Supporting Information for

Trimer-to-monomer disruption mechanism for a potent, protease-resistant antagonist of Tumor Necrosis Factor-α Signaling

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I. Materials and Instrumentations

1). Material Sources

Reagent or Material	Vendor	Catalog No.
SPPS Chemicals and Materials		
α-N-Fmoc-amino acids	Chem Impex	Various
Fmoc β3-amino acids	PepTech	Various
Fmoc-(1S,2S)-2-ACPC-OH	Chem Impex	15073
Fmoc-aminoisobutyric acid	Chem Impex	02547
Dodecanoic acid	Millipore Sigma	L4250
Fmoc-NH-PEG8-CH2-CH2-COOH	ChemPep	280112
SPPS reaction vessel syringes/caps	Torviq	SF-1000/PC-SF
α-Cyano-4-hydroxycinnamic acid (CHCA)	Millipore Sigma	70990
N, N Dimethylformamide (DMF) ACS-grade	Millipore Sigma	319937
N, N Dimethylformamide (DMF) biotech-grade	Millipore Sigma	494488
Piperidine	Millipore Sigma	104094
Trifluoroacetic acid (TFA)	Millipore Sigma	T6508
1,2 ethanedithiol	Millipore Sigma	2390
Thioanisole	Millipore Sigma	T28002
N,N′-Diisopropylcarbodiimide (DIC)	Chem Impex	00110
Ethyl (hydroxyimino) cyanoacetate (Oxyma)	Chem Impex	26426
Rink Amide Resin (Low Loading)	CEM	R002
Acetonitrile	Millipore Sigma	34851
Triisopropylsilane	Millipore Sigma	233781
Protein Expression and Purification Reagents		
BL21 (DE3) Competent *E. coli*	NEB	C2527
S.O.C. Medium	Thermo Fisher	15544034
Plasmid (GST-TEV-TNFα)	Gene Universal	Customized order
TEV protease Plasmid (gift from David Waugh)	Addgene	pDZ2087
Tryptone	Dot Scientific	SD149182
Yeast Extract	Dot Scientific	DS149162
NaCl	Millipore Sigma	S9888
Tris base	Millipore Sigma	77861
2-mercaptoethanol (BME)	Millipore Sigma	444203
L-Arginine	Millipore Sigma	A5006
L-Glutathione reduced	Millipore Sigma	G4251
L-Glutathione oxidized	Millipore Sigma	G4376
Sodium phosphate dibasic	Millipore Sigma	S9763
Potassium phosphate monobasic	Millipore Sigma	P0662
TWEEN-20	Millipore Sigma	P1379

Protein Crystallography Reagents and Materials		
Index crystallization screen	Hampton Research	HR2-144
Crystal Screen crystallization screen	Hampton Research	HR2-110/112
PEGRx HT	Hampton Research	HR2-086
PEG/Ion 400	Hampton Research	HR2-460
crystallization accessories	Hampton Research	various
ELISA Reagents and Materials		
Recombinant hTNF-α	R&D systems	210-TA
Recombinant hTNFR1	R&D systems	636-R1-025
Streptavidin-Alkaline Phosphatase	R&D systems	AR001
hTNF-α Biotinylated Antibody	R&D systems	BAF210
SIGMAFAST p-Nitrophenyl phosphate tablets	Millipore Sigma	N1891
Nunc MaxiSorp Flat-bottom Plate	Invitrogen	44-2402-21
PBS-TWEEN Tablets	Millipore Sigma	524653
BSA(7.5%)	Thermo Fisher	15260037
DPBS	Millipore Sigma	D8537
Cell Assay Reagents and Materials		
WEHI-13VAR cell line	ATCC	CRL-2148
RPMI-1640 Medium	ATCC	30-2001
Actinomycin D	Thermo Fisher	BP-606
Fetal bovine serum (FBS)	Millipore Sigma	F2442
penicillin/streptomycin	HyClone	SV30010
Trypsin EDTA (0.05%)	Corning	25-051-CI
White 96-well plate	Corning	3610
CellTiter-Glo luminescent cell viability assay	Promega	G7571
Software and Illustrations		
GraphPad Prism 8	GraphPad Software	
Origin 7	Origin Software	
PyMOL	Schrodinger	
Chimera	UCSF	
Coot		
Phenix		
CCP4		

2) Instrumentation Acknowledgments

Crystallization and structure solution were supported by the Collaborative Crystallography Core in the Department of Biochemistry, UW-Madison.

ITC data were obtained at the University of Wisconsin – Madison Biophysics Instrumentation Facility, which was established with support from the University of Wisconsin – Madison and grants BIR-9512577 (NSF) and S10 RR13790 (NIH).

Bruker microflex LRF, MALDI-TOF-MS, Generous gift from the Bender Fund

Bruker Advance III-400, NMR Spectrometer, NSF CHE-1048642 2010

II. Methods

1) Peptide synthesis, purification, and characterization

Fmoc-(3S,4R)-4-aminopyrrolidine acid (Fmoc-APC-OH) was synthesized as previously reported.[1] Rink Amide ProTide resin (LL) was added to a Torviq solid-phase peptide synthesis vessel with a stir bar. The resin was swelled in 50:50 DCM:DMF for 15 minutes before the reactions were started. Fmoc amino acids (4 eq., 0.1M) were activated with OXYMA (8 eq.), and DIC (4 eq.) and added to the reaction vessel. Regular coupling cycles were done at 70°C for 4 minutes. The difficult amino acids (Leu, Ile, Val, β-amino acids) were extended (for 8 to 12 minutes) or double coupled. Histidine was coupled at 50°C for 10 minutes to avoid racemization. Then the resin was washed 5 times with DMF. The Fmoc protecting group was removed by combining the resin with 20% v/v piperidine in DMF at 80°C for 2 minutes. To the deprotection solution, 0.1M OXYMA was added to limit aspartimide formation. To append the di-dodecanoyl group, Fmoc-Lys(Fmoc)-OH was first coupled to the N-term after the PEG linker. After deprotecting the two Fmoc groups on the N-term Lys residue, decanoic acid (8 eq.) was activated and coupled with regular coupling condition.

After the sequence was completed, cleavage was performed by mixing the resin with 1% TIPS, 2.5% water, 2.5% EDT, 2.5% thioanisole, and 91.5% TFA at room temperature for 4 hours. Crude cleaved peptide was drained from the reaction vessel into a 50-mL falcon tube. Excess TFA was evaporated under a stream of nitrogen, and about 40 mL of cold diethyl ether was added to precipitate the peptide. The peptide pellet was collected by centrifuging at 3500 g for 5 minutes. The pellet was dried under nitrogen and dissolved in DMSO (8mL per 50 μmol). NH_4OH (50 drops per 50 μmol) was added to adjust the pH for disulfide bond formation. The mixture was stirred at room temperature for at least 3 days.

Oxidized peptide was then filtered through a 0.22 micro filter and purified on a Waters preparative HPLC system with a C18 (5 μm, 19x250 mm) column (solvent A = H_2O + 0.01% TFA, solvent B = acetonitrile + 0.01% TFA, flow rate = 15 mL/min). Collected HPLC fractions were characterized by MALDI-TOF-MS and analytical UPLC (see Section IV).

Collected peptide was freeze-dried for long-term storage. The lyophilized powder was dissolved in H_2O (for crystallization and ITC) or DMSO (for ELISA) and filtered. The peptide concentration was measured via absorbance with a ThermoScientific Nanodrop 2000. The peptide molar extinction coefficient was calculated as previous reported.[2]

2) Protein Expression and Purification

A plasmid containing TEV protease gene pDZ2087 was a gift from David Waugh (Addgene plasmid # 92414). TEV protease was expressed and purified as previous reported.[3] Purified TEV protease was aliquoted and stored at -20°C for future use.

A GST tag plasmid (pGEX-4T-1) containing a TEV cleavage site and codon optimized TNFα gene fragment (with BamHI/NotI insertion) was purchased from Gene Universal and transformed into BL21 competent cells. GST-TNFα was expressed in LB media with 1 mM IPTG induction at 18°C overnight. The cell pellets were harvested the next morning by centrifuging at 8,000 rpm for 30 minutes at 4°C. The collected cell pellets were frozen at -20°C.

Cell pellets were lysed in lysis buffer (50 mM Tris at pH 7.5, 150 mM NaCl, 5% glycerol, 2 mM BME, 2 mM $MgCl_2$) with lysozyme and DNase I. The lysis mixture was stirred at room

temperature for 1 hour and then incubated at 4°C overnight. The lysate was then sonicated and spun down at 8,000 rpm. The supernatant was loaded onto a GSTrap column and purified by washing buffer (50 mM Tris at pH 7.5, 150 mM NaCl, 5% glycerol, 2 mM BME). The GST tag was cleaved on column by injecting 2 mol% TEV onto the column and incubating the column at room temperature overnight. The tag-free TNFα was eluted from GSTrap, and the TEV protease was removed by eluting the solution from a HisTrap column with buffer (50 mM Tris at pH 7.5, 150 mM NaCl). The eluted free TNFα was further purified by size-exclusion column chromatography (Superdex 75). The purified TNFα was added to pre-chilled refolding buffer to final concentration of 20 μg/mL and stirred for three days at 4°C. The refolding buffer contains 50 mM Tris at pH 9.5, 150 mM NaCl, 500 mM L-arginine, 1 mM L-glutathione reduced, 1 mM L-glutathione oxidized. The refolded TNFα was concentrated and further purified on a size-exclusion column in PBS (for ITC) or in 50 mM Tris at pH 7.5, 150 mM NaCl (for crystallization).

3) Crystallization, X-ray Diffraction Data Collection, and Structure Solution

Peptide and TNFα were premixed at 1:1 molar ratio of peptide to TNFα monomer, and the mixture was incubated at room temperature overnight before setting up crystal trays. The initial screens were carried out on 96-well sitting drop vapor diffusion plates with commercial crystal screen conditions. For each condition, 200 nL of TNFα-peptide solution was combined with 200 nL precipitant solution with a TTP LabTech Mosquito. Crystal growth was monitored under a microscope every day for at least two weeks. The initial crystal hits were then optimized with 48-well hanging drop plates.

Diffraction data were collected from LS-CAT and GM/CA beam lines at APS (Argonne National Laboratory). Indexing, integration, and scaling were carried out using XDS/XSCALE and autoPROC programs.[4,5]

The TNFα protein chains in the TNFα-**1** structure were solved by molecular replacement in Phaser-MR using eight single chains of TNFα derived from native human TNFα crystal structure (PDB 1TNF) as the search model.[6–8] The number of copies used in the search was predicted by Matthew's probabilities.[9,10] The TNFα protein chains in the TNFα-**3** structure were solved by AutoBuild using the single chain TNFα as the starting model. The peptides in both models were built manually in Coot using a poly-alanine helix starting model.[11] The non-proteinogenic residues (Aib and β-amino acids) were first built and refined in the program Elbow, introduced to the peptide chains in Coot, and refined in Phenix Refine with defined linkage files.[12]

4) Isothermal Titration Calorimetry (ITC)

Separate solutions of 1 mM peptide and 75 μM TNFα (monomer concentration) were dialyzed against PBS in the same beaker, and each solution was diluted to the experiment concentration with PBS or PBS with 0.05% TWEEN20. All experiments were performed on a MicroCal VP-ITC. In the peptide to protein titration experiment, 300 μM peptide was loaded in the injection syringe, and 30 μM TNFα (monomer concentration) was added in the sample cell. The titrations were performed at 37°C. The control experiments (buffer-to-buffer, peptide-to-buffer, and buffer-to-protein) were carried out at 37°C and repeated three times. The data were processed on Origin 7.

5) Ultra-high Performance Liquid Chromatography-Size Exclusion Chromatography (UPLC-SEC)

Recombinant TNFα expressed and purified in house was non-specifically labeled with fluorescein 5-isothiocyanate (FITC; TCI Chemical, F0026) in 0.1 M sodium carbonate buffer (pH 9.0). The reaction solution was incubated in the dark overnight at 4 °C, and then excess FITC was removed by extensive dialysis. The conformation and bioactivity of the labeled protein, FITC-TNFα, were confirmed by size-exclusion chromatography analysis and WEHI-13VAR cell viability assay.

Solutions containing 3 μM FITC-TNFα or 50 μM peptide were mixed and pre-incubated in PBS at 37 °C in the UPLC sample manager. For each time point, 5 μL of the pre-incubated solution was injected on a Protein BEH SEC column (1.7 μm, 4.6 mm x 150 mm) with PBS as the mobile phase (flow rate = 0.4 mL/min). The results were monitored by fluorescence-UPLC (excitation at 494 nm; emission at 519 nm).

6) TNFα/TNFR1 ELISA

Recombinant human sTNFR1 was diluted in PBS, added to the ELISA plate, and incubated at 4°C overnight to immobilize TNFR1. After overnight immobilization, the plate was washed with PBS + TWEEN (PBST). Then 1% BSA in PBS was added and incubated for 3 hours, and the plate was washed again with PBST. Serial dilutions of peptides were prepared in DMSO and added to 80 ng/mL TNFα (from R&D systems) in PBST. The peptide-TNFα solution was incubated on an orbital shaker for 1 hour and added to the washed ELISA plate. The plate was then incubated for 1 hour, followed by biotinylated anti-TNFα antibody coating, washing, streptavidin-alkaline phosphatase coating, washing and the *p*-nitrophenyl phosphate (pNPP) addition. Then the 405 nm absorbance in each well was recorded every two minutes during the pNPP reaction on BioTek Synergy 2 microplate reader. Each condition was run in duplicate, and each experiment was repeated at least three times. The IC_{50} values were calculated using GraphPad Prism 8.

7) WEHI-13VAR Cell Viability Assay

a) Cell culture: WEHI-13VAR mouse fibrosarcoma cells were cultured in RPMI + 10% FBS at 37 °C with 5% CO_2. When cells reached ~75-80% confluency, cells were trypsinized, counted and reseeded at 120,000 cells/mL density in 100 μL fresh RPMI + 10% FBS medium per well in 96-well white, clear-bottom plates. Plated cells were kept in the incubator for 18-24 hours to adhere before TNFα or TNFα/peptide addition.

b) Pre-incubation: Serial dilutions of peptides were prepared in DMSO. 5 μL of peptide solutions in DMSO were added to 120 μL of 83 ng/mL of TNFα in PBS. The "+ TNFα" control was prepared by mixing 120 μL of 83 ng/mL of TNFα with 5 μL of DMSO; the "- TNFα" control was prepared by mixing 120 μL of PBS with 5 μL of DMSO. The pre-incubation mixtures were incubated for 1, 2, 4, 6, 12, and 24 hours at 37°C before being diluted and added to cells.

c) Addition to cells: After the pre-incubation step was completed, the TNFα/peptide mixture was diluted 350-fold with RPMI (no FBS) + 3.8 μg/mL actinomycin D solution. 50 μL of diluted pre-incubation mixture was added to each well. The final concentration of TNFα was approximately 0.08 ng/mL. The plates were incubated at 37°C with 5% CO_2 for ~18 hours to allow TNFα to induce cell death. For data analysis, the initial inhibitor concentrations before this dilution step were used.

d) CellTiter-Glo Assay: After 18 hours of incubation, the plates were removed from the incubator and allowed to cool to room temperature. 150 µL of CellTiter-Glo reagent (Promega, WI) was then added to each well, and the plate was agitated for 2 minutes on an orbital shaker. The luminescence value was measured on a BioTek Synergy 2 microplate reader. Data were normalized to "- TNFα" control as 100% (represents maximal cell survival) and to "+ TNFα" control as 0% (represents minimal cell survival). IC_{50} values were calculated using GraphPad Prism 8.0. Data points are presented as mean ± SD of three independent experiments.

