, *J*=11.4, 4.0 Hz, 0.5 x CH_2, 1H), 3.44 (dt, *J*=11.4, 1.9 Hz, 0.5 x CH_2, 1H), 2.27 (dddd, *J*=12.3, 7.7, 2.8, 1.8 Hz, 0.5 x CH_2, 1H), 2.06 (ddd, *J*=13.2, 8.6, 4.5 Hz, 0.5 x CH_2, 1H), 1.45 (s, Boc, 9H). ^{13}C NMR: (CD_3OD, 101MHz) δ 176.75 & 176.37 (pair of rotamers, Cq), 156.54 & 156.02 (pair of rotamers, Cq), 81.72 & 81.42 (pair of rotamers, Cq),

70.68 & 70.06 (pair of rotamers, CH), 59.39 & 58.91 (pair of rotamers, CH), 55.85 & 55.51 (pair of rotamers, CH_2), 40.07 & 39.4 (pair of rotamers, CH_2), 28.71 & 28.53 (pair of rotamers, 3 x CH_3). LCMS *(m/z)*: 277.35 $[M + 2Na]^+$.

*Boc-cis-4-hydroxy-D-proline (**vi**)*

The title compound was prepared according to Method A from *cis*-4-hydroxy-D-proline (**ii**, 2.0 g, 15.3 mmol) to yield a white foam (2.42 g) which was reacted directly on for the next step. ^{1}H NMR: (CD_3OD, 300 MHz) δ 4.42 – 4.19 (m, 2H), 3.61 (dd, *J* = 11.1, 5.1 Hz, 1H), 3.40 – 3.34 (m, 1H), 2.53 – 2.33 (m, 1H), 2.13 – 1.97 (m, 1H), 1.47 & 1.43 (s, pair of rotamers, 9H). LCMS *(m/z)*: 277.35 $[M + 2Na]^+$. LCMS t_R: 11.84 min.

*Boc-cis-4-hydroxy-L-proline (**vii**)*

The title compound was prepared according to Method A from *cis*-4-hydroxy-L-proline (**iii**, 0.50 g, 3.8 mmol) to yield a clear oil (0.77 g) which was reacted directly on for the next step. ^{1}H NMR: (CD_3OD, 400 MHz) δ 4.37 – 4.24 (m, 2H), 3.61 (dd, *J* = 11.1, 5.1 Hz, 1H), 3.35 – 3.32 (m, 1H), 2.49 – 2.37 (m, 1H), 2.09 – 2.01 (m, 1H), 1.47 & 1.43 (s, pair of rotamers, 9H).

*Boc-cis-3-hydroxy-L-proline (**viii**)*

The title compound was prepared according to method A from *cis*-3-hydroxy-L-proline (**iv**, 0.50 g, 3.8 mmol) to yield a clear oil (0.72 g) which was reacted directly on for the next step. ^{1}H NMR: (CD_3OD, 400 MHz) δ 4.59 – 4.53 (m, 1H), 4.29 (t, *J* = 7.1 Hz, 1H), 3.61 – 3.54 (m, 1H), 3.47 – 3.38 (m, 1H), 2.10 – 1.91 (m, 2H), 1.46 & 1.42 (s, pair of rotamers, 9H).

*Boc-trans-4-propargyloxy-L-proline (**ix**)*

The title compound was prepared according to method B from **v** (2.80 g, 12.13 mmol) to yield a brown solid (2.97 g) which was directly carried on to the next step. ^{1}H NMR: (CD_3OD, 400 MHz) δ 4.37-4.31 (m, CH, 1H), 4.31-4.21 (m, CH, 1H), 4.19 (d, J=2.4 Hz, CH_2, 2H), 3.64-3.57 (m, 0.5 x CH_2, 1H), 3.57-3.50 (m, 0.5 x CH_2, 1H), 2.94-2.77 (m, CH, 1H), 2.44(tddd, J=14.3, 11.5, 3.0, 1.6 Hz, 0.5 x CH_2, 1H), 2.13- 2.04 (m, 0.5 x CH_2, 1H), 1.45 (s, Boc, 9H). ^{13}C NMR: (CD_3OD, 101MHz) δ 178.33 & 175.54 (pair of rotamers, Cq), 156.04 & 155.93 (pair of rotamers, Cq), 81.64 & 80.9 (pair of rotamers, Cq), 79.31 (CH), 76.2 & 75.85 (pair of rotamers, CH), 75.04 (Cq), 57.90 & 57.87 (pair of rotamers, CH), 56.58 & 56.51 (pair of rotamers, CH_2), 51.93 & 51.18 (pair of rotamers, CH_2), 36.57 & 34.57 (pair of rotamers, CH_2), 28.45 & 28.33 (pair of rotamers, 3 x CH_3). LCMS *(m/z)*: 315.35 $[M + 2Na]^+$.

*Boc-cis-4-propargyloxy-D-proline (**x**)*

The title compound was prepared according to method B from **vi** (2.20 g, 9.50 mmol) to yield a brown solid (2.41 g) which was directly carried on to the next step. ^{1}H NMR: (CD_3OD, 300 MHz) δ 4.41 – 4.23 (m, 2H), 4.15 (s, 2H), 3.63 (dd, *J* = 11.5, 5.3 Hz, 1H), 3.44 (d, *J* = 11.6 Hz, 1H), 2.85 (d, *J* = 1.7 Hz, 1H), 2.53 – 2.32 (m, 1H), 2.32 – 2.17 (m, 1H), 1.47 & 1.43 (s, pair of rotamers, 9H). LCMS *(m/z)*: 315.35 $[M + 2Na]^+$.

*Boc-cis-4-propargyloxy-L-proline (**xi**)*

The title compound was prepared according to method B from **vii**, (0.77 g, 3.3 mmol) to yield a brown residue (0.83 g) which was reacted directly on for the next step. ^{1}H NMR: (CD_3OD, 400 MHz) δ 4.35 – 4.28 (m, 2H), 4.15 – 4.14 (m, 2H), 3.64 (dd, *J* = 11.7, 5.5 Hz, 1H), 3.44 (ddd, *J* = 11.7, 3.0, 1.0 Hz, 1H), 2.83 (t, *J* = 2.4 Hz, 1H), 2.49 – 2.34 (m, 1H), 2.29 – 2.21 (m, 1H), 1.47 & 1.43 (s, pair of rotamers, 9H).

*Boc-cis-3-propargyloxy-L-proline (**xii**)*

The title compound was prepared according to method B from **viii**, **(**0.72 g, 3.1 mmol) yielding a brown residue (0.66 g) which was reacted directly on for the next step. ^{1}H NMR: (CD_3OD, 400 MHz) δ 4.59 – 4.49 (m, 1H), 4.45 – 4.42 (m, 1H), 4.27 – 4.24 (m, 2H), 3.60 – 3.52 (m, 1H), 3.47 – 3.36 (m, 1H), 2.87 – 2.86 (m, 1H), 2.12 – 2.06 (m, 2H), 1.46 & 1.42 (s, pair of rotamers, 9H).

*Fmoc-trans-4-propargyloxy-L-proline (**xiii**)*

The title compound was prepared according to method C using **ix**, (2.97 g, 11.01 mmol) to yield a yellow foam. Purification was achieved by flash chromatography (0-2% MeOH in Chloroform) yielding a white powder (1.31 g, 30%). ^{1}H NMR: (CD_3OD, 400 MHz) δ 7.8 (t, *J*=7.5 Hz, 2H), 7.63 (td, *J*=7.5, 2.4 Hz, 2H), 7.39 (td, *J*=7.4, 4.0 Hz, 2H), 7.35-7.28 (m, 2H), 4.46-4.18 (m, 6H), 4.15 (dd, *J*=4.7, 2.4 Hz, 1H), 3.64-3.5 (m, 2H), 2.89 (t, J=2.4 Hz, 1H), 2.57-2.4 (m, 1H), 2.22-2.05 (m, 1H). ^{13}C NMR: (CD_3OD, 101MHz) δ 175.98 & 175.75 (pair of rotamers, Cq), 156.71 & 156.62 (pair of rotamers, Cq), 145.31, 145.29, 145.12, 145.05 (rotamers, Cq), 142.64, 142.61, 142.56, 142.49 (rotamers, Cq), 128.88 (CH), 128.25 (CH), 126.28, 125.25, 126.16, 126.15 (rotamers, CH), 121.03 & 120.98 (pair of rotamers, CH), 80.58 & 80.57 (pair of rotamers , Cq), 77.92 & 77.15 (pair of rotamers, CH), 76.27 & 77.26 (pair of rotamers, CH), 69.32 & 68.75 (pair of rotamers, CH_2), 59.26 & 59.01 (pair of rotamers, CH), 57.25 & 57.21 (pair of rotamers, CH_2), 53.21 & 52.78 (pair of rotamers, CH_2), 48.39 & 48.33 (pair of rotamers, CH), 37.6 and 36.6 (pair of rotamers, CH_2). LCMS *(m/z)*: 392.30, 100% $[M + H]^+$. HRMS (*m/z*): $C_{23}H_{22}NO_5^+$ requires $[M + H]^+$ 392.1492; found 392.1494.

*Fmoc-cis-4-propargyloxy-D-proline (**xiv**)*

The title compound was prepared according to method C using **x** (2.30 g, 8.54 mmol) to yield a brown oil. Purification was achieved by flash chromatography (SiO_2, MeOH 0-10% in CH_2Cl_2) yielding a white powder (2.25 g, 67%). ^{1}H NMR: (CD_3OD, 400 MHz) δ 7.80 (t, *J* = 8.2 Hz, 2H), 7.70 – 7.58 (m, 2H), 7.44 – 7.36 (m, 2H), 7.36 – 7.27 (m, 2H), 4.47 – 4.31 (m, 4H), 4.24 (dt, *J* = 32.6, 6.7 Hz, 1H), 4.14 (d, *J* = 2.4 Hz, 2H), 3.64 (ddd, *J* = 16.9, 11.8, 5.2 Hz, 1H), 3.57 – 3.47 (m, 1H), 2.86 (dt, *J* = 6.5, 2.4 Hz, 1H), 2.44 – 2.31 (m, 2H). ^{13}C NMR: (CD_3OD, 101MHz) δ 175.30 & 175.05 (pair of rotamers, Cq), 156.64 & 156.54 (pair of rotamers, Cq), 145.36, 145.31, 145.10, 145.07 (rotamers, Cq), 142.61, 142.55, 142.46 (rotamers, Cq), 128.81 (CH), 128.19 (CH), 126.21 & 126.14 (pair of rotamers, CH), 120.93 & 120.88 (pair of rotamers, CH), 80.39 & 80.35 (pair of rotamers, Cq), 77.69 & 76.81 (pair of rotamers, CH), 76.13 & 76.08 (pair of rotamers, CCH), 68.99 & 68.76 (pair of rotamers, CH_2), 59.01 & 58.88 (pair of rotamers, CH), 56.86 & 56.78 (pair of rotamers, CH_2), 53.26 & 52.97 (pair of rotamers, CH_2), 48.40 & 48.34 (pair of rotamers, CH), 36.66 and 35.68 (pair of rotamers, CH_2). LCMS *(m/z)*: 392.35, 100% $[M + H]^+$. HRMS (*m/z*): $C_{23}H_{22}NO_5^+$ requires $[M + H]^+$ 392.1492; found 392.1501.

*Fmoc-cis-4-propargyloxy-L-proline (**xv**)*

The title compound was prepared according to method C using **xi**, (0.83 g, 3.08 mmol) to yield a crude brown oil (1.38 g). Purification was achieved by flash chromatography (Et_2O : Petroleum, 7 : 3, containing 1% AcOH) yielding a white powder (0.71 g, 59%). ^{1}H NMR: (CD_3OD, 400 MHz) δ 7.79 (t, *J* = 8.2 Hz, 2H), 7.68 – 7.58 (m, 2H), 7.44 – 7.36 (m, 2H), 7.36 – 7.27 (m, 3H), 4.48 – 4.30 (m, 4H), 4.23 (dt, *J* = 31.1, 6.6 Hz, 1H), 4.14 (d, *J* = 2.4 Hz, 2H), 3.64 (ddd, *J* = 19.2, 11.8, 5.2 Hz, 1H), 3.51 (td, *J* = 11.9, 1.6 Hz, 1H), 2.85 (dt, *J* = 6.5, 2.4 Hz, 1H), 2.41 – 2.30 (m, 2H). ^{13}C NMR: (CD_3OD, 101MHz) δ 175.51, 175.30, 156.62, 156.52, 145.34, 145.31, 145.07, 145.04, 142.58, 142.52, 142.43, 128.80, 128.18, 126.21, 126.19, 126.16, 126.14, 120.93, 120.91, 120.87, 80.40, 80.36, 77.66, 76.78, 76.14, 76.09, 68.98, 68.72, 59.10, 58.98, 56.86, 56.78, 53.22, 52.94, 48.37, 48.31, 36.68, 35.67. LCMS *(m/z)*: 392.35, 100% $[M + H]^+$. HRMS (*m/z*): $C_{23}H_{22}NO_5^+$ requires $[M + H]^+$ 392.1492; found 392.1501.

