

Supporting Information

A Conditionally Fluorescent Peptide Reporter of Secondary Structure Modulation

Oleta T. Johnson,^[a] Tanpreet Kaur,^[b] and Amanda L. Garner^{*[a, b]}

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Author Contributions

A.G. Conceptualization: Lead; Formal analysis: Supporting; Funding acquisition: Lead; Project administration: Lead; Supervision: Lead; Writing – review & editing: Supporting

O.J. Conceptualization: Supporting; Data curation: Lead; Formal analysis: Lead; Methodology: Lead; Writing – original draft: Lead; Writing – review & editing: Supporting

T.K. Data curation: Supporting; Formal analysis: Supporting; Writing – review & editing: Supporting.

Supporting Information

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A. General Materials and Methods

General chemistry methods. NMR spectra (^1H and ^{13}C) were recorded on a Bruker BioSpin GmbH NMR. Chemical shifts are reported in parts per million and referenced to TMS. Spectra were processed using MestReNova software. Mass spectrometry (HRMS) was performed using an Agilent 6520, Accurate-Mass QTOF LC/MS spectrometer using ESI ionization, with less than 5 ppm error for all HRMS analyses. RP-HPLC was performed using binary gradients of solvents A and B, where A is 0.1% HCO_2H in water and B is 0.1% HCO_2H in acetonitrile or 0.1% HCO_2H in methanol. Analytical RP-HPLC was performed using an Agilent 1260 Infinity HPLC equipped with a ZORBAX Eclipse SB-C18 column (4.6×150 mm; $5\ \mu\text{m}$) at a flow rate of 1 mL/min, with detection at 214 and 254 nm. Preparative RP-HPLC was performed using an Agilent 1260 Infinity HPLC equipped with a PrepHT SB-C18 column (21.2×150 mm; $5\ \mu\text{m}$) at a flow rate of 18.6 mL/min, with detection at 214 and 254 nm. In all cases, fractions were analyzed off-line using an Agilent Q-TOF HPLC-MS. Pure fractions were then pooled and lyophilized.

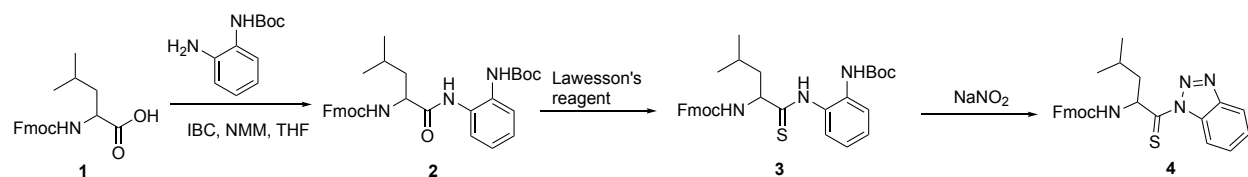
General assay methods. Peptide fluorescence measurements were carried out on a SpectraMax M Series Multimode Plate Reader using excitation/emission wavelengths of 490/520 nm with a 515 nm cutoff and 3 scans/well unless otherwise indicated. Fluorescence was read in Corning 3677 black round-bottom 384-well plates.

Data analysis. All data was analyzed using GraphPad Prism version 7.0 for Mac OS X (GraphPad Software, www.graphpad.com). P-values for fluorescence data were determined using 2-way ANOVA and a 99% confidence interval. EC_{50} values for protein titrations of peptide were calculated using the “Sigmoidal Dose-Response” equation for data fitting.

Materials. Fmoc-protected amino acids and Rink amide MBHA resin were purchased from P3 Biosystems and used as received. pHA-eIF4E (Plasmid #17343) was purchased from Addgene (GenBank ID NM_001968). pMCSG9 vector was provided by the Center for Structural Biology at University of Michigan.

B. Synthesis

The synthesis of α -N-Fmoc-L-thioleucine-benzotriazole (**4**) was adapted from Shalaby *et al.*:^[1]



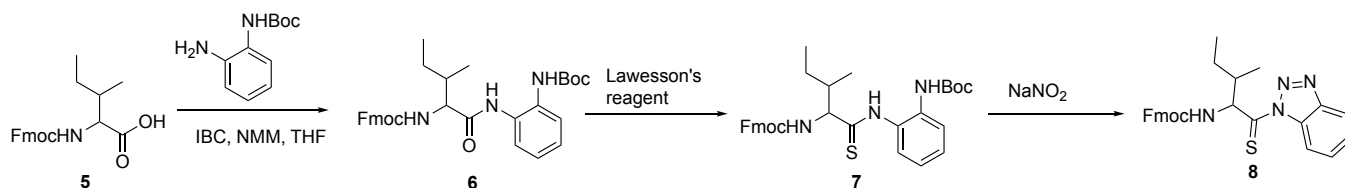
α -N-Fmoc-L-leucine-(N-Boc)-2-aminoanilide (2**):** To a solution of Fmoc-Leu-OH (1.7675 g, 5 mmol) in dry tetrahydrofuran (50 mL) at $-10\ ^\circ\text{C}$, was sequentially added N-methylmorpholine

(NMM, 1.10 mL, 10 mmol) and isobutyl chloroformate (0.65 mL, 5 mmol) with stirring. The reaction mixture was stirred for 15 min at this temperature and followed by slow addition of *N*-Boc-1,2-phenylenediamine (1.0483 g, 5 mmol). The reaction mixture was warmed to 25 °C and stirred overnight. Upon reaction completion by thin layer chromatography, the solvent was evaporated *in vacuo* and extracted 3× with ethyl acetate (~50 mL). The combined organic layers were washed with 30 mL of brine and 20 mL of 5% NaHCO₃ and dried over anhydrous sodium sulfate. The mixture was again concentrated *in vacuo* and the crude product was purified via silica gel column chromatography (eluent: 10%-40% EtOAc/petroleum hexanes) to yield a white foam (75%). ¹H NMR (300 MHz, Chloroform-*d*) δ 8.66 (s, 1H), 7.76 (d, *J* = 7.4 Hz, 2H), 7.58 (d, *J* = 6.4 Hz, 3H), 7.38 (d, *J* = 6.5 Hz, 3H), 7.28 (s, 1H), 7.18 (t, *J* = 7.6 Hz, 1H), 7.08 (d, *J* = 15.1 Hz, 1H), 7.01 (s, 1H), 5.61 (d, *J* = 7.3 Hz, 1H), 5.30 (s, 1H), 4.40 (s, 3H), 4.18 (dt, *J* = 14.7, 6.7 Hz, 2H), 1.71 (d, *J* = 41.2 Hz, 3H), 1.49 (s, 10H), 1.07 – 0.92 (m, 6H). HRMS (ESI-TOF) *m/z* calculated for C₃₂H₃₇N₃O₅: [M + H]⁺ 544.2811; observed: 544.2801.