III. Supporting Figures and Tables

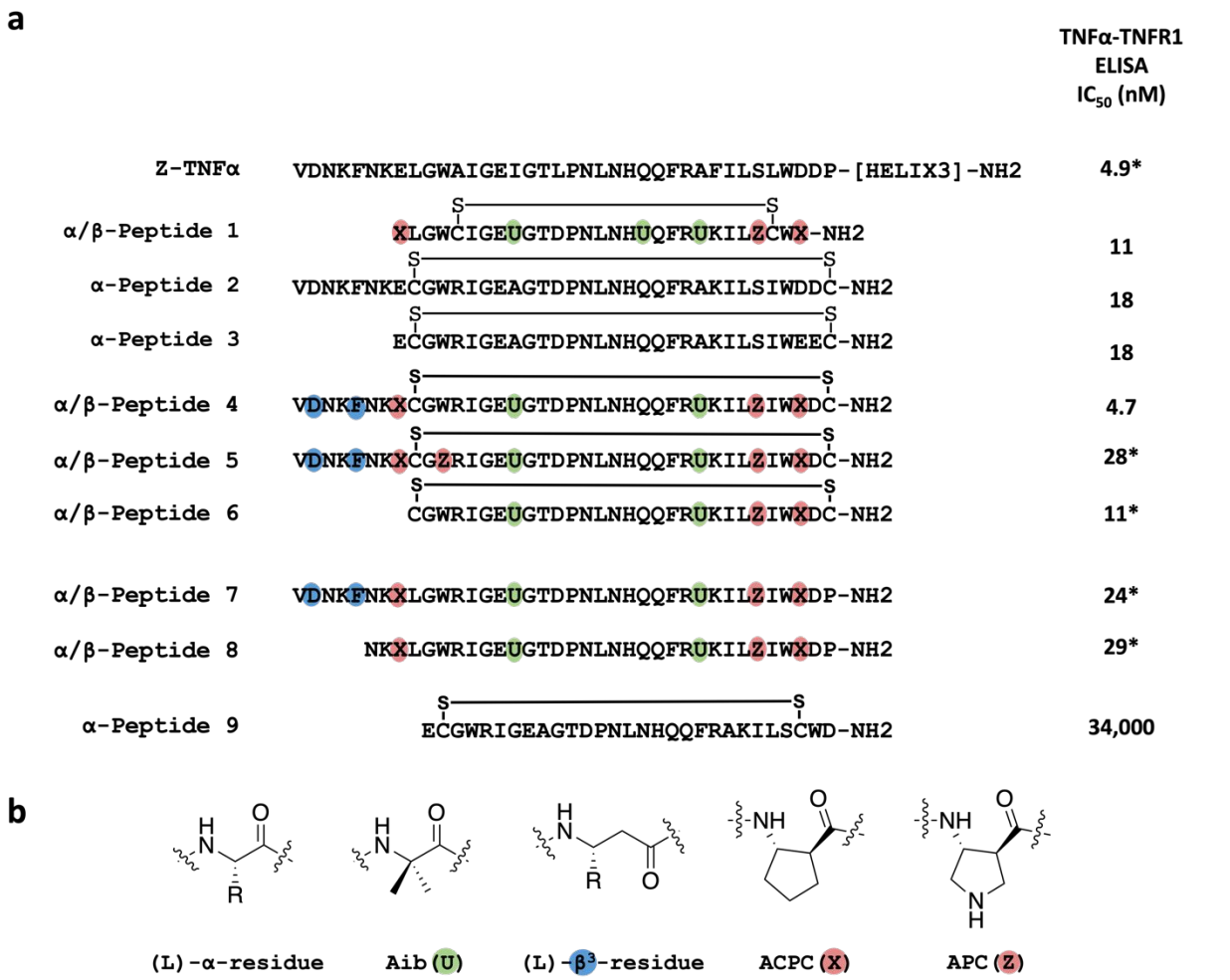


Figure S1: List of α and α/β-peptides (**1-9**) derived from Z-TNFα and corresponding IC_{50} values determined by TNFα/TNFR1 ELISA competition assays. α/β-peptides **4-8** were used for TNFα co-crystallization but failed to give high resolution data sets. The α-peptide **9** was synthesized and tested in parallel with α-peptide **3** as part of our effort to shorten α-peptide **2** and obtain high-resolution X-ray crystallography data. *IC_{50} values that were reported previously.[13] [Helix3] = SQSANLLAEAKKLNDAQAPK. (**b**) Structures of a generic (L)-α-residue, an Aib residue (U) (green), a (L)-β^3-residue (blue) a cyclic β-residue ACPC residue (X) (red), and a cyclic β-residue APC residue (Z) (red).

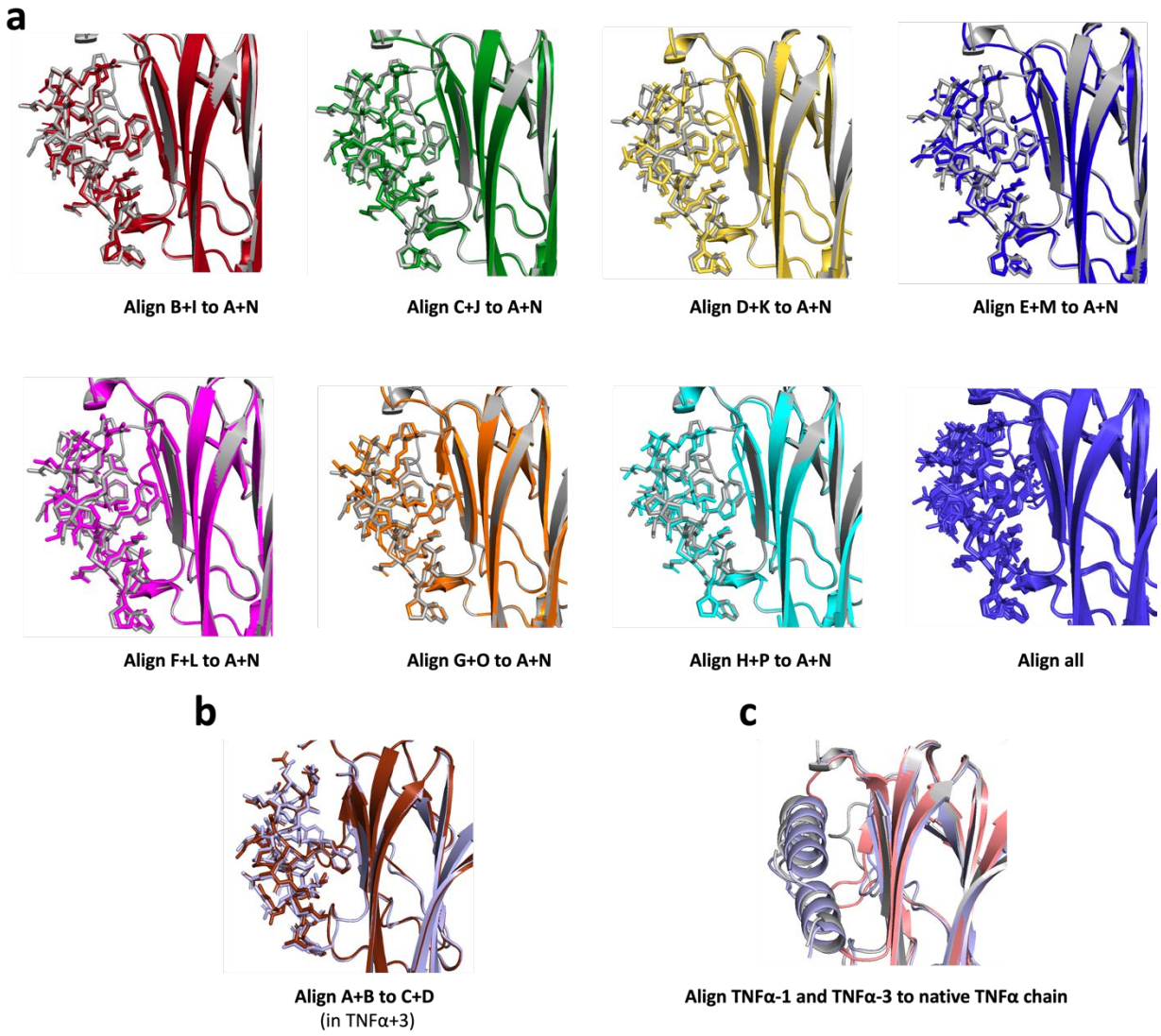


Figure S2: (**a**) Alignments of eight TNFα+**1** protomers found in the asymmetric unit in 7TA6. (**b**) Alignment of two TNFα+**3** protomers found in the asymmetric unit in 7TA3. (**c**) Alignment of TNFα+**1** (light grey) and TNFα+**3** (light purple) to native TNFα monomer (salmon). All atoms in TNFα-**1** protomer (TNFα+peptide **1)** and TNFα-**3** protomer (TNFα+peptide **3**) were aligned to native TNFα monomer (PDB ID: 1TNF). The displacements (RMSD) of each alignment are summarized in Table **S1** and **S2**, calculated using command align on Pymol.

Table S1. Comparison of TNFα+peptide protomers in the asymmetric unit

	RMSD[α] (Å)	
Chain NO.	All atoms	C-alpha
Aligned to A+N in TNFα+1		
A+N in TNFα+**1**	--	--
B+I in TNFα+**1**	0.394	0.302
C+J in TNFα+**1**	0.341	0.247
D+K in TNFα+**1**	0.358	0.290
E+M in TNFα+**1**	0.395	0.305
F+L in TNFα+**1**	0.356	0.288
G+O in TNFα+**1**	0.278	0.206
H+P in TNFα+**1**	0.369	0.268
Aligned to A+B in TNFα+3		
A+B in TNFα+**3**	--	--
C+D in TNFα+**3**	0.415	0.334
A+N in TNFα+1	**0.579**	**0.320**

[α] Root mean square deviation (RMSD) between corresponding atoms after pairwise superposition of TNFα+peptide chains found in the asymmetric units from complexes TNFα+**1** and TNFα+**3**.

Table S2. Comparison of TNFα chains in the asymmetric unit

Aligned to native TNFα single chain	**RMSD[α] (Å)**	
Chain NO.	All atoms	C-alpha
A in TNFα+**1**	0.710	0.534
B in TNFα+**1**	0.680	0.523
C in TNFα+**1**	0.714	0.514
D in TNFα+**1**	0.718	0.553
E in TNFα+**1**	0.698	0.605
F in TNFα+**1**	0.709	0.538
G in TNFα+**1**	0.697	0.528
H in TNFα+**1**	0.656	0.527
Average in TNFα+1	**0.698 (±0.019)**	**0.540 (±0.027)**
B in TNFα+**3**	0.705	0.582
D in TNFα+**3**	0.734	0.539
Average in TNFα+3	**0.720 (±0.015)**	**0.561 (±0.022)**

[α] Root mean square deviation (RMSD) between corresponding atoms after pairwise superposition of TNFα chains found in the asymmetric units from complexes TNFα+**1** and TNFα+**3** to native TNFα monomer in 1TNF.

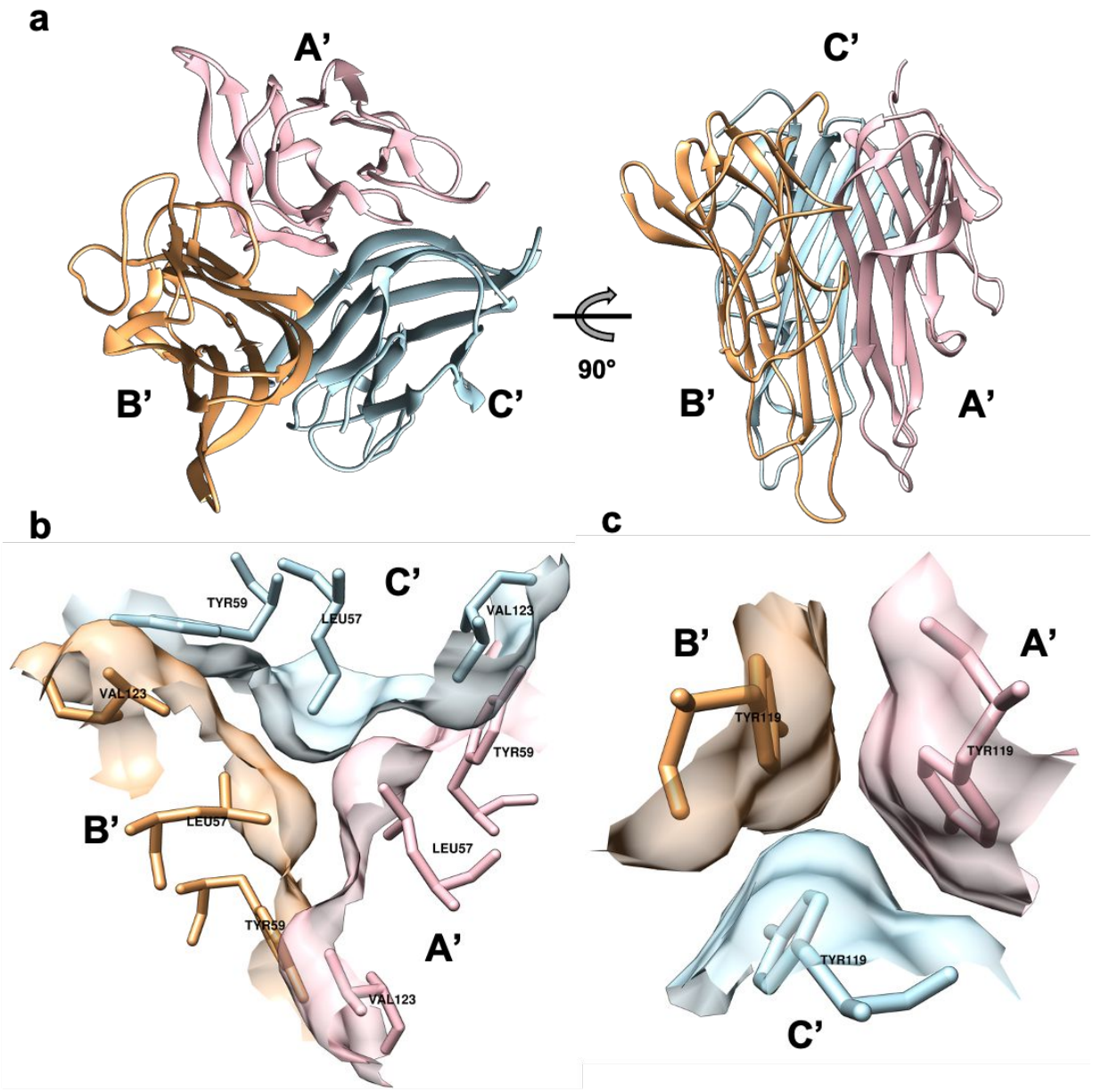


Figure S3: The structure of native TNFα (PDB code: 1TNF).[8] (**a**) Three subunits were arbitrarily assigned as A' (pink), B' (orange) and C' (blue). (**b**) and (**c**) Top and bottom zoom-in views highlighting some of the hydrophobic interactions that facilitate TNFα trimeric conformation. Parts of the main chains were hidden for visual clarity.