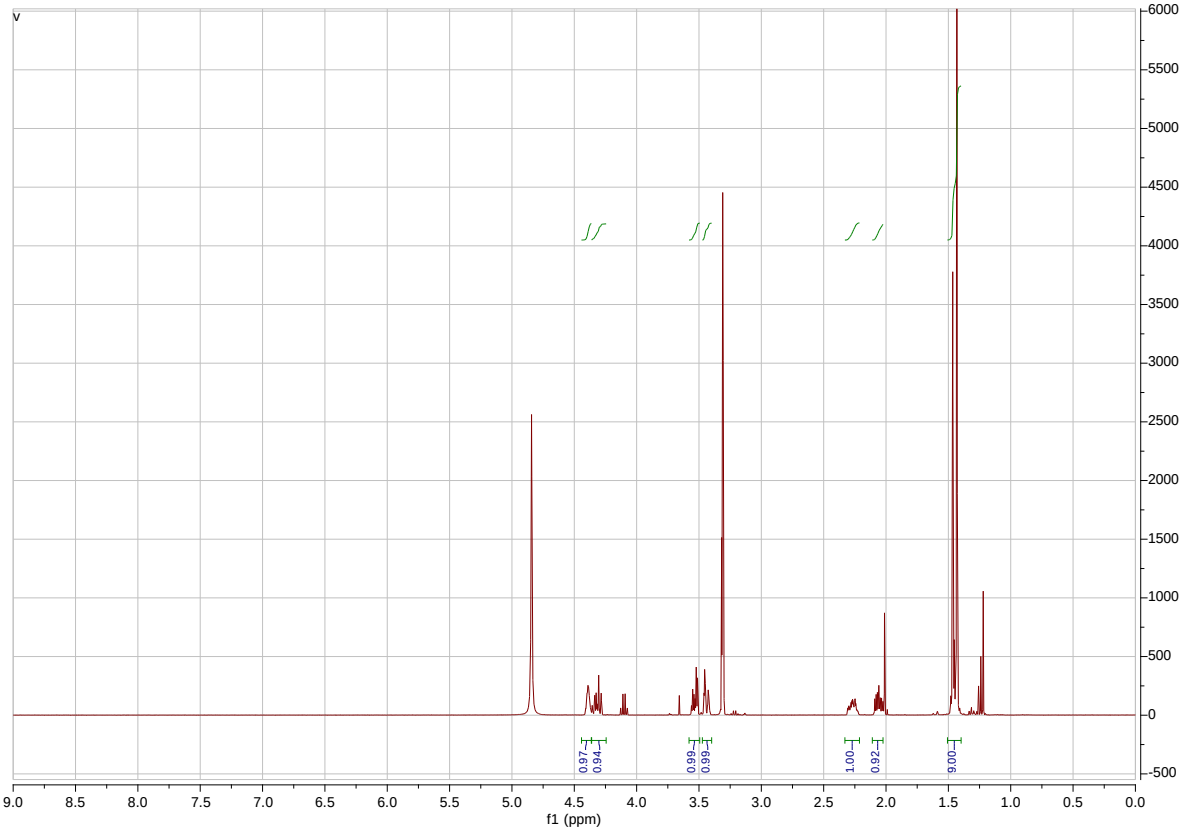
*Fmoc-cis-3-propargyloxy-L-proline (**xvi**)*

The title compound was prepared according to method C using **xii**, (0.33 g, 1.23 mmol) to yield a crude brown oil. Purification was achieved by flash chromatography (Et_2O : Petroleum, 7 : 3, containing 1% AcOH) yielding a white powder (0.29, 60%). ^{1}H NMR: (CD_3OD, 400 MHz) δ 7.80 (dd, *J* = 7.5, 3.1 Hz, 2H), 7.68 – 7.60 (m, 2H), 7.39 (t, *J* = 7.5 Hz, 2H), 7.36 – 7.28 (m, 2H), 4.59 – 4.46 (m, 2H), 4.45 – 4.30 (m, 2H), 4.30 – 4.15 (m, 3H), 3.63 – 3.54 (m, 1H), 3.53 – 3.40 (m, 1H), 2.88 (dt, *J* = 9.6, 2.4 Hz, 1H), 2.16 – 2.06 (m, 2H). ^{13}C

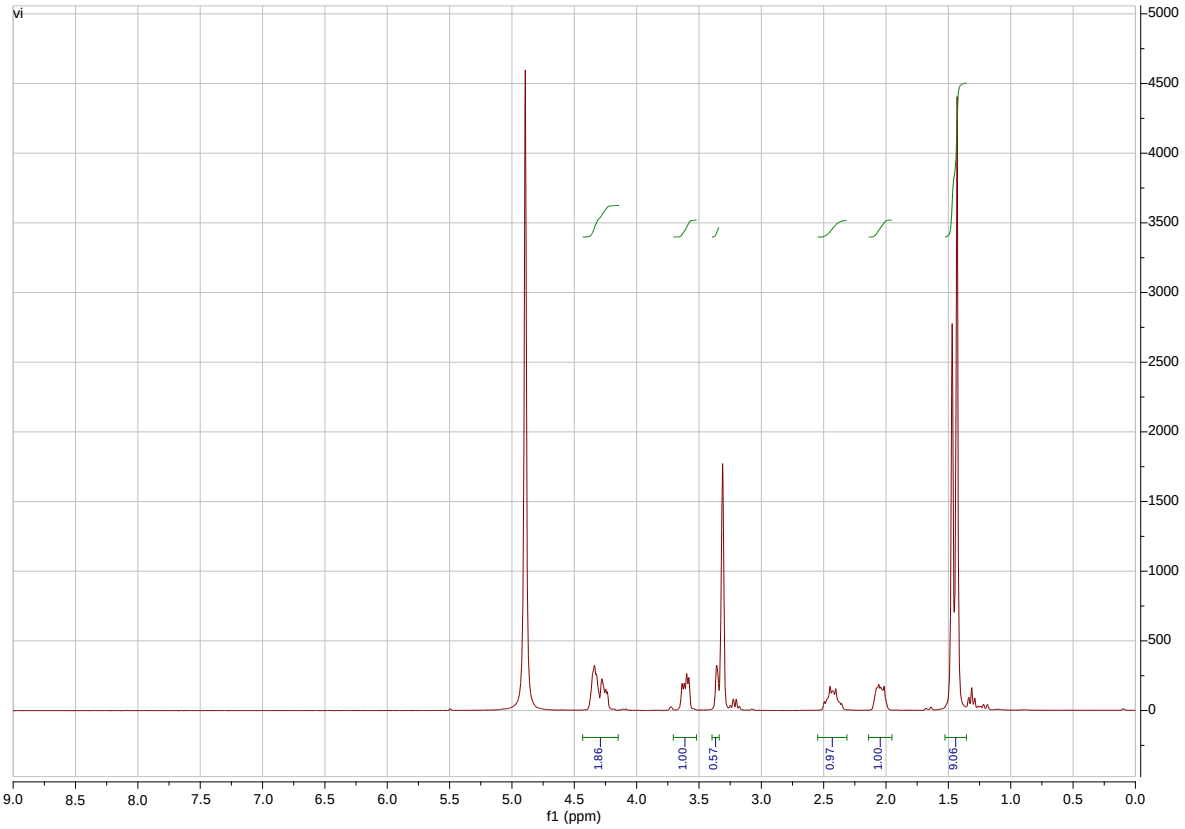
NMR: (CD_3OD, 101MHz) δ 172.89, 172.60, 156.51, 156.40, 145.25, 145.04, 144.99, 142.57, 142.52, 142.43, 128.82, 128.22, 128.17, 126.23, 126.20, 126.12, 120.94, 120.90, 80.18, 80.16, 79.50, 78.74, 76.32, 76.27, 69.13, 68.69, 63.37, 62.97, 58.36, 58.30, 48.38, 48.30, 45.34, 45.15, 30.81, 30.00. LCMS *(m/z)*: 392.35, 100% $[M + H]^+$. HRMS (*m/z*): $C_{23}H_{22}NO_5^+$ requires $[M + H]^+$ 392.1492; found 392.1498.

