Studies of Thioamide Positional Effects on Serine Protease Activity Enable Two-Site Stabilization of Cancer Imaging Peptides

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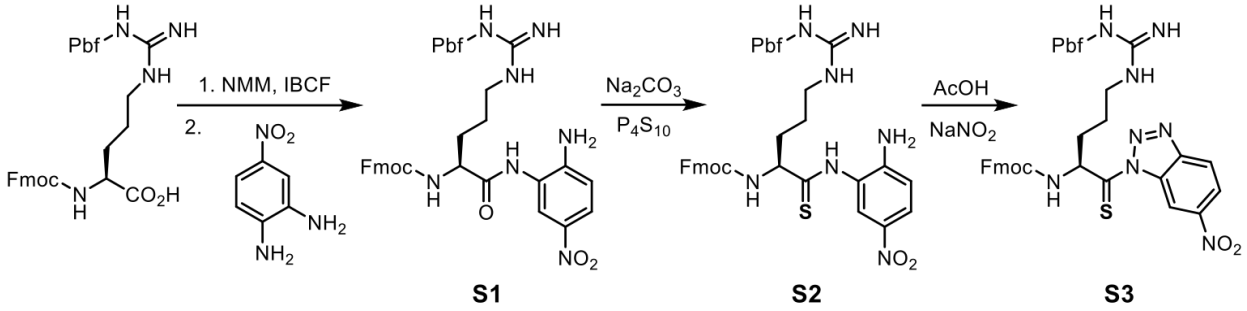
General Information. Fmoc-β-(7-methoxycoumarin-4-yl)-Ala-OH was purchased from Bachem (Torrance, CA, USA). All other Fmoc protected amino acids and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Novabiochem (currently EMD Millipore; Billerica, MA, USA). Piperidine was purchased from American Bioanalytical (Natick, MA, USA). 7-Azabenzotriazol-1-yloxy)tripyrrolidino-phosphonium hexafluorophosphate (PyAOP) was purchased from ChemImpex (Wood Dale, IL, USA). Sigmacote®, *N*,*N*-diisopropylethylamine (DIPEA), N-benzoyl-L-arginine 4-nitroanilide, trypsin (lyophilized powder; from porcine pancreas; Type II-S; 1,000 – 2,000 units/mg dry solid), chymotrypsin (lyophilized powder; from bovine pancreas; Type II; greater than or equal to 40 units/mg dry solid) and mouse serum (M5905) were ordered from Sigma Aldrich. Kallikrein (in Tris HCl buffer; from human plasma; 15 units/mg protein) was ordered from Millipore Sigma. PathHunter® eXpress NPY1R CHO-K1 β-Arrestin GPCR Assay kits were purchased from DiscoverX (Fremont, CA, USA). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA) unless specified otherwise. Milli-Q filtered (18 MΩ) water was used for all solutions (EMD Millipore). Time-course UV-Vis absorbance and fluorescence data were obtained with a Tecan Infinite® M1000 PRO plate reader (San Jose, CA, USA). Peptides were purified with a Varian ProStar High-Performance Liquid Chromatography (HPLC) with a diode array detector (currently Agilent Technologies), an Agilent 1260 Infinity II Preparative HPLC system, a Biotage Isolera one (Biotage, LLC, Charlotte NC) and analyzed with an Agilent 1100 or Agilent 1260 Infinity II Series Analytical HPLC system. Peptide mass spectrometry was collected with a Bruker Ultraflex III MALDI-MS mass spectrometer (Billerica, MA, USA). Nuclear magnetic resonance (NMR) spectra were collected with a Bruker DMX 500 MHz instrument (Billerica, MA). High resolution electrospray ionization mass spectra (ESI-HRMS) were obtained

on a Waters LCT Premier XE liquid chromatograph/mass spectrometer (Milford, MA). Cellular images were obtained using a VT-iSIM confocal microscope (VisiTech International Ltd., Sunderland, U.K.). Fluorescence lifetime measurements were made by time correlated single photon counting (TCSPC) using a Photon Technologies International (PTI) QuantaMaster40 fluorometer (currently Horiba Scientific, Edison, NJ, USA).

Chemical Synthesis and Characterization.



Scheme S1. Synthesis of Thioarginine Precursor.

Synthesis of N_α-Fmoc-N_ω-Pbf-L-arginine-2-amino-5-nitroanilide (S1). Under argon atmosphere, Fmoc-Arg(Pbf)-OH (6.48 g, 10 mmol, 1 equiv) was dissolved in 50 mL of dry tetrahydrofuran (THF) and equilibrated to -10 °C in a salt ice bath (NaCl/ice = 1:3). Under magnetic stirring, *N*-methylmorpholine (NMM, 2.20 mL, 20 mmol, 2 equiv) and isobutylchloroformate (IBCF, 1.97 mL, 10 mmol, 1 equiv) were added to the solution dropwise by a pipette. The reaction was allowed to proceed for 30 min at -10 °C with stirring. 4-nitro-1,2-pheylenediamine (1.53 g, 10 mmol, 1 equiv) was added and the reaction was stirred at -10 °C until the ice melted, then at room temperature overnight. After removal of solvent by rotary evaporation, the residue was dissolved in 50 mL of ethyl acetate and extracted with 50 mL each of 1 M Na_2HPO_4, Brine, and 5% $NaHCO_3$. The organic layer was dried over magnesium sulfate and