α-*N*-Fmoc-*L*-thioleucine-(*N*-Boc)-2-aminoanilide (3): To a solution of **2** (1.86 g, 3.42 mmol) in THF (50 mL), Lawesson's Reagent (1.80 g, 4.45 mmol) was added and refluxed at 70 °C under an argon environment for 6 h. After completion of the reaction (monitored with TLC), the solvent was evaporated *in vacuo*. The crude reaction mixture was purified by silica gel column chromatography (eluent: 10%-40% EtOAc/petroleum hexanes) to yield **3** (1.6 g, 84% yield). ¹H NMR (300 MHz, Chloroform-*d*) δ 9.69 (s, 1H), 7.78 (d, *J* = 7.8 Hz, 3H), 7.61 (t, *J* = 6.5 Hz, 3H), 7.40 (dt, *J* = 13.8, 6.7 Hz, 3H), 7.32 (d, *J* = 7.5 Hz, 2H), 7.23 – 7.16 (m, 1H), 5.63 (s, 1H), 4.75 – 4.67 (m, 1H), 4.54 (d, *J* = 7.8 Hz, 1H), 4.46 (d, *J* = 6.9 Hz, 1H), 4.25 (t, *J* = 7.1 Hz, 1H), 1.81 (d, *J* = 21.2 Hz, 4H), 1.48 (s, 10H), 1.03 (s, 5H). HRMS (ESI-TOF) *m/z* calculated for C₃₂H₃₇N₃O₄S: [M + H]⁺ 560.2583; observed: 560.2570.

α-*N*-Fmoc-*L*-thioleucine-benzotriazolide (4): To an ice-cold solution of **3** (1.83 g, 3.27 mmol), 25% TFA in CH₂Cl₂ (40 mL) was added and stirred for 2 h. After completion of the reaction, the solvent was removed *in vacuo* and dried on a high vacuum overnight. The resulting orange solid was treated with 20 mL of 95% glacial acetic acid and cooled to 0 °C. Sodium nitrite (0.3384 g, 4.90 mmol) was added in small portions over 5 min. After 30 min, the reaction was quenched with 75 mL of ice-water. The resulting precipitate was collected, dried and purified via silica gel column chromatography (eluent: 10%-20% EtOAc/petroleum hexanes) to yield **4** as a yellow solid (0.85 g, 55% yield). ¹H NMR (300 MHz, Chloroform-*d*) δ 8.83 (d, *J* = 8.4 Hz, 1H), 8.19 (d, *J* = 8.1 Hz, 1H), 7.81 – 7.56 (m, 6H), 7.38 (dd, *J* = 17.4, 7.5 Hz, 4H), 6.44 – 6.34 (m, 1H), 5.72 (d, *J* = 24.6 Hz, 1H), 4.56 – 4.47 (m, 1H), 4.45 – 4.36 (m, 1H), 4.27 (d, *J* = 7.0 Hz, 1H), 1.99 – 1.81 (m, 2H), 1.79 – 1.65 (m, 1H), 1.15 (d, *J* = 5.5 Hz, 3H), 0.99 (d, *J* = 6.1 Hz, 3H). HRMS (ESI-TOF) *m/z* calculated for C₂₇H₂₇N₄O₂S: [M + H]⁺ 471.1855; observed: 471.1821.

The synthesis of α -*N*-Fmoc-*L*-thioisoleucine-benzotriazolide^[1] (**8**) was adapted from Shalaby *et al.*:



α -*N*-Fmoc-*L*-isoleucine-(*N*-Boc)-2-aminoanilide (6**):** Same as for α -*N*-Fmoc-*L*-leucine-(*N*-Boc)-2-aminoanilide. White foam (85%). ¹H NMR (300 MHz, Chloroform-*d*) δ 8.57 (s, 1H), 7.76 (d, *J* = 7.4 Hz, 2H), 7.57 (d, *J* = 6.2 Hz, 2H), 7.49 (d, *J* = 7.8 Hz, 1H), 7.39 (s, 3H), 7.26 – 7.07 (m, 3H), 7.01 (s, 1H), 5.62 (d, *J* = 8.7 Hz, 1H), 4.54 – 4.35 (m, 2H), 4.23 (d, *J* = 6.2 Hz, 2H), 1.48 (s, 9H), 1.09 – 0.98 (m, 4H), 0.98 – 0.88 (m, 4H). HRMS (ESI-TOF) *m/z* calculated for C₃₂H₃₇N₃O₅: [M + H]⁺ 544.2811; observed: 544.2831.

α -*N*-Fmoc-*L*-thioisoleucine-(*N*-Boc)-2-aminoanilide (7**):** Same as for α -*N*-Fmoc-*L*-thioleucine-(*N*-Boc)-2-aminoanilide. Yellowish foam (75%). ¹H NMR (300 MHz, Chloroform-*d*) δ 7.78 (d, *J* = 7.3 Hz, 3H), 7.59 (d, *J* = 6.3 Hz, 3H), 7.46 – 7.30 (m, 4H), 7.20 (d, *J* = 7.6 Hz, 1H), 4.44 (d, *J* = 3.5 Hz, 2H), 4.26 (d, *J* = 6.6 Hz, 1H), 1.48 (s, 9H), 1.11 – 0.90 (m, 6H). HRMS (ESI-TOF) *m/z* calculated for C₃₂H₃₇N₃O₄S: [M + H]⁺ 560.2583; observed: 560.2575.

α -*N*-Fmoc-*L*-thioisoleucine-benzotriazolide (8**).** Same as for α -*N*-Fmoc-*L*-thioleucine-benzotriazolide. Yellow solid (50%). ¹H NMR (300 MHz, Chloroform-*d*) δ 8.85 (d, *J* = 8.3 Hz, 1H), 8.21 (d, *J* = 7.9 Hz, 1H), 7.88 – 7.54 (m, 7H), 7.47 – 7.30 (m, 4H), 6.32 – 6.18 (m, 1H), 5.81 (d, *J* = 9.9 Hz, 1H), 4.35 (dt, *J* = 55.0, 6.7 Hz, 4H), 2.14 (s, 1H), 1.92 – 1.60 (m, 2H), 1.13 – 0.75 (m, 7H). HRMS (ESI-TOF) *m/z* calculated for C₂₇H₂₆N₄O₂S: [M + Na]⁺ 493.1674; observed: 493.1640.

C. Peptide Synthesis

FITC-4E-BP1 peptide sequences without additional thioamides were synthesized on a Liberty Blue peptide synthesizer on 0.1-mmol scale using Rink Amide resin. All amino acid coupling reactions were performed using 5 excess equivalents of Fmoc-AA-OH with 1:1:1 of AA:DIC:Oxyma in DMF for 4 min at 90°C for all amino acids. Fmoc deprotections were performed for 1 min at 90°C in 20% piperidine in DMF. FITC was coupled to the unprotected N-terminus using a previously reported method.^[2]

FITC-4E-BP1 peptide sequences containing additional thioamides were synthesized manually in a 20-mL fritted syringe using MBHA Rink resin. After the initial deprotection in 20% piperidine in DMF, the peptide was synthesized using standard SPPS procedures with HBTU/DIPEA activation. For coupling of thioamide-containing amino acid precursors, 3.5 equiv of the amino

acid was dissolved in a minimal volume (~2 mL) of dry DCM with 10 equiv of DIPEA^[1b, 3]. Thioamide amino acid couplings were carried out for 2 h and repeated for a total of 3 couplings before checking thioamide addition to the peptide by a TFA test cleavage and mass spectrometry. After confirmation of thioamide incorporation, Fmoc was removed using 2% DBU in DMF to reduce side products^[3]. FITC was coupled to the unprotected N-terminus using a previously reported method.^[2] All peptides were cleaved from the resin by agitation in a 90:0.4:0.4:0.2 by volume solution of TFA:thioanisole:TIPS:H₂O for 4 h at 25 °C. The cleaved peptide in TFA solution was then precipitated in ice-cold diethyl ether, and the precipitate was centrifuged, dissolved, and purified via RP-HPLC.

