Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Synthesis of Tumor Necrosis Factor α for Use as a Mirror-Image Phage Display Target.

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1. Reagents and materials

Fmoc-protected L-amino acids were purchased from Protein Technologies Inc. D-amino acids were purchased from Peptides International and CBL Patras. 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and 2-chlorotrityl chloride resin (200-400 mesh, 1.2 mmol/g) were purchased from ChemPep. TentaGel R RAM resin (0.19 mmol/g) was obtained from RAPP Polymere, and Oxyma Pure was purchased from Novabiochem. N,N-dimethylformamide (DMF), dichloromethane (DCM), methanol (MeOH), diethyl ether, and HPLC-grade acetonitrile were from purchased from Fisher Scientific. Recombinant TNFα was purchased from GoldBio. CellTiter 96® AQueous One Solution Cell Proliferation Assay kit was purchased from Promega. All other reagents were purchased from Sigma-Aldrich.

2. General experimental and analytical methods

2.1. Preparation of 2-Chlorotrityl hydrazine resin[1, 2]

Fresh batches of 2-Chlorotrityl hydrazine resin were prepared for each synthesis and used within 24 h. For a 30 μmol scale synthesis, 150 mg of 2-Chlorotrityl chloride resin in an 8 mL fritted solid-phase extraction tube was swollen in DMF (2 mL) for 30 min. The DMF was drained, and the resin was treated with 5% hydrazine in DMF (2 mL) for 30 min. The solid-phase extraction tube was drained, and another aliquot of 5% hydrazine in DMF (2 mL) was added for 30 min. The resin was washed with DMF (3 × 2 mL), then any unreacted resin was deactivated by treatment with 5% MeOH in DMF (2 mL) for 10 min. The 2-

Chlorotrityl hydrazine resin was washed with DMF (6 × 2 mL) and transferred to a reaction vessel for peptide synthesis.

2.2. Peptide synthesis: TNF N-terminal and middle segments

The TNF N-terminal and middle segments were synthesized as C-terminal hydrazides on 2-Chrolotrityl hydrazine resin. The first (C-terminal) glycine residue was manually attached to the resin to a desired density of ~ 0.2 mmol/g by dissolving 0.03 mmol amino acid and 0.03 mmol Oxyma Pure in 1 mL 1:1 dimethylformamide (DMF):DCM, followed by the addition of 0.06 mmol N,N′-diisopropylcarbodiimide. This solution was allowed to react for 10 min before addition to 150 mg of resin for 1 h. The resin was then washed with DMF (6 × 2 mL), and unreacted sites were capped by treatment with 1:1 acetic anhydride: 0.6 M N-methylmorpholine (NMM) in DMF for 15 min. After capping, the density of the first amino acid (desired ~ 0.2 mmol/g) was confirmed by measuring Fmoc release after 30-min treatment with 20% piperidine in DMF (using Fmoc ε_{301} = 7,800 $M^{-1} \cdot cm^{-1}$). The remaining amino acids were coupled using an automated peptide synthesizer (Prelude, Protein Technologies) according to the following parameters. For Fmoc deprotection: 2 mL of 20% piperidine in DMF (3 cycles for 3 min each). For amino acid coupling: Fmoc-protected amino acid in NMP (0.66 mL, 200 mM), HATU in DMF (0.66 mL, 198 mM), and NMM in DMF (0.5 mL, 600 mM) were mixed for 25 min. For DMF washing (performed between deprotection and coupling steps): 2 mL of DMF (3 cycles for 30 s each, new solvent delivered

for each mixing cycle). Upon completion of the peptide chain, resins were washed with DCM and dried (using vacuum) for >10 min.

2.3. Peptide synthesis: TNF C-terminus

The C-terminal segment was synthesized on TentaGel® R RAM resin (0.18 mmol/g). All amino acids were coupled using an automated peptide synthesizer (Prelude; Protein Technologies) according to the following parameters. For Fmoc deprotection: 2 mL of 20% piperidine/0.1M Oxyma Pure in DMF (3 cycles for 3 min each). For amino acid coupling: Fmoc-protected amino acid in NMP (0.66 mL, 200 mM), HATU in DMF (0.66 mL, 198 mM), and NMM in DMF (0.5 mL, 600 mM) were mixed for 25 min. For DMF washing (performed between deprotection and coupling steps): 2 mL of DMF (3 cycles for 30 s each, new solvent delivered for each mixing cycle). Upon completion of the peptide chain, resins were washed with DCM and dried (using vacuum) for >10 min.

2.4. Resin cleavage and peptide global deprotection

Peptides were cleaved from resin for 2.5 h using a TFA mixture specific to the peptide sequence. N-terminal and middle segments: 3.8 mL TFA, 100 µL water, and 100 µL TIS; C-terminal segment: 3.7 mL TFA, 100 µL water, 100 µL TIS, and 100 µL EDT. Following cleavage, the mixture was isolated from the resin via filtration, precipitated in cold diethyl ether (40 mL), washed with additional diethyl ether (3 × 25mL), and finally, peptides were collected by centrifugation. Pelleted

peptides were dried overnight in a vacuum desiccator before dissolution and HPLC purification.

2.5. HPLC and LC-MS of peptides

Peptides were purified on RP-HPLC columns (Phenomenex) Jupiter 4 μm Proteo C12 90 Å and Jupiter 5 μm C4 300 Å (250 × 21.4 mm). Analytical traces were obtained on Jupiter 4 μm Proteo C12 90 Å (150 × 4.6 mm) with linear gradient 10 to 90% B over 30 min, or on an Aeris 3.6 μm C4 300 Å (50 × 2.1 mm), with linear gradient 25 to 60% B over 15 min; A: 0.1% TFA in water and B: 0.1% TFA in 90% acetonitrile/10% water. MS data were acquired on an AB Sciex API 3000 LC/MS/MS system: buffer A: 0.1% FA in water and buffer B: 0.1% FA in acetonitrile.

2.6. Native chemical ligation

For a typical ligation reaction, the N-terminal peptide segment (containing C-terminal hydrazide for in situ conversion to azide and then thioester) was dissolved (0.5–5.0 mM) in activation buffer [6 M GuHCl, 100 mM sodium phosphate (pH 3.0)] and prechilled to −20 °C for 10 min. Next, the hydrazide was converted to an azide by the addition of sodium nitrite to a final concentration of 5–50 mM from a 200 mM aqueous stock (pH adjusted to 3.8– 4.2). This solution was mixed and then incubated at −20 °C for 20 min. During the activation step, the C-terminal peptide segment (containing N-terminal cysteine) was dissolved in ligation buffer [6 M GuHCl, 200 mM 4-mercaptophenylacetic acid (MPAA), 200

mM sodium phosphate, pH adjusted to 7.0–7.2]. Following activation of the N-terminal segment, the solutions containing N- and C-terminal segments were combined, pH adjusted to 6.8–7.0 with 2 M NaOH, and allowed to react at 25°C for >2 h. Following consumption of the cysteine containing peptide, the reactions were quenched by addition of an equal volume of a freshly prepared 100 mM aqueous solution of tris(2-carboxyethyl)phosphine (TCEP) and incubated for >10 min. The quenched reaction solution was diluted to twice the volume with 6 M GuHCl in 5% AcOH, and then the volume doubled once more with 5% AcOH (final pH 2.0–3.0). Samples were vortexed and sonicated thoroughly then clarified via centrifugation prior to HPLC purification using a Phenomenex Jupiter column (5 μm, C4 300 Å, 250 × 10 mm).

2.7. Desulfurization[3, 4]

Desulfurization buffer [6 M GuHCl, 100 mM phosphate, pH 6.5] was sparged with helium and used to prepare a 550 mM TCEP stock and a 120 mM reduced glutathione stock. A 240 mM VA-044 stock was prepared in sparged water. The cysteine-containing peptide was dissolved in 500 μL of desulfurization buffer at a concentration of ~3 mM (6 mM thiol) in a 5 mL eppendorf tube. Reagents were added to the peptide solution in the following order: 204 μL glutathione stock, 204 μL VA-044 stock, and 720 μL TCEP stock. The pH was readjusted with NaOH to 6.5. The reaction was covered with argon, heated to 50 °C in a water bath for 2 h, and then incubated on a tube rotisserie at 37°C until completion. The volume was increased to 4 mL with 6 M GuHCl in 5% AcOH and then brought to a total

volume of 8 mL with 5% AcOH. This solution was vortexed and sonicated, then clarified via centrifugation prior to HPLC purification using a Phenomenex Jupiter column (5 μm, C4 300 Å, 250 × 10 mm).

2.8. Acm removal[5]

The peptide, as a lyophilized powder, was dissolved in 50% AcOH/water at a concentration of ~1 mM. To this solution was added silver acetate to a final concentration of 20 mM, and the reaction was incubated on a tube rotisserie for >2 h. Following complete removal of the Acm groups, the reaction was quenched by the addition of an equal volume of a 1 M DTT solution in 6 M GuHCl in 5% AcOH. This cloudy solution was clarified by centrifugation, the supernatant removed and pellet extracted twice with 1 mL aliquots of the 1 M DTT solution in 6 M GuHCl in 5% AcOH. The combined supernatants were diluted with 5% AcOH to a final GuHCl concentration of 3 M, vortexed and sonicated, then clarified via centrifugation prior to HPLC purification.

2.9. Oxidative folding of L- and D-TNFα

Full-length TNFα was incubated in 400 μL of oxidation buffer [6 M GuHCl, 50 mM phosphate, 2% DMSO, pH 8.0] at a concentration of ~50-100 μM for 2 h. Following complete oxidation of the disulfide bond, the solution was transferred to a 0.5 mL Slide-A-Lyzer minidialysis cassette (3500 molecular weight cutoff (MWCO)) and dialyzed first against 500 mL of 2 M GuHCl, 50 mM phosphate, pH 8.0 for 2 h, then against 1 L of 0.5 M GuHCl, 50 mM phosphate, pH 8.0 for 2 h,

and finally against 1 L of 50 mM ammonium bicarbonate for 16 h. The dialyzed material was clarified by centrifugation at 14,000 rpm for 10 min then purified by SEC.

2.10. Size-exclusion chromatography

SEC purification was conducted using a Superdex 200 10/300 column from GE Life Sciences in 50 mM ammonium bicarbonate, pH 8.0, at flow rate of 0.5 mL/min using 500 µL injections. Fractions containing the L-synthetic product were combined and used directly for CD and for functional assays. Fractions containing D-synthetic material were used directly for CD.