Figure S4 **NMR spectra for compounds v - xvi**

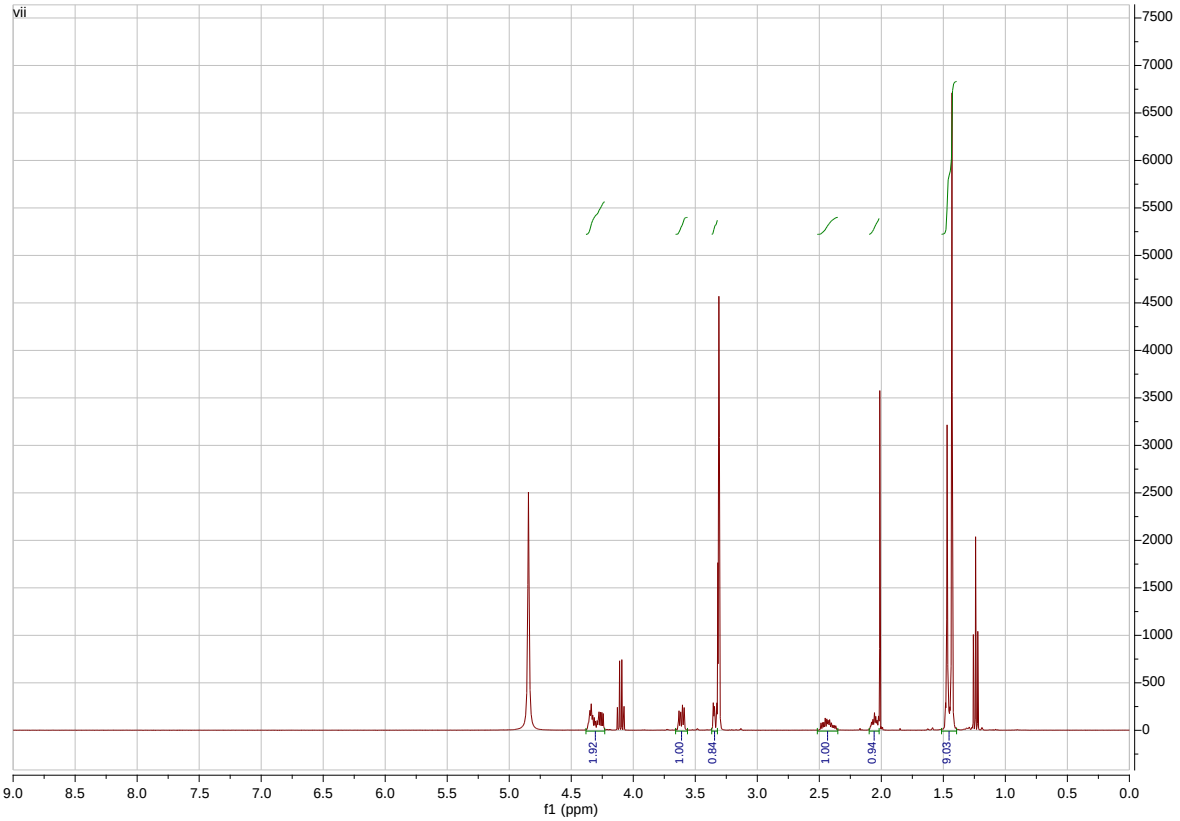
Boc-trans-L-4-hydroxyproline, ***v***



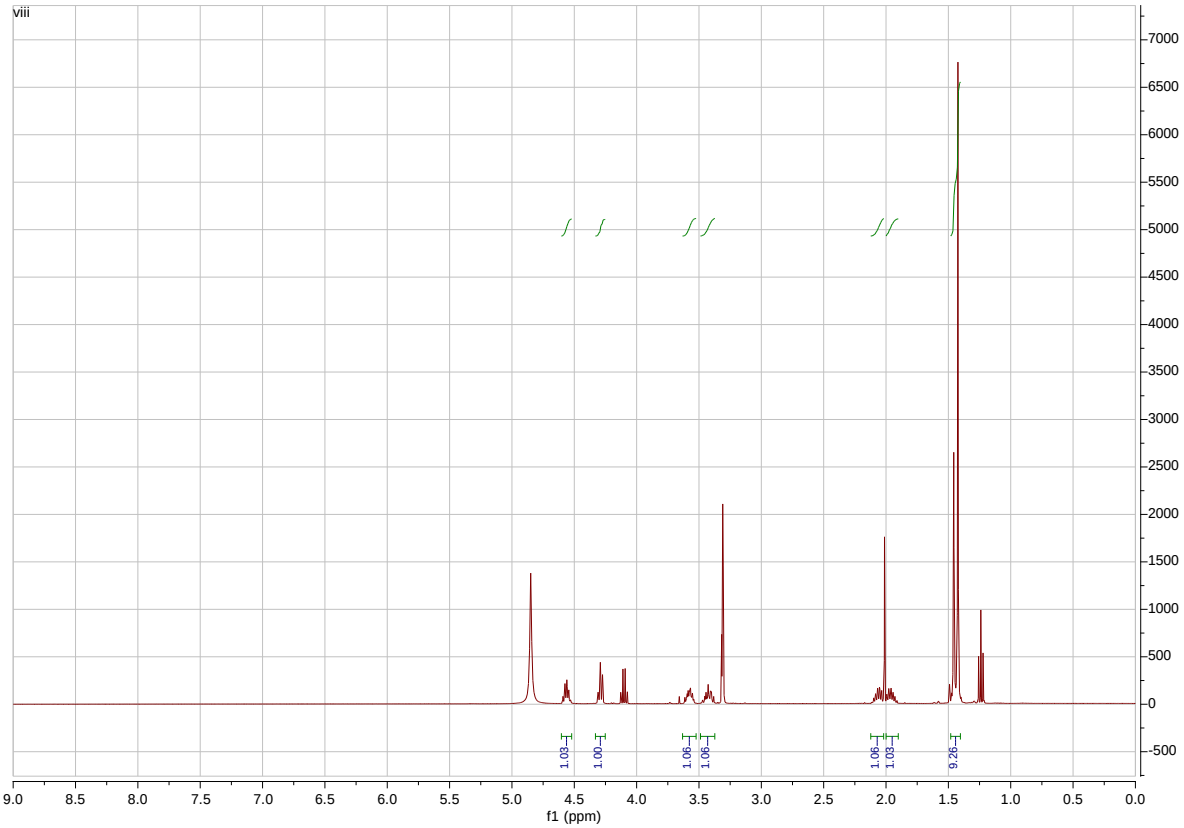
Boc-cis-D-4-hydroxyproline, ***vi***



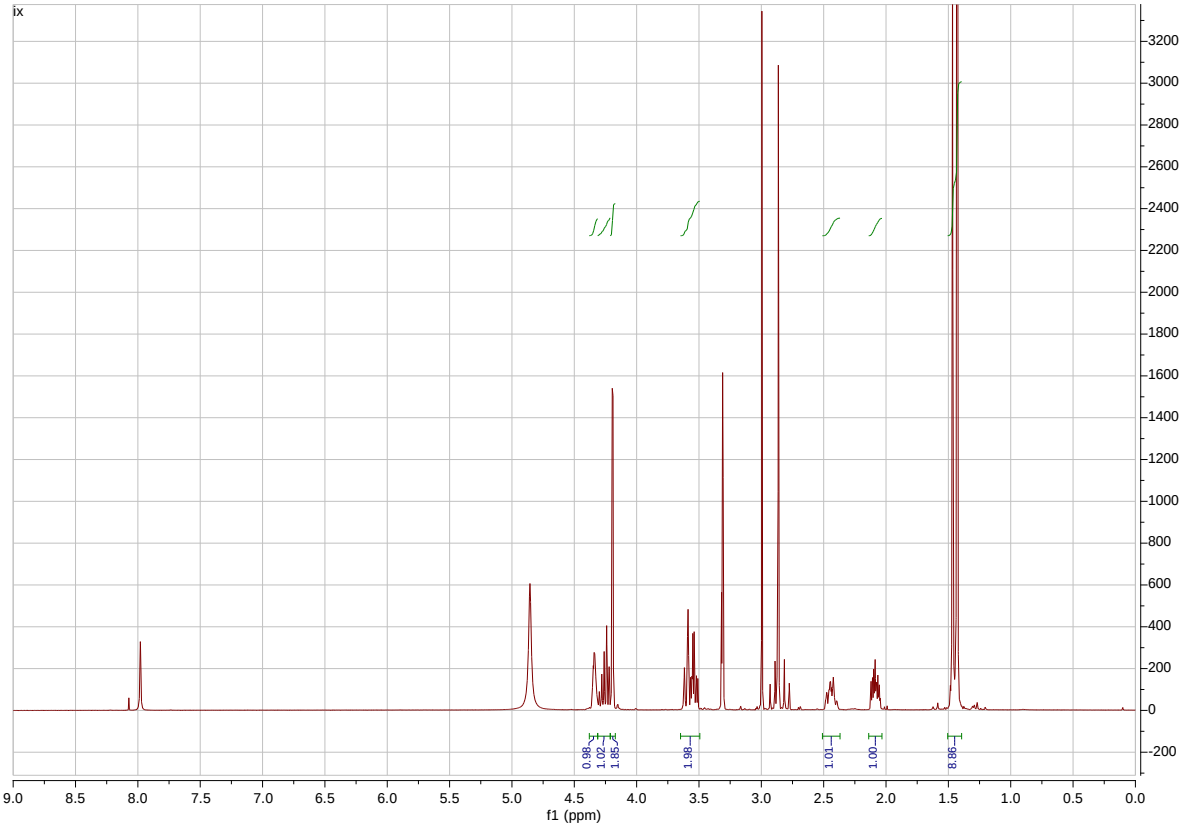
Boc-cis-L-4-hydroxyproline, ***vii***



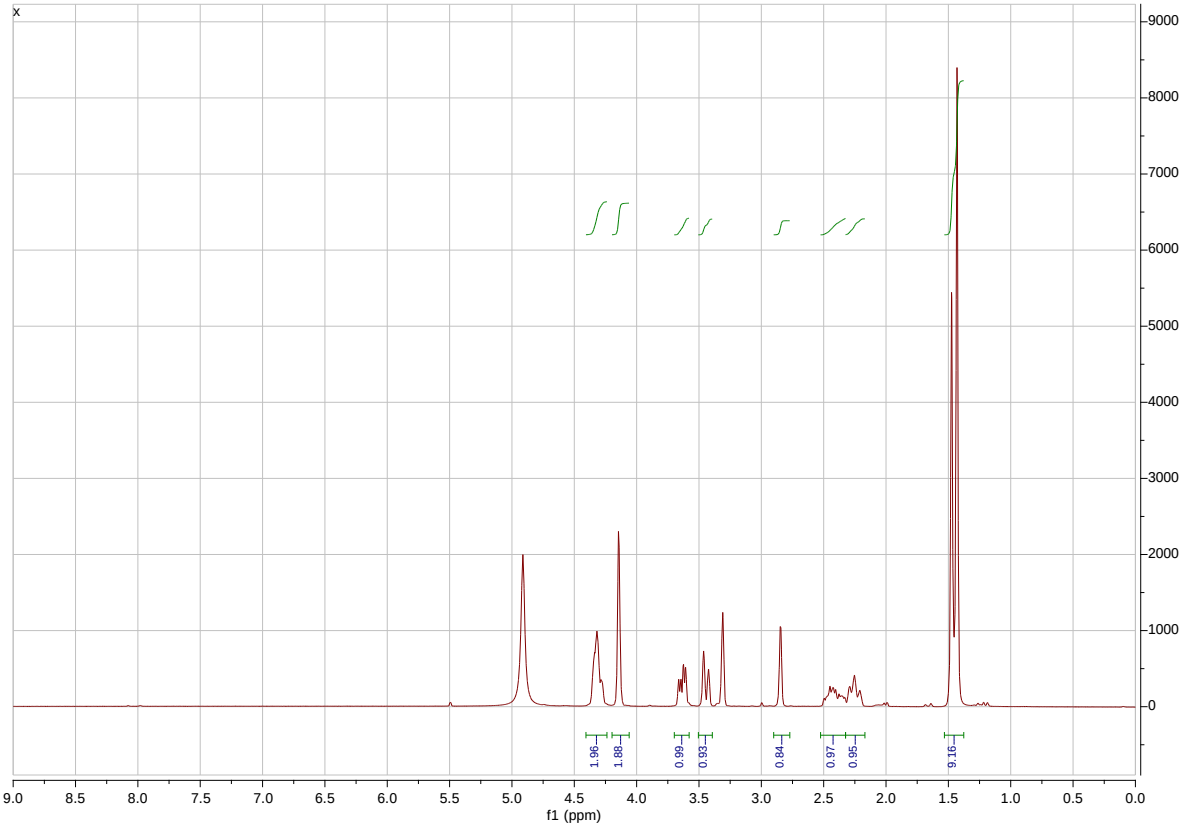
Boc-cis-L-3-hydroxyproline, ***viii***



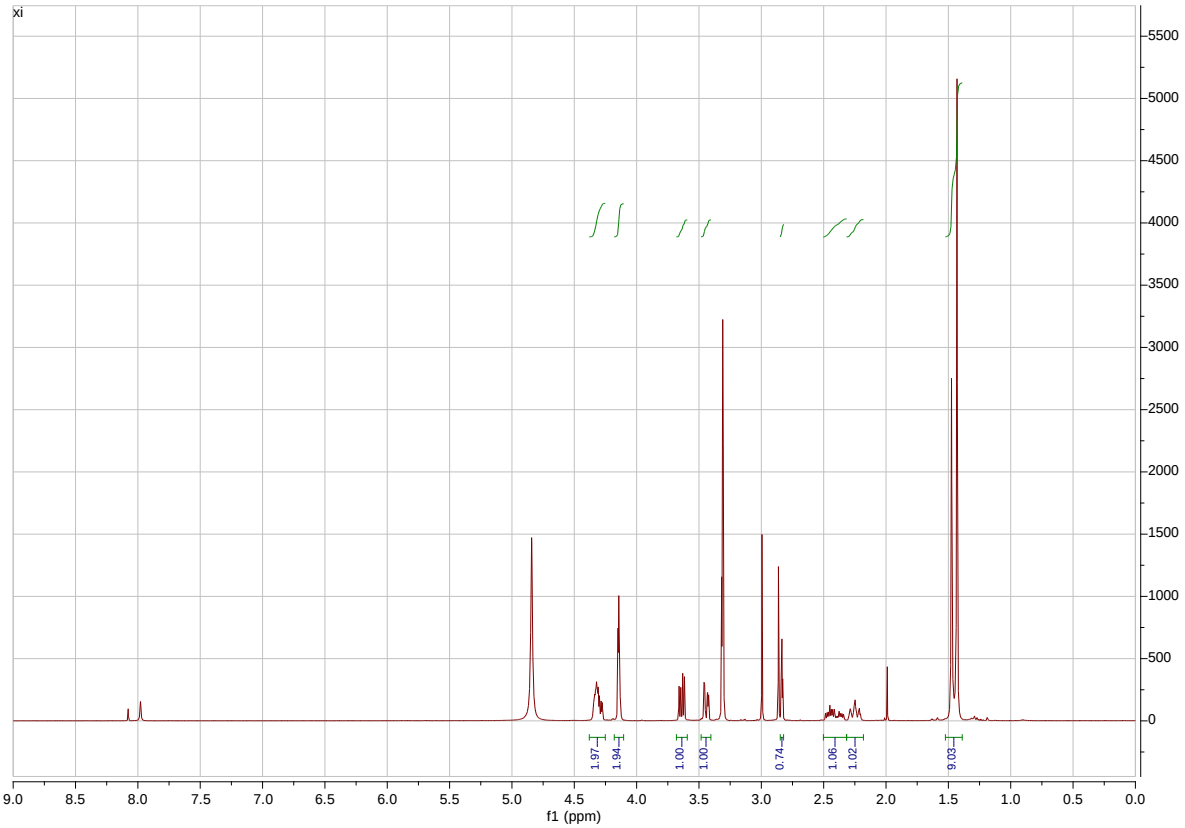
Boc-trans-L-4-propargyloxyproline, ***ix***



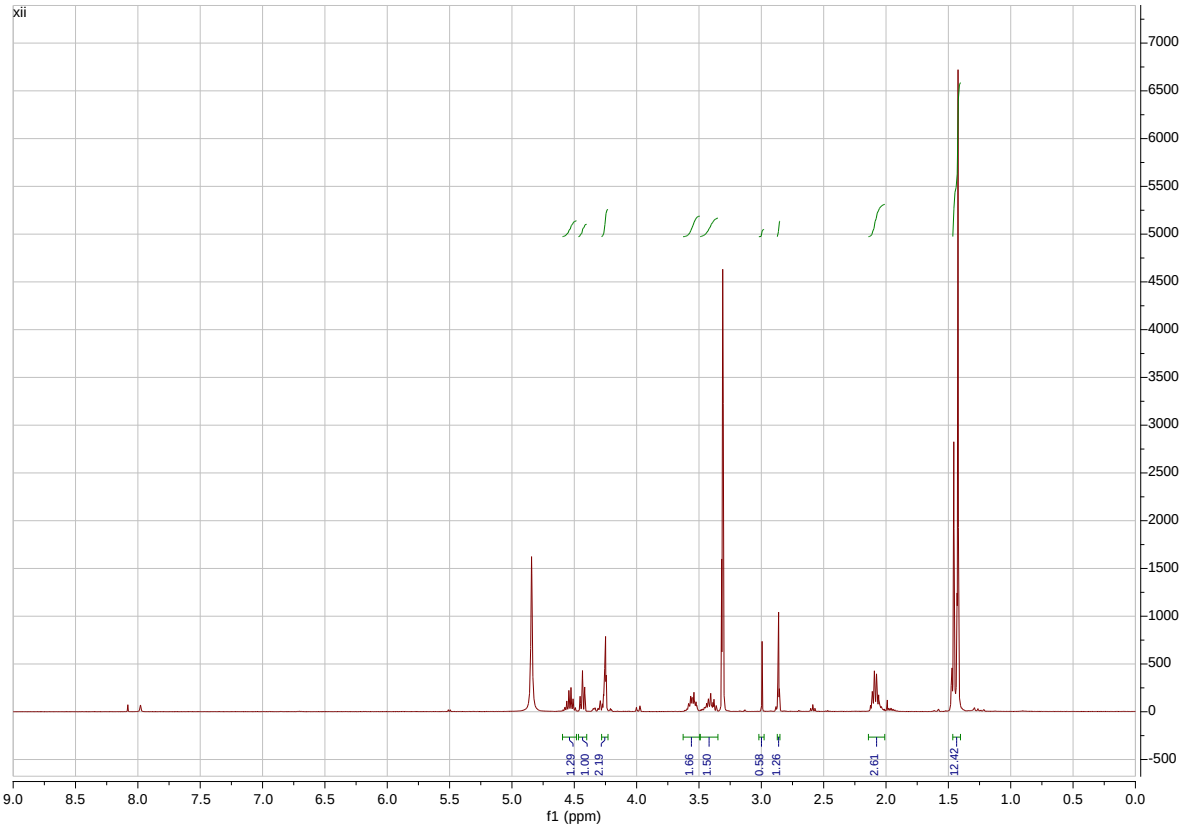
Boc-cis-D-4-propargyloxyproline, ***x***