solvent was taken off by rotary evaporation and high vacuum. An orange solid was obtained as the final product (6.791 g, 8.67 mmol, 87% yield) in 75% purity (determined by HPLC). R_f = 0.51 in 9:1 ethyl acetate/hexanes. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.34 (s, 1H), 8.16 (d, J = 2.4 Hz, 1H), 7.87 – 7.80 (m, 3H), 7.69 (q, J = 6.9 Hz, 3H), 7.37 (t, J = 7.4 Hz, 2H), 7.29 (t, J = 7.4 Hz, 2H), 6.74 (d, J = 9.1 Hz, 1H), 4.28 (d, J = 7.0 Hz, 2H), 4.24 – 4.17 (m, 2H), 4.13 (dd, J = 14.3, 6.9 Hz, 2H), 3.09 – 3.01 (m, 2H), 2.46 (p, J = 1.8 Hz, 31H), 2.39 (s, 3H), 1.95 (s, 3H), 1.35 (s, 6H). ^{13}C NMR (101 MHz, CD_3CN) δ 172.42, 158.89, 157.30, 157.04, 150.51, 144.58, 144.50, 141.69, 138.61, 137.76, 133.99, 132.82, 128.29, 127.68, 125.78, 125.58, 124.60, 124.26, 121.28, 120.55, 118.16, 117.88, 117.68, 114.63, 87.09, 78.73, 67.13, 55.80, 47.60, 43.08, 40.55, 30.48, 29.05, 28.23, 26.15, 19.16, 17.91, 12.22, 1.52, 1.46, 1.37, 1.31, 1.25, 1.16, 1.11, 1.05, 1.01, 0.90, 0.84, 0.81, 0.69, 0.63, 0.60, 0.49, 0.39, 0.28. ESI$^+$-HRMS $[M+H]^+$ Calculated: 784.313 m/z, Observed: 784.314 m/z.

Synthesis of N_α-Fmoc-N_ω-Pbf-L-thioarginine-2-amino-5-nitroanilide (S2) Anhydrous Na_2CO_3 (0.676 g, 6.38 mmol, 1 equiv) and P_4S_{10} (2.84 g, 6.38 mmol, 1 equiv) were dissolved in 50 mL of dry THF and stirred at room temperature under argon for 30 min until the mixture became a clear pale yellow solution. The solution was then cooled to 0 °C over an ice bath. Compound **S1** (5.00 g, 6.38 mmol, 1 equiv) was added to the solution and allowed to react under argon atmosphere overnight at 0 °C, warming to room temperature. Upon completion, the solvent was removed by rotary evaporation. The crude reaction mixed was filtered through Celite to remove excess phosphorous pentasulfide. The filtrate was dry packed onto a 340 g silica SNAP column from Biotage (Charlotte, NC) and purified by normal phase chromatography using an ethyl acetate/hexanes gradient (50% to 100% ethyl acetate over 12 column volumes) to yield an orange foam (1.518 g, 1.899 mmol, 30% yield) in 100% purity (determined by HPLC). R_f = 0.24 in 9:1

ethyl acetate:hexanes. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.27 (s, 2H), 8.46 (d, J = 2.4 Hz, 1H), 7.93 (dd, J = 9.1, 2.6 Hz, 3H), 7.86 (t, J = 9.1 Hz, 9H), 7.78 (dd, J = 8.7, 2.5 Hz, 1H), 7.70 (dd, J = 15.9, 7.5 Hz, 5H), 7.38 (t, J = 7.4 Hz, 5H), 7.29 (t, J = 7.4 Hz, 5H), 6.77 (d, J = 9.1 Hz, 3H), 6.51 (d, J = 8.7 Hz, 2H), 6.32 (s, 4H), 4.40 (q, J = 6.5 Hz, 2H), 4.34 – 4.26 (m, 3H), 4.25 – 4.17 (m, 5H), 3.99 (dd, J = 8.6, 6.6 Hz, 3H), 3.72 (qq, J = 7.1, 4.0, 3.6 Hz, 2H), 3.51 – 3.45 (m, 1H), 3.14 (s, 2H), 3.08 (q, J = 6.5 Hz, 5H), 2.91 (s, 5H), 2.41 (s, 7H), 1.97 (s, 7H), 1.95 (s, 2H), 1.36 (s, 15H), 1.14 (t, J = 7.1 Hz, 2H), 1.02 (d, J = 6.7 Hz, 6H), 0.82 (d, J = 6.7 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 206.48, 171.40, 159.44, 157.00, 156.46, 148.88, 143.77, 143.74, 143.69, 141.45, 141.39, 141.34, 138.71, 138.00, 132.59, 128.02, 127.98, 127.94, 127.36, 127.30, 127.22, 125.55, 125.33, 125.28, 125.19, 125.13, 122.58, 120.23, 120.20, 118.09, 115.10, 86.91, 86.82, 82.58, 77.42, 67.61, 60.81, 60.63, 53.64, 47.28, 47.24, 47.14, 43.26, 40.62, 33.08, 32.14, 31.80, 29.92, 29.87, 29.58, 28.75, 28.49, 28.43, 25.89, 22.91, 22.76, 21.29, 20.34, 19.99, 19.94, 19.55, 18.21, 14.41, 14.35, 12.73, 12.67, 12.50. ESI^{+}-HRMS $[M+H]^{+}$ Calculated: 800.290 m/z, Observed: 800.2917 m/z.

Synthesis of N_α-Fmoc-N_ω-Pbf-L-thioarginine-nitrobenzotriazolide (S3) Compound **S2** (1.503 g, 1.899 mmol, 1 equiv) was added to 40 mL of neat glacial acetic acid at 0 °C and stirred until fully dissolved. $NaNO_2$ (0.164 g, 2.374 mmol, 1.25 equiv) was added in small portions with stirring over 5 min. The reaction was allowed to stir for 30 min at 0 °C, then quenched with 200 mL of ice water, resulting in an orange precipitate. The precipitate was collected by filtration, rinsed with cold water, and dried overnight by lyophilization. An orange solid was obtained as the final product (1.104 g, 1.363 mmol, 72% yield) in 80% purity (determined by HPLC). ^{1}H NMR (500 MHz, Chloroform-d) δ 9.53 (s, 1H), 8.78 (d, J = 8.2 Hz, 1H), 8.48 – 8.32 (m, 1H), 8.30 (s, 1H), 8.24 – 8.15 (m, 1H), 7.68 (d, J = 7.6 Hz, 2H), 7.55 (t, J = 7.0 Hz, 3H), 4.41 (d, J = 7.1 Hz,

2H), 4.29 (d, *J* = 8.5 Hz, 2H), 4.12 (td, *J* = 7.2, 4.1 Hz, 2H), 2.88 (s, 3H), 2.51 (d, *J* = 12.2 Hz, 4H), 2.44 (d, *J* = 12.0 Hz, 5H), 2.04 (d, *J* = 12.4 Hz, 7H), 1.42 (s, 10H). ^{13}C NMR (101 MHz, DMSO) δ 202.51, 176.16, 158.89, 158.74, 157.97, 157.93, 156.53, 156.33, 154.43, 147.42, 145.16, 144.39, 144.22, 144.01, 143.90, 143.82, 141.26, 141.20, 141.12, 138.93, 138.44, 137.79, 137.66, 134.61, 134.51, 133.12, 132.61, 132.57, 131.98, 131.94, 131.88, 131.84, 128.08, 127.53, 125.65, 125.25, 125.22, 124.78, 124.73, 124.69, 121.46, 120.55, 120.51, 117.25, 117.04, 116.81, 116.76, 116.71, 116.67, 87.21, 87.17, 86.69, 86.64, 66.21, 55.34, 47.18, 47.13, 42.93, 42.72, 28.69, 28.65, 19.47, 19.43, 19.38, 18.34, 18.12, 18.05, 12.73, 12.68, 12.66. ESI$^+$-HRMS $[M+H]^+$ Calculated: 811.270 m/z, Observed: 811.270 m/z.