D. Protein Expression and Purification^[4]

Human eIF4E cDNA was cloned into a pMCSG9 from pHA-eIF4E via LIC cloning for expression of an MBP-tagged eIF4E protein. MBP-eIF4E plasmid was transformed into Rosetta cells using a standard transformation protocol. Single colonies were picked to inoculate 6 × 6 mL overnight cultures in LB media containing 50 mg/mL ampicillin (amp) that were incubated shaking at 37 °C. Overnight cultures were used to inoculate 1 L of LB media containing 50 mg/mL amp, and that culture was grown to an OD₆₀₀ of 0.6. At this stage, the culture was induced with 1 mM IPTG and incubated shaking at 16 °C for 16 h. Cells were pelleted by centrifugation at 6000× g for 15 min at 25 °C, re-suspended in lysis buffer (50 mM Tris pH 7.5, 500 mM NaCl, 1 mM PMSF, 1 mM EDTA, 2 mM DTT), and lysed by sonication. Lysate was then clarified by centrifugation at 16,000× g for 20 min at 4 °C. Clarified lysate was incubated 15 min on Ni-NTA resin in a Bio-Rad column, then the flow-through was collected. The Ni-NTA resin was washed 3 × with 10 column volumes of wash buffer (50 mM Tris pH 7.5, 200 mM NaCl, 1 mM EDTA, 2 mM DTT), and eluted with the same buffer containing a gradient of 0–500 nM imidazole. The protein was dialyzed 3 × against 2 L of storage buffer (50 mM Sodium Phosphate pH 7.4, 200 mM NaCl, 1 mM EDTA, 2 mM DTT, 15% glycerol), distributed into 15-mL aliquots, and flash frozen until use. Before using protein for experiments, aliquots were thawed on ice, concentrated to 5 mL, purified by size-exclusion chromatography (SEC) to remove aggregates, and concentrated as needed, with concentration monitored by A₂₈₀. Purified protein was stored at 4 °C for up to 5 days after SEC, after which it was discarded.

E. Circular Dichroism (CD) Spectroscopy

Peptides, which were dissolved as 1 mM stocks in 50 mM Sodium Phosphate pH 7.4 or 8.5 and 30% acetonitrile, were diluted to 100–150 μM with additional phosphate buffer or phosphate buffer with TFE for a final TFE concentration of 50%. CD measurements were carried out on a JASCO J-1500 CD spectrophotometer. Baseline measurements were conducted using 50 mM Sodium Phosphate pH 7.4 or 8.5 and 3% acetonitrile. 50% TFE was added to the baseline solution

for peptide measurements in TFE. The baseline was automatically subtracted by the Jasco software. CD spectra for the peptides were measured from 190–260 nm over 3 scans. Helicity was determined by molar ellipticity of each peptide at 222 nm using the equations below.^[5]

Equation 1:

$$\%_H = (([\theta]_{obs222} - [\theta]_C)/([\theta]_{\infty222} - [\theta]_C)) * 100$$

Equation 2:

$$[\theta]_C = 2220 - 53T$$

Equation 3:

$$[\theta]_{\infty222} = (-44000 + 250T)(1 - \frac{k}{N_p})$$

Where,

$\%_H$ = percent α -helix content

$[\theta]_{obs222}$ = observed molar ellipticity at 222 nm

$[\theta]_C$ = Random coil molar ellipticity

$[\theta]_{\infty222}$ = Infinite molar α -helix molar ellipticity

T = temperature in degrees Celsius

k = finite length correction

N_p = Number of peptide units

F. Peptide Fluorescence Measurements

Peptides, which were dissolved as 1 mM stocks in Assay Buffer (50 mM Sodium Phosphate pH 7.4 or 8.5, 200 mM NaCl, 1mM DTT, 1mM EDTA) and 30% DMF, were diluted to appropriate working concentrations for subsequent experiments. For fluorescence measurements, peptides were diluted to 20 nM working solutions with buffer A. For TFE experiments, the working solutions were further diluted to 10 nM using either buffer A or TFE. To each well, 10 μ L of the 10 nM peptide working solution was added, and the fluorescence was read immediately using excitation/emission wavelengths of 490/520 nm with a 515 nm cutoff and 3 scans per well. For MBP-eIF4E and α -synuclein titrations, 10 μ L of the working solution was titrated serially with 10 μ L of the protein solutions for a final peptide concentration of 10 nM, and final protein concentrations of 0–500 nM and 0–900 nM for MBP-eIF4E and α -synuclein, respectively. All conditions were measured in triplicate.

Fluorescence lifetime experiments were conducted on a PTI TimeMaster Fluorescence Lifetime Spectrometer using 3 mL of 10 μ M peptide in solutions of either Assay Buffer or Assay Buffer containing 50% TFE (ex/em: 494/520). Tail-fitting analysis was done in the accompanying software. Peptide absorbance spectra were conducted on a Cary UV50 UV/Vis Spectrometer using

3 mL of 10 μ M peptide in solutions of either Assay Buffer or Assay Buffer containing 50% TFE. Absorbance was monitored over the range of 300-800 nm.