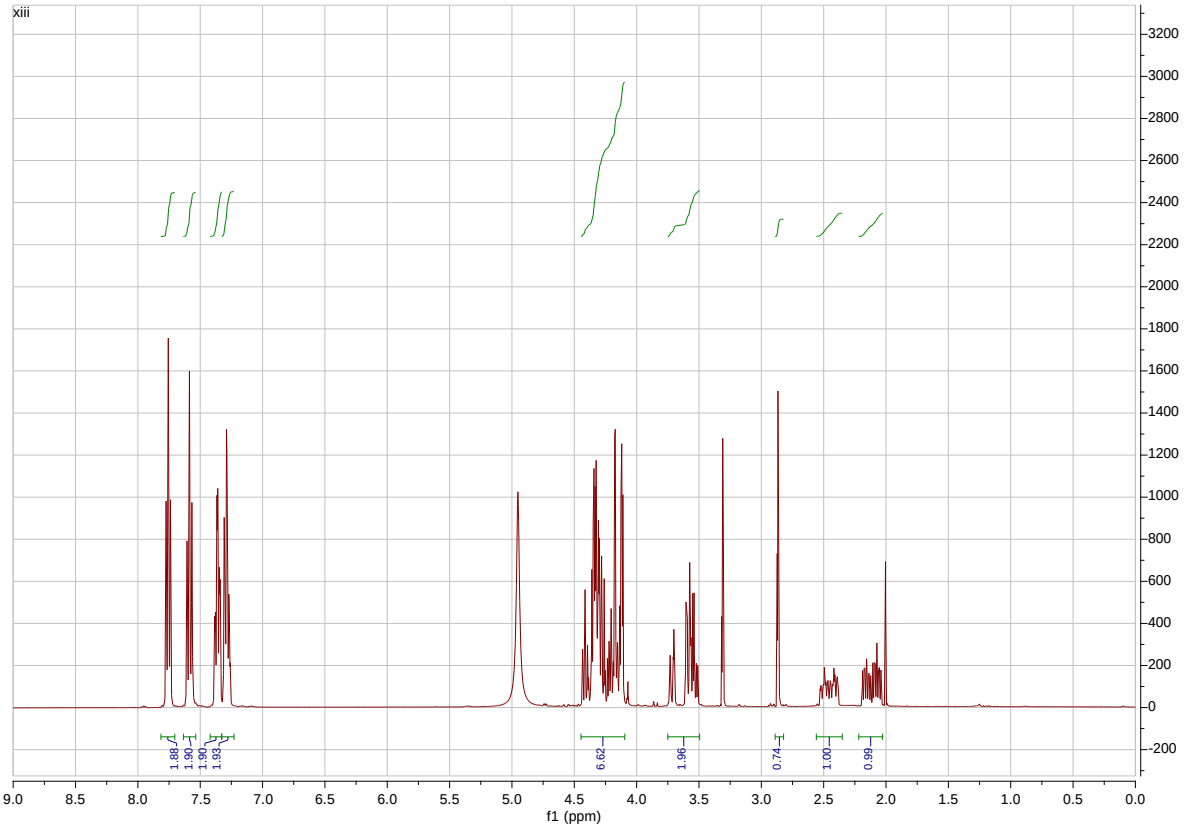
Boc-cis-L-4-propargyloxyproline, ***xi***

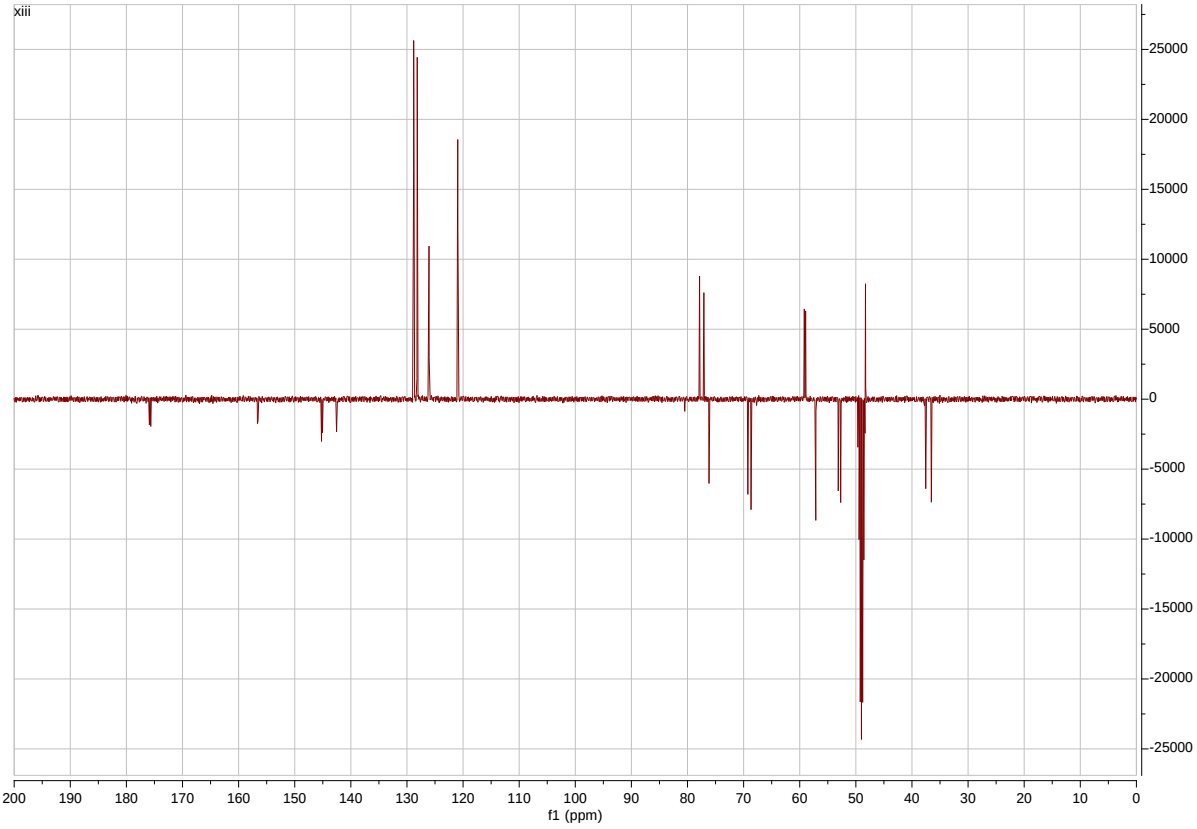


Boc-cis-L-3-propargyloxyproline, ***xii***

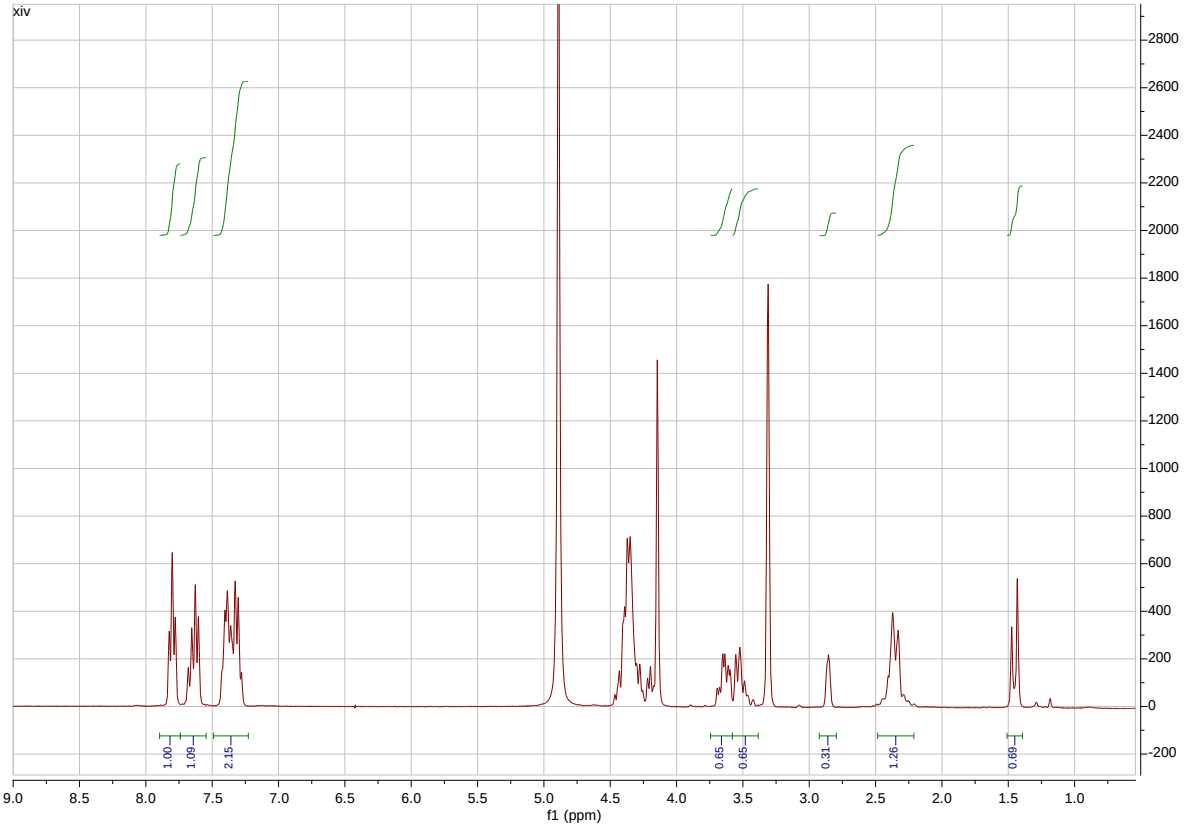


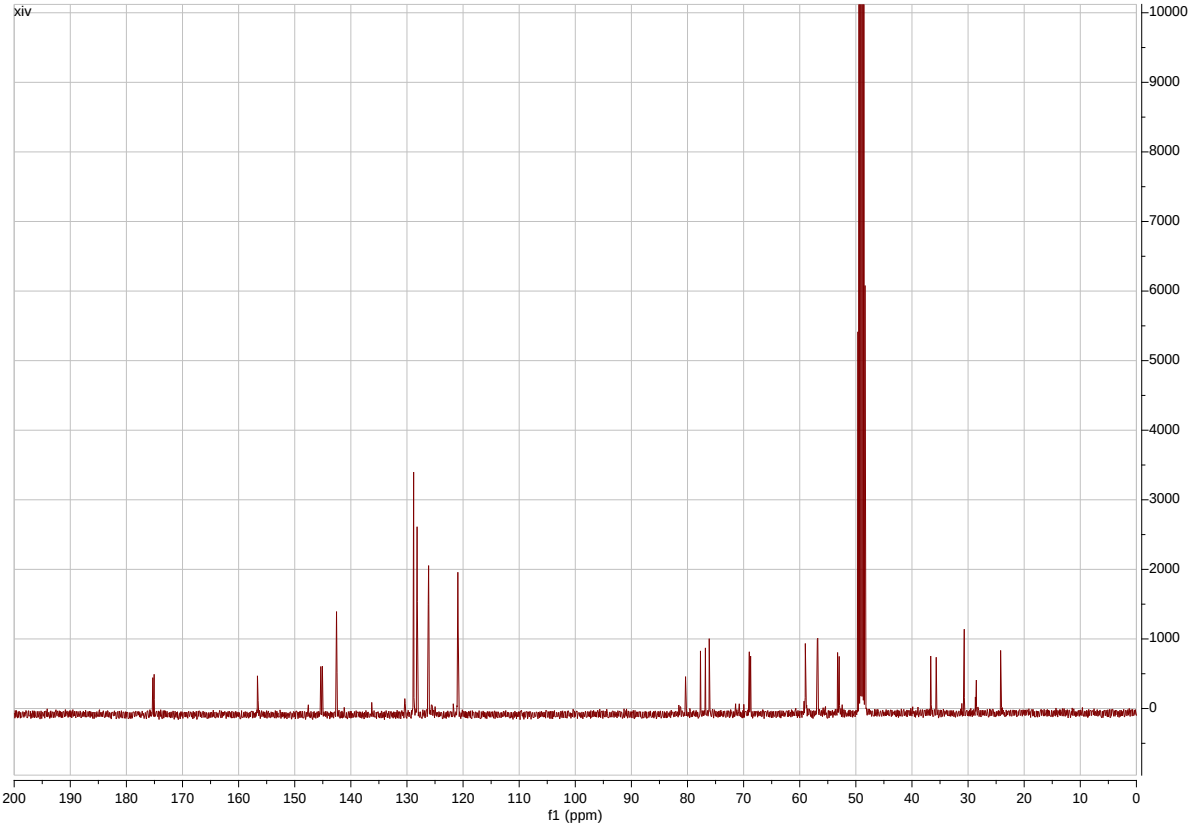
Fmoc-trans-L-4-Propargyloxyproline-OH, ***xiii***