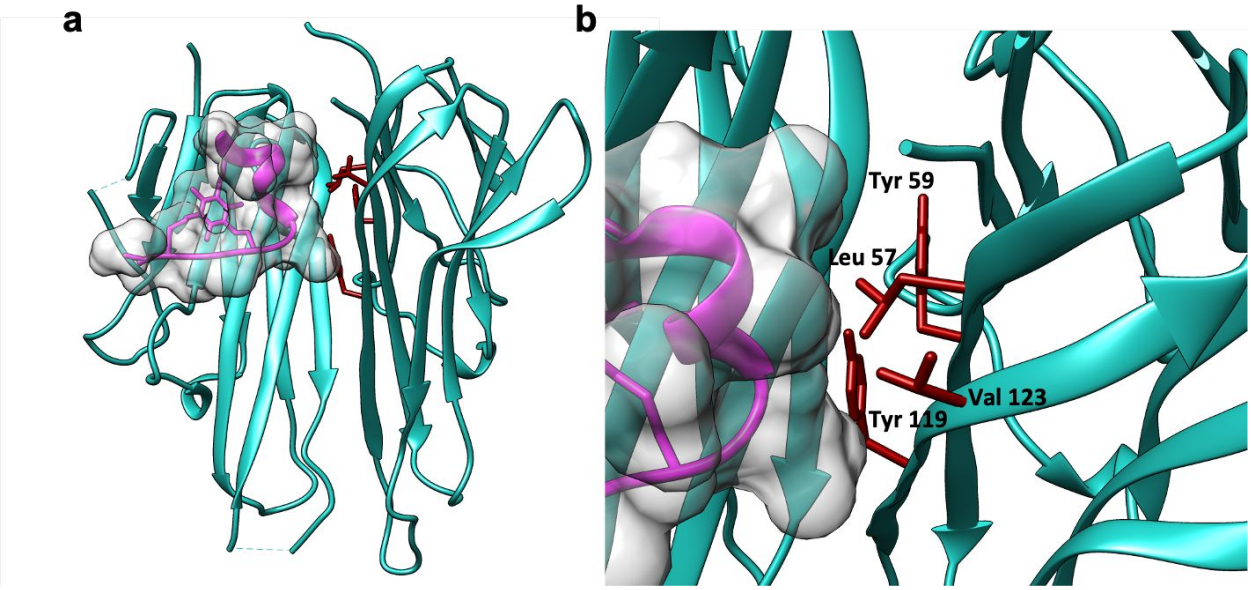


Figure S4: (**a**) Co-crystal structure of cyclic peptide M21 (in magenta ribbon and light grey volume) bound to dimeric TNFα (in cyan) (PDB ID: 4TWT).[14] (**b**) Zoom-in view of the surface of peptide M21 bound to dimeric TNFα. The peptide volume (in grey) does not clash with the key TNFα interface residues (Leu^{57}, Tyr^{59}, Tyr^{119}, and Val^{123}), in contrast to the clashes we would expect for α/β-peptide **1** (Figure 3) or α-peptide **3** (Figure 4).

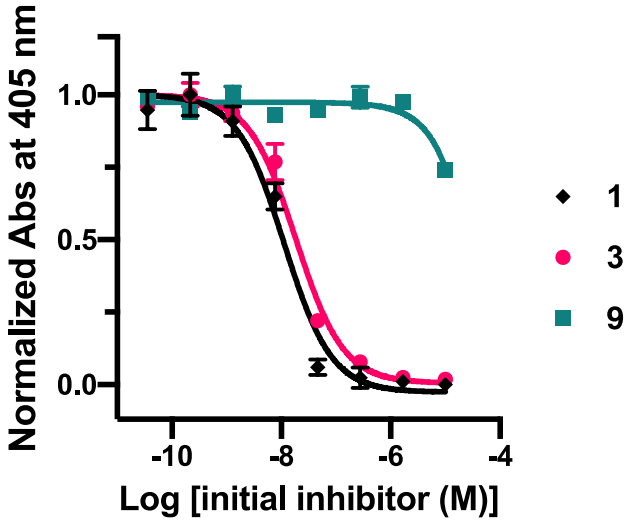


Figure S5: Dose response curves of α/ β -peptide **1**, α-peptide **3** and α-peptide **9** in the TNFα/TNFR1 competition ELISA assay. The calculated IC_{50} values of **1**, **3** and **9** are 11, 18 and 34,000 nM, respectively. Data points represent standard derivation (SD) from the mean. Each experiment was repeated at least three times.

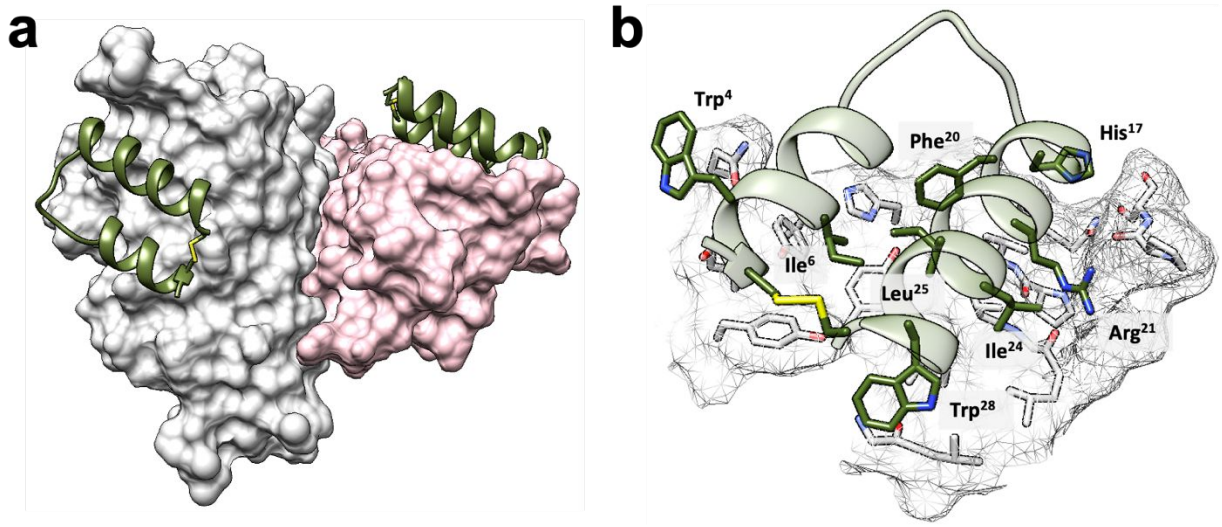


Figure S6. Crystal structure of α-peptide **3** bound to TNFα with a 1:1 stoichiometry (PDB ID: 7TA3). **(a)** Front views of the asymmetric unit, which contains two copies α-peptide **3** (in green, intramolecular disulfide in yellow) and two copies of TNFα monomer (light grey and pink). Two TNFα subunits pack against each other perpendicularly, in contrast to the parallel packing in the native TNFα trimer. **(b)** Side chains of the α-peptide **3** that contact the TNFα surface are shown, including those from Leu^{2}, Trp^{4}, Ile^{6}, His^{17}, Phe^{20}, Arg^{21}, Ile^{24}, Leu^{25}, and Trp^{28}, which are conserved with **1**.

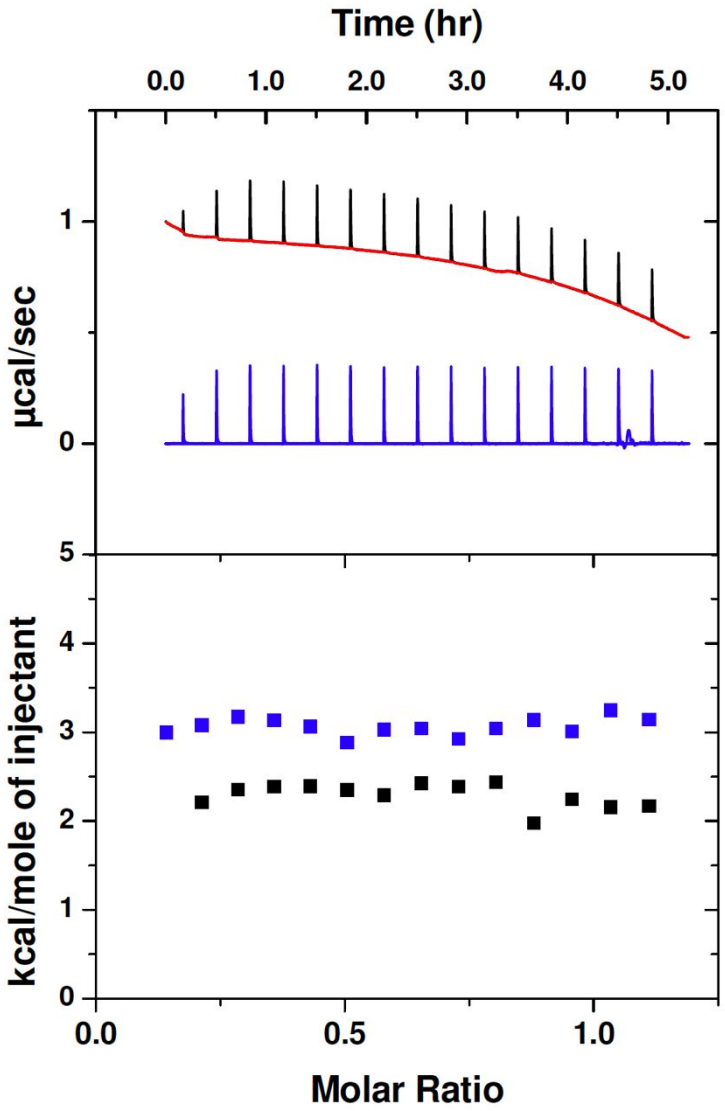


Figure S7: Controls for the isothermal titration calorimetry (ITC) assay for peptide **3** titrated into buffer (**blue**) and into TNFα (**black**). ***Blue:*** In the peptide-to-buffer control, a large endothermic dilution heat (represented by positive titration peaks) was observed, presumable due to the solvation of the highly charged peptide. Similar effects were observed with the other peptides. ***Black:*** When the titrations were initially performed in PBS without TWEEN-20, only the peptide dilution heat was detected, presumably due to the slow binding (with trimer dissociation) of TNFα with the peptide in the absence of TWEEN-20 as reported previously.[13] When 0.05% TWEEN-20 was included in the buffer (Figure **4c**), noticeable exothermic events (represented by negative titration peaks) were detected by the calorimeter, suggesting that peptide binding occurs and is fast enough to be detected, although the endothermic peptide dilution heat was seen as well (in **blue**). The dependency on TWEEN-20 in these ITC assays is consistent with our hypothesis that peptide-TNF*α* binding is accelerated by detergents like TWEEN-20.[13]

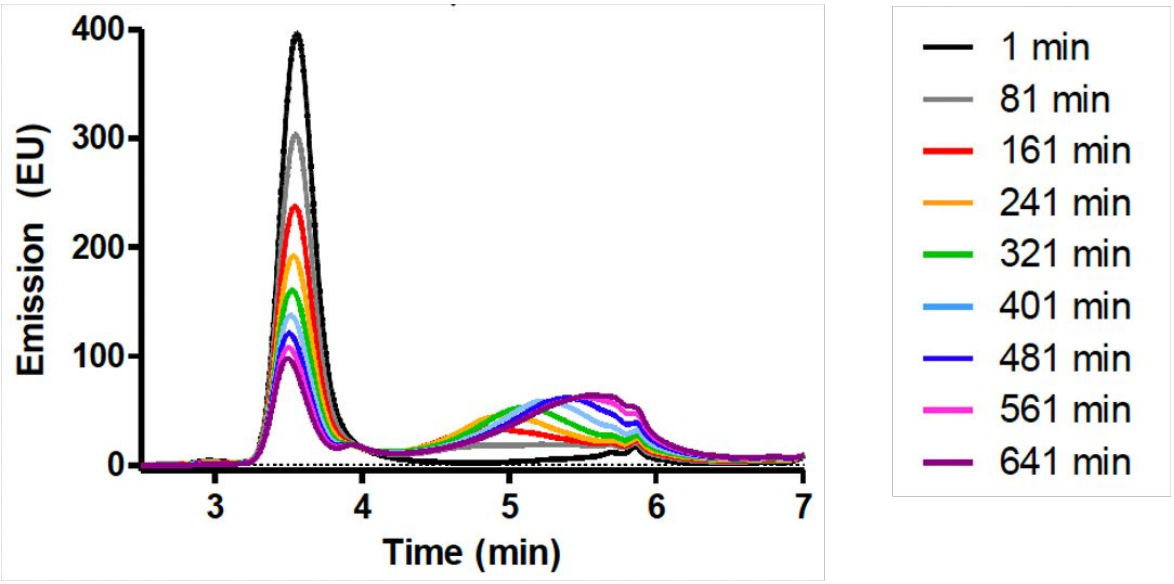


Figure S8: UPLC-SEC analysis of TNFα dissociation induced by peptide **2** in PBS at 37 °C. TNFα was labeled with FITC to enable fluorescence UPLC-SEC. Elution was monitored at 495 nm (emission at 519 nm). The FITC-TNFα trimer eluted at 3.5 minutes. Upon peptide **2** addition, the size of the trimeric peak (eluted at 3.5 min) slowly decreases over time, and the other peak (eluted between 4.5 to 6 min) grows over time. The later peak (eluted between 4.5 to 6 min) corresponds to dissociated TNFα, although we cannot determine from these measurements whether the dissociated form is a monomer, a dimer or a combination of monomer and dimer. This observation suggests that our peptides induce dissociation of the TNFα trimer to a smaller species in solution. ([FITC-TNFα] = 3 μM; [peptide **2**] = 50 μM. The TNFα and peptide preincubation and size-exclusion chromatography were both carried out in PBS at 37 °C. Stationary phase: Protein BEH SEC, 1.7 μm, 4.6 mm x 150 mm.)

Table S3. IC_{50} Values of Peptide Antagonists and Hydrophobically Modified Derivatives Measured by TNFα – TNFR1 Competition ELISA

(pre-inc. for 1 hr, at RT, with 0.05% TWEEN20)

Peptide	***IC_{50} (nM)***
1	11
$(C_{12})_2$-PEG$_{16}$-***1***	450
2	18
$(C_{12})_2$-PEG$_{16}$-***2***	270
4	4.7
$(C_{12})_2$-PEG$_{16}$-***4***	33

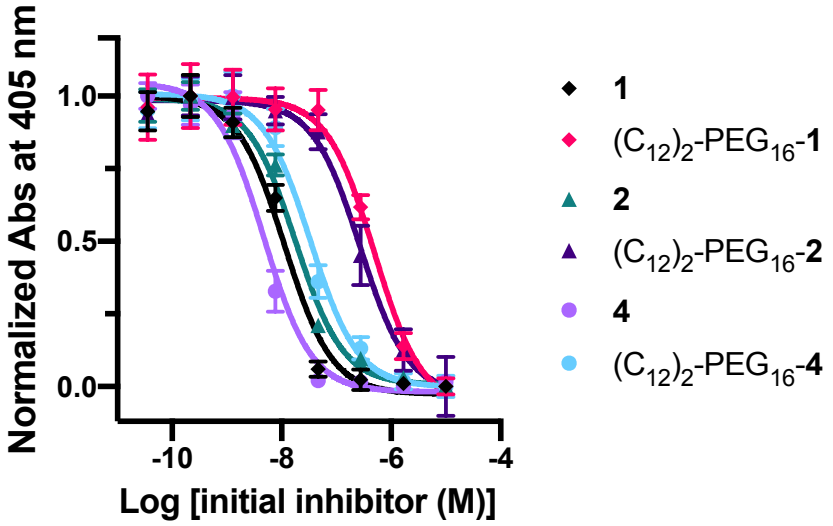


Figure S9. Evaluation of peptides **1**, **2**, **4** and their hydrophobically modified analogues in their abilities to block TNFα-TNFR1 interaction by TNFα-TNFR1 competition ELISA. IC_{50} values are summarized in Table S3. Peptides were pre-incubated with 80 ng/mL TNFα in PBS containing 0.05% TWEEN20 at room temperature for 1 hour. Data points presented as mean ± SD with three independent experiments (N = 3).