G. Supplemental Figures

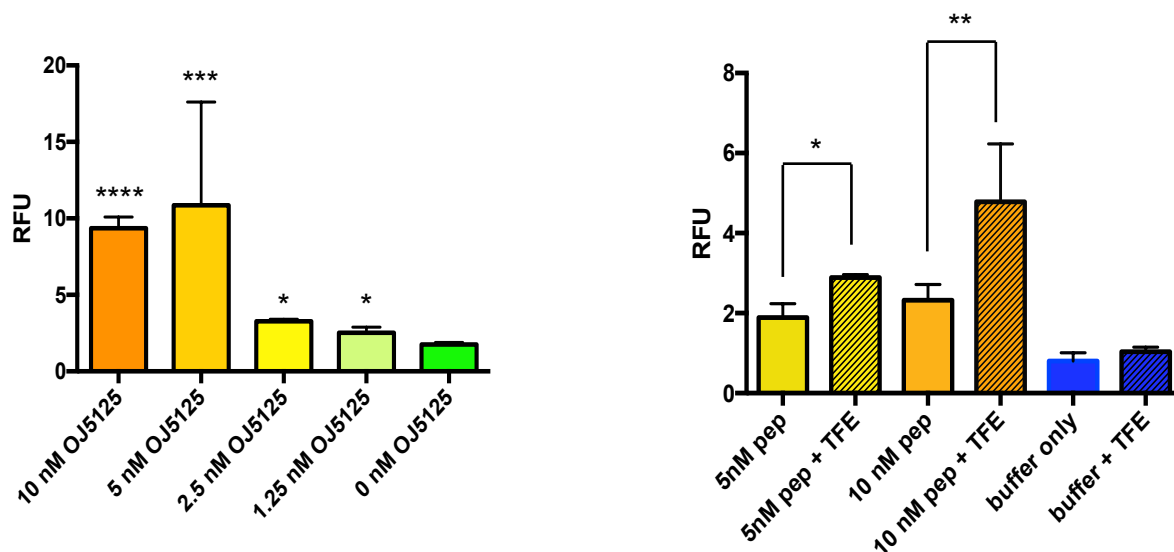


Figure S1. Optimization of fluorescent peptide concentration. 10 μ L solutions of FITC-4E-BP1 peptide were prepared in assay buffer pH 7.4 at concentrations from 1—10 nM and fluorescence signal (ex/em: 494/520 nm) was read to determine the minimum concentration required to give significant fluorescence signal over assay buffer alone. Accordingly, solutions of 5 nM and 10 nM peptide were prepared with and without the presence of 50% TFE, to determine which peptide concentration would give the best signal-to-background ratio, treating peptide + TFE as signal and peptide – TFE as background. Based on this data, 10 nM peptide was used for all future fluorescence measurements. Fluorescence measurements were done in triplicate and p-values were generated in GraphPad using 2-way ANOVA. **** p-value < 0.0001; *** p-value = 0.001; **p-value = 0.02; *p-value = not statistically significant.

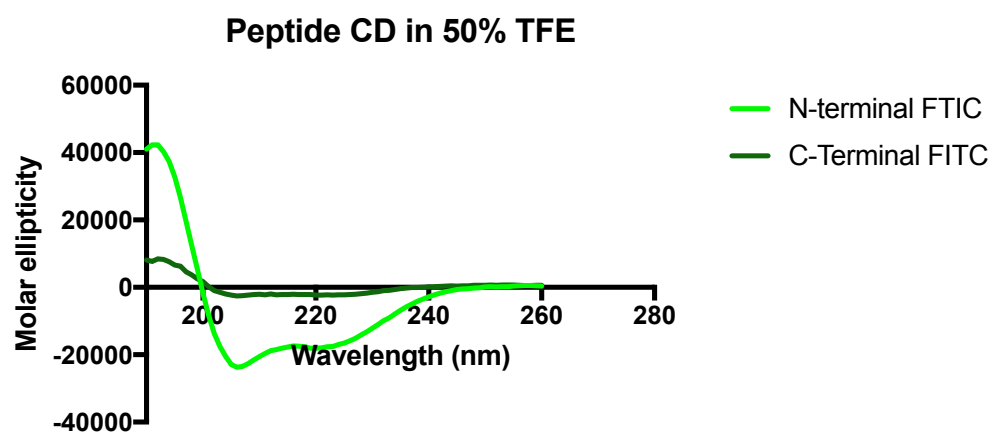


Figure S2. FITC conjugation to the C-terminus of the 4E-BP peptide significantly diminishes helical propensity.

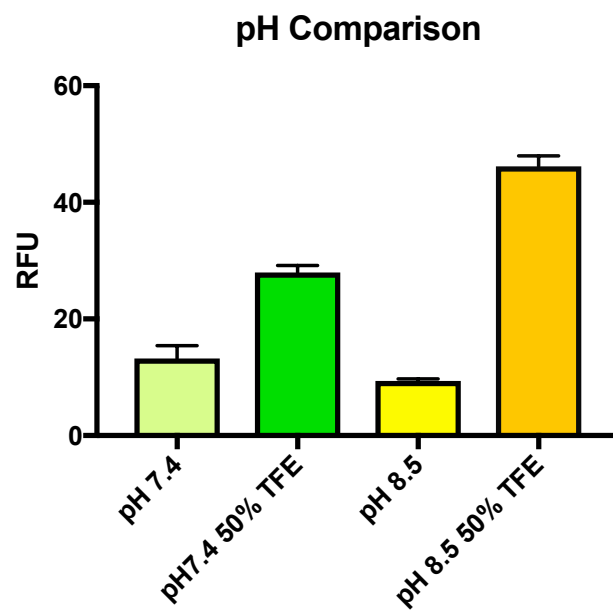


Figure S3. Increasing buffer pH results in better S/B. S/B at pH 7.4 = 1.5; S/B at pH 8.5 = 4.5.

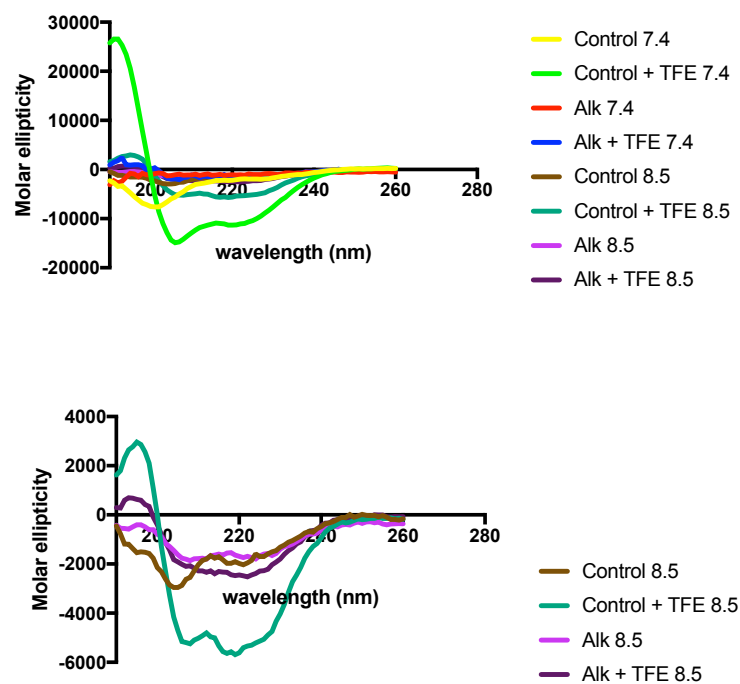


Figure S4. Trends in peptide helicity are unchanged in Sodium Phosphate buffer pH 8.5. Elevated pH enhances peptide dimerization at Cys62 in CD spectroscopy, which is intolerant of reducing agent. This dimerization reduces the effective concentration of strictly α -helical peptide, yielding spectra with an imperfect appearance. Despite this, helicity calculations confirm the presence of a α -helical peptide population, which follows the same general trend in response to TFE and/or alkylation observed at pH 7.4.

Table S1. CD of FITC-4E-BP1. The difference in the percent change in TFE helical ordering at pH 8.5 is attributed to the increased probability of Cys dimerization at elevated pH, in combination with the lack of reducing agent, as is a requirement for CD measurements.