Synthesis of Other Thioaminoacid Precursors. N_α-Fmoc-N_e-Boc-L-thiolysine-nitrobenzo triazolide, N_α-Fmoc-L-thioleucine-nitrobenzotriazolide, N_α-Fmoc-L-thiophenylalanine-nitrobenzo triazolide, and N_α-Fmoc-L-thioalanine-nitrobenzotriazolide were synthesized as previously reported by our laboratory.[1-2]

Peptide Synthesis, Purification, and Characterization.

Peptide Synthesis. Each peptide was manually synthesized on a 25 μmol scale on 2-chlorotrityl resin using our established protocols.[3] Prior to use, the glass peptide reaction vessel (RV) was coated with Sigmacote® and dried under vacuum. For a typical synthesis, 2-chlorotrityl resin was added to a dry RV and swelled in 5 mL dimethylformamide (DMF) for 30 min with magnetic stirring. Between each reaction, the resin was washed extensively with adequate DMF. For the first amino acid coupling, 5 equiv of amino acid was dissolved in 2 mL of DMF and added to the RV, following an addition of 10 equiv of DIPEA. The reaction ran for 30 min under stirring. After

washing, the resin was incubated with 2 mL of 20% piperidine solution in DMF for 20 min under stirring for deprotection. The same deprotection procedure was followed for all the subsequent standard amino acids. For a typical coupling reaction, 5 equiv of amino acid and 5 equiv of HBTU or PyAOP was dissolved in DMF, and added to the RV, with an addition of 10 equiv of DIPEA. Thioamide residues were coupled and deprotected with slightly different procedures. Thioamides were coupled through pre-activated precursors, where 2 equiv of thioamide was dissolved in 1.5 mL of dry dichloromethane (DCM) with 2 equiv DIPEA, and stirred for 30 min. This procedure was repeated prior to deprotection. For the deprotection of thioamides, 2% DBU (1,8-diazabicyclo(5.4.0)undec-7-ene) in DMF was added to the RV and reacted three times for two min each with extensive washing with DMF and DCM between each deprotection step.

Peptide Cleavage and Purification. Upon completion of the synthesis, the resin was dried with DCM under vacuum. Peptides were cleaved off resin by treatment with a 2 mL fresh cleavage cocktail of trifluoroacetic acid (TFA), water, and triisopropylsilane (TIPSH) (95:2.5:2.5 v/v) for 45 min with stirring. After treatment, the cocktail solution was expelled from the RV with nitrogen and reduced to a volume of less than 1 mL by rotary evaporation. This TFA solution was then treated with over 10 mL of cold ethyl ether in order to precipitate the peptides from the cleavage solution. This precipitate was redissolved in 2mL 50:50 CH_3CN/H_2O, flash frozen with liquid nitrogen, and evaporated using lyophilization. The crude was diluted in CH_3CN/H_2O (1:1 v/v) and then purified on a Vydac 218TP C18 prep column (Grace/Vydac; Deerfield, IL, USA) by HPLC using the following gradients (**Table S1**, **Table S2**) at a flow rate of 12 mL/min. MALDI-MS was used to confirm peptide identities (**Table S3**). Purified peptides were dried on a lyophilizer (Labconco; Kansas City, MO, USA) or in a vacuum centrifuge (Savant/Thermo

Scientific; Rockford, IL, USA). If needed, peptides were subjected to multiple rounds of purification until 99% purity was achieved.

Table S1. Peptide Purification Methods and Retention Time.

Peptide	Gradient	Retention Time
μLLKAAAμ	1	35 min
μL^{S}LKAAAμ	1	33 min
μLL^{S}KAAAμ	1	35 min
μLLK^{S}AAAμ	1	35 min
μLLKA^{S}AAμ	2	28 min
μLLKAA^{S}Aμ	2	28 min
μLLKAAA^{S}μ	2	28 min
μLLRAAAμ	1	35 min
μL^{S}LRAAAμ	3	18 min
μLL^{S}RAAAμ	2	22 min
μLLR^{S}AAAμ	1	35 min
μLLRA^{S}AAμ	3	21 min
μLLRAA^{S}Aμ	3	20 min
μLLRAAA^{S}μ	3	23 min
μKAAFAAAμ	4	23 min
μK^{S}AAFAAAμ	5	20 min
μKA^{S}AFAAAμ	5	20 min
μKAA^{S}FAAAμ	5	23 min
μKAAF^{S}AAAμ	5	22 min
μKAAFA^{S}AAμ	5	22 min
μKAAFAA^{S}Aμ	5	23 min
μKAAFAAA^{S}μ	5	24 min

* μ: 7-methoxycoumarinylalanine; A^{S}: thioalanine; L^{S}: thioleucine; K^{S}: thiolysine; R^{S}: thioarginine; F^{S}: thiophenylalanine

Table S2. HPLC Gradients for Peptide Purification.

No.	Time (min)	%B	No.	Time (min)	%B
1	0:00	2	2	0:00	2
	6:00	2		6:00	2
	8:00	15		8:00	20
	37:00	40		28:00	40
	40:00	100		31:00	100
	45:00	100		36:00	100
	50:00	2		41:00	2
3	0:00	10	4	0:00	2
	2:00	10		6:00	2
	5:00	20		9:00	20
	25:00	40		24:00	40
	26:00	40		28:00	100
	27:00	100		33:00	100
	30:00	100		48:00	2
	35:00	10			
5	0:00	2			
	6:00	2			
	8:00	27			
	24:00	35			
	26:00	100			
	31:00	100			
	36:00	2			

* Solvent A: 0.1 % TFA in water; Solvent B: 0.1 % TFA in acetonitrile

Table S3. Calculated and Observed Masses of Peptides.