Table S4. IC_{50} values and Maximal Efficacy of Peptide 4 and $(C_{12})_2$-PEG_{16}-4 in WEHI-13VAR Viability Assays as a Function of Pre-Incubation Time

(Pre-inc for 1-24 hr, at 37 °C, without TWEEN 20)

Pre-Inc Time (hr)	Peptide	IC_{50} (nM)[α]	Max. Efficacy (%)[β]
1	**4**	ND[γ]	0
	$(C_{12})_2$-PEG_{16}-**4**	360	19
2	**4**	ND	0
	$(C_{12})_2$-PEG_{16}-**4**	970	36
4	**4**	1.9	15
	$(C_{12})_2$-PEG_{16}-**4**	190	64
6	**4**	7.7	25
	$(C_{12})_2$-PEG_{16}-**4**	210	73
12	**4**	4.7	64
	$(C_{12})_2$-PEG_{16}-**4**	61	95
24	**4**	4.0	85
	$(C_{12})_2$-PEG_{16}-**4**	21	91

α. Concentrations necessary for 50% protection from TNFα-induced death (IC_{50}) measured with WEHI-13VAR cells after the indicated time period of TNFα–peptide pre-incubation.
β. Maximal efficacy (%) is the relative percentage of cells rescued from TNFα-induced cell death after the indicated time period of TNFα–peptide pre-incubation.
γ. Not determined (ND)

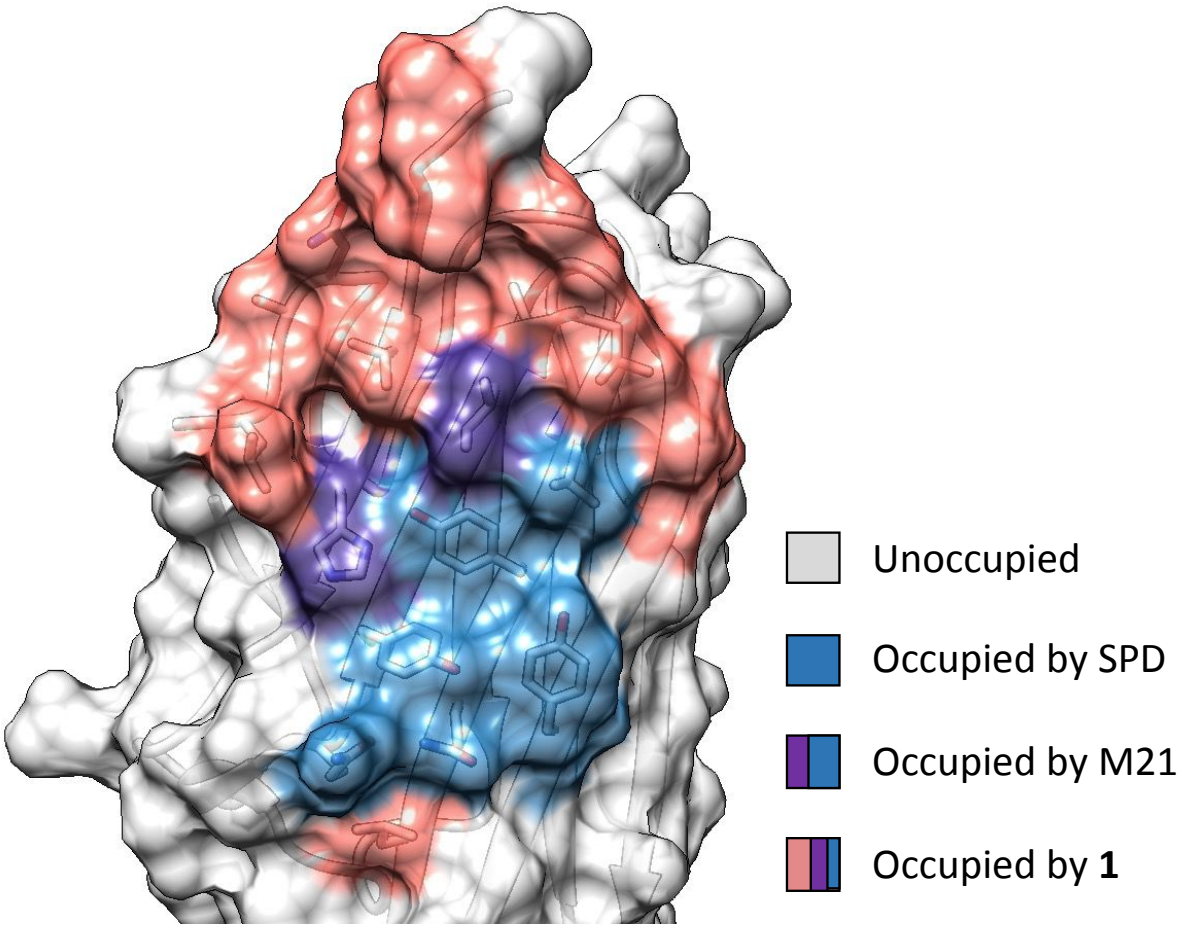


Figure S10: Comparison of surface areas on a TNFα monomer that are covered by SPD, M21 or α/β-peptide **1**.

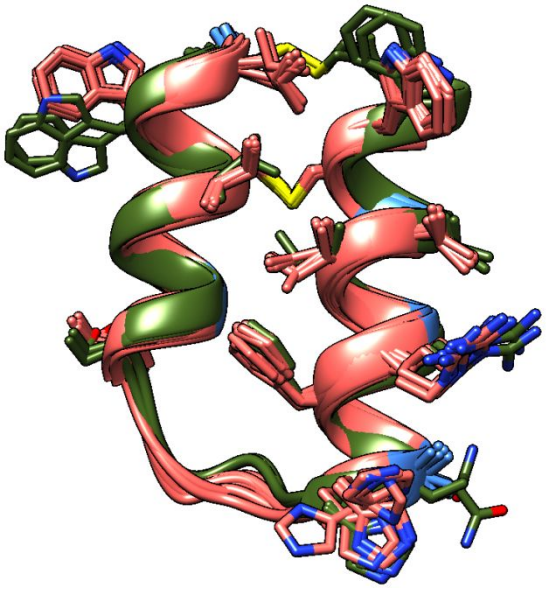
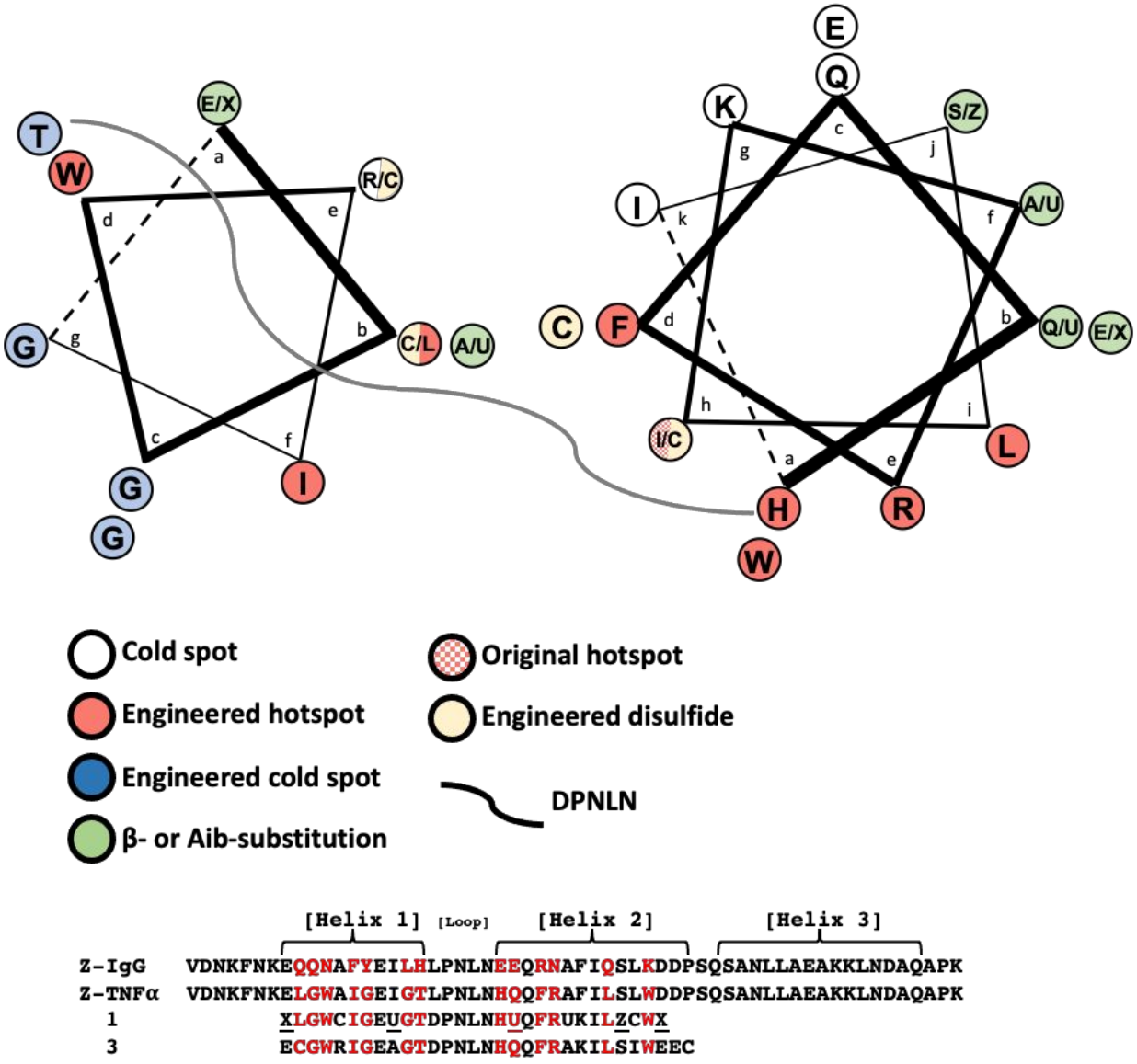


Figure S11: The alignments of all peptides found in the asymmetric units from structures TNFα+**1** (**1** in pink) and TNFα+**3** (**3** in green). Note that the locations of the disulfide crosslink differ between **1** and **3**.

Table S5. Comparison of peptide chains in the asymmetric unit

	RMSD[α] (Å)	
Chain NO.	All atoms	C-alpha
Aligned to chain I in TNFα+1		
I in TNFα+**1**	--	--
J in TNFα+**1**	0.321	0.293
K in TNFα+**1**	0.442	0.373
L in TNFα+**1**	0.412	0.330
M in TNFα+**1**	0.359	0.273
N in TNFα+**1**	0.366	0.325
O in TNFα+**1**	0.459	0.382
P in TNFα+**1**	0.414	0.374
Aligned to chain A in TNFα+3		
A in TNFα+**3**	--	--
C in TNFα+**3**	0.328	0.231
I in TNFα+**1**	0.823	0.710

[α] Root mean square deviation (RMSD) between corresponding atoms after pairwise superposition of peptide chains found in the asymmetric units from complexes TNFα+**1** and TNFα+**3**.




```
                    [Helix 1] [Loop]    [Helix 2]        [Helix 3]
Z-IgG    VDNKFNKEQQNAFYEILHLPNLNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDAQAPK
Z-TNFα   VDNKFNKELGWAIGEIGTLPNLNHQQFRAFILSLWDDPSQSANLLAEAKKLNDAQAPK
  1              XLGWCIGEUGTDPNLNHUQFRUKILZCWX
  3              ECGWRIGEAGTDPNLNHQQFRAKILSIWEEC
```

Figure S12: (**TOP**) "Helix-loop-helix" diagrams of peptides **1** and **3**. The TNFα-contacting residues are designated "hotspots," and the residues that do not form contacts with TNFα are designated "cold spots." (**BOTTOM)** Peptide sequences of original Z domain, Z-IgG, that was used as a starting point in the phage display study; the phage display-derived TNFα-targeting Z domain, Z-TNFα; and peptides **1** and **3**.[15] The positions highlighted in red were varied in the phage display process.

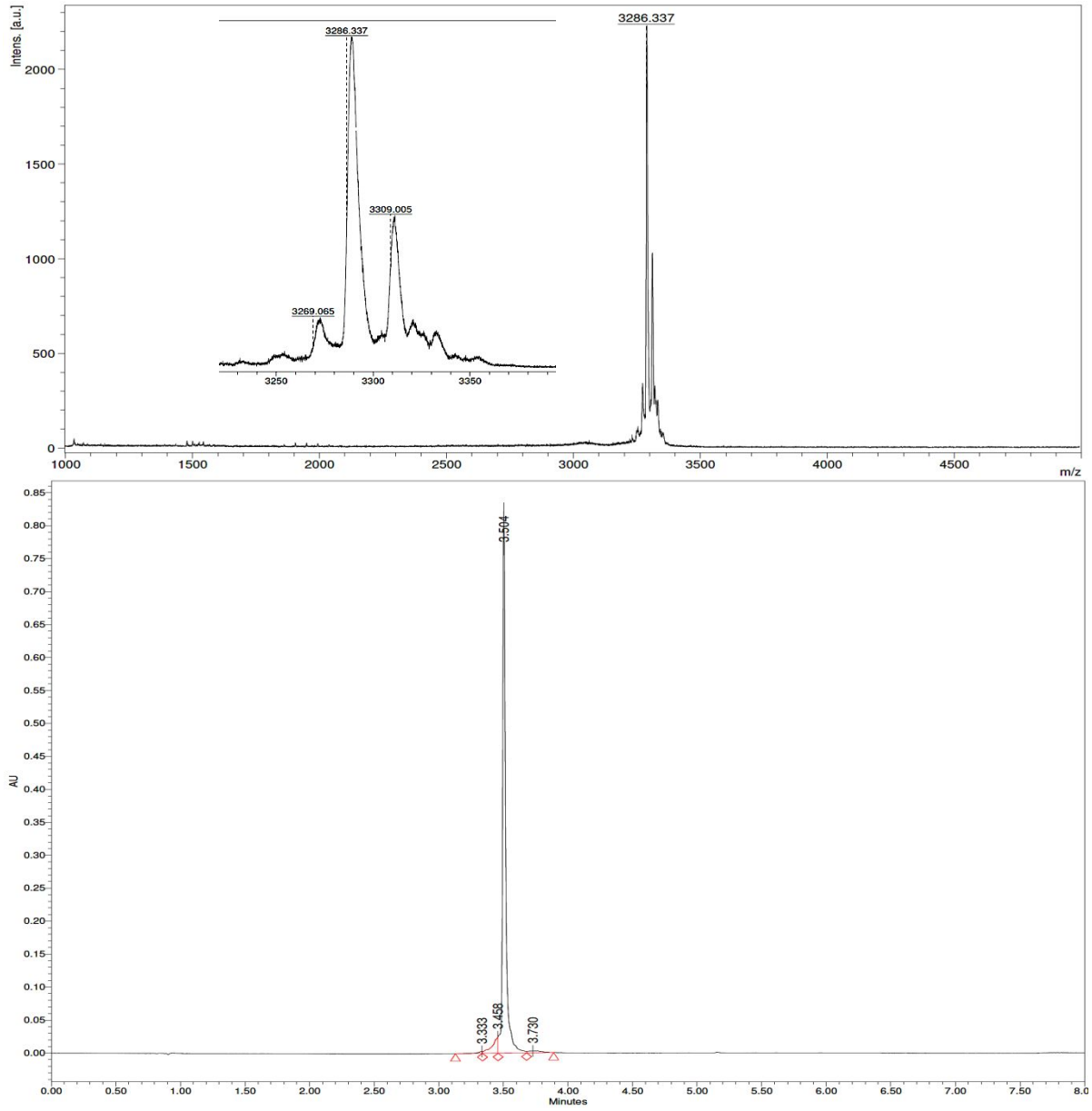
Table S6. TNFα+1 Data Collection and Refinement Statistics