Peptide	% helicity	TFE	Buffer pH
N-Term FITC	7	-	7.4
N-Term FITC	33	+	7.4
C-Term FITC	-3	-	7.4
C-Term FITC	7	+	7.4
N-Term FITC Cys-alkylated	3	-	7.4
N-Term FITC Cys-alkylated	5	+	7.4
N-Term FITC	6	-	8.5
N-Term FITC	17	+	8.5
N-Term FITC Cys-alkylated	5	-	8.5
N-Term FITC Cys-alkylated	8	+	8.5

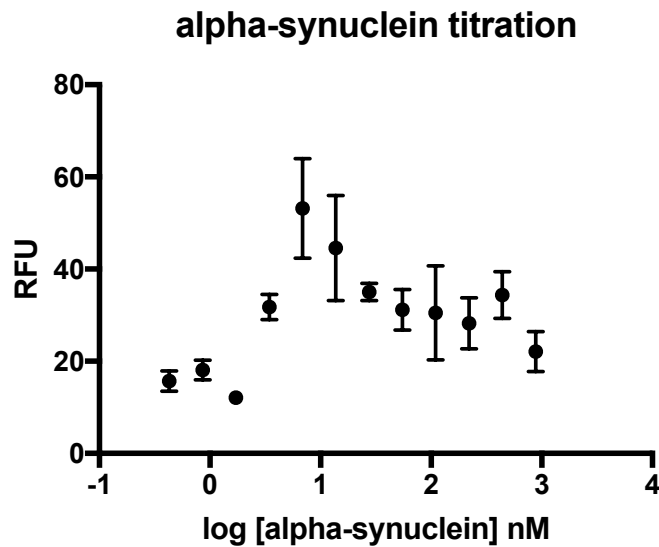


Figure S5. 10 μ L of peptide working solution was titrated serially with 10 μ L of α -synuclein solutions for a final peptide concentration of 10 nM, and final protein concentrations of 0–900 nM in Assay Buffer at pH 8.5. Addition of α -synuclein to the peptide does not give the dose-dependent response expected for a specific binding interaction, indicating that observed response for MBP-eIF4E titrations is likely due to specific binding, as opposed to a non-specific and/or aggregation induced phenomena.

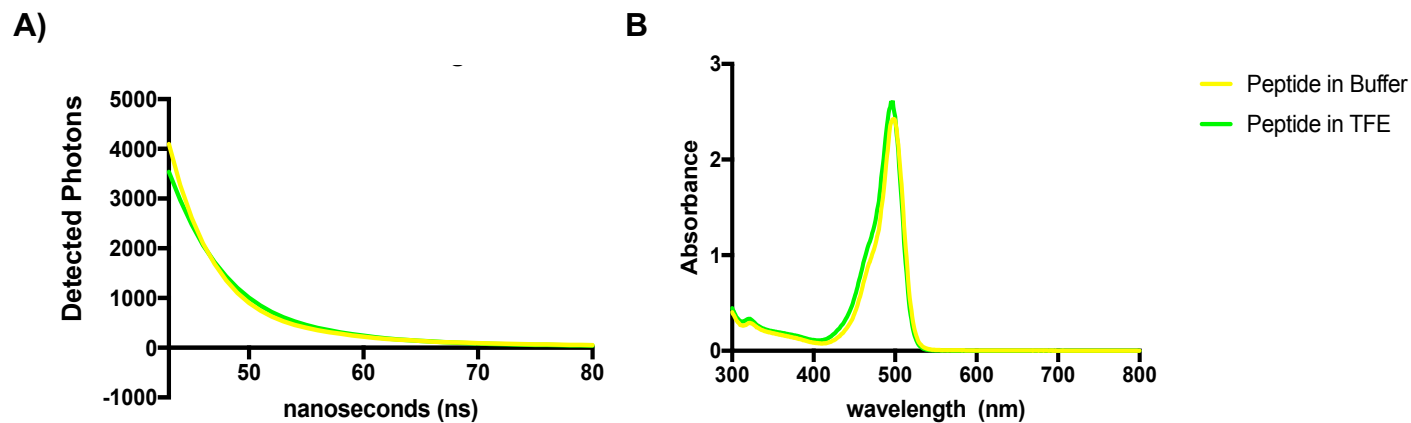


Figure S6. A) Tail-fitting analyses of TCSPC experiments for N-term FITC 4E-BP1 peptides shows an increase in fluorescence lifetime from 4.029 ns Assay Buffer pH 8.5 to 4.956 ns in Assay Buffer pH 8.5 containing 50% TFE. B) The overall absorbance spectra of the N-term FITC 4E-BP1 peptide is unchanged, with a 3 nm blueshift in maximum fluorescence in TFE that is often observed as a result of structural transitions of fluorophore conjugates.^[6]