Peptide	$[M+H]^+$ (m/z)		$[M+Na]^+$ (m/z)		$[M+K]^+$ (m/z)	
	Calculated	**Observed**	**Calculated**	**Observed**	**Calculated**	**Observed**
μLLKAAAμ	1076.53	1075.81	1098.51	1097.79	1114.49	1113.77
μL^{S}LKAAAμ	1092.51	1092.44	1114.49	1114.42	1130.46	1130.39
μLL^{S}KAAAμ	1092.51	1092.42	1114.49	1114.40	1130.46	1130.37
μLLK^{S}AAAμ	1092.51	1092.87	1114.49	1114.85	1130.46	1130.84
μLLKA^{S}AAμ	1092.51	1092.43	1114.49	1114.41	1130.46	1130.38
μLLKAA^{S}Aμ	1092.51	1092.38	1114.49	1114.36	1130.46	1130.43
μLLKAAA^{S}μ	1092.51	1092.52	1114.49	1114.51	1130.46	1130.47
μLLRAAAμ	1104.54	1104.11	1126.52	1126.11	1142.49	1142.08
μL^{S}LRAAAμ	1120.51	1120.48	1142.50	1142.57	1158.47	1158.57
μLL^{S}RAAAμ	1120.51	1120.64	1142.50	1142.61	1158.47	1158.59
μLLR^{S}AAAμ	1120.51	1120.54	1142.50	1142.53	1158.47	1158.50
μLLRA^{S}AAμ	1120.51	1120.40	1142.50	1142.58	1158.47	1158.41
μLLRAA^{S}Aμ	1120.51	1120.42	1142.50	1142.47	1158.47	1158.58
μLLRAAA^{S}μ	1120.51	1120.83	1142.50	1142.88	1158.47	1158.82
μKAAFAAAμ	1139.50	1139.77	1161.49	1161.80	1177.46	1177.79
μK^{S}AAFAAAμ	1155.48	1155.54	1177.46	1177.57	1193.44	1193.55
μKA^{S}AFAAAμ	1155.48	1155.68	1177.46	1177.68	1193.44	1193.67
μKAA^{S}FAAAμ	1155.48	1155.12	1177.46	1177.12	1193.44	1193.11
μKAAF^{S}AAAμ	1155.48	1155.80	1177.46	1177.88	1193.44	1193.93
μKAAFA^{S}AAμ	1155.48	1155.73	1177.46	1177.72	1193.44	1193.68
μKAAFAA^{S}Aμ	1155.48	1155.71	1177.46	1177.74	1193.44	1193.73
μKAAFAAA^{S}μ	1155.48	1155.54	1177.46	1177.45	1193.44	1193.72

* μ: 7-methoxycoumarinylalanine; A^S: thioalanine; L^S: thioleucine; K^S: thiolysine; R^S: thioarginine; F^S: thiophenylalanine

Sensor Peptide Analysis

Steady State Protease Assays. In a typical trial, a 7.5 μM solution of peptide was incubated in the presence or absence of 0.2 mg/mL chymotrypsin in 100 mM Tris-HCl, pH 7.8 at 25 °C, or in the presence or absence of 25 μg/mL trypsin in 67 mM sodium phosphate, pH 7.6 at 25 °C, or in the presence or absence of 4.3 μg/mL kallikrein in 20 mM Tris-HCl, 100 mM NaCl, pH 7.5 buffer at 25 °C. The fluorescence was monitored as a function of time at 390 nm with an excitation wavelength of 325 nm on the Tecan plate reader. Three independent trials were performed for each assay to ensure reproducibility. Primary data are shown in **Figures S1-S4**. The data from the fluorescent trials were averaged and cleavage percentages were calculated by dividing the fluorescence change of each time point by the fluorescence change of complete cleavage (measured up to 6 h). Initial rates were determined by fitting a line to early timepoints (first 2 min) and later timepoints (between min 10 to 20) if a biphasic curve was observed. These are reported in **Table S4a/b** (with standard error) and normalized rates are given in the main text.

Table S4a. Initial Rates of Peptides Cleavage by Serine Proteases.

Trypsin Lys Peptides	Rate (μM•Min^{-1})	Trypsin Arg Peptides	Rate (μM•Min^{-1})
Lys Control	1.900 ± 0.101	Arg Control	3.320 ± 0.902
Lys P3	0.939 ± 0.037	Arg P3	1.838 ± 0.101
Lys P2	1.787 ± 0.019	Arg P2	3.458 ± 0.093
Lys P1	0.177 ± 0.017[a] 0.011 ± 0.002[b]	Arg P1	0.016 ± 0.001
Lys P1'	0.990 ± 0.073	Arg P1'	3.222 ± 0.304
Lys P2'	1.140 ± 0.031	Arg P2'	5.405 ± 0.125
Lys P3'	1.048 ± 0.021	Arg P3'	4.953 ± 0.166

[a]Cleavage rate during first five min. [b]Cleavage rate during 10-40 min.

Table S4b. Initial Rates of Peptides Cleavage by Serine Proteases.

Chymotrypsin Peptides	**Rate (μM•Min^{-1})**	**Kallikrein Peptides**	**Rate (μM•Min^{-1})**
Phe Control	6.049 ± 0.469	Arg Control	0.634 ± 0.021
Phe P3	1.311 ± 0.181	Arg P3	0.022 ± 0.006
Phe P2	0.010 ± 0.001	Arg P2	0.390 ± 0.016
Phe P1	0.012 ± 0.001	Arg P1	0.002 ± 0.001
Phe P1'	10.050 ± 0.440	Arg P1'	0.544 ± 0.065
Phe P2'	7.826 ± 0.532	Arg P2'	0.601 ± 0.013
Phe P3'	9.452 ± 0.118	Arg P3'	0.690 ± 0.019