PDB accession code	**7TA6**
Beam line	GM/CA @APS
Wavelength	1.033
Resolution range	39.74 - 2.672 (2.767 - 2.672)
Space group	C 1 2 1
Unit cell	143.774 143.552 76.888 90 97.847 90
Total reflections	298794 (30736)
Unique reflections	43722 (4361)
Multiplicity	6.8 (7.0)
Completeness (%)	99.86 (99.98)
Mean I/sigma(I)	10.58 (0.99)
Wilson B-factor	75.67
R-merge	0.1127 (1.816)
R-meas	0.1222 (1.959)
R-pim	0.04671 (0.7318)
CC1/2	0.998 (0.383)
CC*	0.999 (0.744)
Reflections used in refinement	43691 (4360)
Reflections used for R-free	1997 (200)
R-work	0.2481 (0.4121)
R-free	0.2701 (0.4226)
CC(work)	0.935 (0.558)
CC(free)	0.957 (0.599)
Number of non-hydrogen atoms	10387
macromolecules	10109
ligands	481
solvent	45
Protein residues	1304
RMS(bonds)	0.002
RMS(angles)	0.55
Ramachandran favored (%)	93.78
Ramachandran allowed (%)	5.87
Ramachandran outliers (%)	0.35
Rotamer outliers (%)	0.66
Clashscore	3.76
Average B-factor	92.41
macromolecules	92.83
ligands	77.79
solvent	73.89
Number of TLS groups	16

Table S7. TNFα+3 Data Collection and Refinement Statistics

PDB accession code	**7TA3**
Beam line	LS-CAT @APS
Wavelength	1.127
Resolution range	43.28 - 2.5 (2.589 - 2.5)
Space group	P 43 21 2
Unit cell	86.56 86.56 143.73 90 90 90
Total reflections	518153 (51336)
Unique reflections	19581 (1910)
Multiplicity	26.5 (26.9)
Completeness (%)	99.65 (99.16)
Mean I/sigma(I)	21.82 (1.47)
Wilson B-factor	69.46
R-merge	0.112 (1.816)
R-meas	0.1142 (1.851)
R-pim	0.02214 (0.3545)
CC1/2	0.999 (0.872)
CC*	1 (0.965)
Reflections used in refinement	19527 (1894)
Reflections used for R-free	1934 (191)
R-work	0.2460 (0.3920)
R-free	0.2764 (0.4330)
CC(work)	0.943 (0.819)
CC(free)	0.930 (0.722)
Number of non-hydrogen atoms	2782
macromolecules	2767
ligands	6
solvent	13
Protein residues	353
RMS(bonds)	0.004
RMS(angles)	0.58
Ramachandran favored (%)	96.50
Ramachandran allowed (%)	3.50
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	0.34
Clashscore	6.94
Average B-factor	90.48
macromolecules	90.56
ligands	119.92
solvent	70.55
Number of TLS groups	15

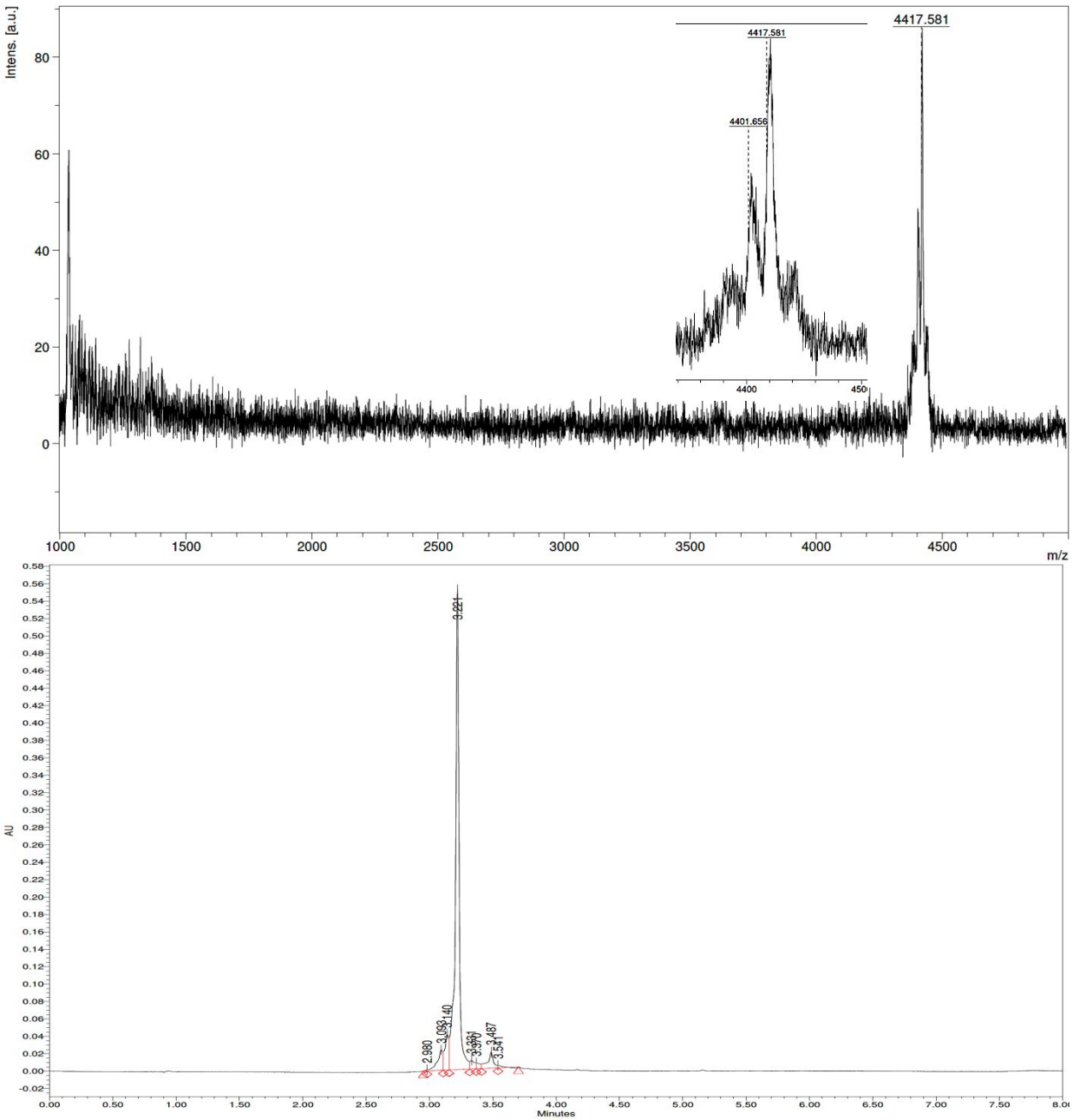
IV. MALDI-TOF-MS Spectra and UPLC Data for Synthetic Peptides



1) α/β-peptide 1: XLGWCIGEUGTDPNLNHUQFRUKILZCWX-NH_2

MALDI-TOF-MS (m/z): calculated monoisotopic $[M+H]^+$ = 3287.693, $[M+Na]^+$ = 3309.675
observed monoisotopic $[M+H]^+$ = 3286.337, $[M+Na]^+$ = 3309.005
(-16 m/z peak may correspond to aspartimide formation)

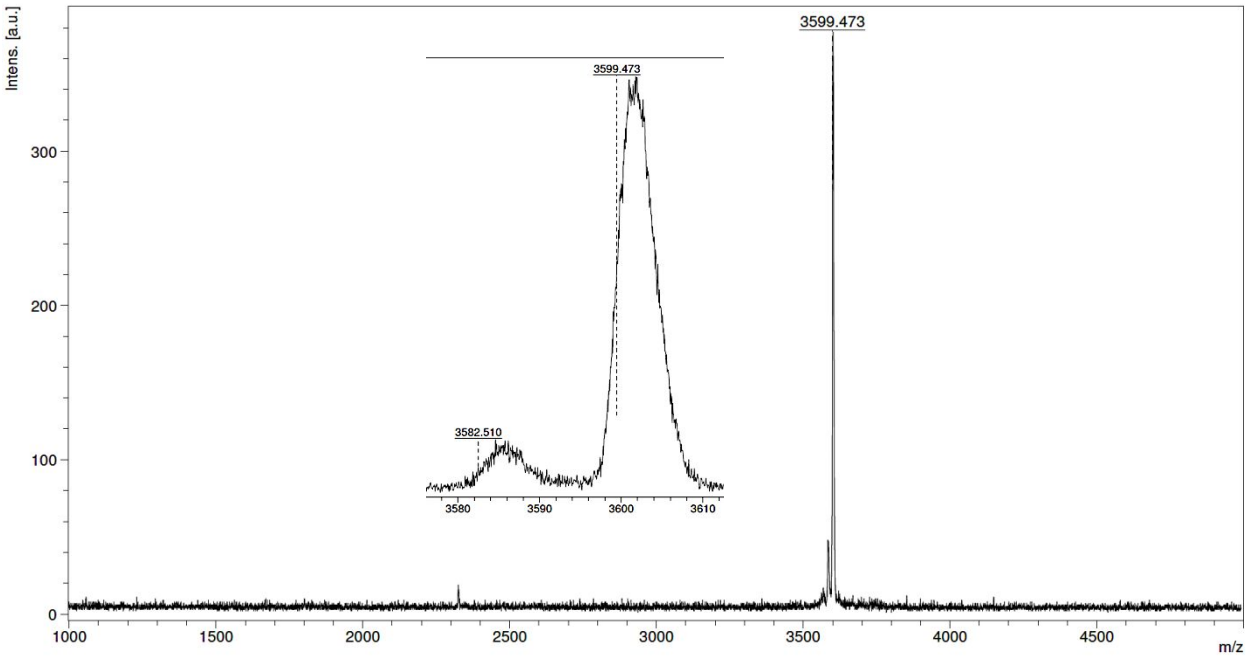
UPLC: H_2O/MeCN + 0.1% TFA, 10-95% MeCN, 5 min, 0.3 mL/min on a BEH C18 (1.7 μm, 2.1 x 50 mm) column
Purity = 93.73%

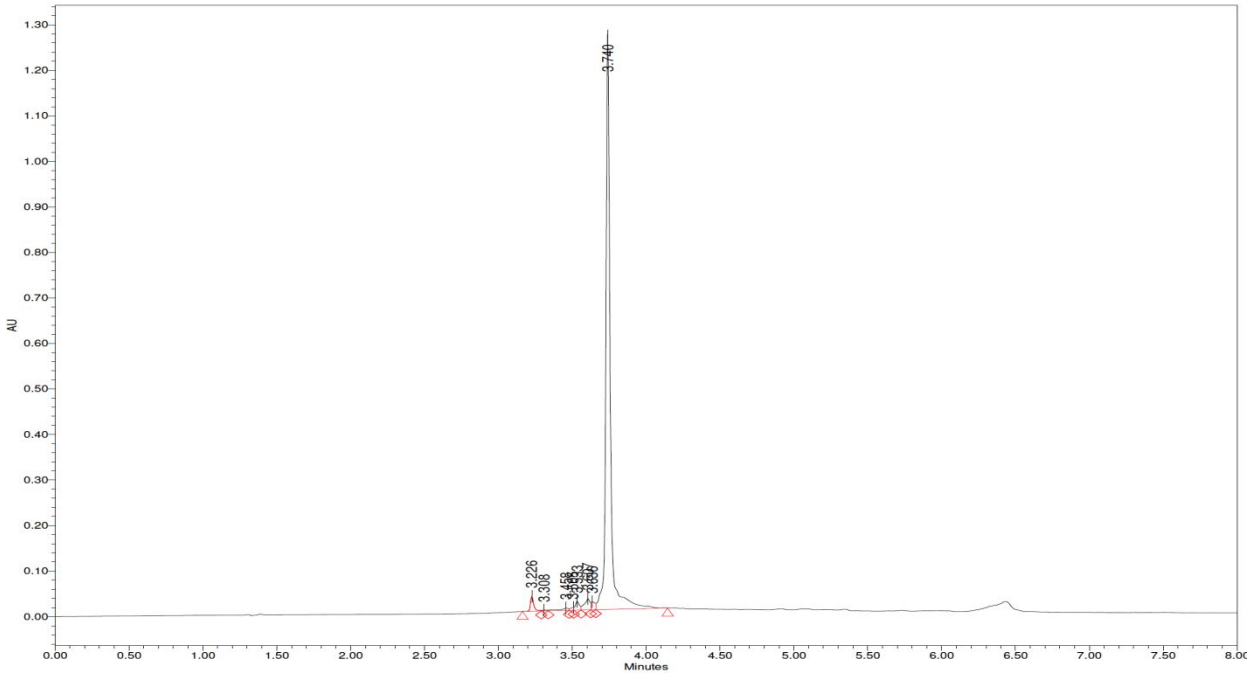


2) α-peptide 2: VDNKFNKECGWRIGEAGTDPNLNHQQFRAKILSIWDDC-NH_2

MALDI-TOF-MS (m/z): calculated monoisotopic $[M+H]^+$ = 4415.125
observed monoisotopic $[M+H]^+$ = 4417.581
-16 m/z peak may correspond to aspartimide formation

UPLC: H_2O/MeCN + 0.1% TFA, 10-95% MeCN, 5 min, 0.3 mL/min on a BEH C18 (1.7 µm, 2.1 x 50 mm) column
Purity = 80.81%

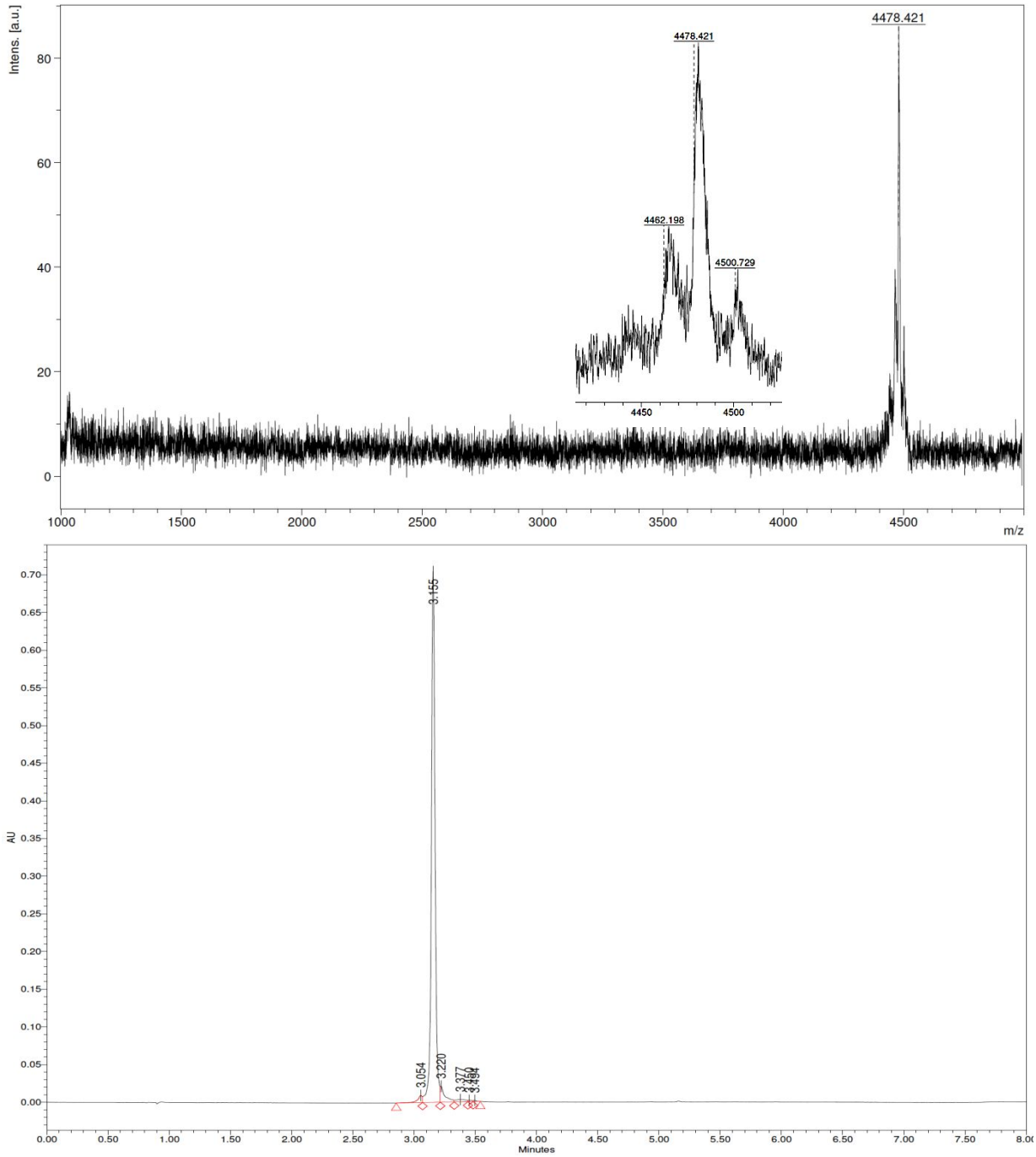




3) α-peptide 3: ECGWRIGEAGTDPNLNHQQFRAKILSIWEEC-NH_2

MALDI-TOF-MS (m/z): calculated monoisotopic $[M+H]^+$ = 3597.717
observed monoisotopic $[M+H]^+$ = 3599.473
-16 m/z peak may correspond to N-terminal pyroglutamate formation or loss of sidechain amine