2.11. Circular dichroism

All CD spectra were recorded on an AVIV Model 410 spectrophotometer in 50 mM ammonium bicarbonate, pH 8.0, in a 1 mm QS quartz cuvette (Starna) at 25°C. Wavelength scans were performed at 1 nm resolution with 1 s averaging time. Data from triplicate scans were averaged, blank-subtracted, and normalized to calculate mean residue ellipticity by the following equation: $[\theta] = 100 \times \theta/C \times l \times (n - 1)$, where C is protein concentration in mM, l is path length in cm, and n is the number of residues in the protein. The protein concentrations used for CD experiments were 10 µM for recombinant TNFα, 4.2 µM for synthetic L-TNFα, and 4.9 µM for synthetic D-TNFα

2.12. Quantitative SDS-PAGE gel assay

Prior to biological assay, the concentration of our synthetic L-TNF stock solution was quantitated by SDS-PAGE. Specifically, a 10 μM stock solution of commercial TNFα was diluted to a concentration of 80 μg/mL, and a standard curve was generated by serial two-fold dilutions ranging from 80 μg/ml to 2.5 μg/mL. Synthetic TNFα was run next to the standard curve on a NuPAGE 12% bis-tris gel (Invitrogen). The gel was stained with SimplyBlue SafeStain (Invitrogen) and visualized at 700 nm using a Licor Odyssey Infrared Scanner. By comparison to the standard curve, the concentration of the synthetic TNFα stock was ~10 μg/mL. This solution and a 10 μg/mL stock of commercial TNFα were sterilized by centrifugation at 14,000 rpm for 20 min, then diluted to 100 ng/mL in complete media and used directly in the L929 cell assay (section 2.13)

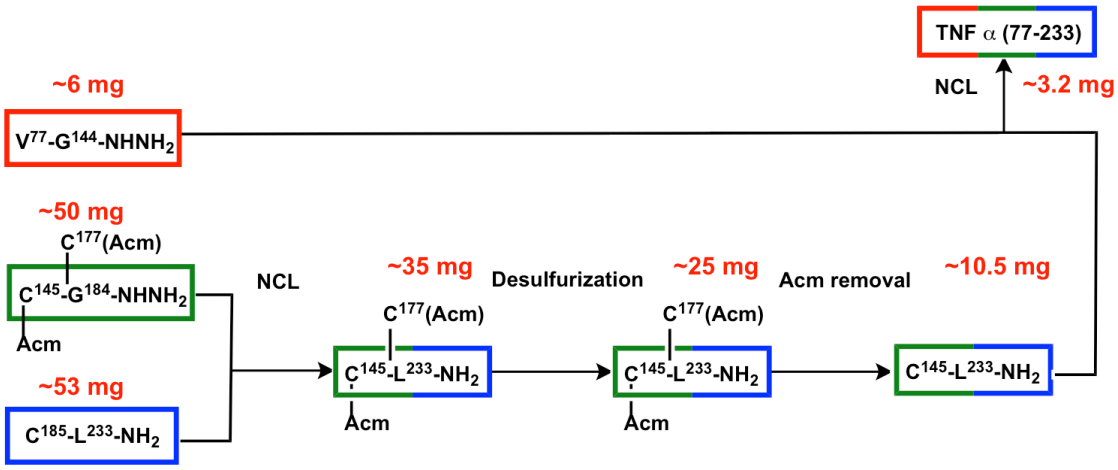
2.13. L929 cell viability assay[6]

Activity of synthetic and commercially purchased recombinant TNFα in a L929 cell viability assay was determined according to the protocol published by Promega.[6] Murine L929 cells were grown in complete media [Dulbecco's RPMI media supplemented with 10% FBS and 1% L-glutamine, Gibco]. Cells were harvested at ~90% confluence with trypsin. Cell number and viability were determined by trypan blue exclusion then seeded into a 96 well plate (100 uL, 2×10^4 cells per well). The 96 well plate was incubated at 37°C for 24 h in a 5% CO_2, humidified atmosphere. Media was aspirated from each well and replaced by media containing 1 μg/mL actinomycin plus a serial 3-fold dilution of either synthetic or recombinant TNFα starting with a high concentration of 10 ng/mL.

The treated plate was incubated at 37 °C for 20 h in a 5% CO_2, humidified atmosphere. Cell viability was determined by the addition of 20 µL of CellTiter 96® AQueous One Solution Cell Proliferation Assay reagent and visualized at 490 nm, 4 h post treatment.

3. Synthesis of L-TNFα

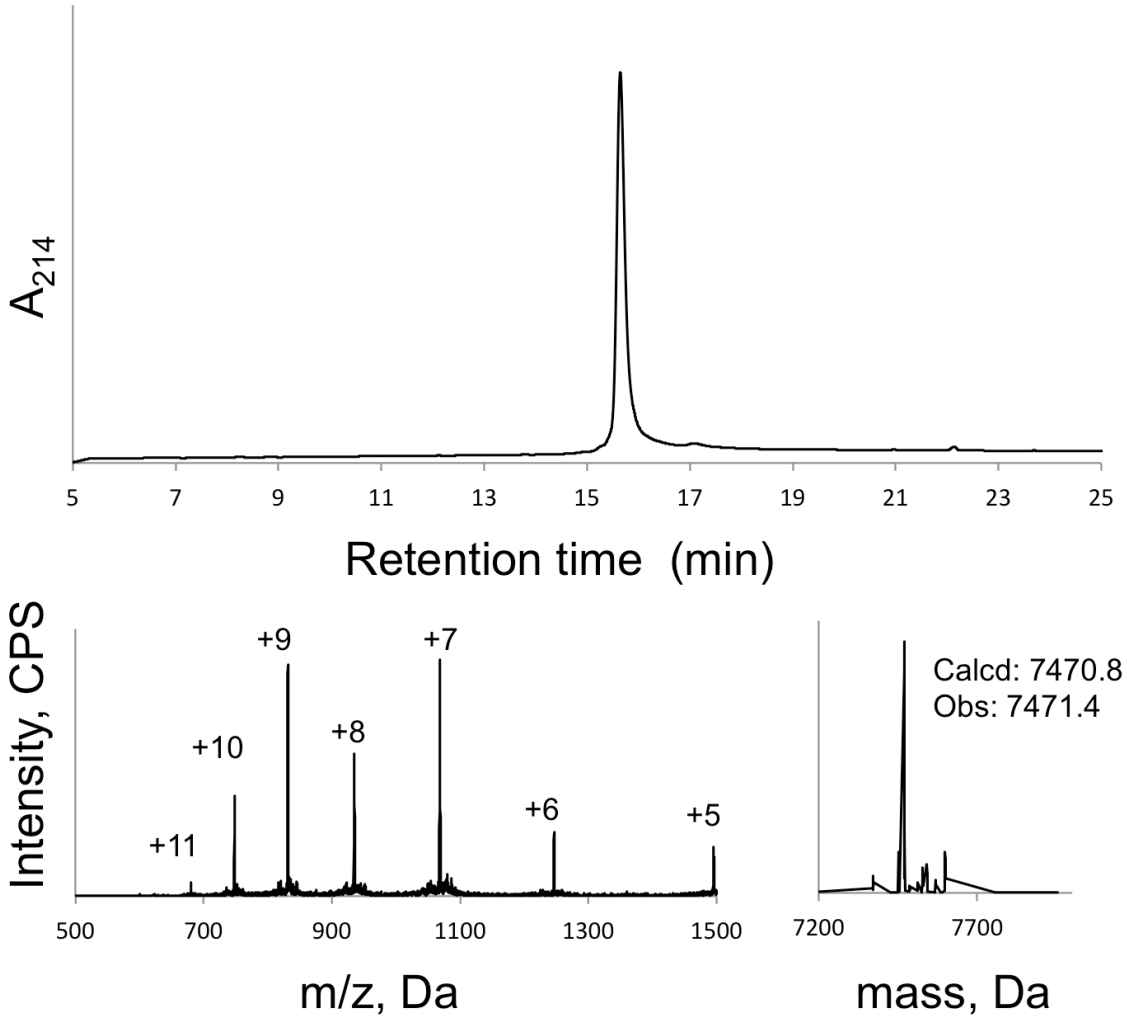
3.1. Yields for L-synthesis



3.2. L-TNF N-terminus (77-144)

Peptide sequence:

VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQL VVPSEGLYLIYSQVLFKGQG-$NHNH_2$



Analytical HPLC trace of purified product:

- **Column** = Phenomenex C12 proteo (4.6 × 150 mm)
- **Gradient method** = 10-90% buffer B over 30 min, Flow = 1 mL/min

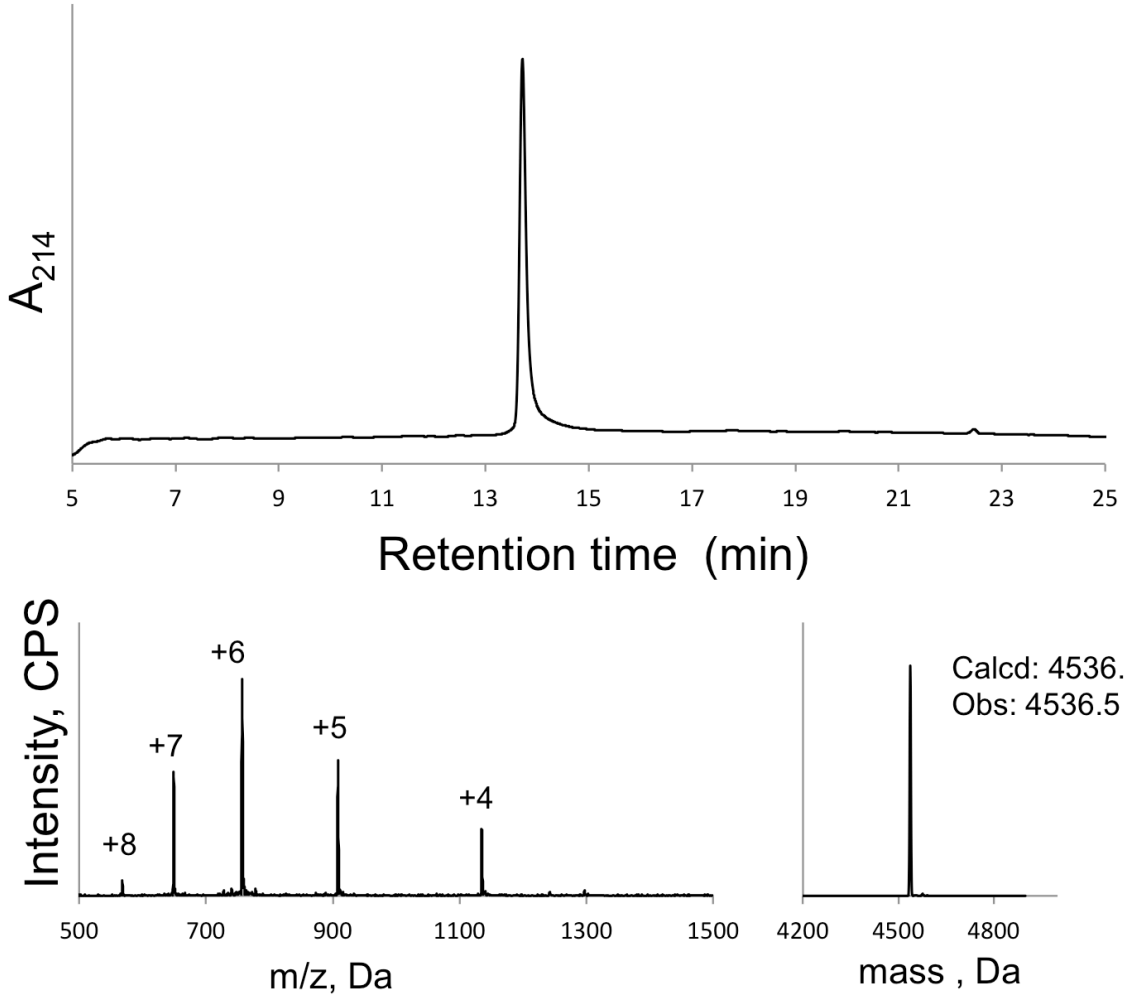
Mass spec analysis of purified product:

- **Expected mass based on sequence** = 7470.8 Da
- **Major observed deconvoluted mass** = 7471.4 Da
- **Major observed ions** = 680.3 (+11), 748.4 (+10), 831.4 (+9), 935.0 (+8), 1068.4 (+7), 1246.3 (+6), 1495.3 (+5).

3.3. L-TNF middle segment (145-184)

Peptide sequence:

C(Acm)PSTHVLLTHTISRIAVSYQTKVNLLSAIKSPC(Acm)QRETPEG-$NHNH_2$



Analytical HPLC trace of purified product:

- **Column** = Phenomenex C12 proteo (4.6 × 150 mm)
- **Gradient method** = 10-90% buffer B over 30 min, Flow = 1 mL/min

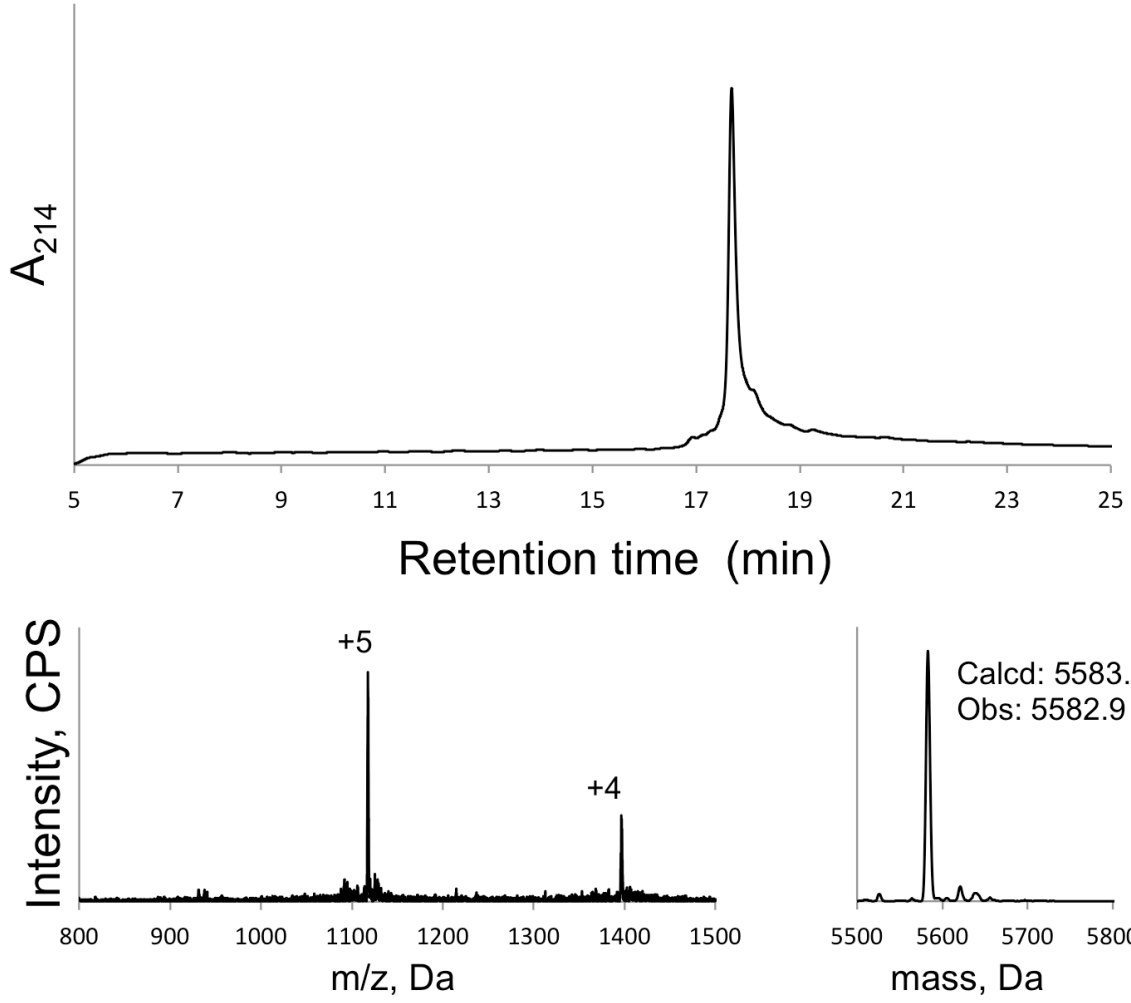
Mass spec analysis of purified product:

- **Expected mass based on sequence** = 4536.0 Da
- **Major observed deconvoluted mass** = 4536.5 Da
- **Major observed ions** = 568.2 (+8), 649.3 (+7), 757.2 (+6), 908.3 (+5), 1135.2 (+4).

3.4. L-TNF C-terminus (185-233)

Peptide sequence:

CEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL-NH_2



Analytical HPLC trace of purified product:

- **Column** = Phenomenex C12 proteo (4.6 × 150 mm)
- **Gradient method** = 10-90% buffer B over 30 min, Flow = 1 mL/min

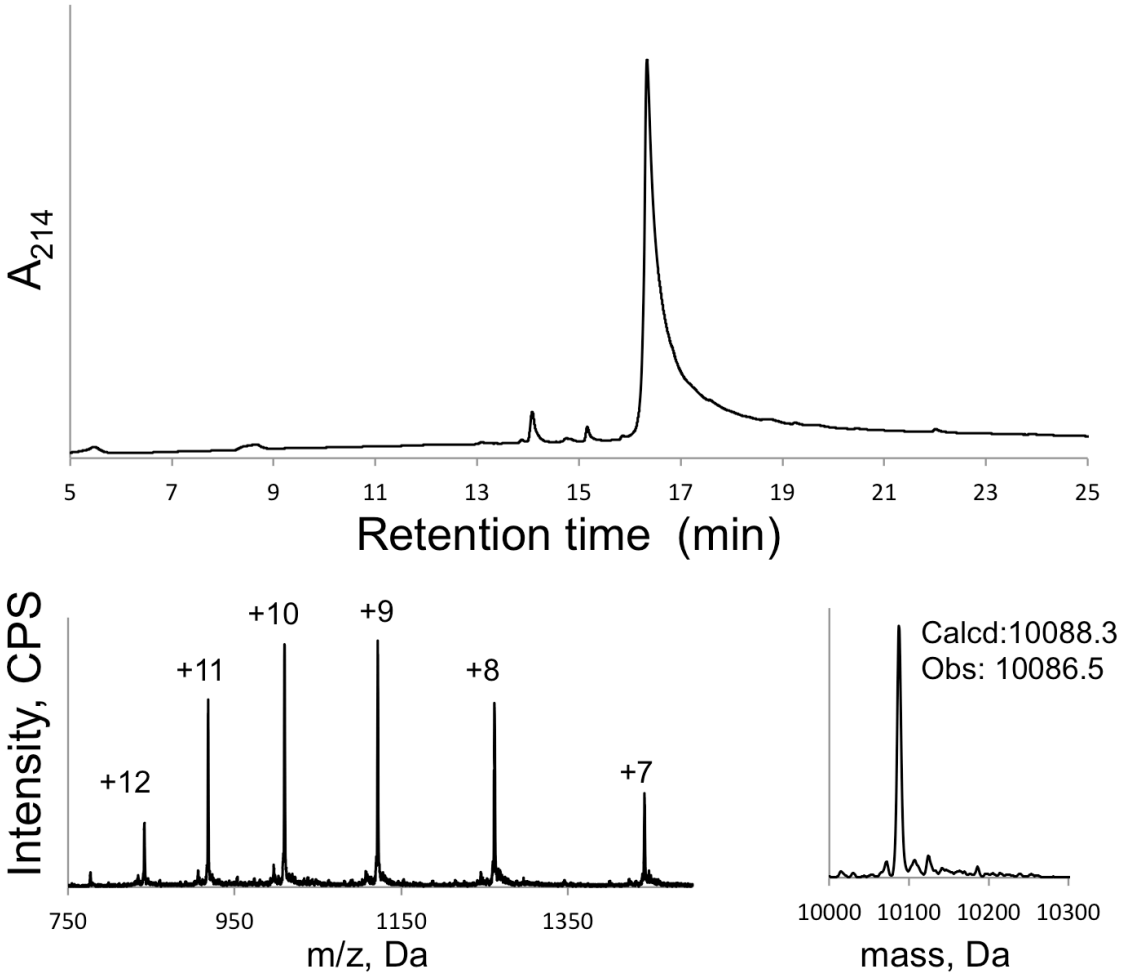
Mass spec analysis of purified product:

- **Expected mass based on sequence** = 5583.2 Da
- **Major observed deconvoluted mass** = 5582.9 Da
- **Major observed ions** = 1117.6 (+5), 1396.4 (+4).