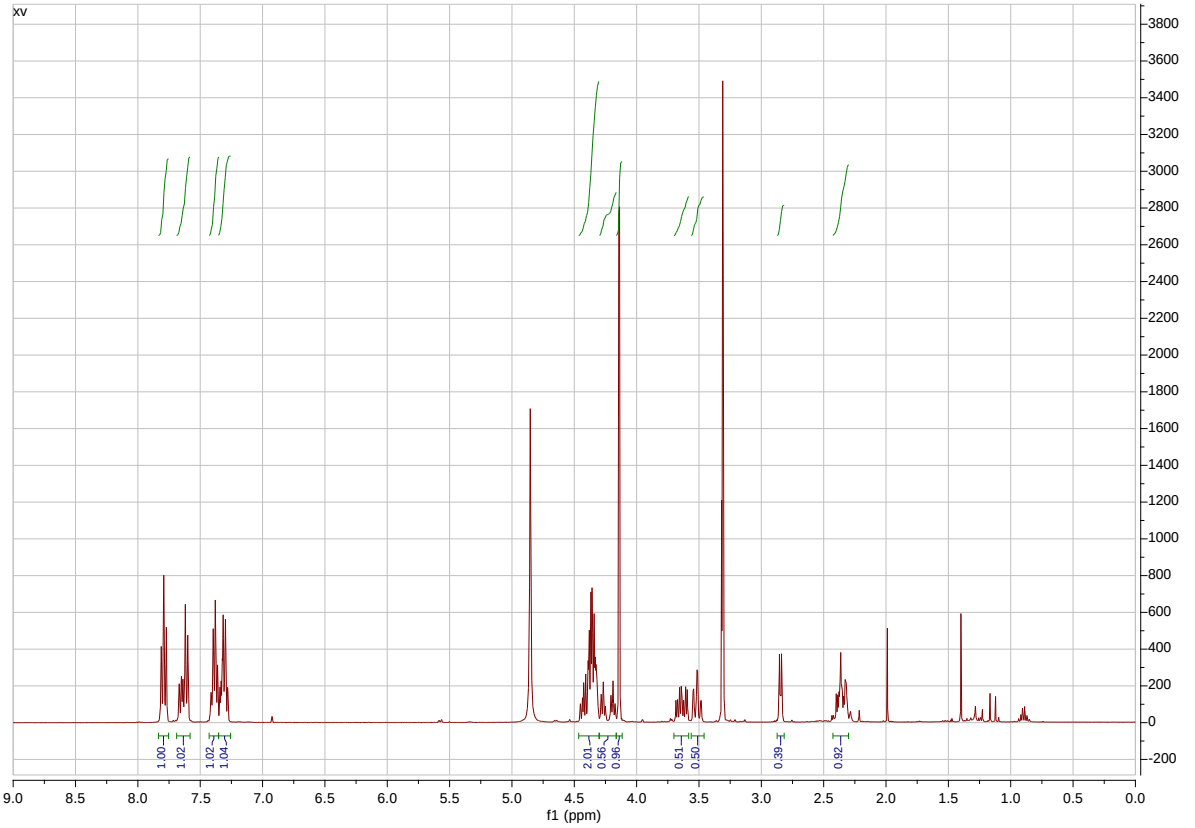


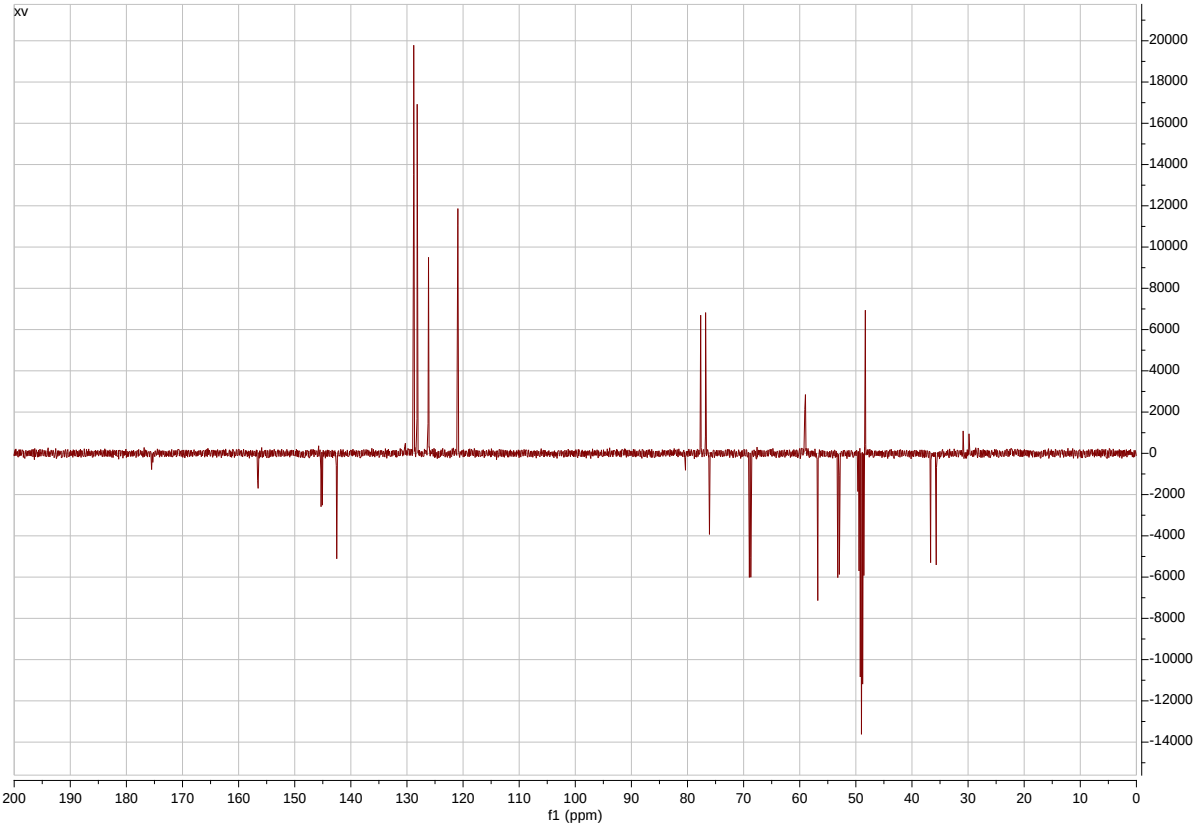
*Fmoc-cis-4-propargyloxy-D-proline (**xiv**)*