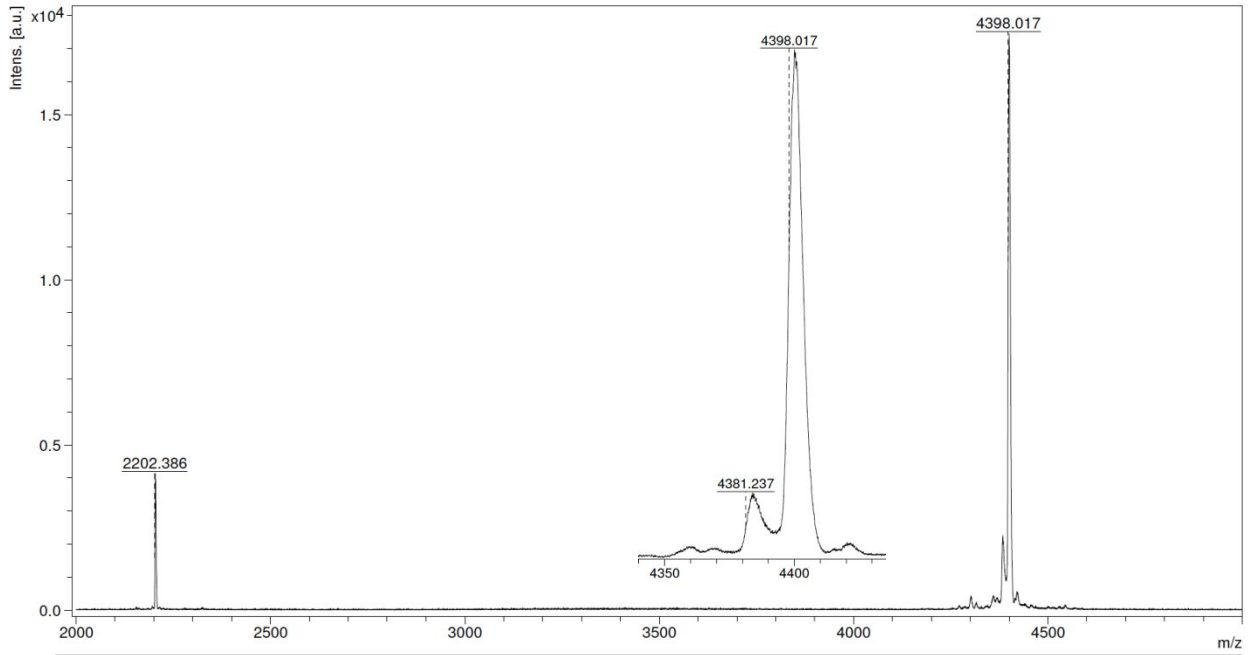
UPLC: H_2O/MeCN + 0.1% TFA, 10-95% MeCN, 5 min, 0.3 mL/min on a BEH C18 (1.7 µm, 2.1 x 50 mm) column
Purity = 92.30%

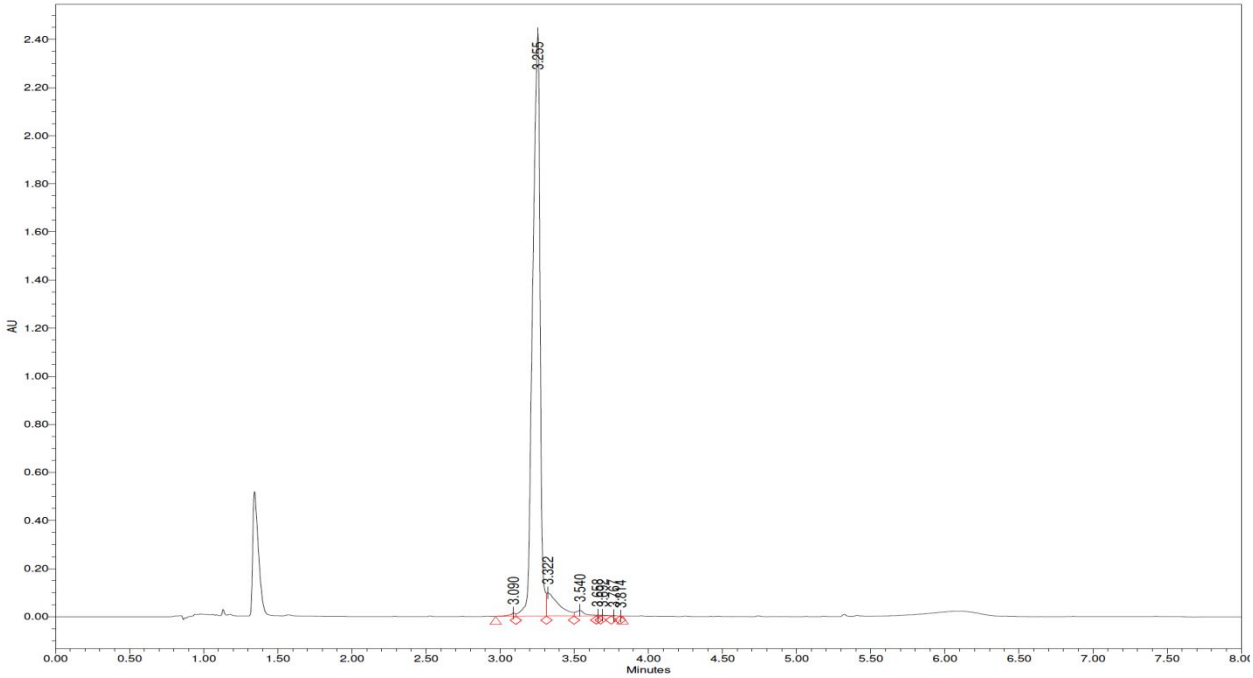


4) α/β-peptide 4: V<u>D</u>NK<u>F</u>NKXCGWRIGEUGTDPNLNHQQFRUKILZIWXDC-NH_2

<u>MALDI-TOF-MS (m/z)</u>: calculated monoisotopic $[M+H]^+ = 4475.298$, $[M+Na]^+ = 4497.280$
observed monoisotopic $[M+H]^+ = 4478.421$, $[M+Na]^+ = 4500.729$
-16 m/z peak may correspond to aspartimide formation

<u>UPLC</u>: H_2O/MeCN + 0.1% TFA, 10-95% MeCN, 5 min, 0.3 mL/min on a BEH C18 (1.7 µm, 2.1 x 50 mm) column
Purity = 93.53%

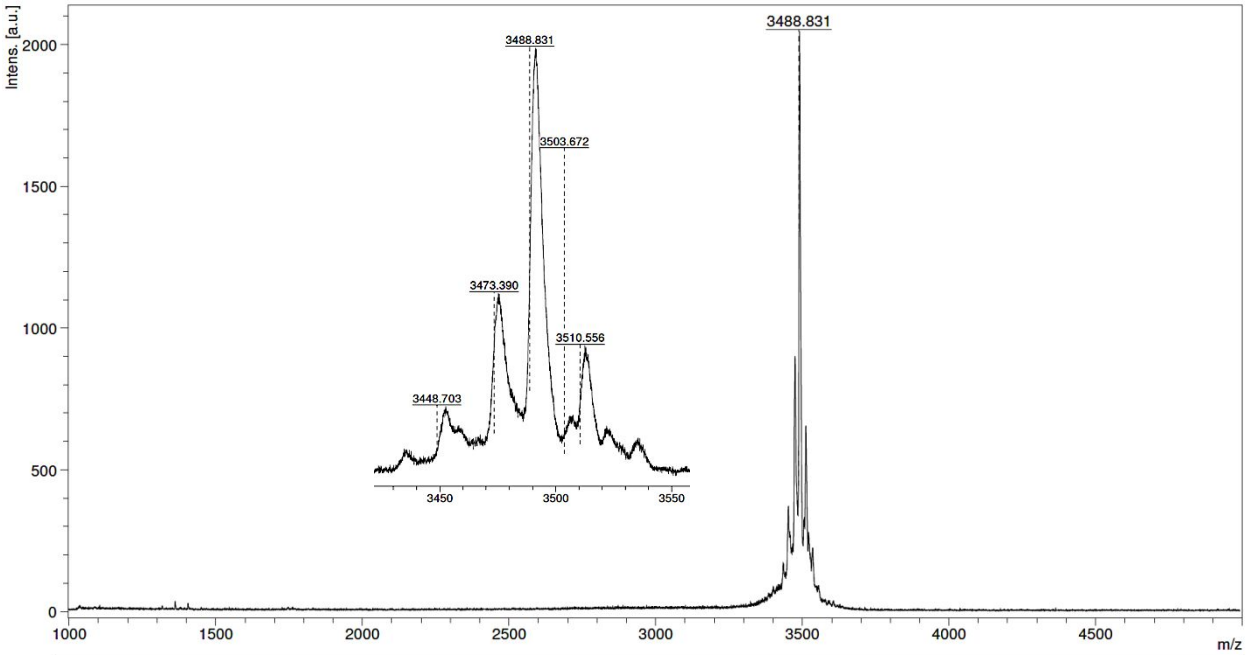


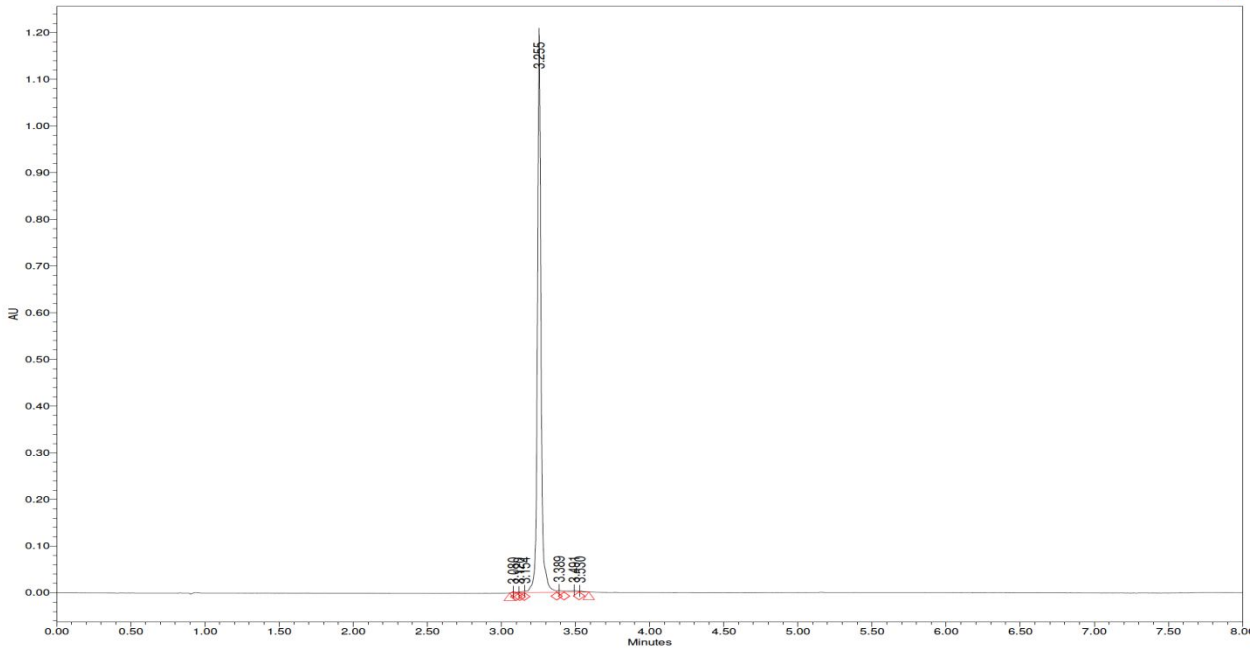


5) α/β-peptide 5: V<u>D</u>NK<u>F</u>NKXCGZRIGEUGTDPNLNHQQFRUKILZIWXDC-NH_2

<u>MALDI-TOF-MS (m/z)</u>: calculated monoisotopic $[M+H]^+$ = 4401.287, $[M+2H]^{2+}$ = 2201.147
observed monoisotopic $[M+H]^+$ = 4398.017, $[M+2H]^{2+}$ = 2202.386
-17 m/z peak may correspond to aspartimide formation

<u>UPLC</u>: H_2O/MeCN + 0.1% TFA, 10-95% MeCN, 5 min, 0.3 mL/min on a BEH C18 (1.7 µm, 2.1 x 50 mm) column
Purity = 92.23%