3.5. Ligation product of TNF C-terminus and middle segment (L-TNF, 145-233)

Peptide Sequence:

C(Acm)PSTHVLLTHTISRIAVSYQTKVNLLSAIKSPC(Acm)QRETPEGCEA KPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL-NH_2



Analytical HPLC trace of purified product:

- **Column** = Phenomenex C12 proteo (4.6 × 150 mm)
- **Gradient method** = 10-90% buffer B over 30 min, Flow = 1 mL/min

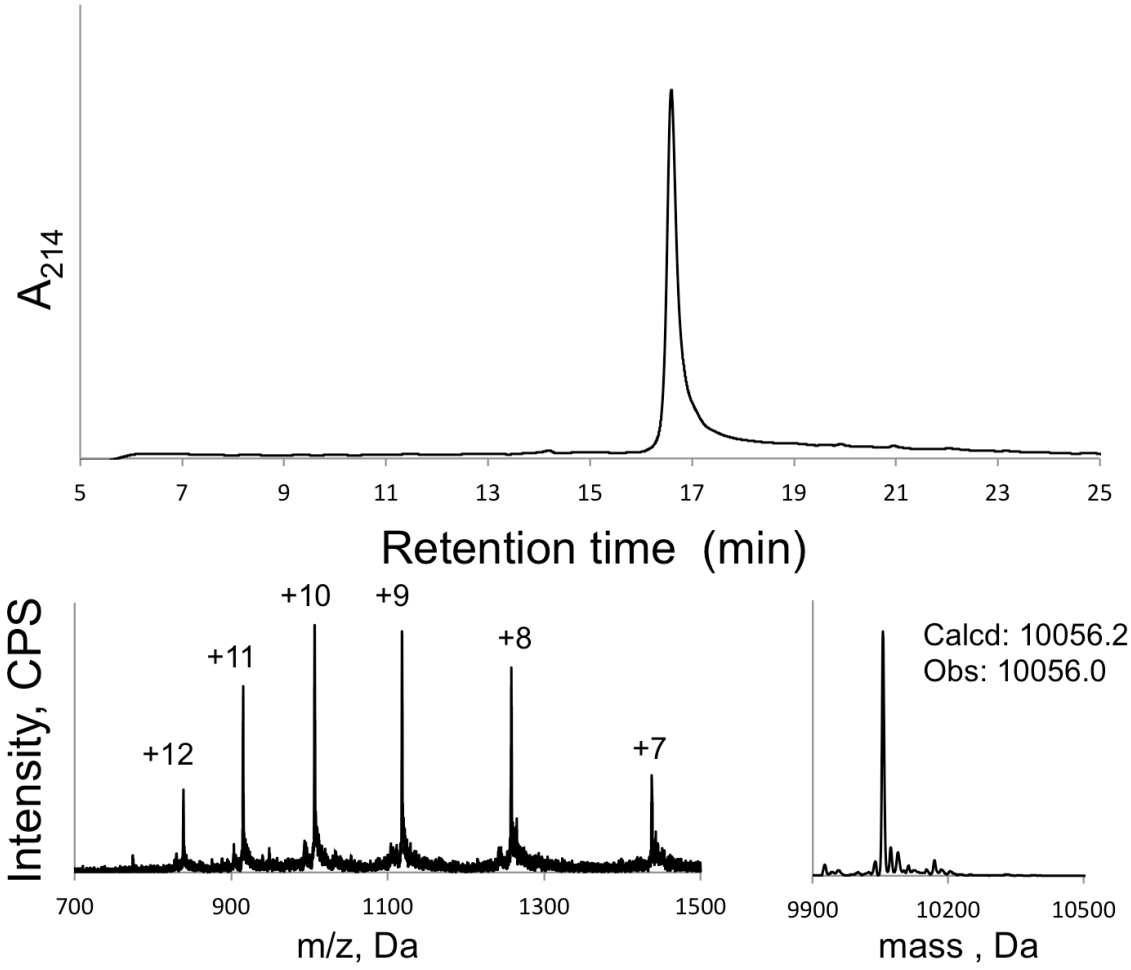
Mass spec analysis of purified product:

- **Expected mass based on sequence** = 10088.3 Da
- **Major observed deconvoluted mass** = 10086.5 Da
- **Major observed ions** = 841.7 (+12), 918.2 (+11), 1010.2 (+10), 1122.0 (+9), 1262.2 (+8), 1442.2 (+7).

3.6. Desulfurization of (L-TNF, 145-233)

Peptide Sequence:

C(Acm)PSTHVLLTHTISRIAVSYQTKVNLLSAIKSPC(Acm)QRETPEGAEA KPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL-NH_2



Analytical HPLC trace of purified product:

- **Column** = Phenomenex C12 proteo (4.6 × 150 mm)
- **Gradient method** = 10-90% buffer B over 30 min, Flow = 1 mL/min

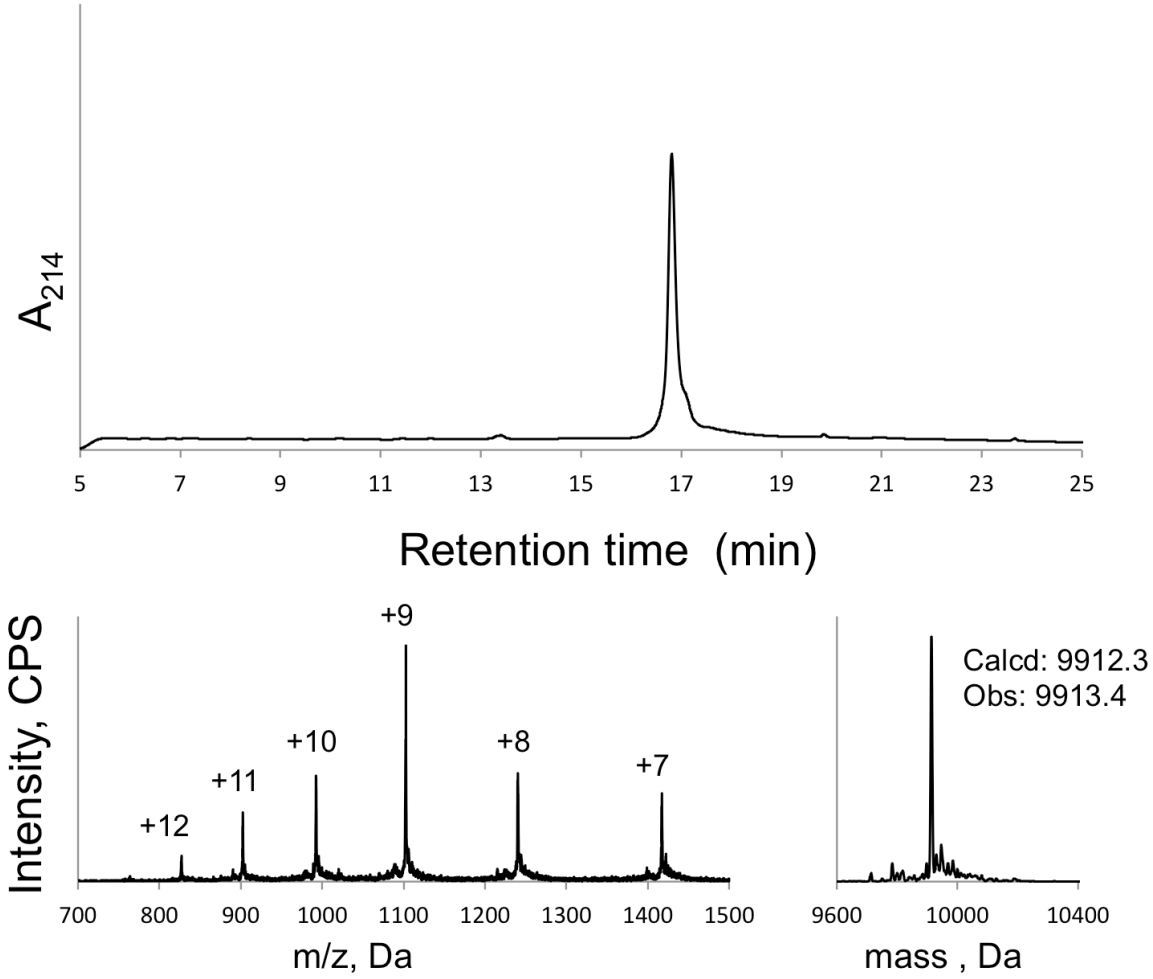
Mass spec analysis of purified product:

- **Expected mass based on sequence** = 10056.2 Da
- **Major observed deconvoluted mass** = 10056.0 Da
- **Major observed ions** = 839.0 (+12), 915.3 (+11), 1006.5 (+10), 1118.2 (+9), 1258.0 (+8), 1437.5 (+7).

3.7. Acm removal from (L-TNF, 145-233)

Peptide Sequence:

CPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIY LGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL-NH_2



Analytical HPLC trace of purified product:

- **Column** = Phenomenex C12 proteo (4.6 × 150 mm)
- **Gradient method** = 10-90% buffer B over 30 min, Flow = 1 mL/min

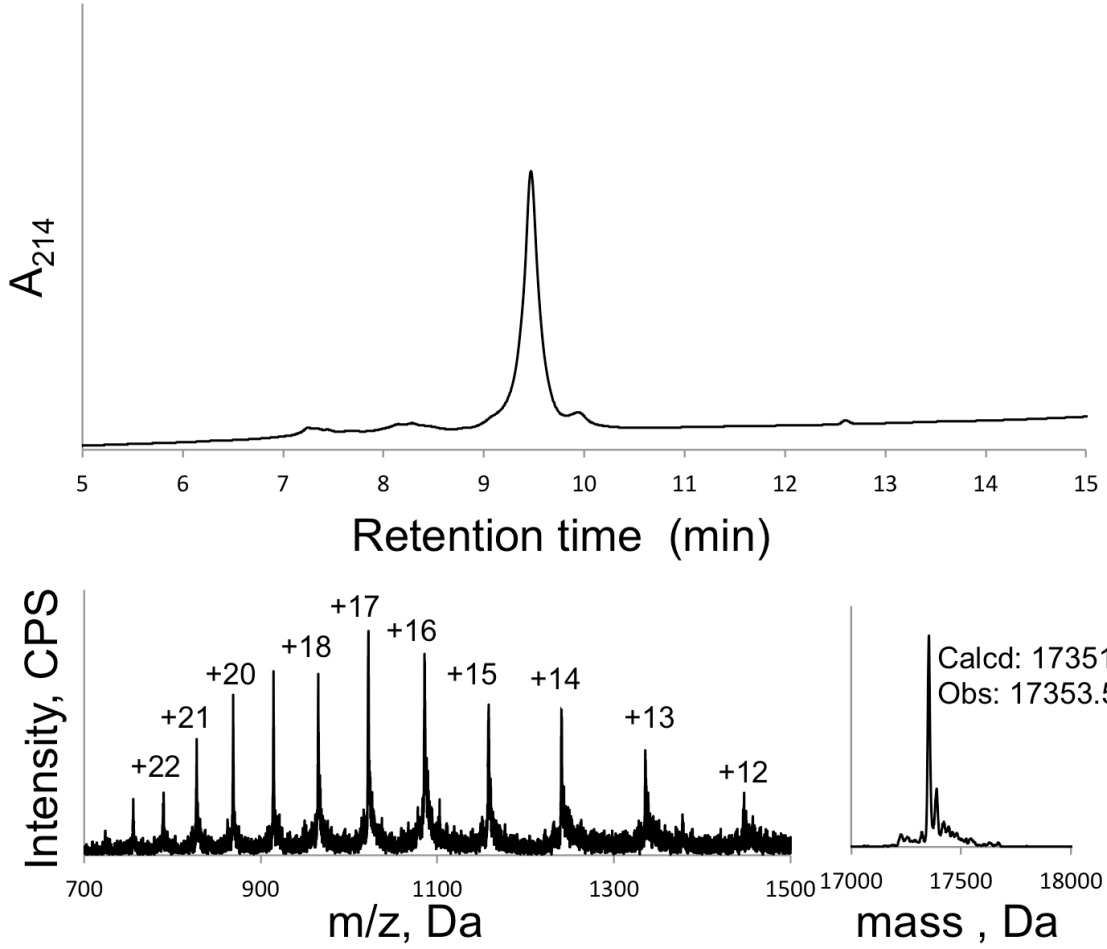
Mass spec analysis of purified product:

- **Expected mass based on sequence** = 9912.3 Da
- **Major observed deconvoluted mass** = 9913.4 Da
- **Major observed ions** = 827.2 (+12), 902.6 (+11), 992.4 (+10), 1102.6 (+9), 1240.4 (+8), 1417.2 (+7).