H. NMR Spectra

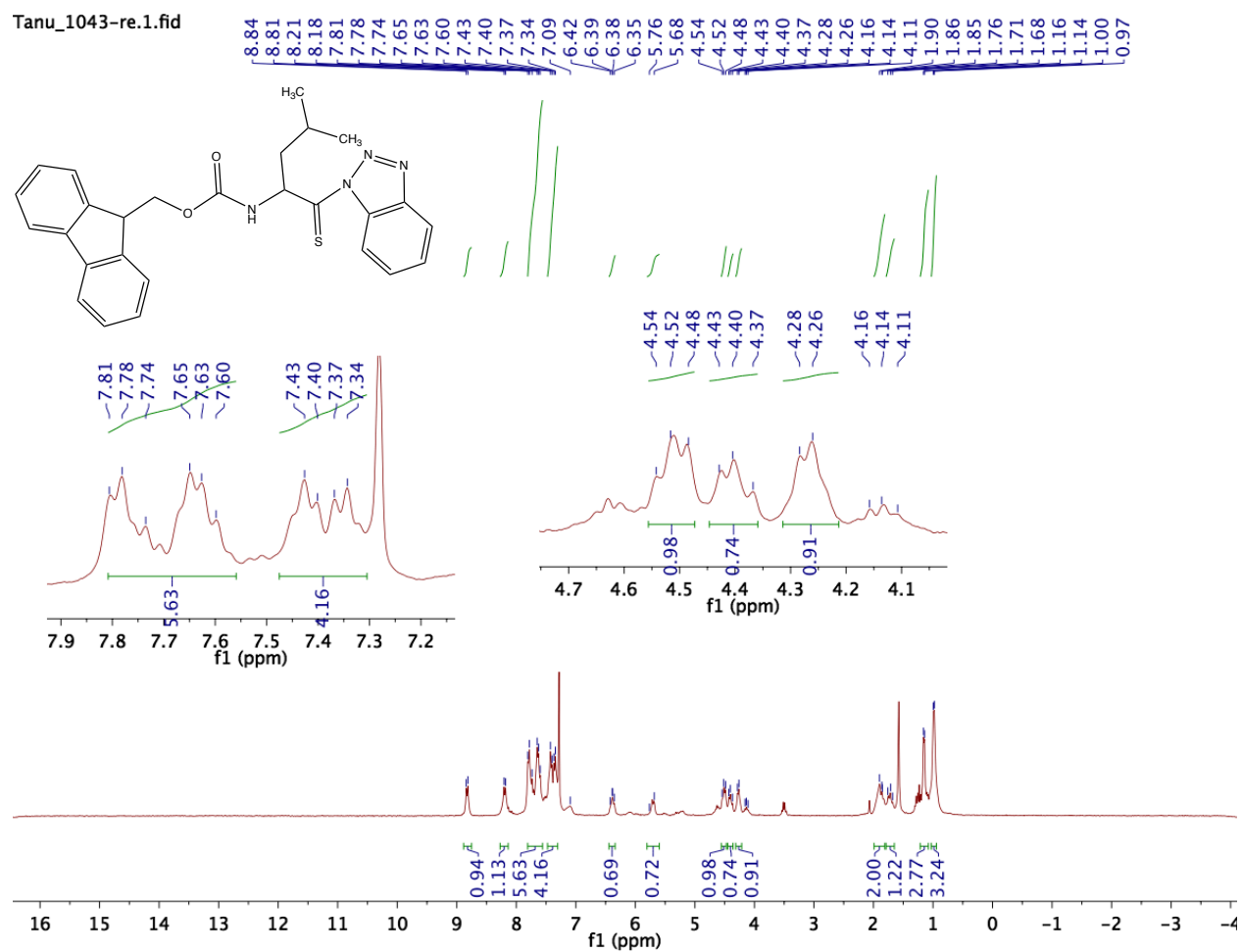


Figure S7. NMR of α -N-Fmoc-L-thioleucine-benzotriazolide

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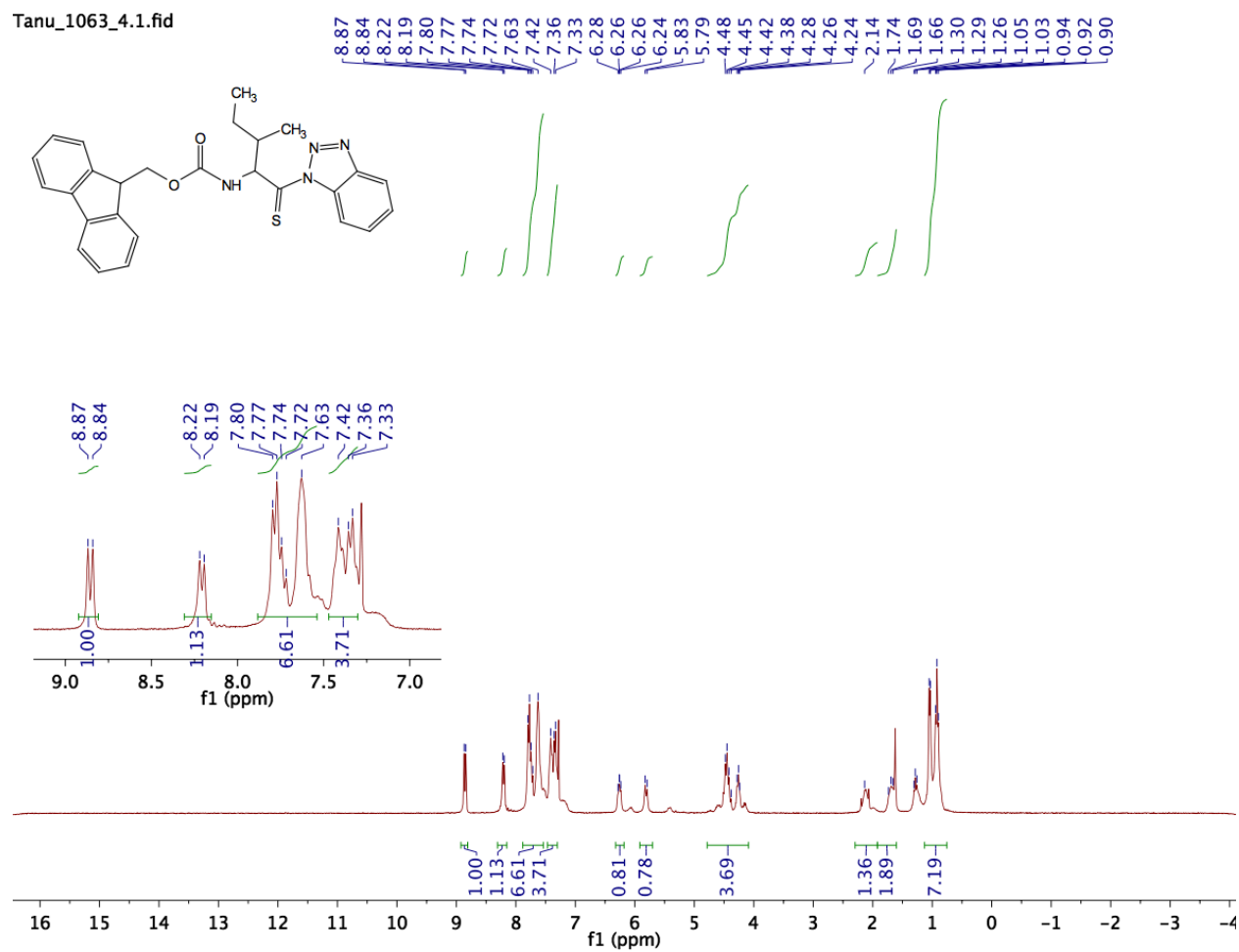
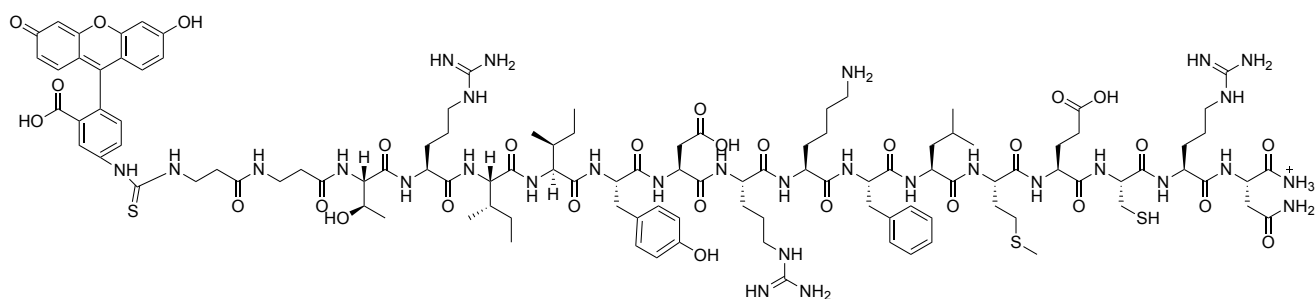


Figure S8. NMR of α -N-Fmoc-L-thioisoleucine-benzotriazolidine

I. Peptide QTOF-MS



Chemical Formula: $C_{112}H_{163}N_{30}O_{29}S_3^+$
 Exact Mass: 2488.14
 Molecular Weight: 2489.90

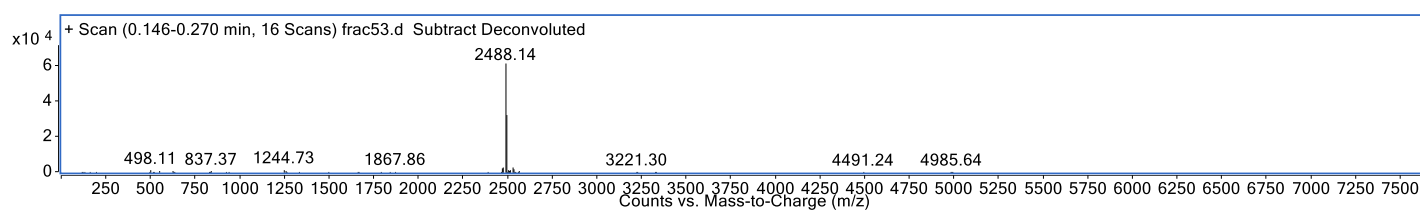


Figure S9. N-terminal FITC conjugated 4E-BP1 peptide Q-TOF-MS.