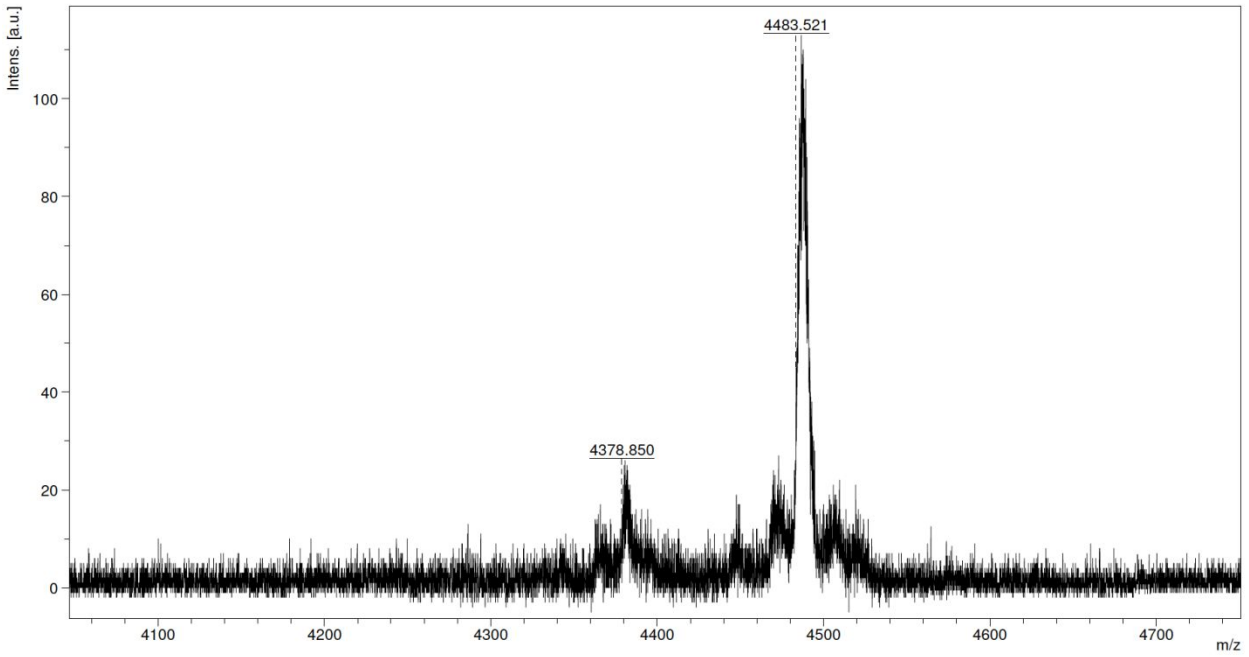


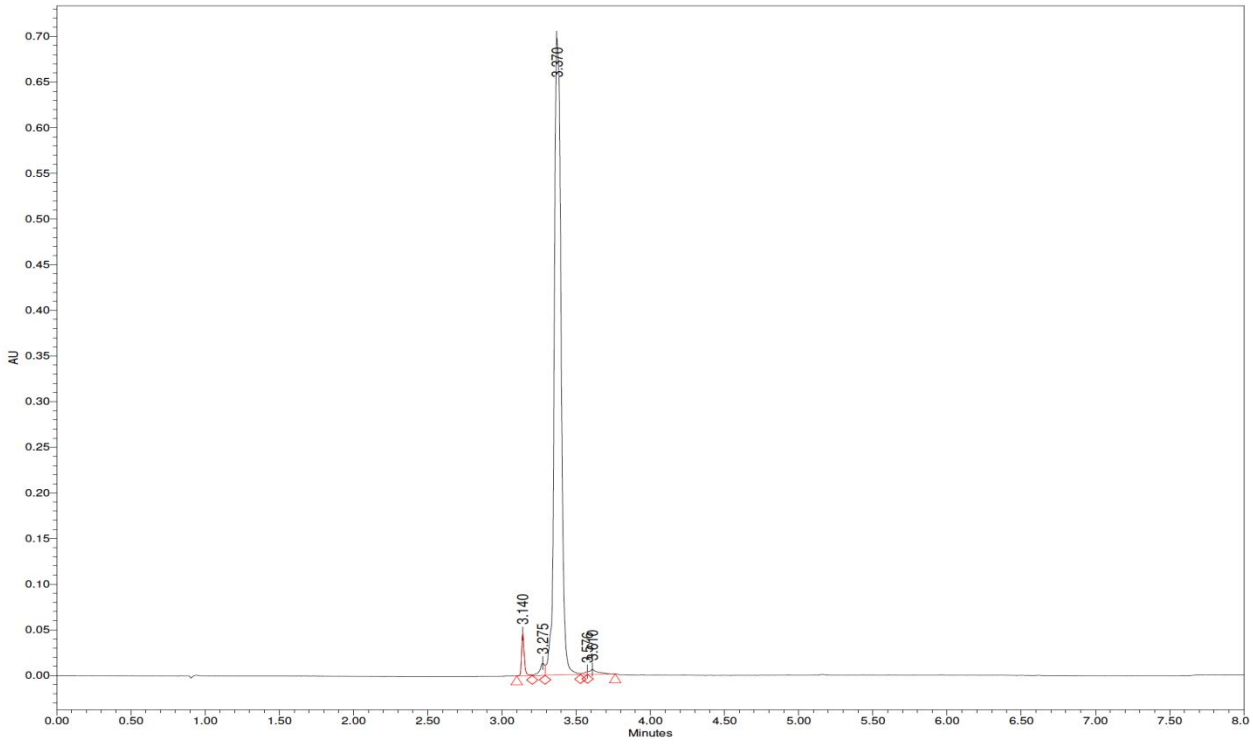


6) α/β-peptide 6: CGWRIGEUGTDPNLNHQQFRUKILZIWXDC-NH_2

MALDI-TOF-MS (m/z): calculated monoisotopic $[M+H]^+$ = 3490.690, $[M+Na]^+$ = 3512.672
observed monoisotopic $[M+H]^+$ = 3488.831, $[M+Na]^+$ = 3510.556
-16 m/z peak may correspond to aspartimide formation

UPLC: H_2O/MeCN + 0.1% TFA, 10-95% MeCN, 5 min, 0.3 mL/min on a BEH C18 (1.7 µm, 2.1 x 50 mm) column
Purity = 97.99%



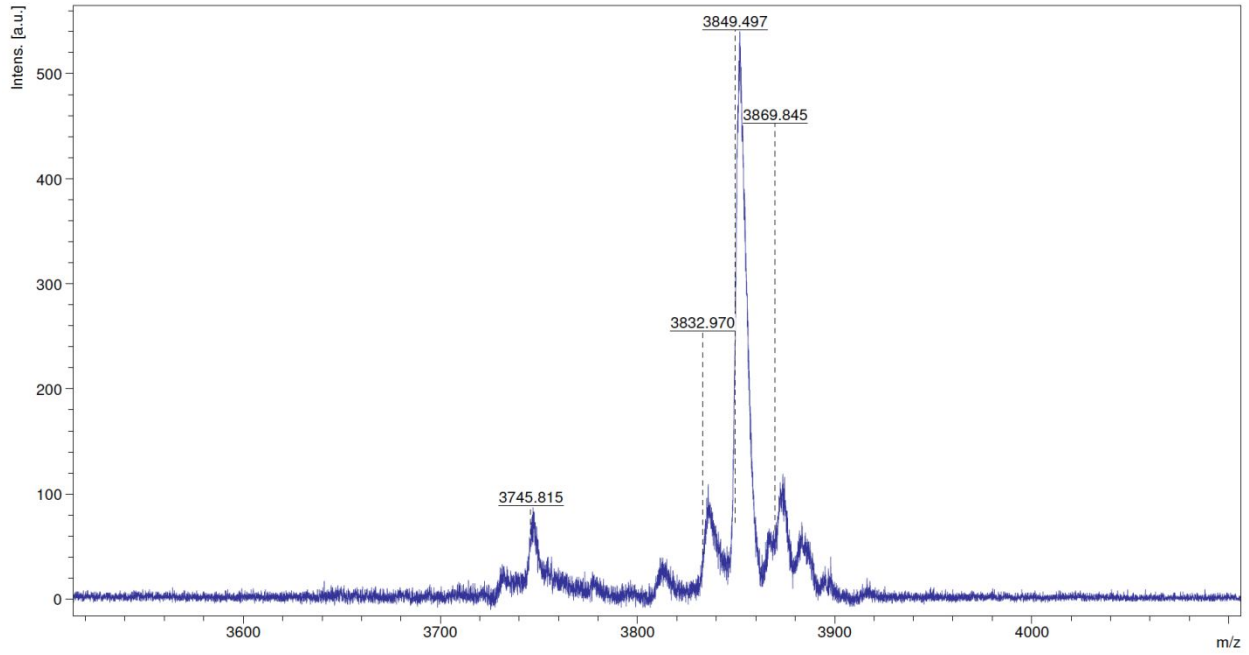


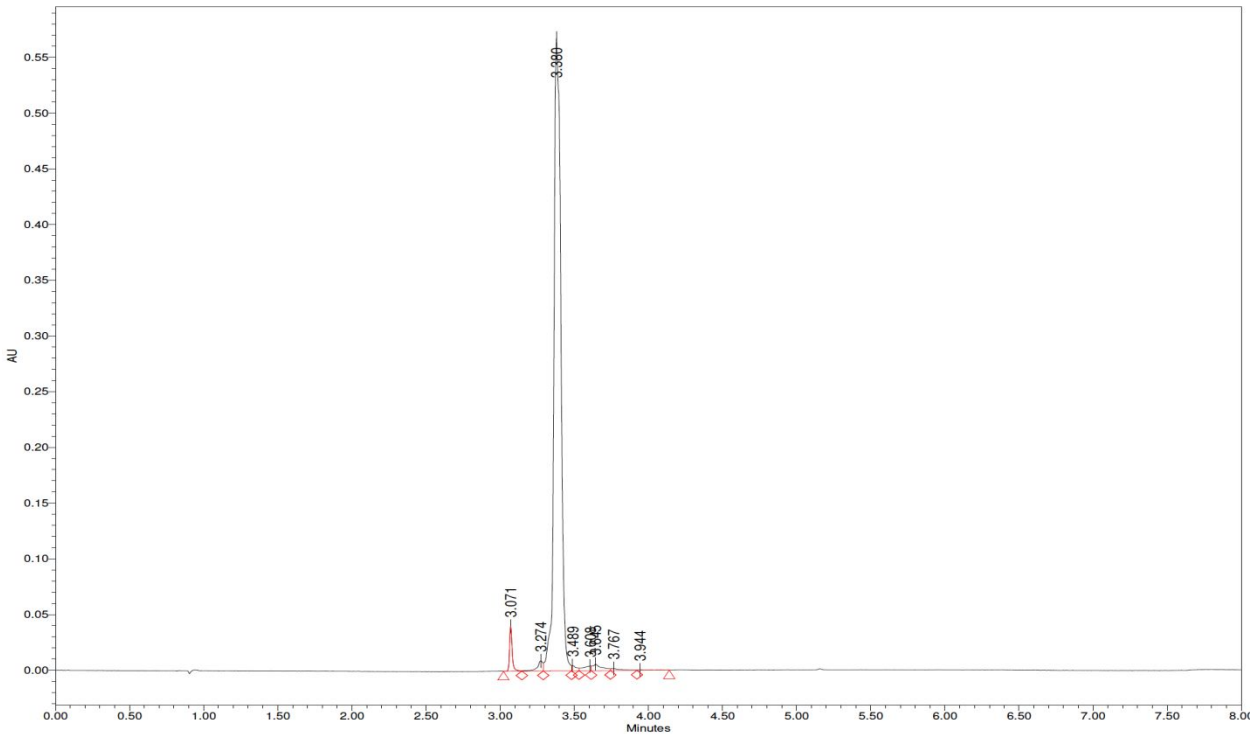
7) α/β-peptide 7: V<u>D</u>NK<u>F</u>NKXLGWRIGEUGTDPNLNHQQFRUKILZIWXDP-NH$_2$

<u>MALDI-TOF-MS (m/z)</u>: calculated monoisotopic $[M+H]^+$ = 4481.301
observed monoisotopic $[M+H]^+$ = 4483.521

<u>UPLC</u>: H_2O/MeCN + 0.1% TFA, 10-95% MeCN, 5 min, 0.3 mL/min on a BEH C18 (1.7 µm, 2.1 x 50 mm) column

Purity = 95.03%

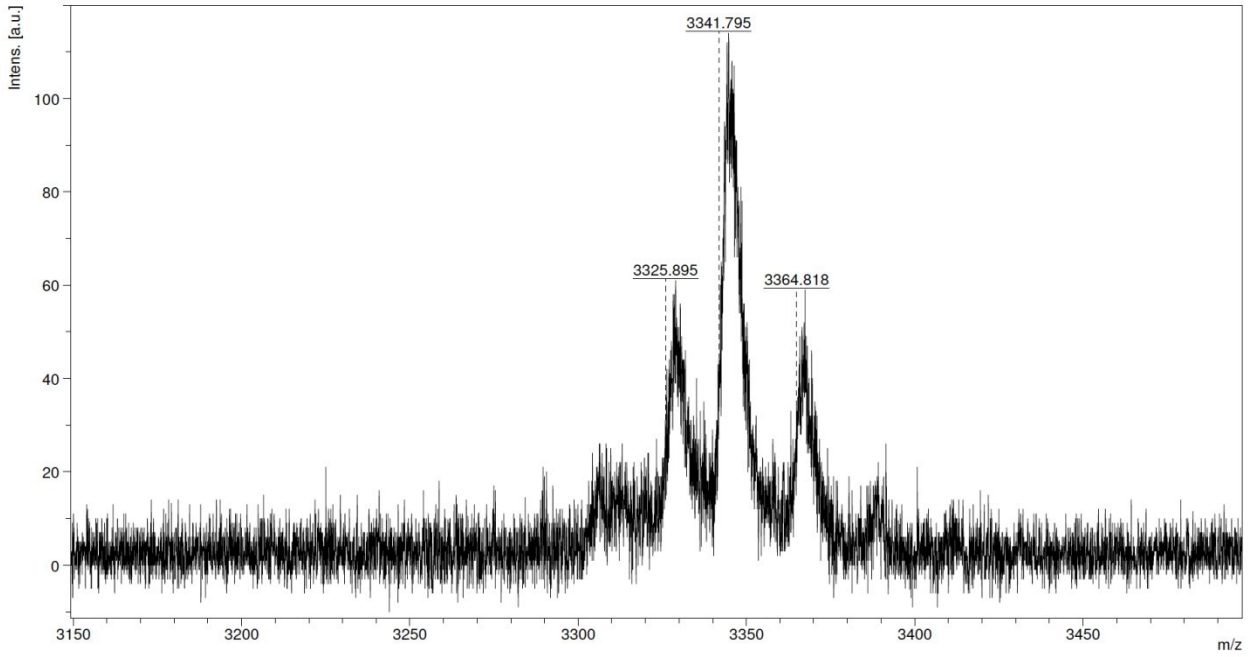


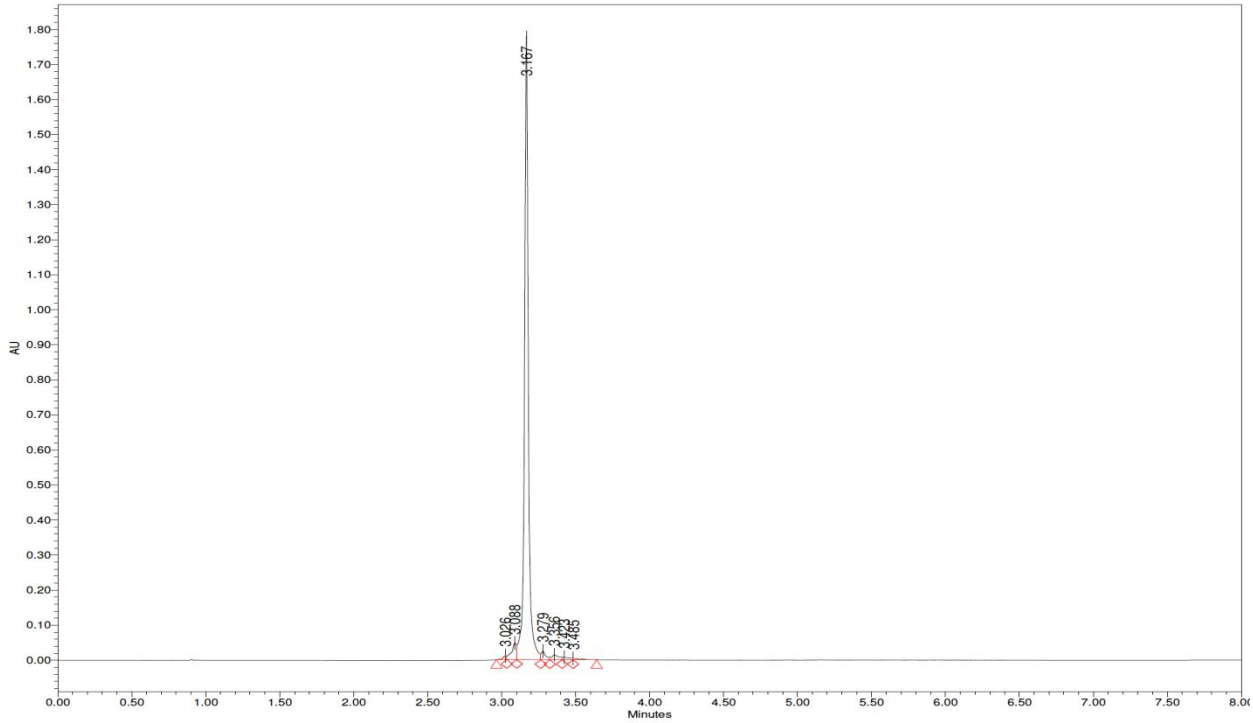


8) α/β-peptide 8: NKXLGWRIGEUGTDPNLNHQQFRUKILZIWXDP-NH_2

MALDI-TOF-MS (m/z): calculated monoisotopic $[M+H]^+$ = 3849.999, $[M+Na]^+$ = 3871.981
observed monoisotopic $[M+H]^+$ = 3849.497, $[M+Na]^+$ = 3869.845