3.8. Final ligation product (L-TNF, 77-233)

Peptide sequence:

VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPS
EGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAK
PWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL-NH_2



Analytical HPLC trace of purified product:

- **Column** = Phenomenex C12 proteo (4.6 × 150 mm)
- **Gradient method** = 10-90% buffer B over 30 min, Flow = 1 mL/min

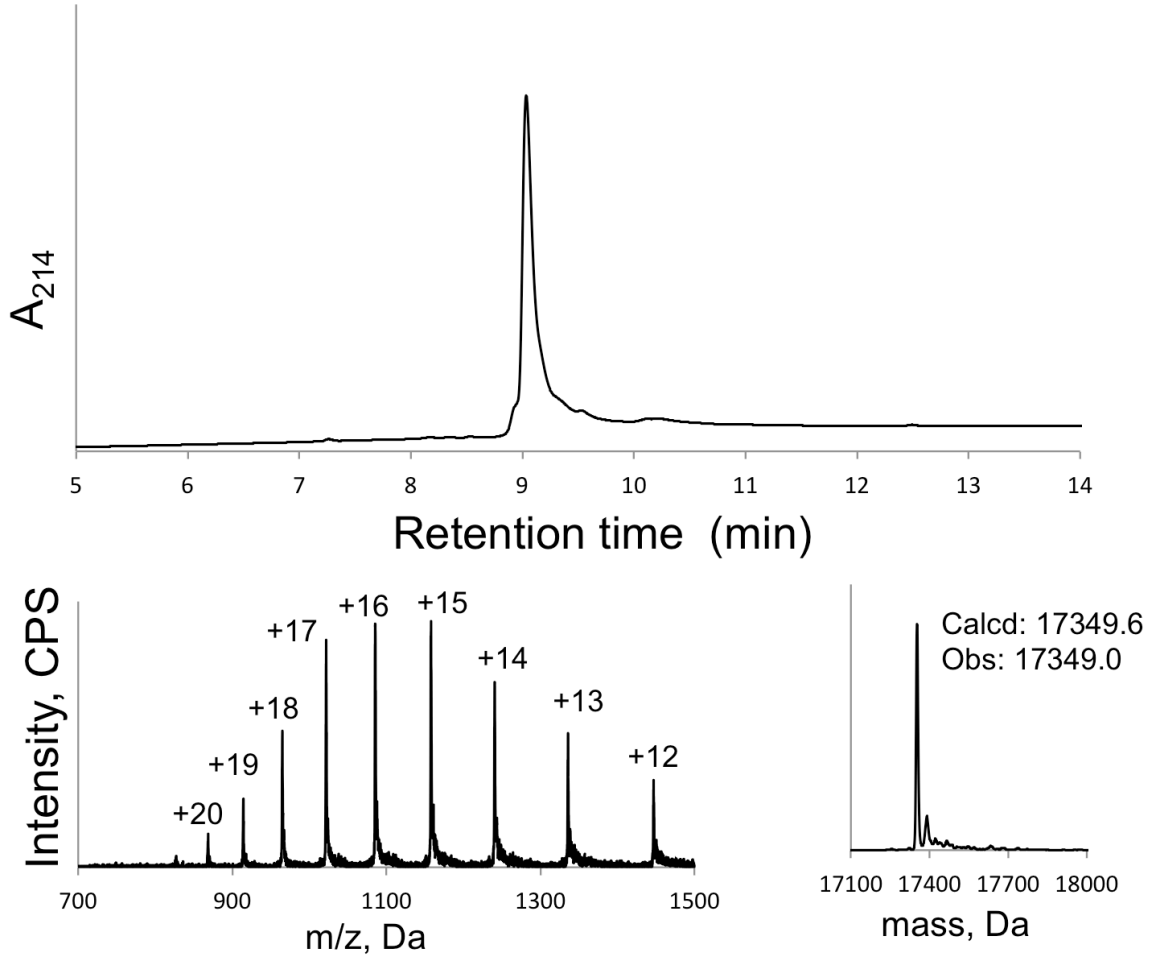
Mass spec analysis of purified product:

- **Expected mass based on sequence** = 17351.5 Da
- **Major observed deconvoluted mass** = 17353.5 Da
- **Major observed ions** = 755.9 (+23), 789.9 (+22), 827.2 (+21), 868.7 (+20), 914.4 (+19), 965.0 (+18), 1021.9 (+17), 1085.8 (+16), 1158.0 (+15), 1240.5 (+14), 1335.8 (+13), 1447.4 (+12).

3.9. Oxidized (L-TNF, 77-233)

Peptide sequence:

VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPS
EGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAK
PWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL-NH_2



Analytical HPLC trace of purified product:

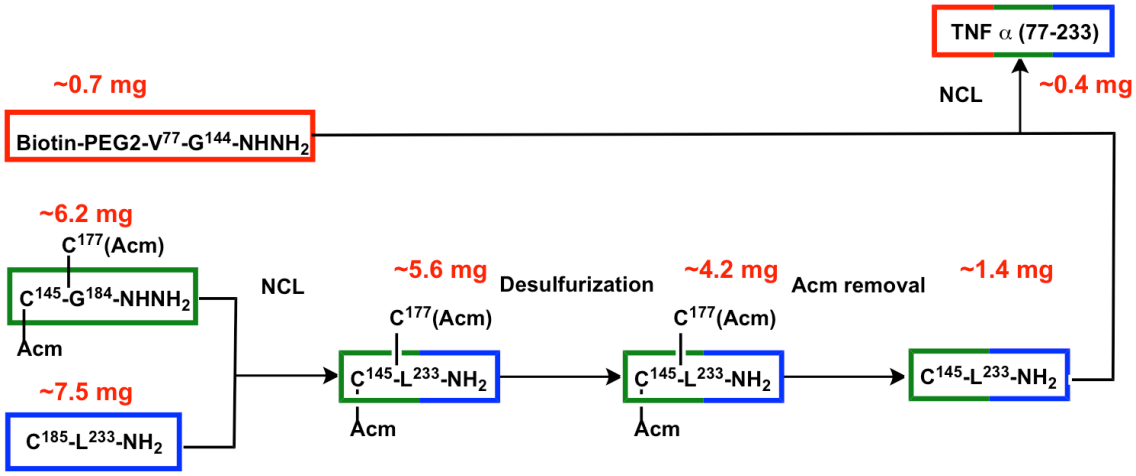
- **Column** = Phenomenex C4 Aeris widepore (2.1 × 50 mm)
- **Gradient method** = 25-60% buffer B over 15 min, Flow = 0.8 mL/min

Mass spec analysis of purified product:

- **Expected mass based on sequence** = 17349.6 Da
- **Major observed deconvoluted mass** = 17349.0 Da
- **Major observed ions** = 868.7 (+20), 914.3 (+19), 965.0 (+18), 1021.8 (+17), 1085.6 (+16), 1158.0 (+15), 1240.5 (+14), 1335.9 (+13), 1446.8 (+12).

4. Synthesis of D-TNFα

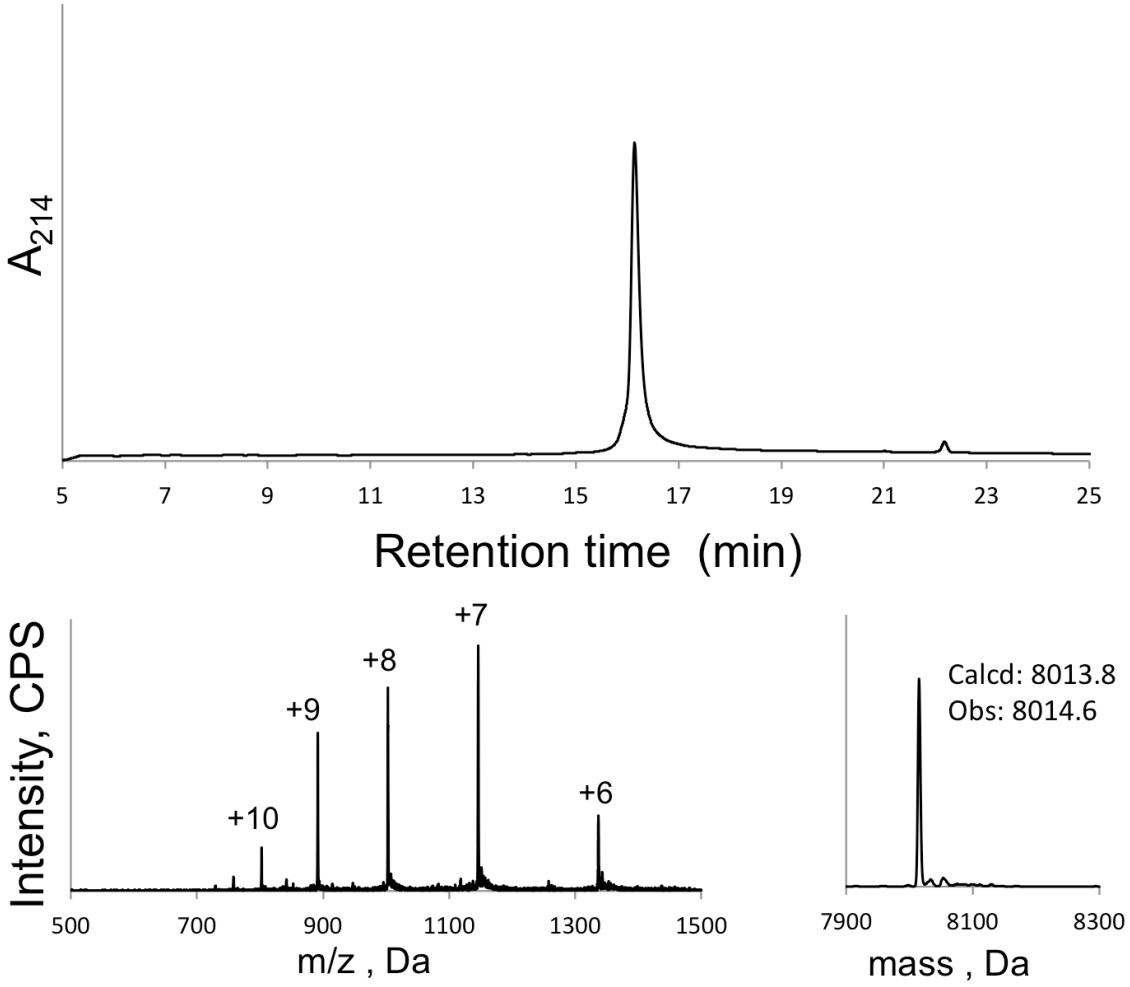
4.1. Yields for D-synthesis



4.2. D-TNF N-terminus (Biotin-PEG2-77-144)

Peptide sequence:

Biotin-PEG2-VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALL ANGVELRDNQLVVPSEGLYLIYSQVLFKGQG-$NHNH_2$



Analytical HPLC trace of purified product:

- **Column** = Phenomenex C12 proteo (4.6 × 150 mm)
- **Gradient method** = 10-90% buffer B over 30 min, Flow = 1 mL/min

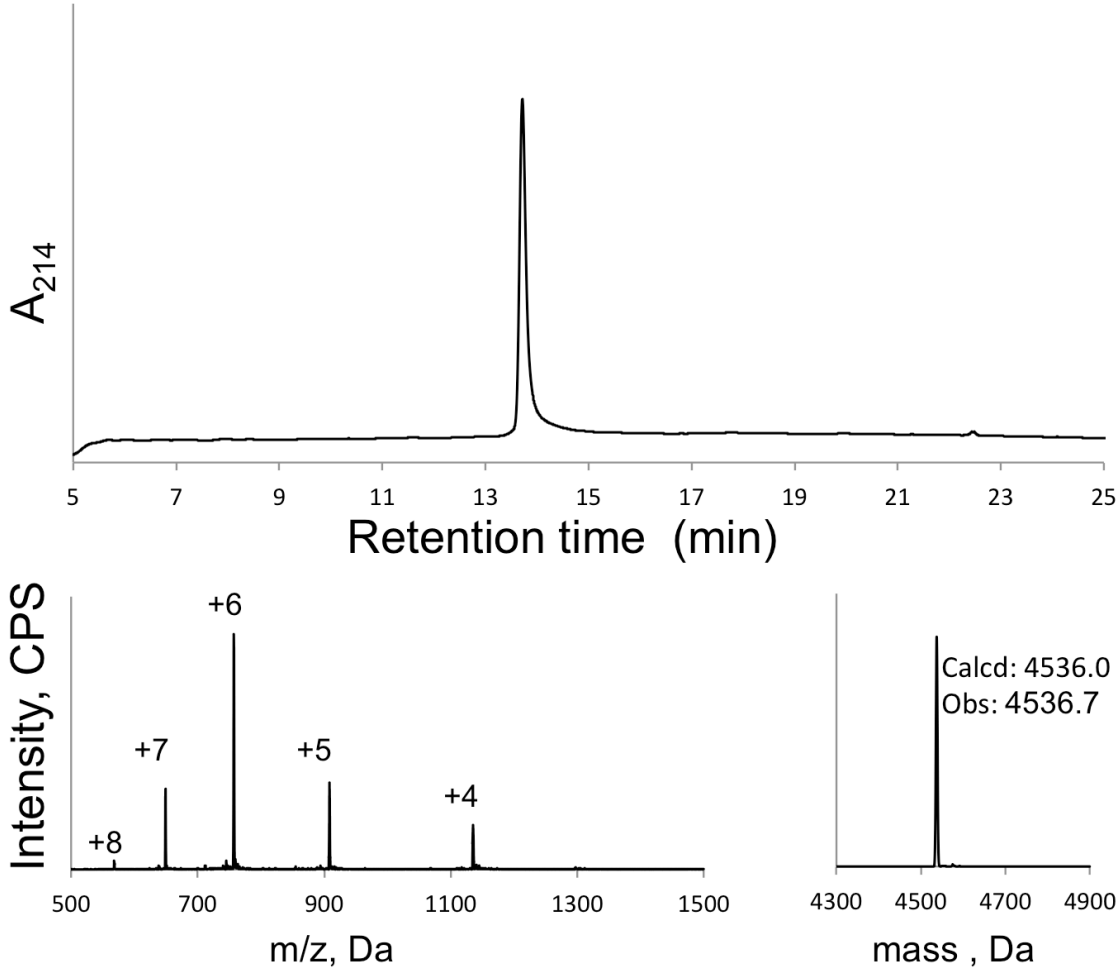
Mass spec analysis of purified product:

- **Expected mass based on sequence** = 8013.8 Da
- **Major observed deconvoluted mass** = 8014.6 Da
- **Major observed ions** = 802.5 (+10), 891.7 (+9), 1002.8 (+8), 1146.1 (+7), 1336.7 (+6).

4.3. D-TNF middle segment (145-184)

Peptide sequence:

C(Acm)PSTHVLLTHTISRIAVSYQTKVNLLSAIKSPC(Acm)QRETPEG-$NHNH_2$



Analytical HPLC trace of purified product:

- **Column** = Phenomenex C12 proteo (4.6 × 150 mm)
- **Gradient method** = 10-90% buffer B over 30 min, Flow = 1 mL/min

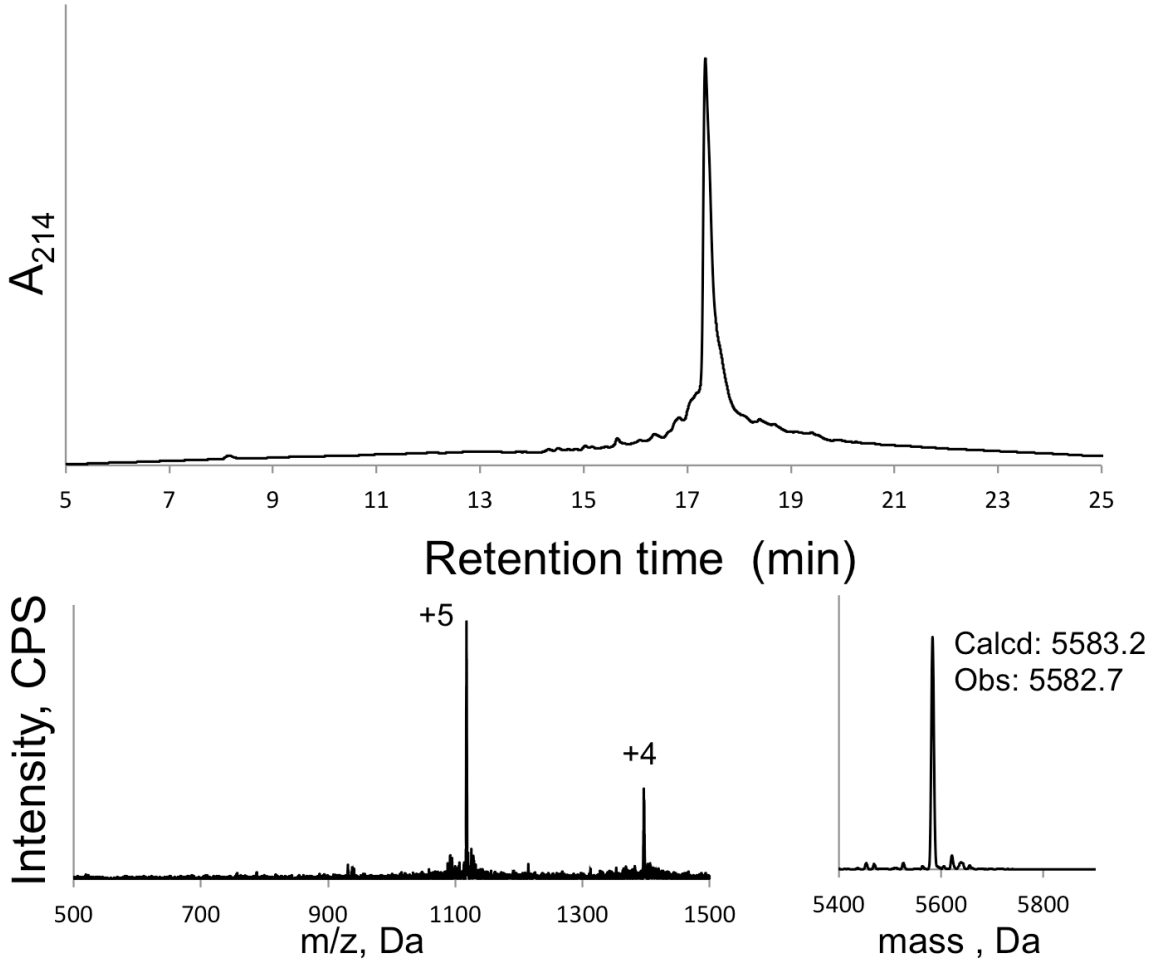
Mass spec analysis of purified product:

- **Expected mass based on sequence** = 4536.0 Da
- **Major observed deconvoluted mass** = 4536.7 Da
- **Major observed ions** = 568.2 (+8), 649.3 (+7), 757.2 (+6), 908.3 (+5), 1135.2 (+4).