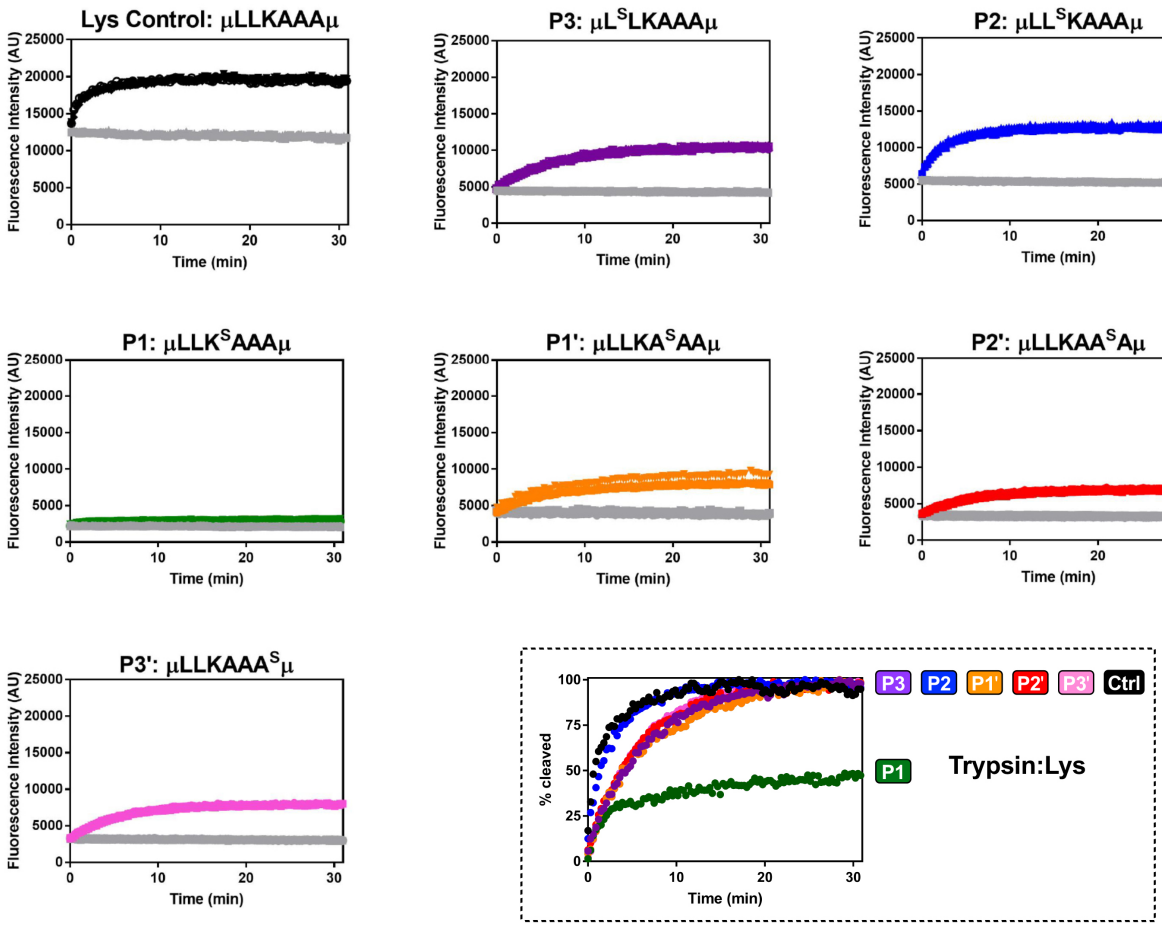


Figure S1. Trypsin Proteolysis of Lys Peptides Monitored by Fluorescence. A 7.5 μM solution of peptide was incubated in the presence or absence of 25 μg/mL of trypsin in 67 mM sodium phosphate buffer (pH 7.6) at 25 °C. Three independent trials for each peptide are shown. Black trace: All amide Lys Control peptide in the presence of protease; Colored traces: Lys thioamide peptides in the presence of protease; Grey traces: Thioamide peptides or control peptide in the absence of protease. Inset: Normalized cleavage data for control and thioamide peptides colored as indicated.