UPLC: H_2O/MeCN + 0.1% TFA, 10-95% MeCN, 5 min, 0.3 mL/min on a BEH C18 (1.7 µm, 2.1 x 50 mm) column
Purity = 93.16%

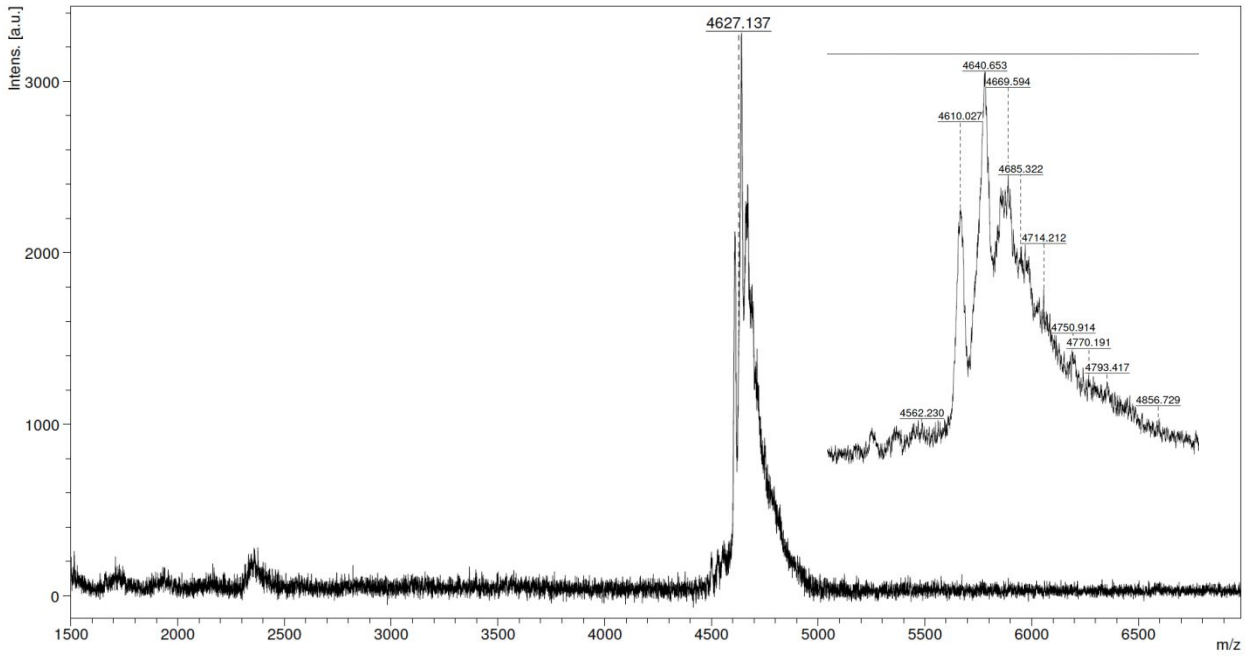


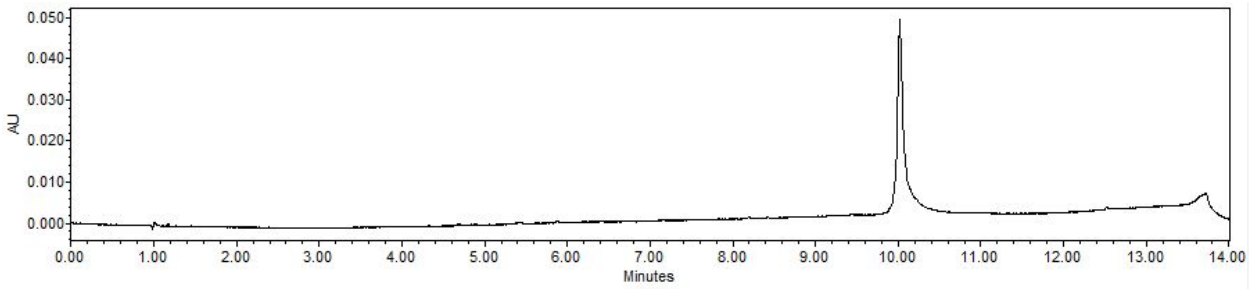


9) α/β-peptide 9: ECGWRIGEAGTDPNLNHQQFRAKILSCWD-NH_2

MALDI-TOF-MS (m/z): calculated monoisotopic $[M+H]^+$ = 3341.575, $[M+Na]^+$ = 3363.557
observed monoisotopic $[M+H]^+$ = 3341.795, $[M+Na]^+$ = 3364.818
-16 m/z peak may correspond to N-terminal pyroglutamate formation or Lys side chain loss due to laser irradiation

UPLC: H_2O/MeCN + 0.1% TFA, 10-95% MeCN, 5 min, 0.3 mL/min on a BEH C18 (1.7 µm, 2.1 x 50 mm) column
Purity = 92.38%

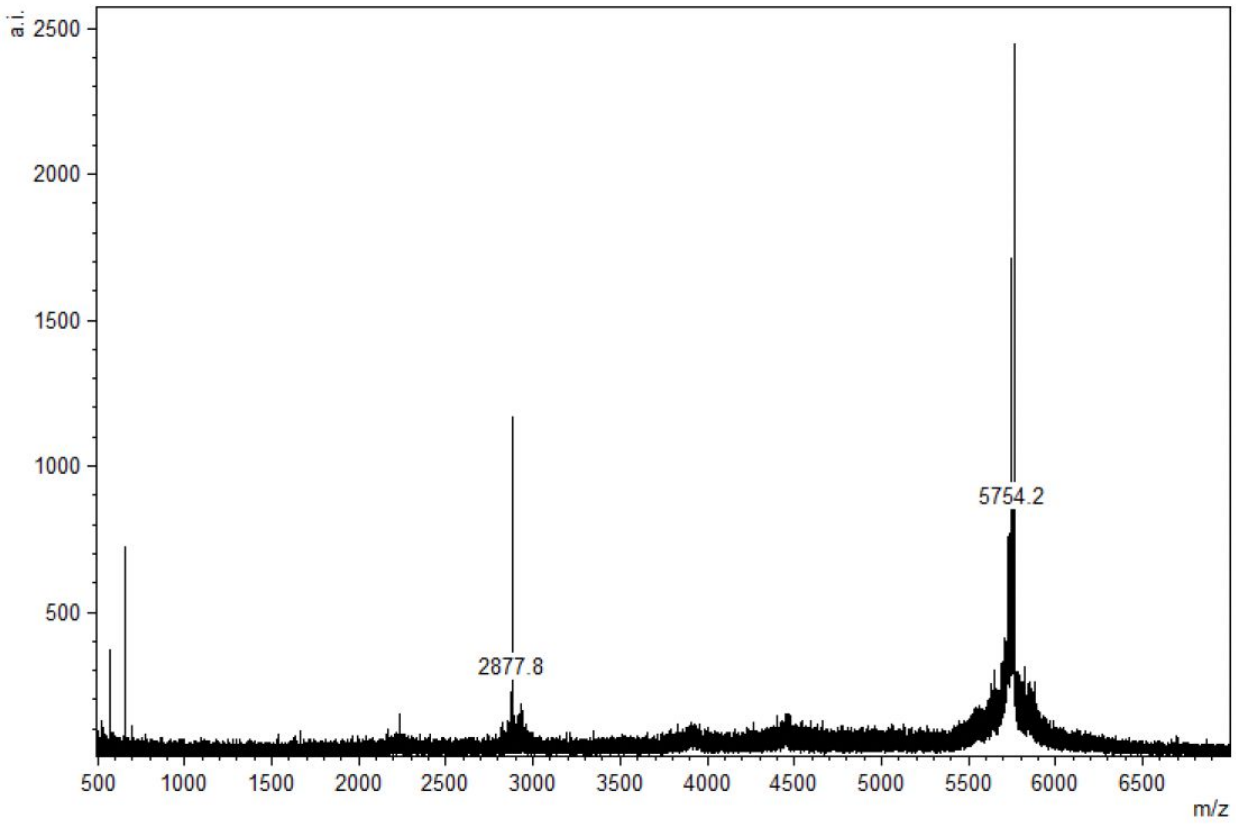


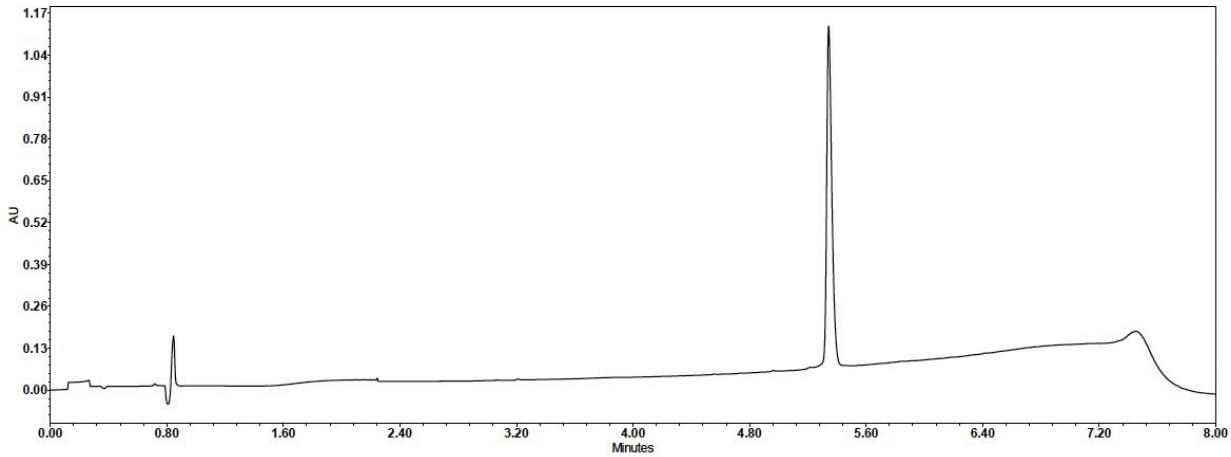


10) $(C_{12})_2$-PEG_{16}-α/β-peptide 1:

MALDI-TOF-MS (m/z): calculated monoisotopic $[M+H]^+ = 4626.626$
observed monoisotopic $[M+H]^+ = 4627.137$
-16 m/z peak may correspond to N-terminal pyroglutamate formation or Lys side chain loss due to laser irradiation
larger m/z peaks may correspond to salt adducts

UPLC: H_2O/MeCN + 0.1% TFA, 10-95% MeCN, 5 min, 0.3 mL/min on a BEH C18 (1.7 µm, 2.1 x 50 mm) column

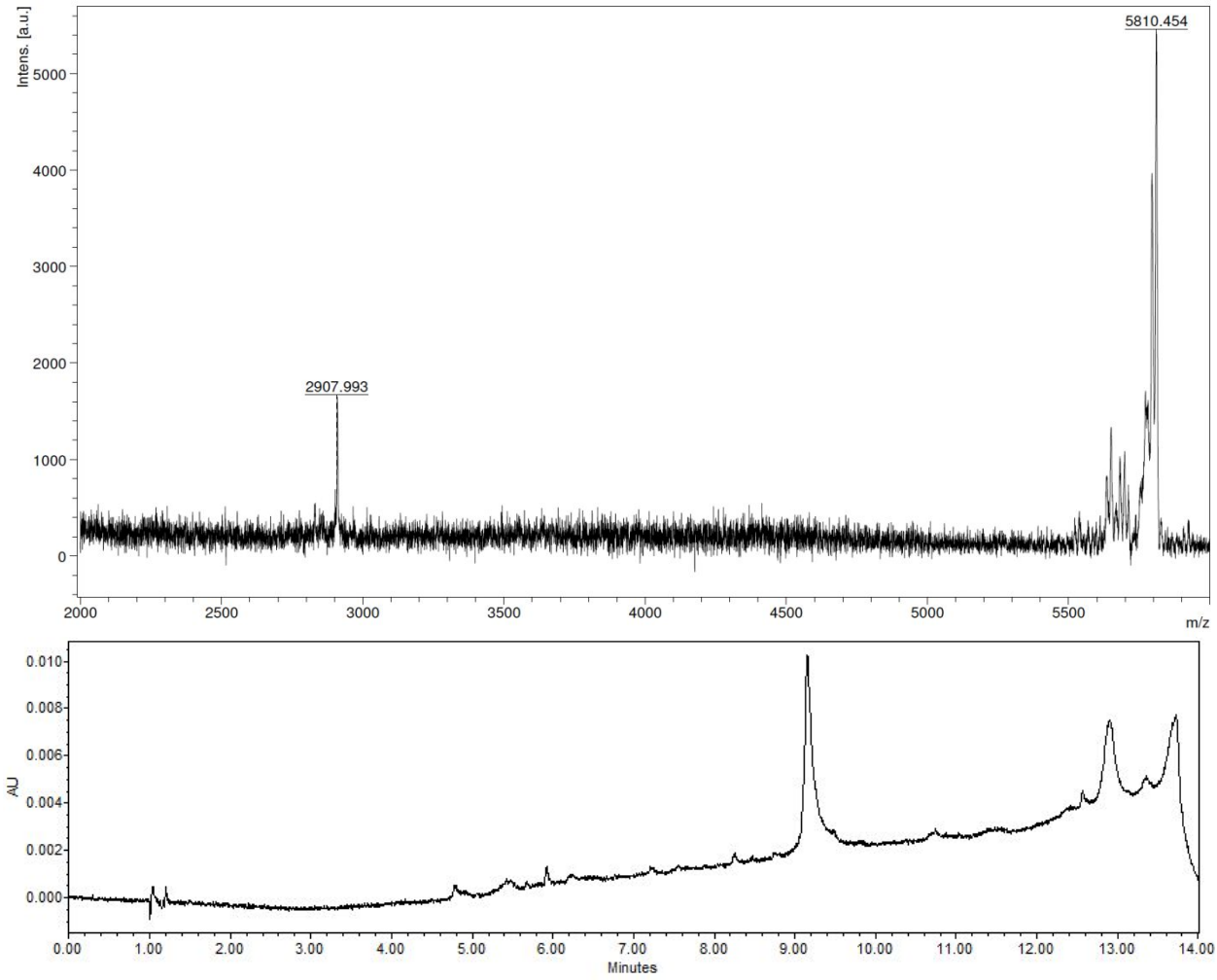




12) $(C_{12})_2$-PEG_{16}-α/β-peptide 2:

MALDI-TOF-MS (m/z): calculated monoisotopic $[M+H]^+ = 5754.0$, $[M+2H]^+ = 2877.5$
observed monoisotopic $[M+H]^+ = 5754.2$, $[M+2H]^+ = 2877.8$

UPLC: H_2O/MeCN + 0.1% TFA, 10-95% MeCN, 5 min, 0.3 mL/min on a BEH C18 (1.7 µm, 2.1 x 50 mm) column



12) $(C_{12})_2$-PEG_{16}-α/β-peptide 4:

MALDI-TOF-MS (m/z): calculated monoisotopic $[M+H]^+ = 5814.231$, $[M+2H]^+ = 2907.619$
observed monoisotopic $[M+H]^+ = 5810.454$, $[M+2H]^+ = 2907.993$
smaller m/z peaks may correspond to laser fragmentation, since high laser power was needed for detection

UPLC: H_2O/MeCN + 0.1% TFA, 10-95% MeCN, 5 min, 0.3 mL/min on a BEH C18 (1.7 μm, 2.1 x 50 mm) column

The peaks eluted at 13 and 14 minutes are aggregated peptide washed off from the column.

V. References:

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