4.4. D-TNF C-terminus (185-233)

Peptide sequence:

CEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL-NH_2



Analytical HPLC trace of purified product:

- **Column** = Phenomenex C12 proteo (4.6 × 150 mm)
- **Gradient method** = 10-90% buffer B over 30 min, Flow = 1 mL/min

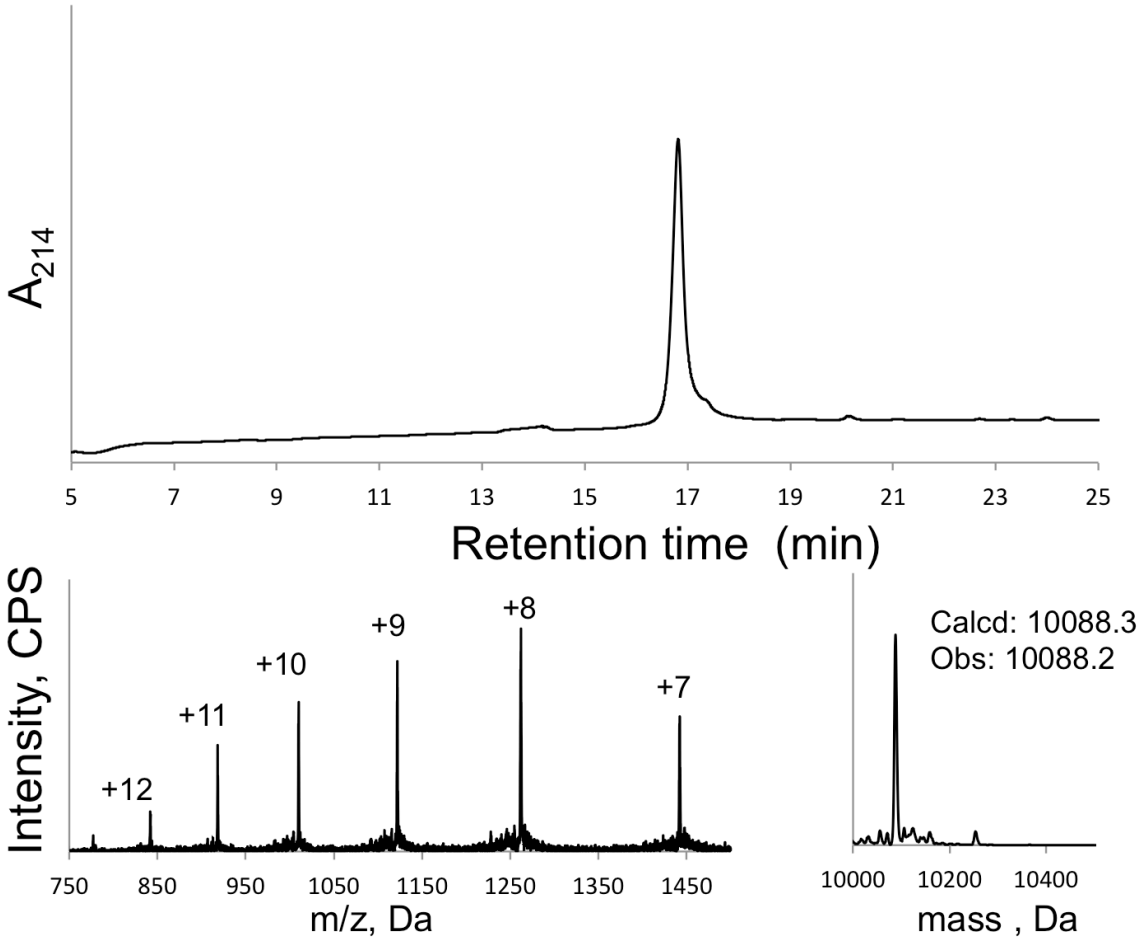
Mass spec analysis of purified product:

- **Expected mass based on sequence** = 5583.2 Da
- **Major observed deconvoluted mass** = 5582.7 Da
- **Major observed ions** = 1117.6 (+5), 1396.4 (+4).

4.5. Ligation product of TNF C-terminus and middle segment (D-TNF, 145-233)

Peptide Sequence:

C(Acm)PSTHVLLTHTISRIAVSYQTKVNLLSAIKSPC(Acm)QRETPEGCEA KPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL-NH_2



Analytical HPLC trace of purified product:

- **Column** = Phenomenex C12 proteo (4.6 × 150 mm)
- **Gradient method** = 10-90% buffer B over 30 min, Flow = 1 mL/min

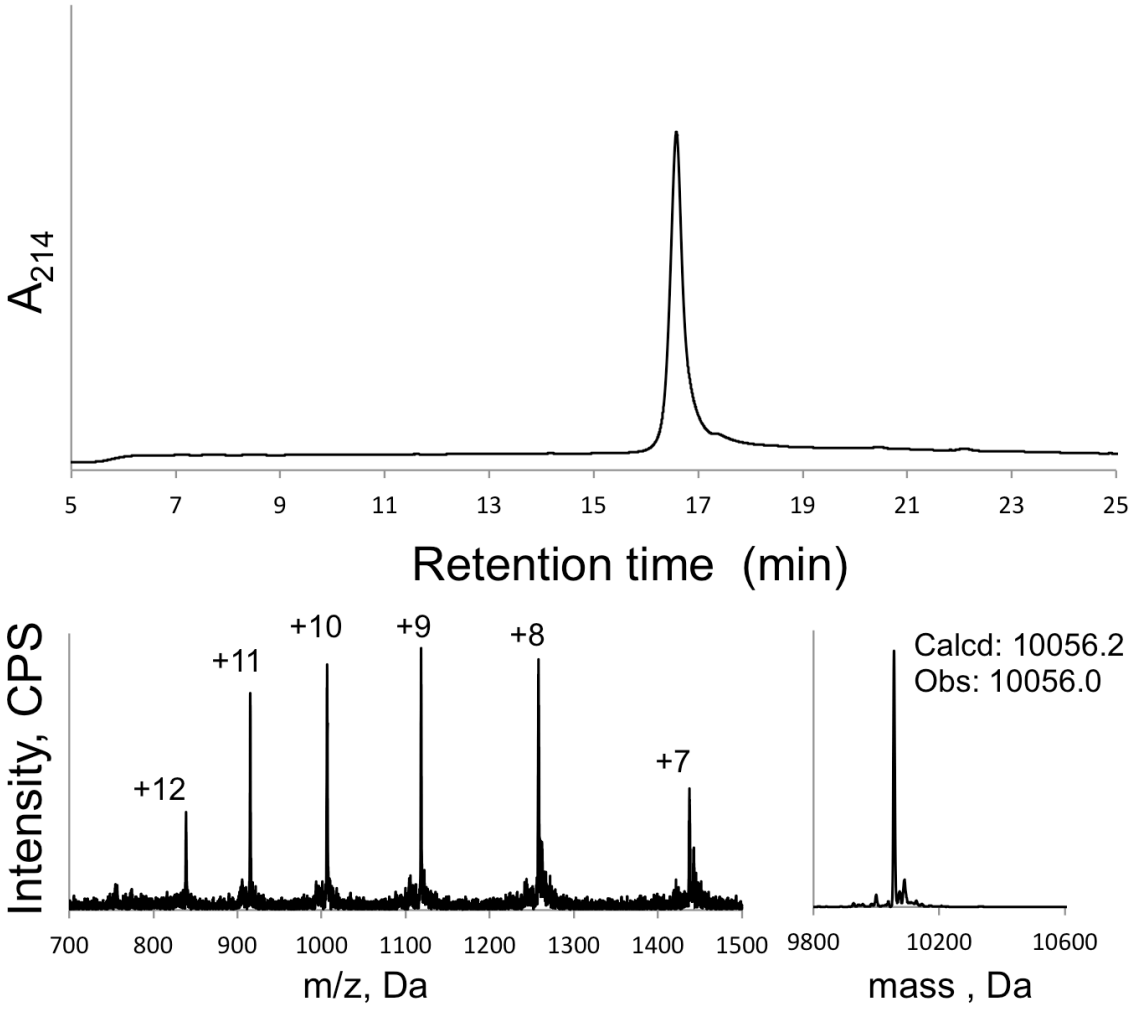
Mass spec analysis of purified product:

- **Expected mass based on sequence** = 10088.3 Da
- **Major observed deconvoluted mass** = 10088.2 Da
- **Major observed ions** = 841.7 (+12), 918.2 (+11), 1010.2 (+10), 1122.0 (+9), 1262.2 (+8), 1442.2 (+7).

4.6. Desulfurization of (D-TNF, 145-233)

Peptide Sequence:

C(Acm)PSTHVLLTHTISRIAVSYQTKVNLLSAIKSPC(Acm)QRETPEGAEA KPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL-NH_2



Analytical HPLC trace of purified product:

- **Column** = Phenomenex C12 proteo (4.6 × 150 mm)
- **Gradient method** = 10-90% buffer B over 30 min, Flow = 1 mL/min

Mass spec analysis of purified product:

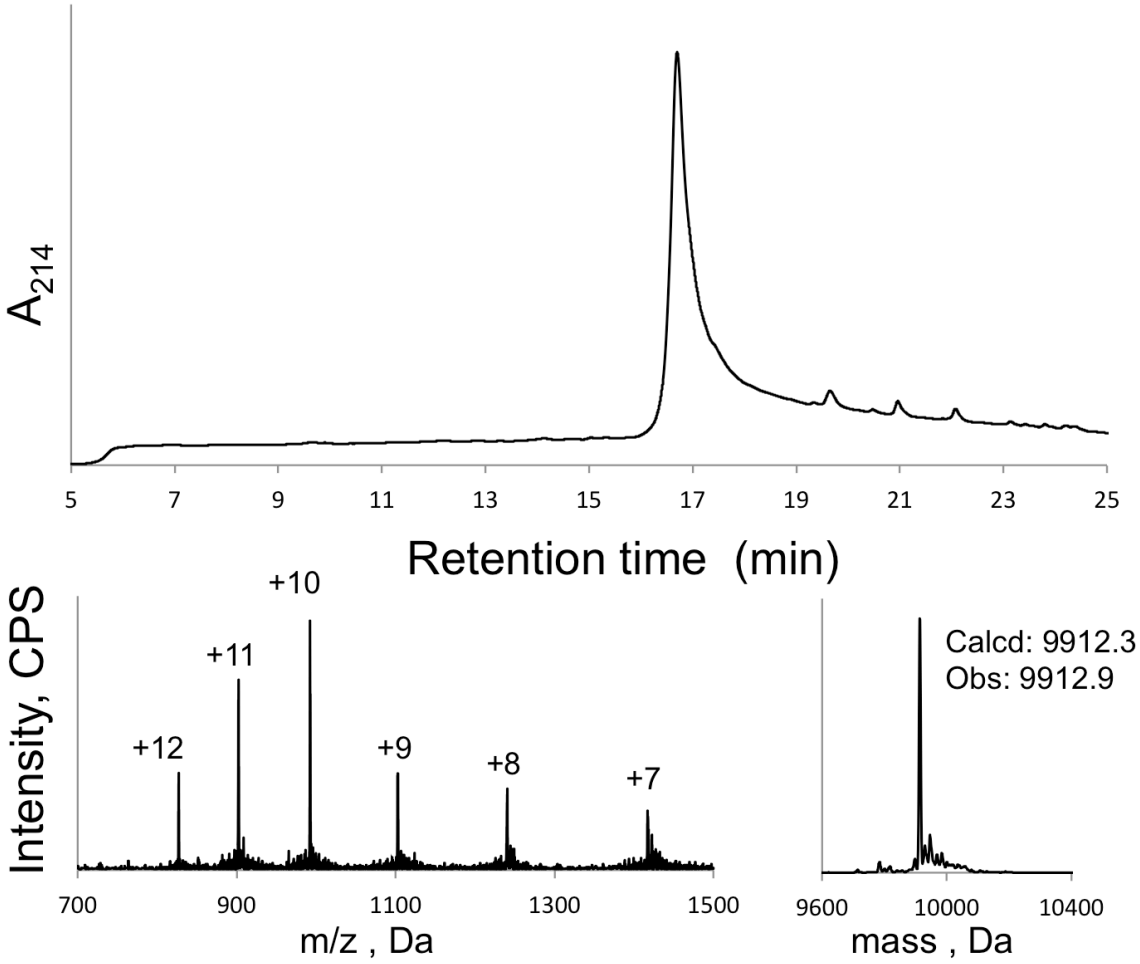
- **Expected mass based on sequence** = 10056.2 Da
- **Major observed deconvoluted mass** = 10056.0 Da

Major observed ions = 839.0 (+12), 915.3 (+11), 1006.5 (+10), 1118.2 (+9), 1258.0 (+8), 1437.5 (+7).