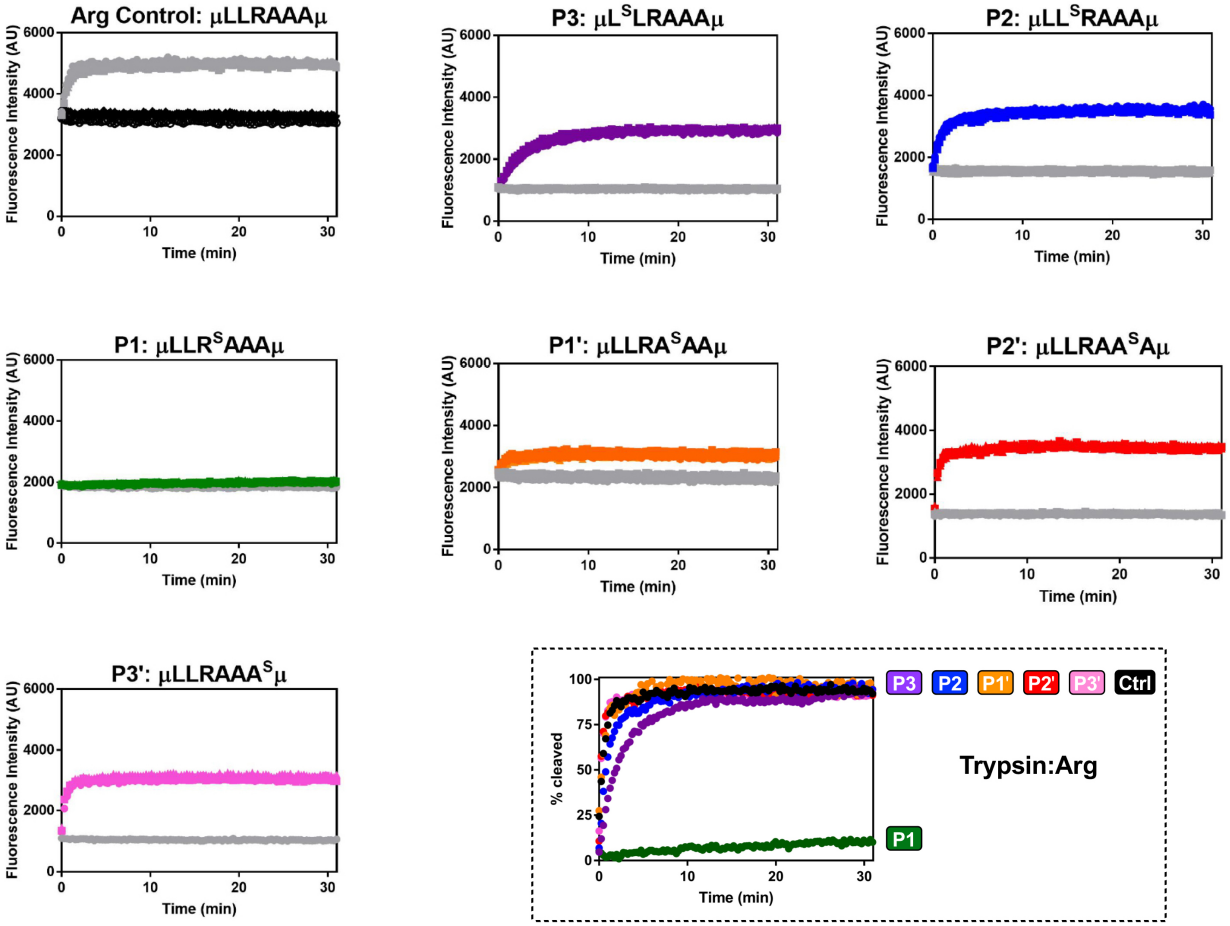


Figure S2. Trypsin Proteolysis of Arg Peptides Monitored by Fluorescence. A 7.5 μM solution of peptide was incubated in the presence or absence of 25 μg/mL of trypsin in 67 mM sodium phosphate buffer (pH 7.6) at 25 °C. Three independent trials for each peptide are shown. Black trace: All amide Arg Control peptide in the presence of protease; Colored traces: Arg thioamide peptides in the presence of protease; Grey traces: Thioamide peptides or control peptide in the absence of protease. Inset: Normalized cleavage data for control and thioamide peptides colored as indicated.

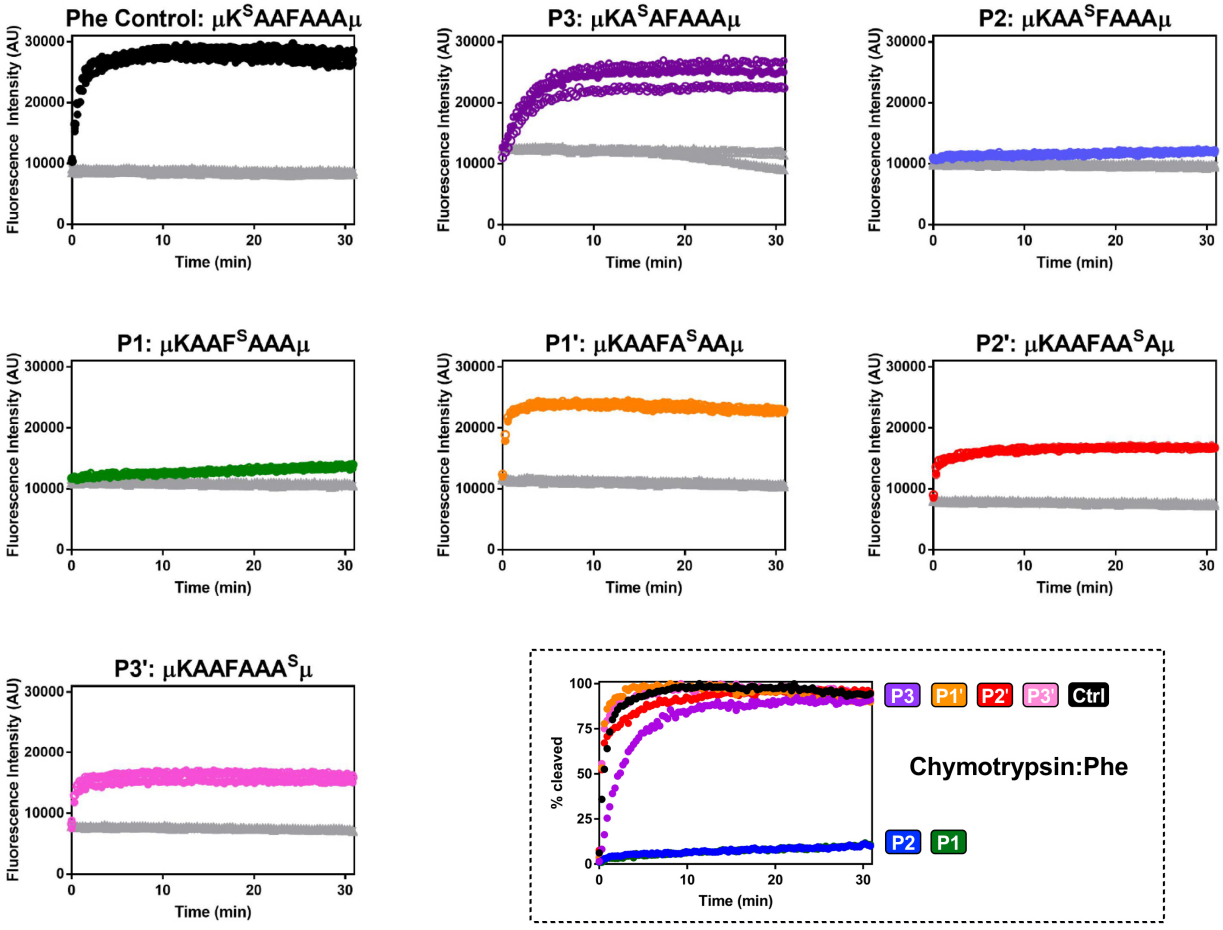


Figure S3. Chymotrypsin Proteolysis of Phe Peptides Monitored by Fluorescence. A 7.5 μM solution of peptide was incubated in the presence or absence of 0.2 mg/mL chymotrypsin in 100 mM Tris-HCl, pH 7.8 at 25 °C. Three independent trials for each peptide are shown. Black trace: P4 thioamide Phe Control peptide in the presence of protease; Colored traces: Phe thioamide peptides in the presence of protease; Grey traces: Thioamide peptides or control peptide in the absence of protease. Inset: Normalized cleavage data for control and thioamide peptides colored as indicated.