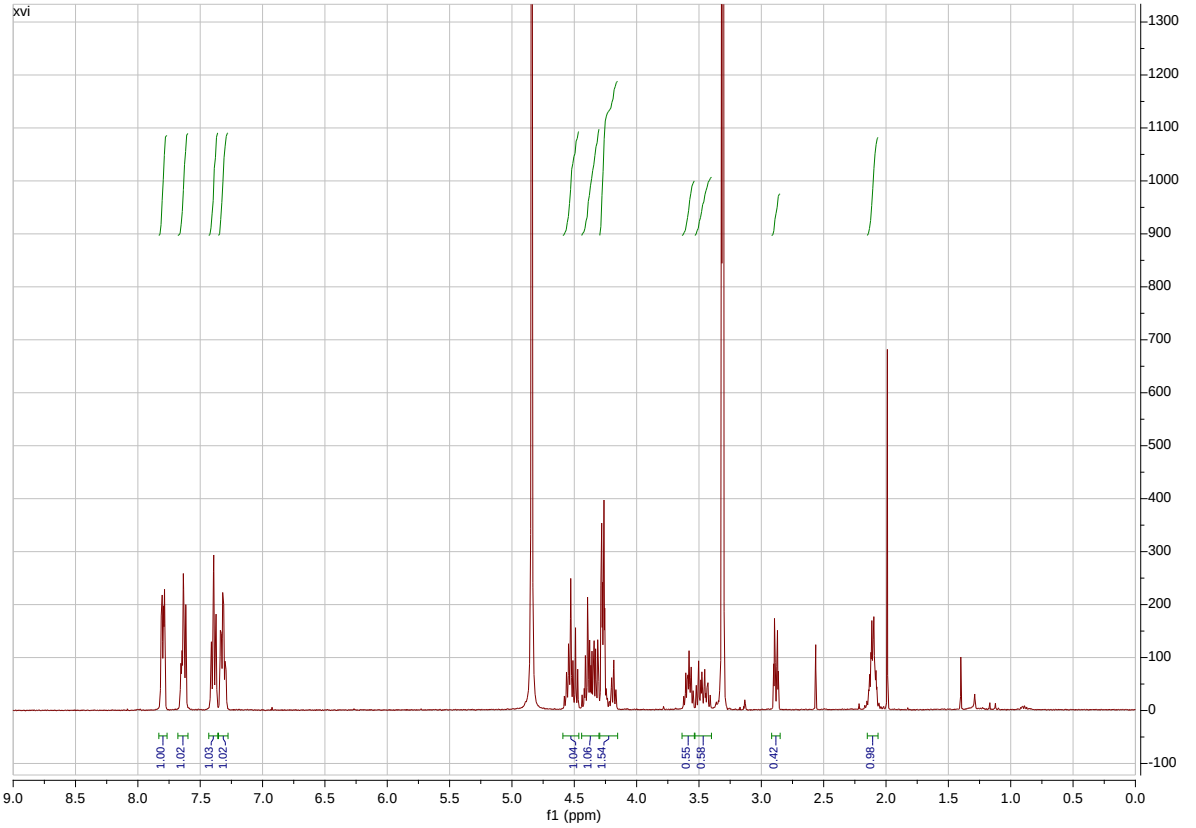


*Fmoc-cis-4-propargyloxy-L-proline (**xv**)*





*Fmoc-cis-3-propargyloxy-L-proline (**xvi**)*



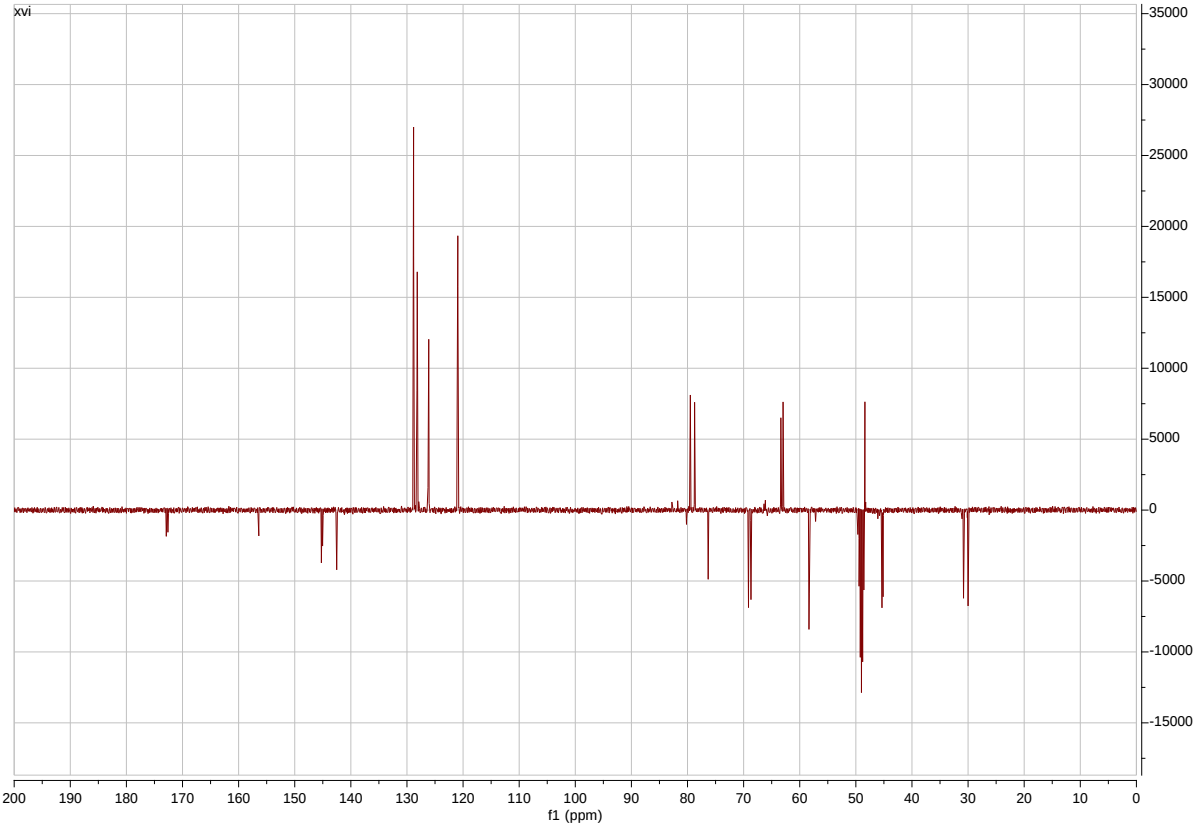
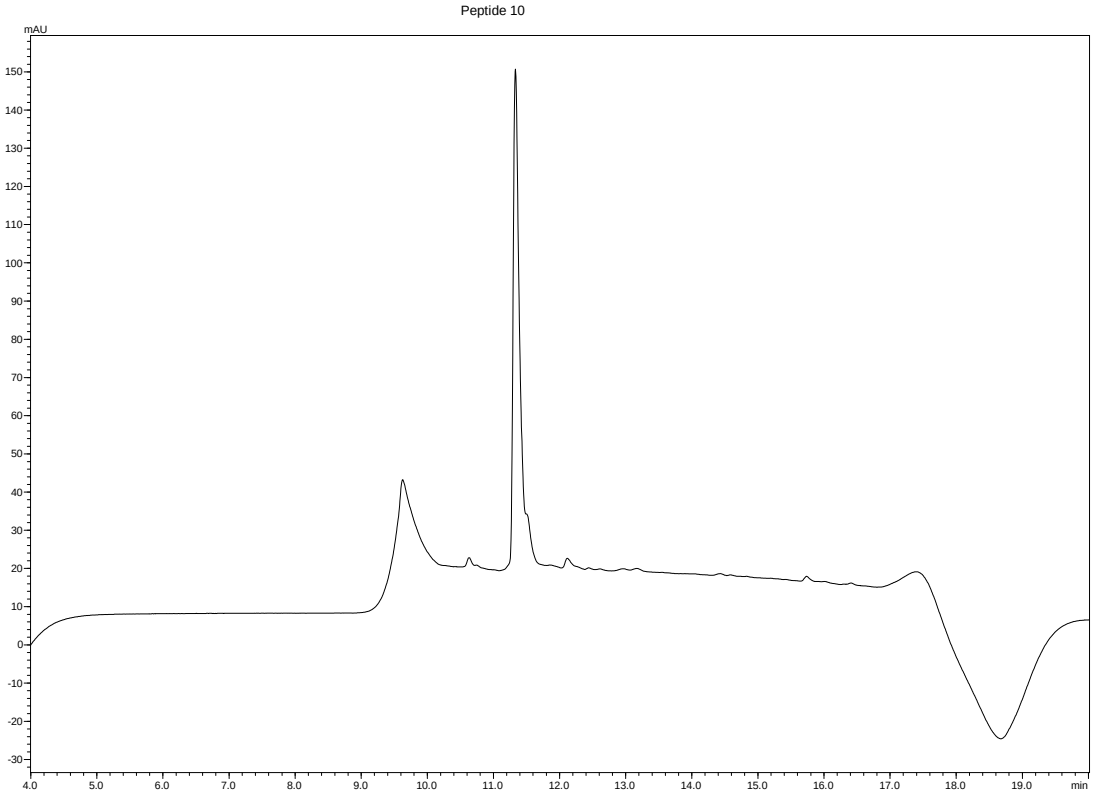
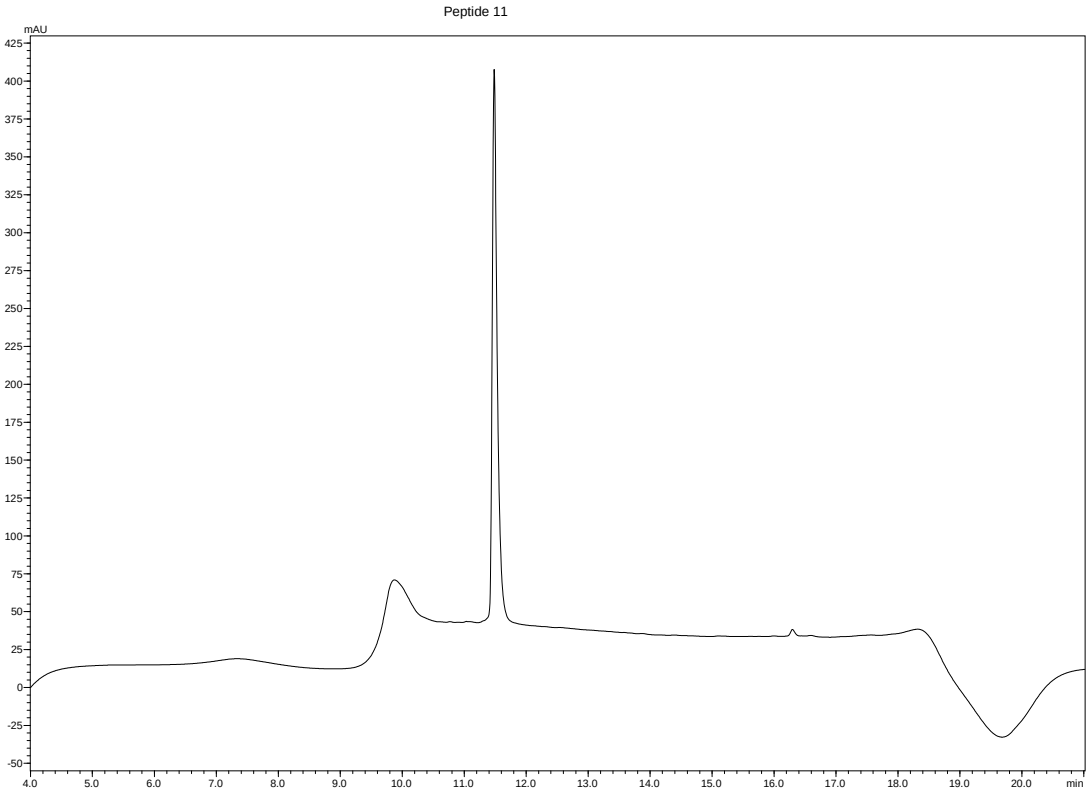
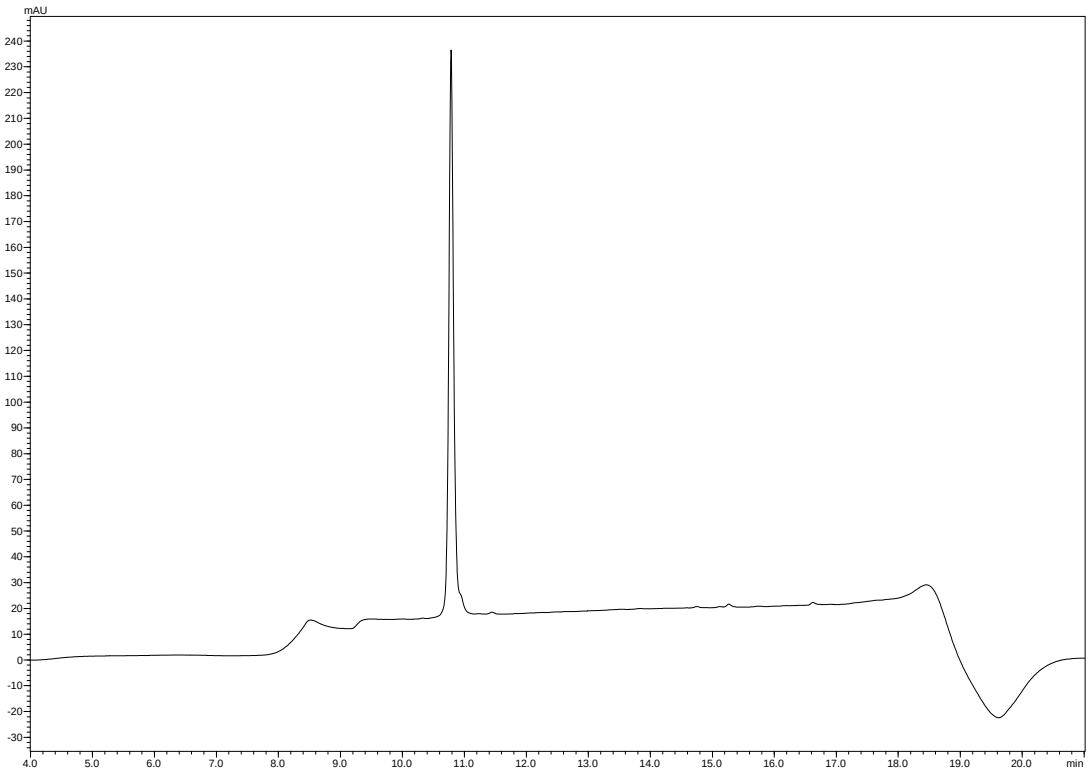