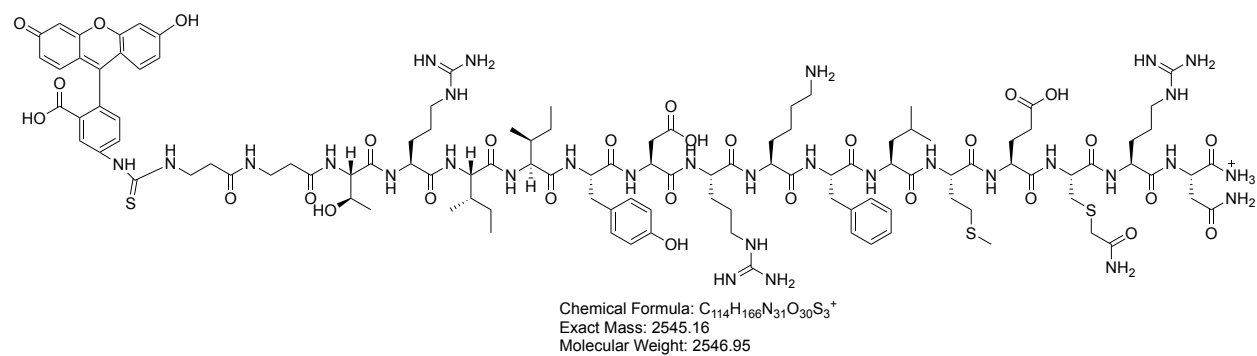
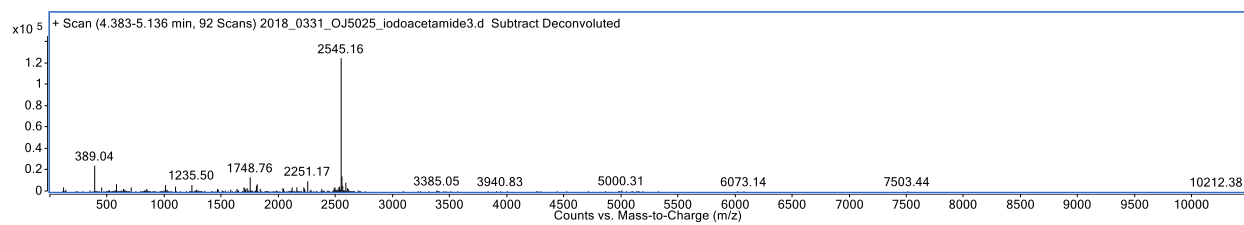


Figure S10. N-terminal FITC conjugated 4E-BP1 peptide alkylated at cysteine Q-TOF-MS.

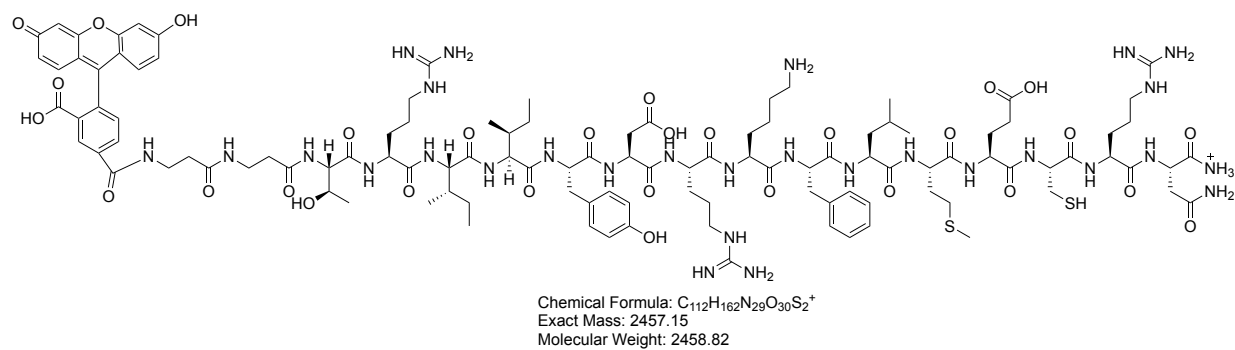
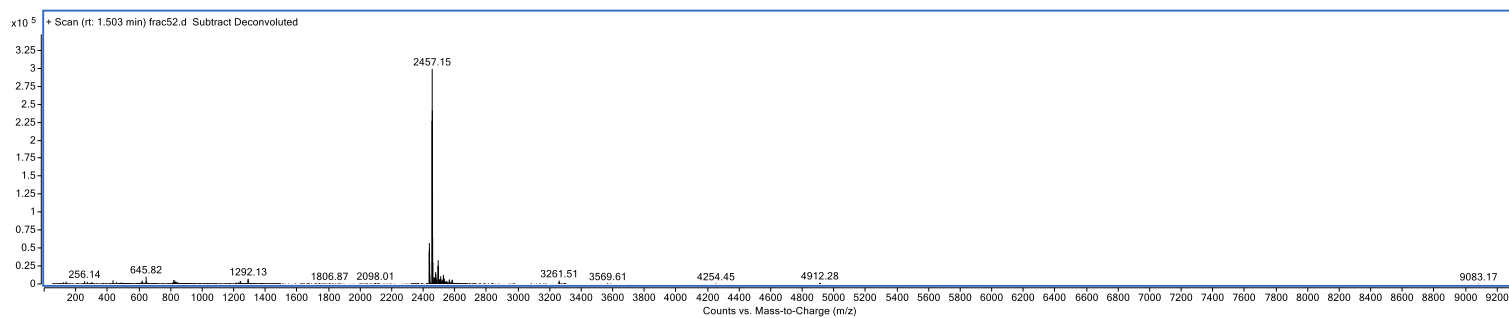


Figure S11. N-terminal FAM conjugated 4E-BP1 peptide Q-TOF-MS.