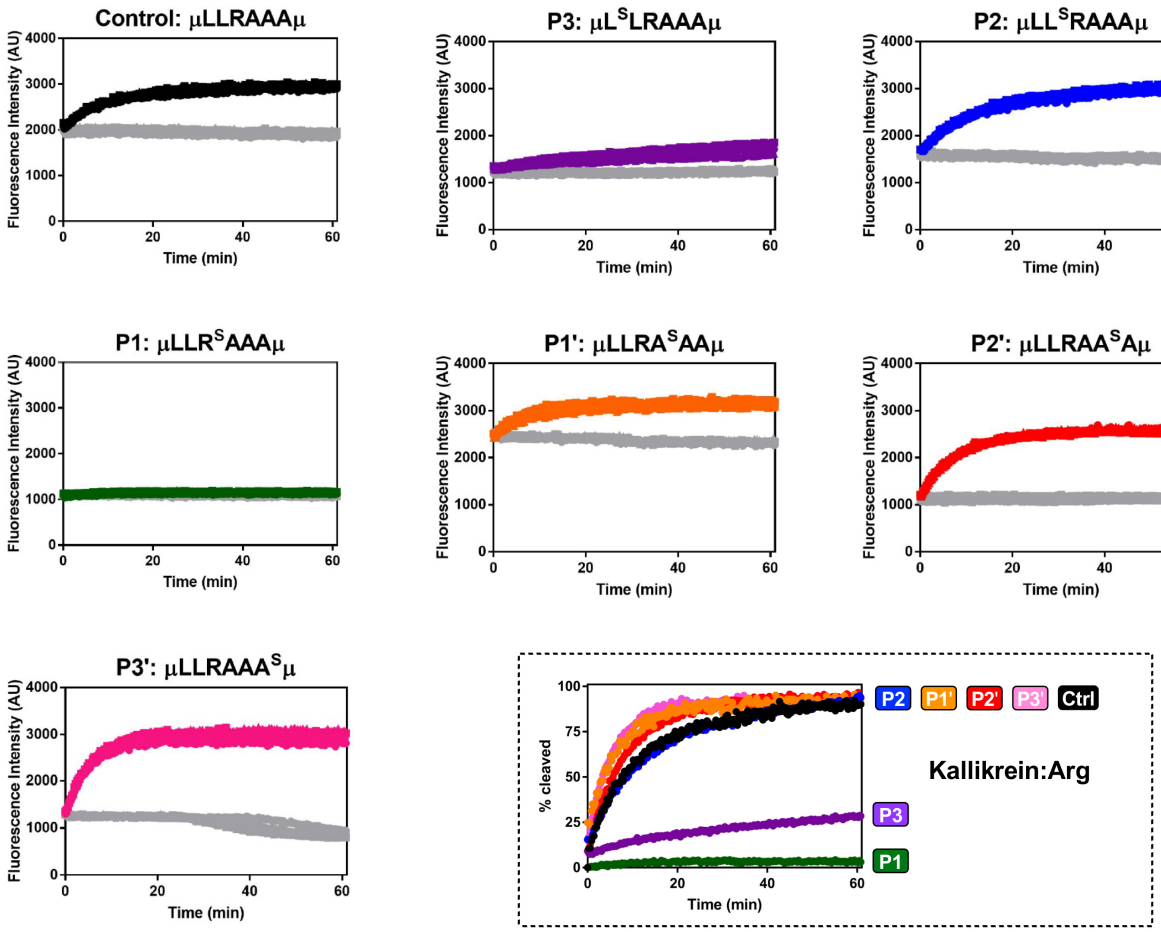


Figure S4. Kallikrein Proteolysis of Arg Peptides Monitored by Fluorescence. A 7.5 µM solution of peptide was incubated in the presence or absence of 4.26 µg/mL kallikrein in 20 mM Tris-HCl, 100 mM NaCl, pH 7.5 buffer at 25 °C. Three independent trials for each peptide are shown. Black trace: All amide Arg Control peptide in the presence of protease; Colored traces: Arg thioamide peptides in the presence of protease; Grey traces: Thioamide peptides or control peptide in the absence of protease. Inset: Normalized cleavage data for control and thioamide peptides colored as indicated.

Kinetic Analysis of P1 Thioamide Peptides

Kinetic Assays at Varying Substrate Concentrations. Various concentration of the Lys or Arg Control peptides were treated with 0.1 μM of trypsin in 67 mM sodium phosphate buffer, pH 7.6, at 25 °C in a 96-well plate. Various concentrations of the P1 thiopeptides were treated with 10 μM of trypsin in 67 mM sodium phosphate buffer, pH 7.6, at 25°C in a 96-well plate. The fluorescence of the reaction was monitored as a function of time at 390 nm with an excitation wavelength of 325 nm on the Tecan plate reader. Three independent trials were performed for each assay to ensure reproducibility. Data were analyzed by subtracting an average of the fluorescence of the no protease control (F_{Min}) from the average fluorescence of the plus protease wells at time t, F(t). Then, fluorescence was converted to concentration of product (in μM) using **Eq. S1**, where F_{Max} is the average fluorescence of the plus protease wells over the final 1000 s of the assay, and $[S]_0$ is the initial concentration of substrate.

$$[\text{Product}] = [S]_0 \times \frac{(F(t) - F_{Min})}{(F_{Max} - F_{Min})} \qquad \textbf{(Eq. S1)}$$

Kinetic Parameter Estimation in COPASI. Since limitations in the solubility of substrates and the low activity of trypsin toward the thioamide substrates precluded using a Michaelis-Menten model, the data were numerically fit using COPASI software to a reversible mass action model (**Eq. S2**) to determine k_1 and k_{-1}, and k_{cat}.[4] In order to fit the data, progress curves were imported into COPASI, and the Parameter Estimation task was used. Initial concentrations of enzyme (E) and substrate (S) were specified as those used in the experiment, while concentrations of the ES complex and product (P) were set to zero. Initial parameter estimations were done using evolutionary programming with 200 generations and a population size of 20. The starting values

for k_1, k_{-1}, and k_{cat} were set to 0.1 with upper and lower bounds set to 1 x $10^{\pm 100}$. Once the values converged and had a small standard deviation, the parameter estimation was run again with 1000 generations and a population size of 100 to confirm the robustness of these values. The goodness of fit can be observed in the relatively small standard deviations in the fitted parameters (**Table S5**) and by inspection of **Figs. S5-S6**.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad \textbf{(Eq. S2)}$$