Figure S5 **HPLC traces or compounds 10-17, 24-30**

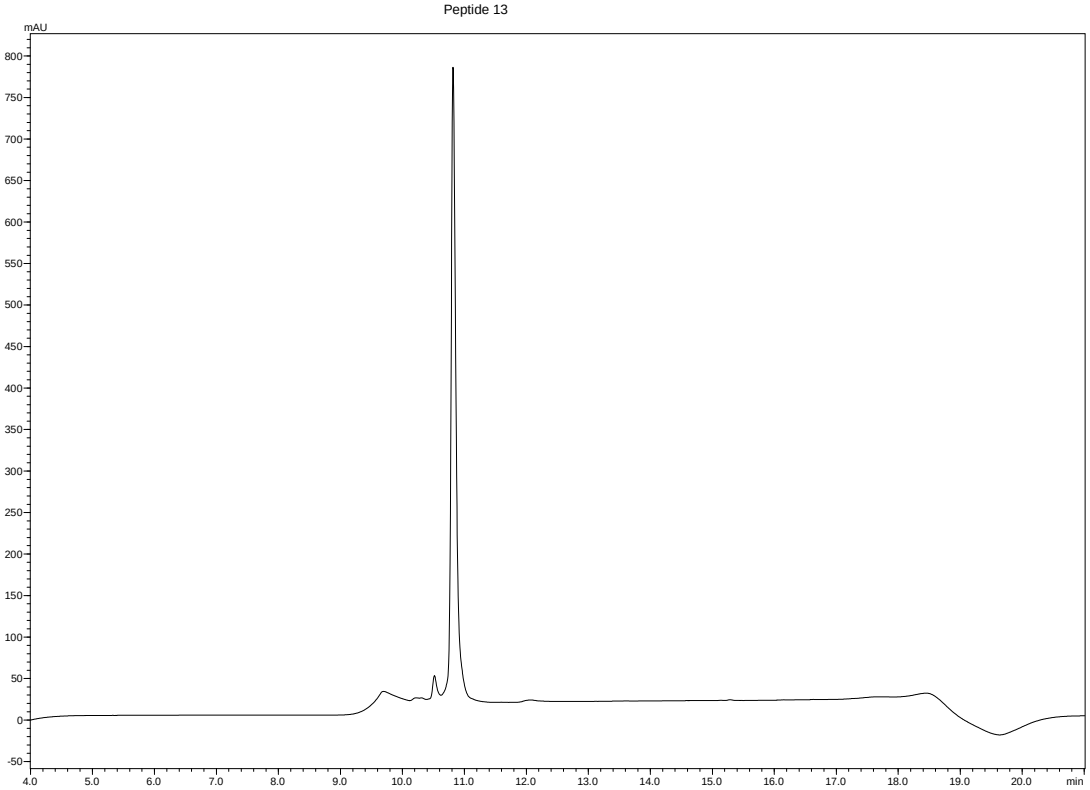




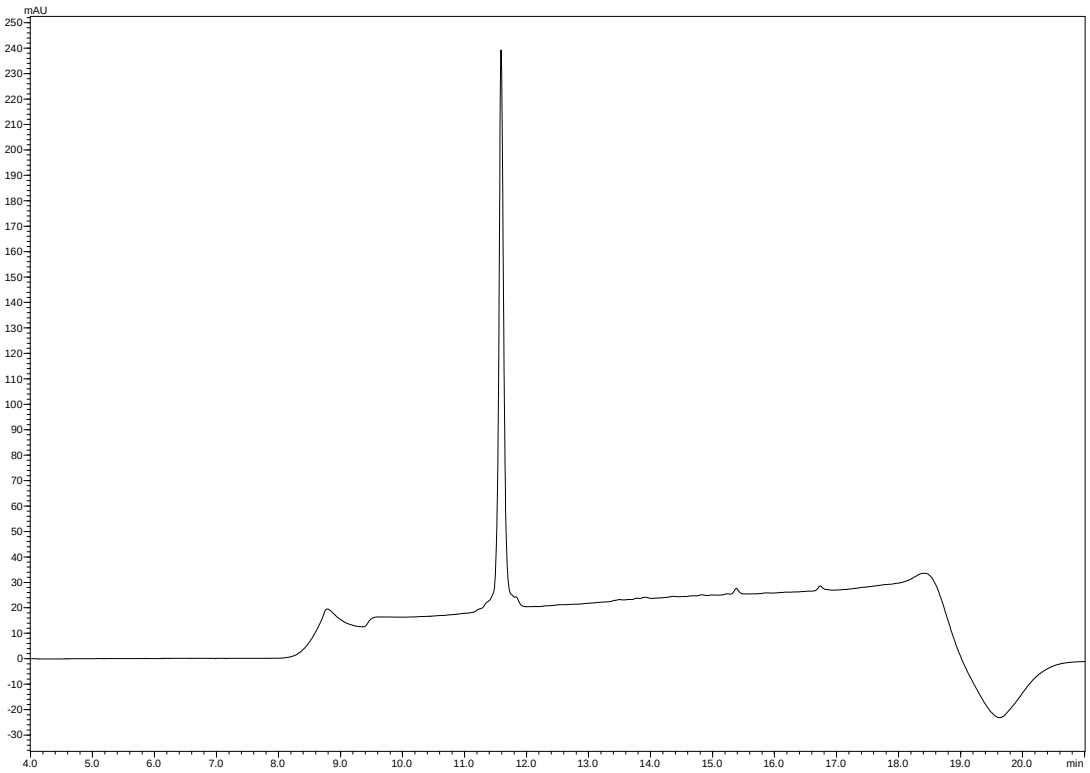
Peptide 12



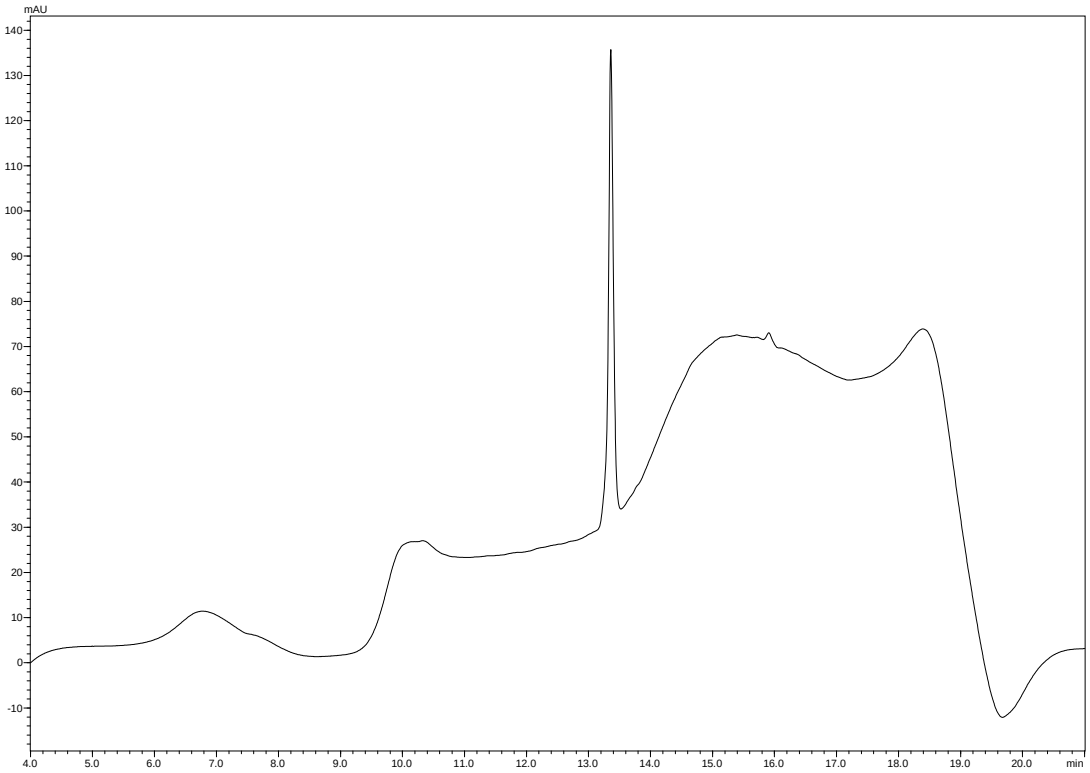
Peptide 13



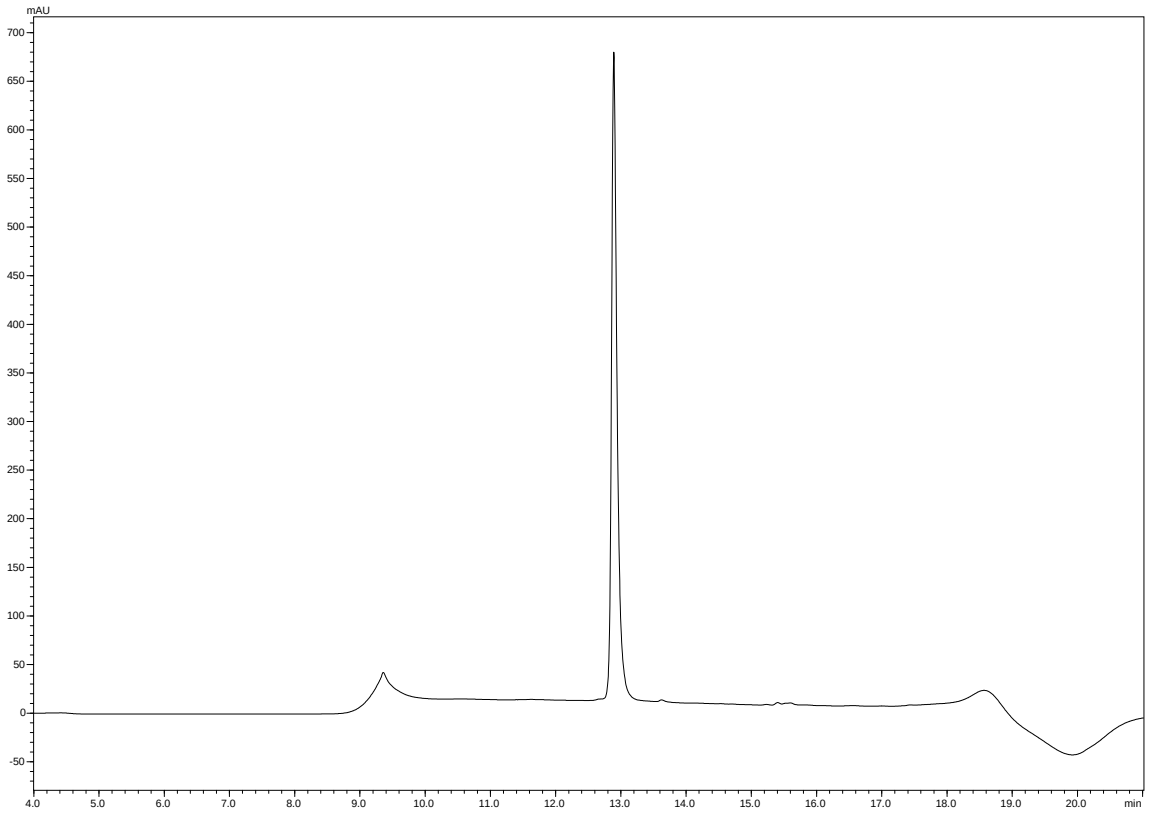
Peptide 14



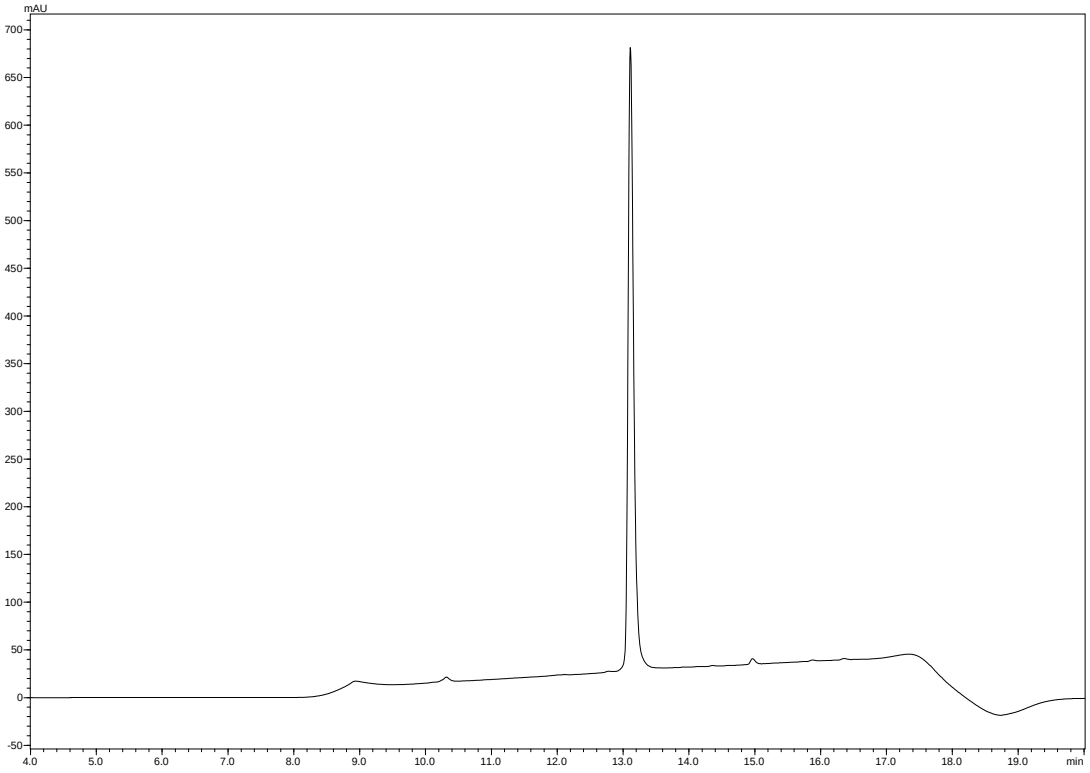
Peptide 15



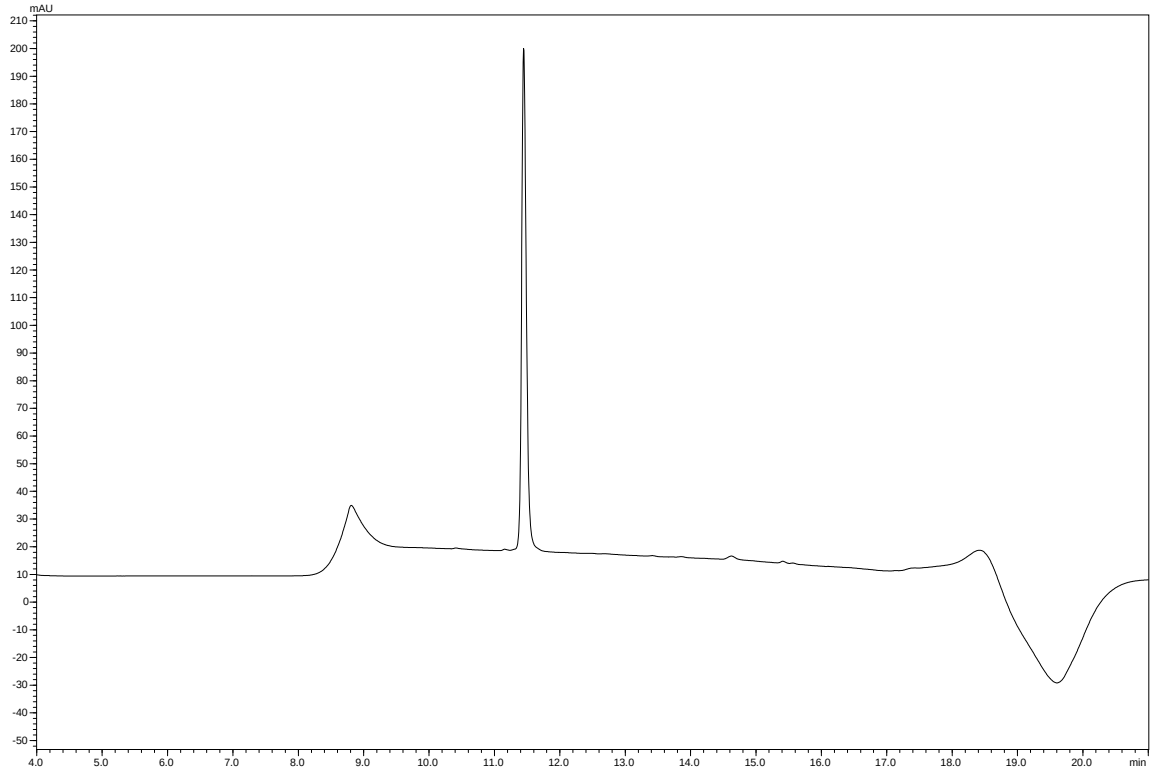
Peptide 16



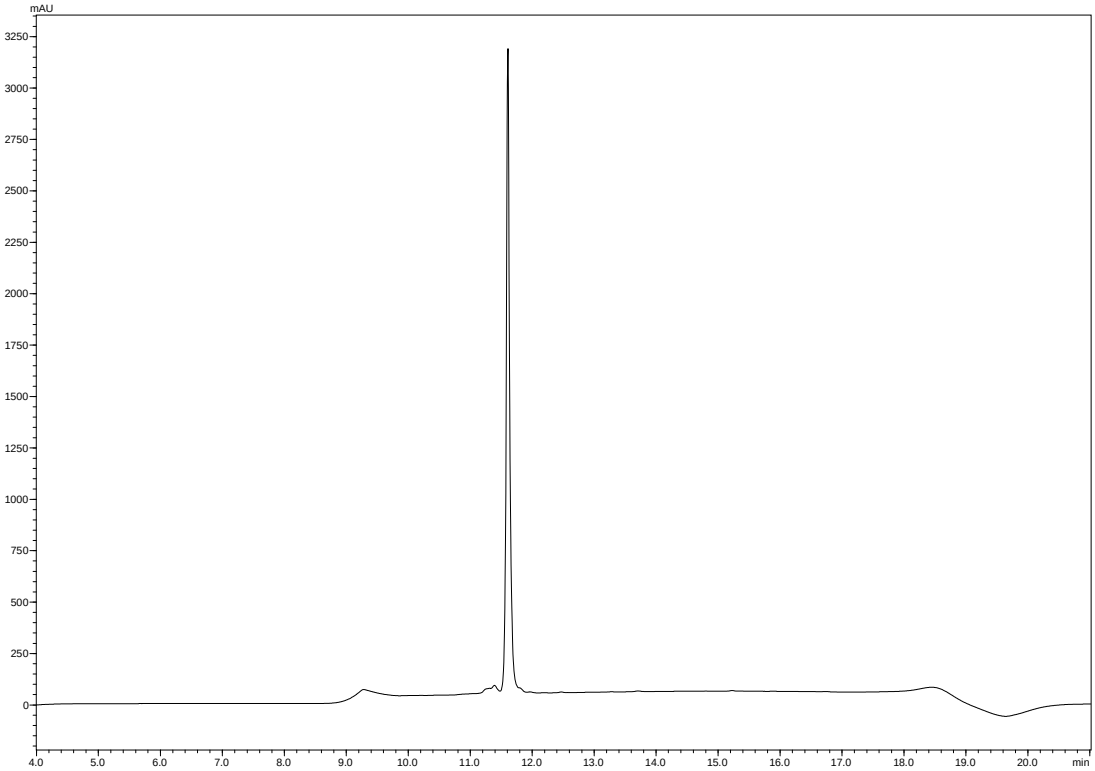
Peptide 17



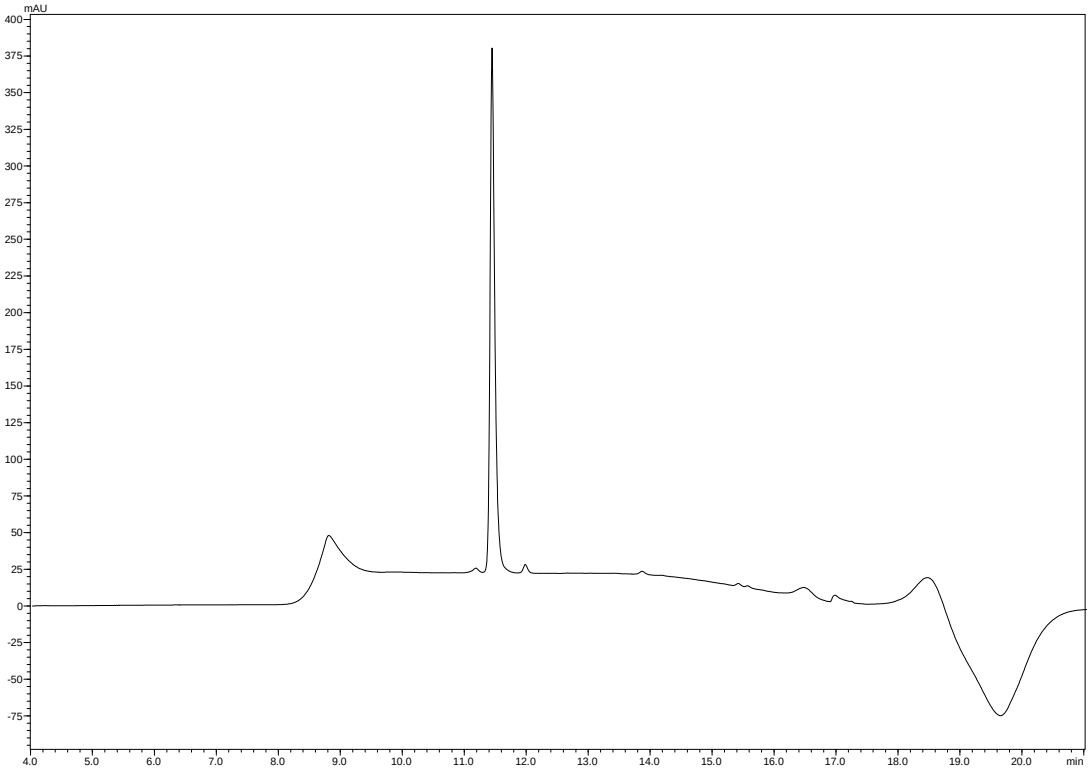
Peptide 24



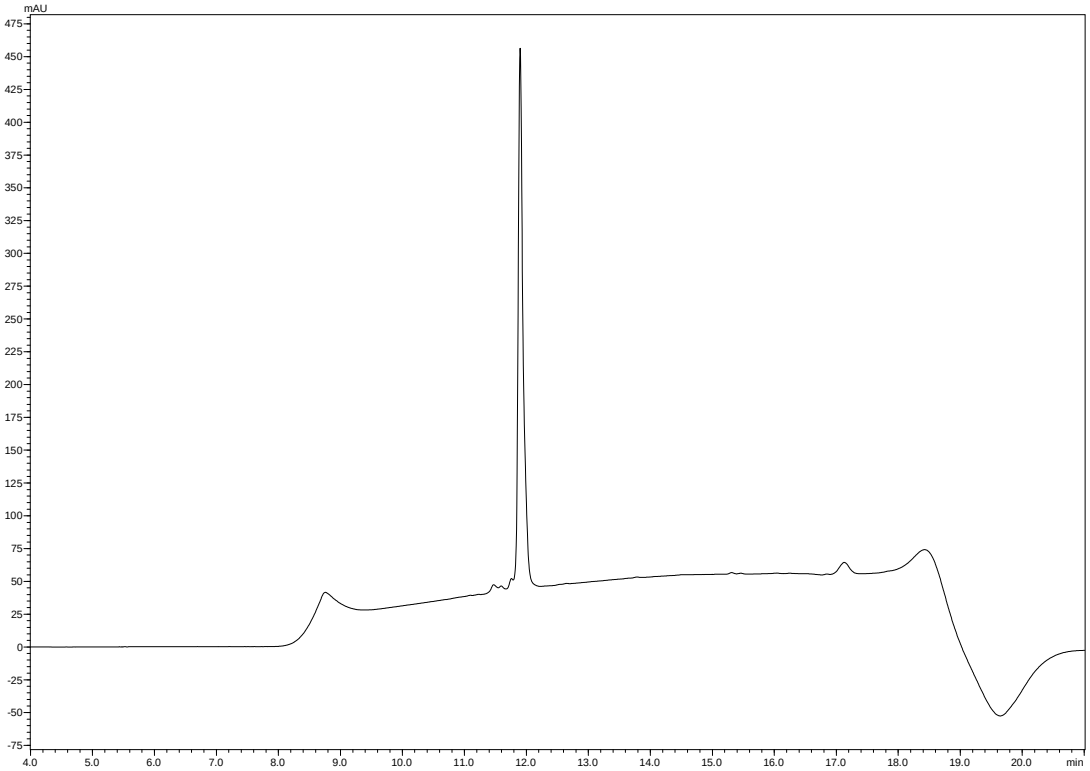