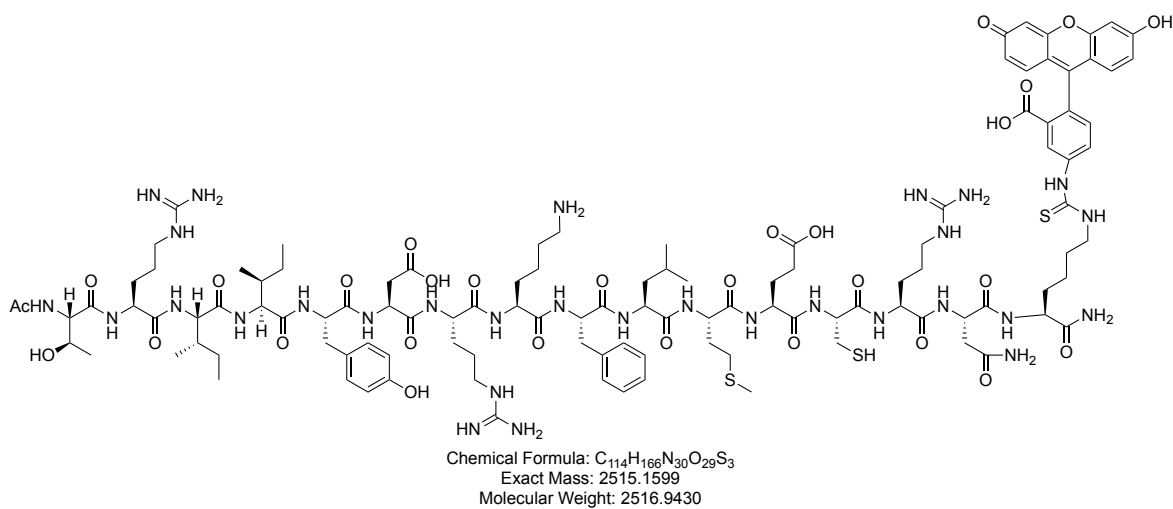
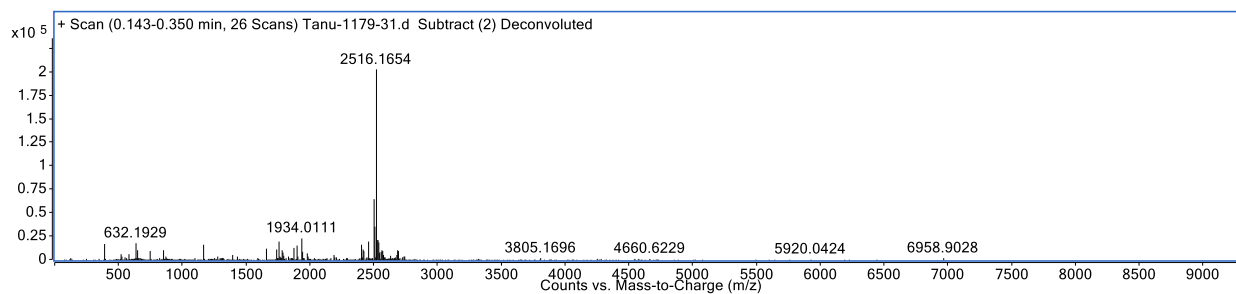


Figure S12. C-terminal FITC conjugated 4E-BP1 peptide Q-TOF-MS.

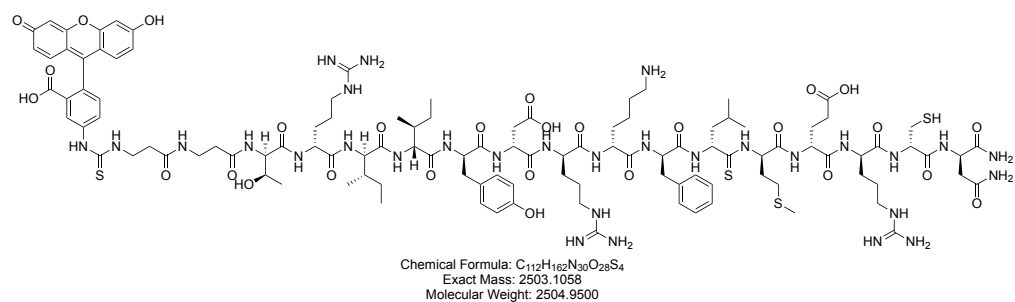
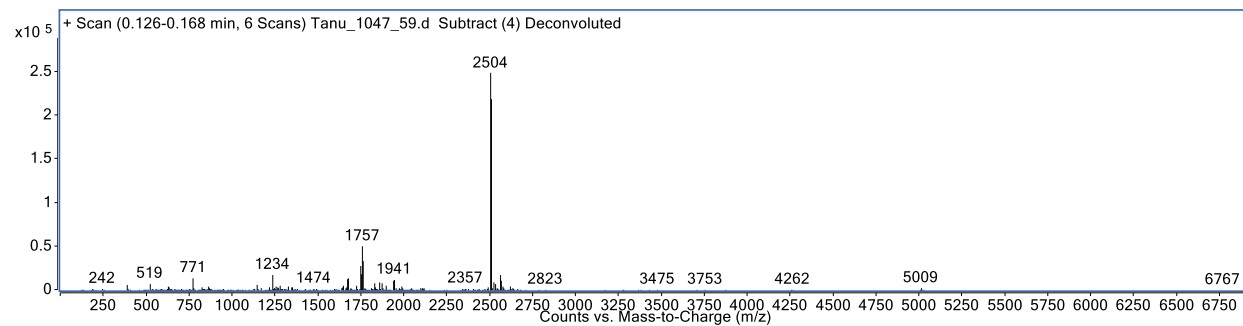


Figure S13. N-terminal thioleucine 4E-BP1 peptide Q-TOF-MS.

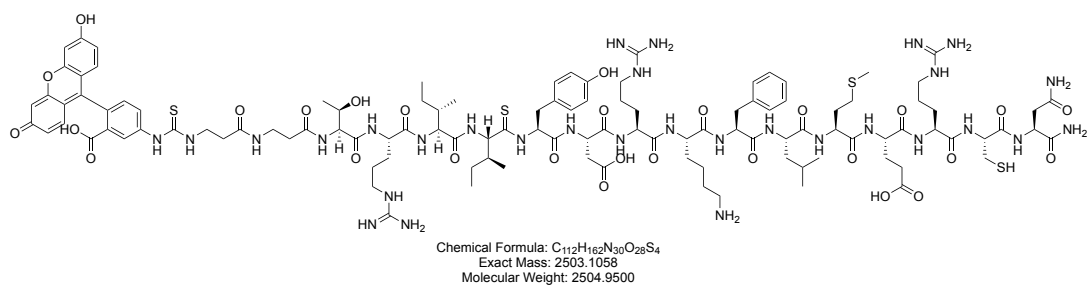
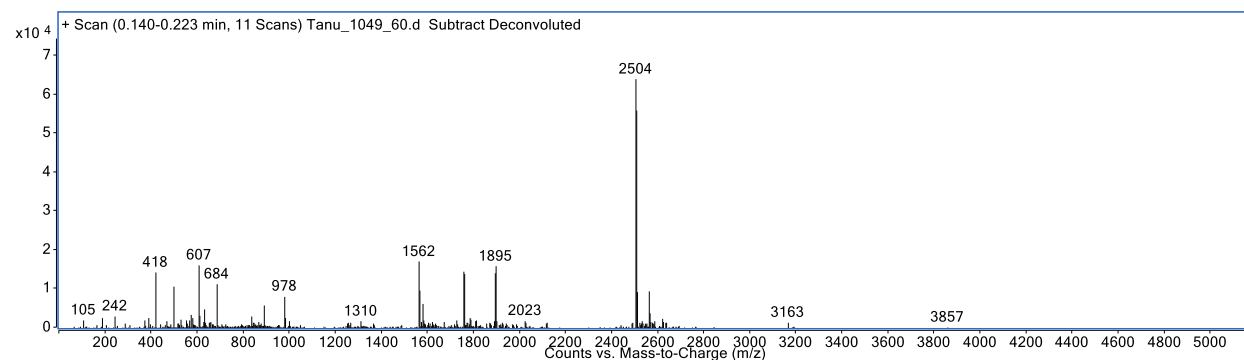


Figure S14. N-terminal thioisoleucine 4E-BP1 peptide QTOF.

J. References

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