4.7. Acm removal from (D-TNF, 145-233)

Peptide Sequence:

CPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIY LGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL-NH_2



Analytical HPLC trace of purified product:

- **Column** = Phenomenex C12 proteo (4.6 × 150 mm)
- **Gradient method** = 10-90% buffer B over 30 min, Flow = 1 mL/min

Mass spec analysis of purified product:

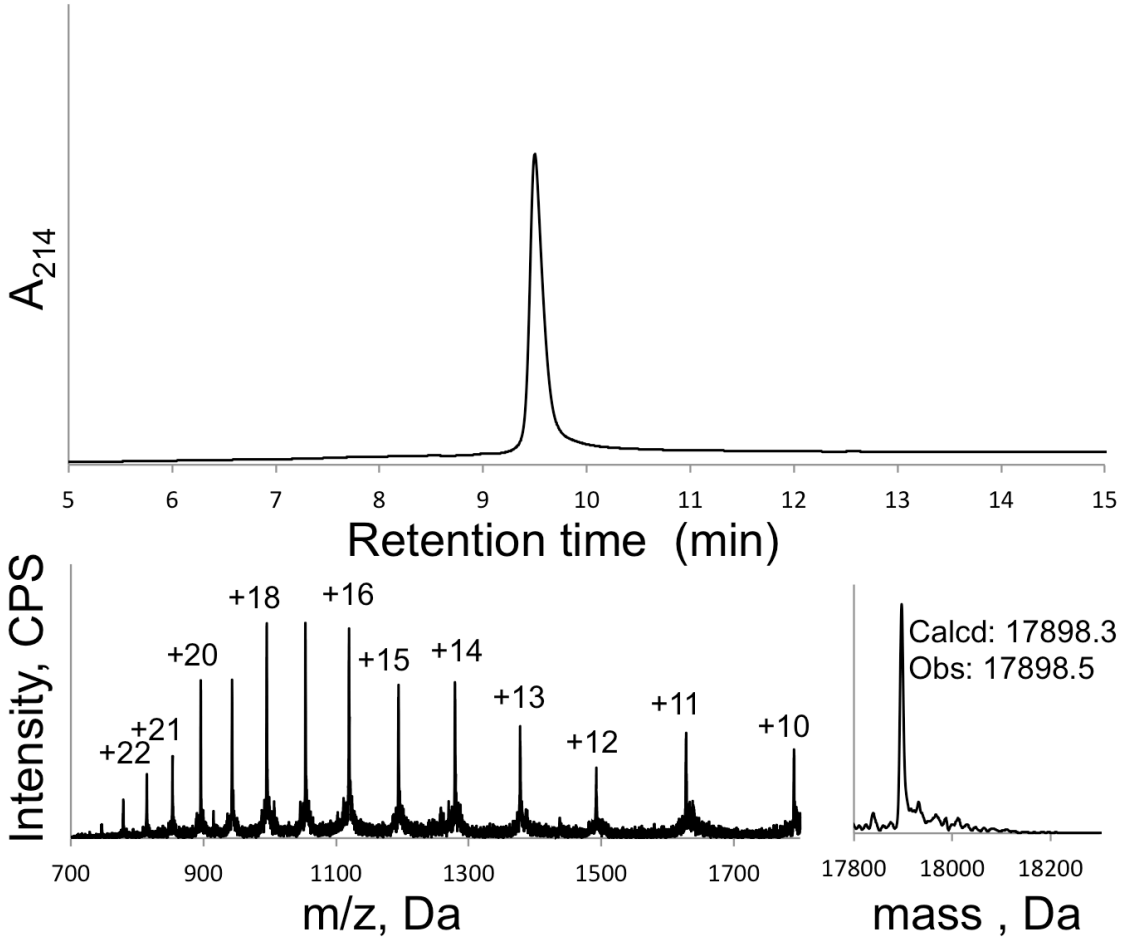
- **Expected mass based on sequence** = 9912.3 Da
- **Major observed deconvoluted mass** = 9912.9 Da

Major observed ions = 827.2 (+12), 902.6 (+11), 992.4 (+10), 1102.6 (+9), 1240.4 (+8), 1417.2 (+7).

4.8. Final ligation product (D-TNF, Biotin-PEG2-77-233)

Peptide sequence:

Biotin-PEG2-VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANG
VELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPC
QRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL-NH_2



Analytical HPLC trace of purified product:

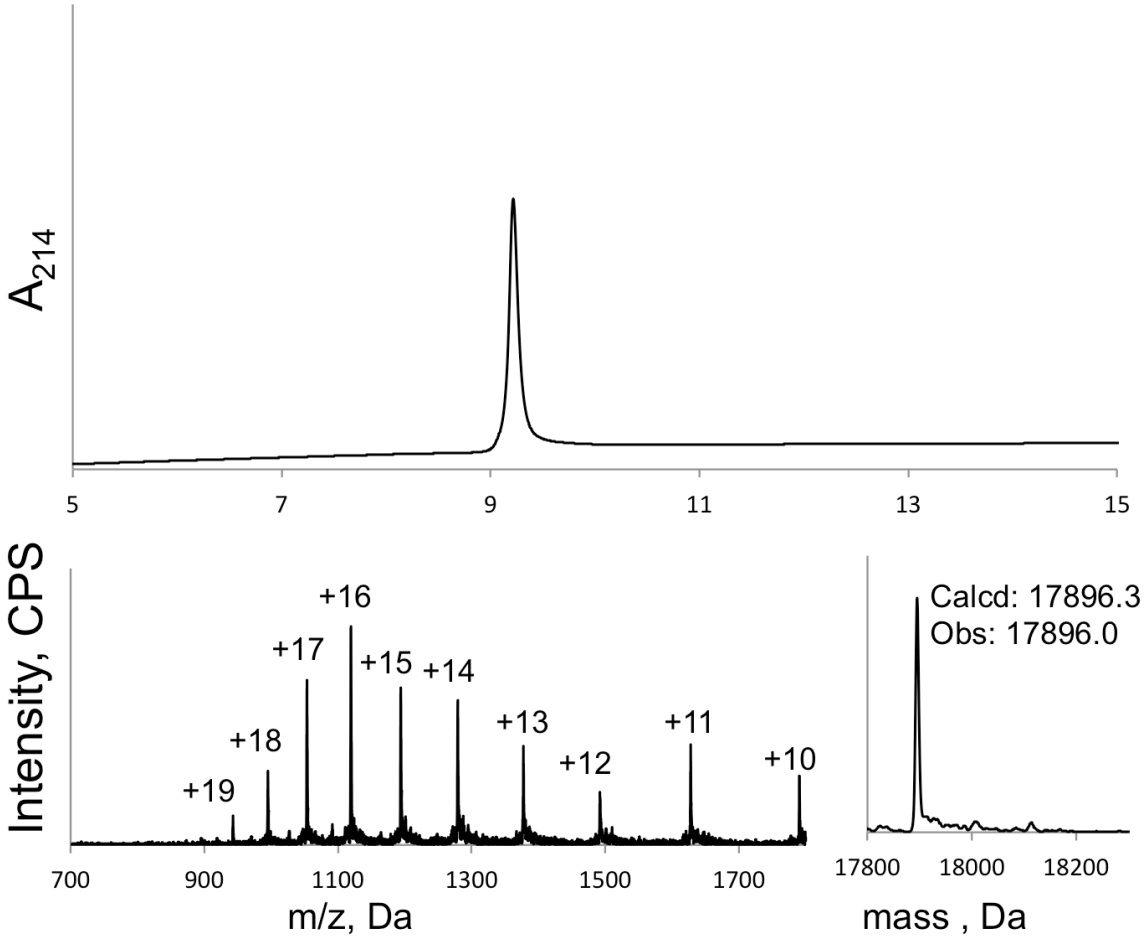
- **Column** = Phenomenex C4 Aeris widepore (2.1 × 50 mm)
- **Gradient method** = 25-60% buffer B over 15 min, Flow = 0.8 mL/min

Mass spec analysis of purified product:

- **Expected mass based on sequence** = 17898.3 Da
- **Major observed deconvoluted mass** = 17898.5 Da
- **Major observed ions** = 778.9 (+23), 814.6 (+22), 853.2 (+21), 895.9 (+20), 943.1 (+19), 995.4 (+18), 1053.7 (+17), 1119.6 (+16), 1194.2 (+15), 1279.2 (+14), 1377.5 (+13), 1492.6 (+12), 1627.7 (+11), 1790.8 (+10).

4.9. Oxidized (D-TNF, Biotin-PEG2-77-233)

Biotin-PEG2-VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANG
VELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPC
QRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL-NH_2



Analytical HPLC trace of purified product:

- **Column** = Phenomenex C4 Aeris widepore (2.1 × 50 mm)
- **Gradient method** = 25-60% buffer B over 15 min, Flow = 0.8 mL/min

Mass spec analysis of purified product:

- **Expected mass based on sequence** = 17896.3 Da
- **Major observed deconvoluted mass** = 17896.0 Da
- **Major observed ions** = 943.0 (+19), 995.1 (+18), 1053.5 (+17), 1119.2 (+16), 1194.0 (+15), 1279.3 (+14), 1377.5 (+13), 1491.9 (+12), 1627.8 (+11), 1790.7 (+10).

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