Peptide 25



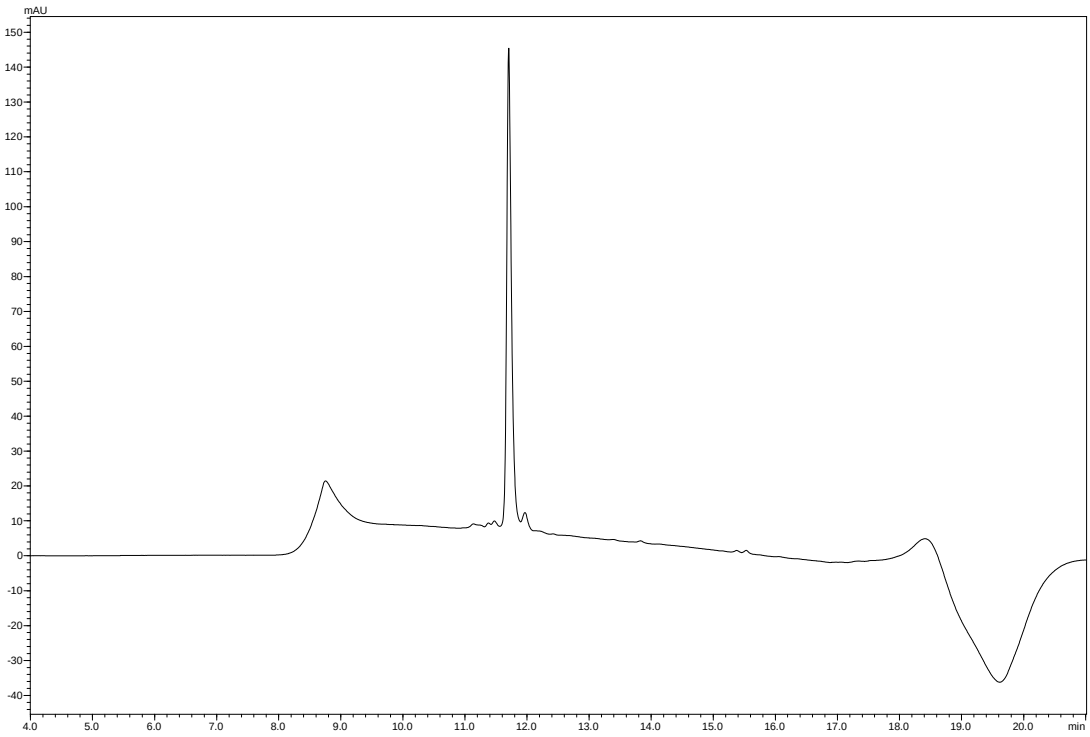
Peptide 26



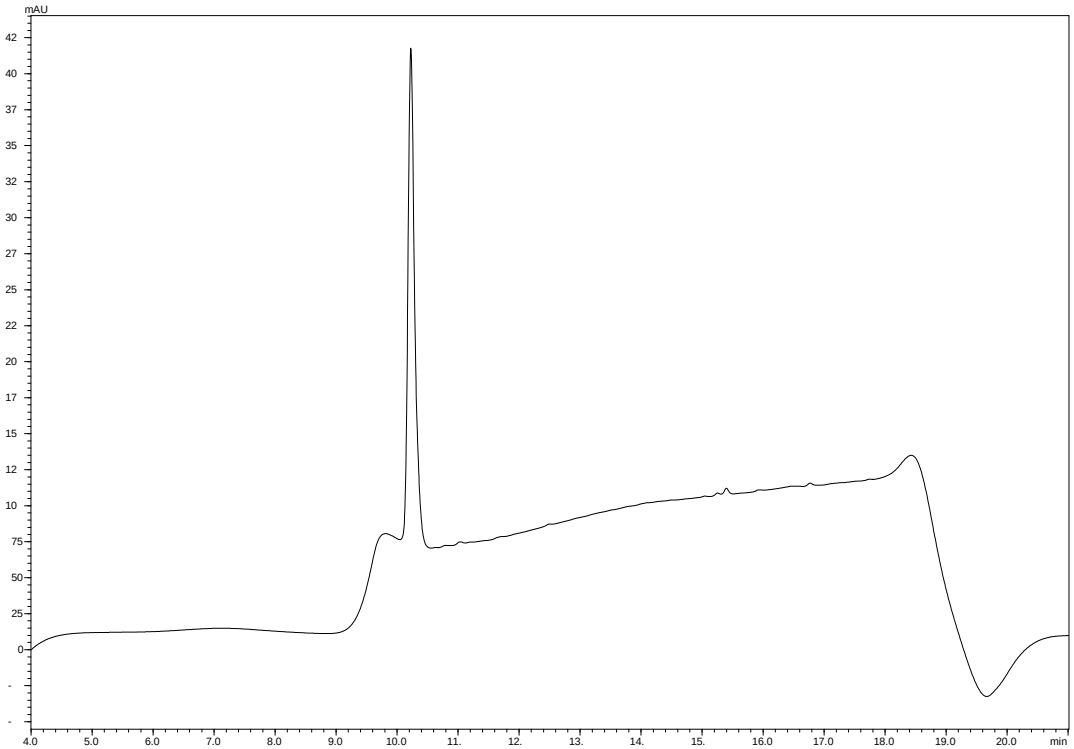
Peptide 27

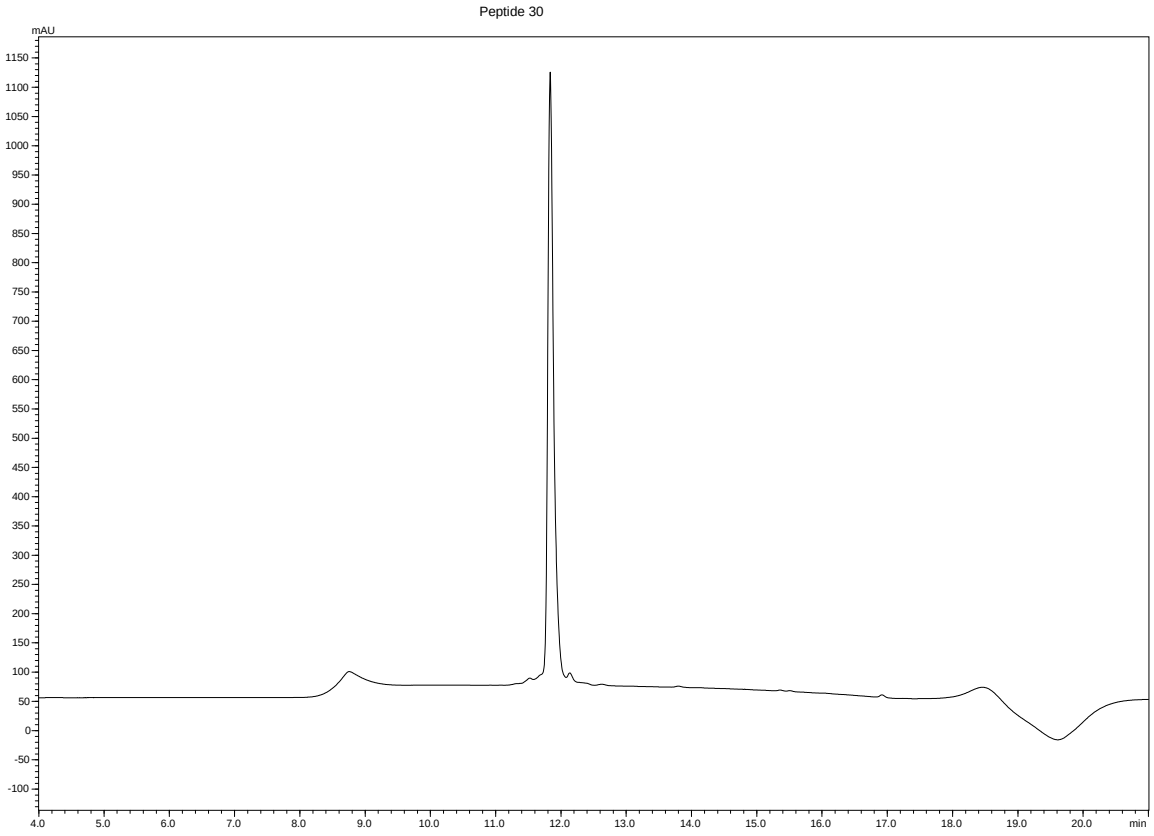


Peptide 28



Peptide 29




Peptide 30
mAU
1150
1100
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950
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350
300
250
200
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-100
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19.0
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min