Table S5. Kinetic Parameters Estimated from Mass Action Analysis

Substrate	k_1 ($\mu M^{-1} \cdot min^{-1}$)	k_{-1} (min^{-1})	k_2 (min^{-1})
µLLKAAAµ	1.026 ± 0.007	<2.8 x 10^{-6}	106.2 ± 0.9
µLLK^{S}AAAµ	0.408 ± 0.007	46.56 ± 0.82	1.308 ± 0.014
µLLRAAAµ	5.724 ± 0.114	<8.4 x 10^{-5}	273.6 ± 3.1
µLLR^{S}AAAµ	0.438 ± 0.156	2.880 ± 0.960	0.126 ± 0.002

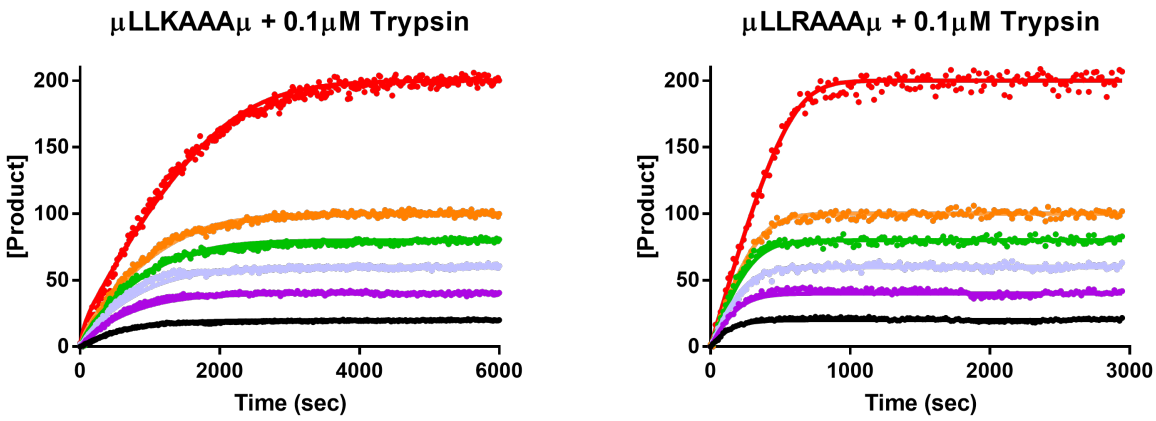


Figure S5. Kinetic Data Fitting for Control Peptides. Progress curves with COPASI fits of µLLKAAAµ (left) and µLLRAAAµ (right) treated with trypsin. Various concentrations of peptide were incubated in the presence of 0.10 µM trypsin in 67 mM sodium phosphate buffer (pH 7.6) at 25 °C. [peptide]: 20 µM, 40 µM, 60 µM, 80 µM, 100 µM, 200 µM in black, purple, lavender, green, orange, and red traces, respectively.

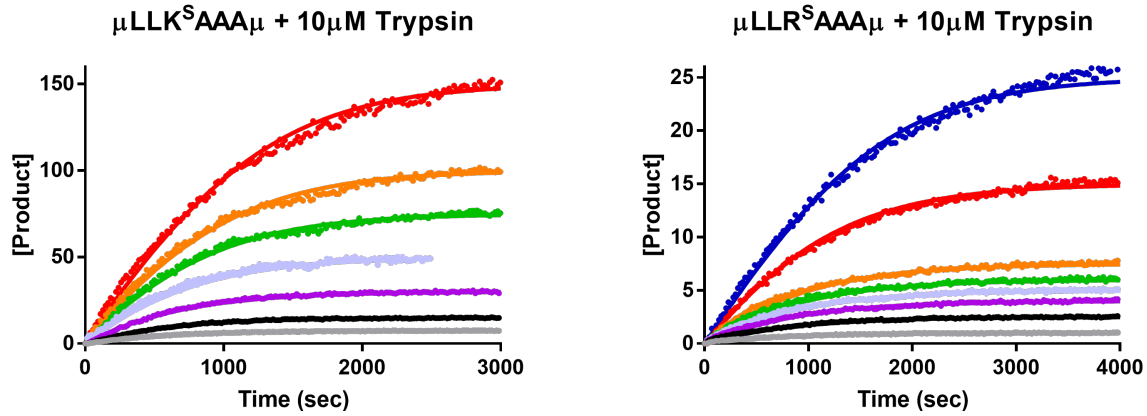


Figure S6. Kinetic Data Fitting for P1 Thioamide Peptides. Progress curves with COPASI fits of µLLK^{S}AAAµ (left) and µLLR^{S}AAAµ (right) treated with trypsin. Various concentrations of peptide were incubated in the presence of 10 µM trypsin in 67 mM sodium phosphate buffer (pH 7.6) at 25 °C. [µLLK^{S}AAAµ]: 7.5, 15, 30, 50, 75, 100, and 150 µM in gray, black, purple, lavender, green, orange, and red traces, respectively. [µLLR^{S}AAAµ]: 1.0, 2.5, 4.0, 5.0, 6.0, 7.5, 15, and 25 µM in gray, black, purple, lavender, green, orange, red, and blue traces, respectively.

Inhibition Assay. Various concentrations of trypsin substrate *N*-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) (50 μM, 100 μM, 500 μM, 1 mM, 1.5 mM, 2.0 mM, and 3.0 mM) were reacted with 25 μg/mL trypsin in 67 mM sodium phosphate buffer, pH 7.6, at 25 °C in a 96-well plate. For thioamide inhibition, 100 μM of Lys P1 or 80 μM Arg P1was incubated with 25 μg/mL trypsin for 15 min at 25 °C to allow for full interactions before adding the BAPNA substrates. The reaction was monitored by UV-Vis absorbance at 410 nm by the plate reader. Three trials were performed to ensure reproducibility. From the extinction coefficient of *p*-nitroaniline (8800 $M^{-1}\cdot cm^{-1}$), the absorbance was converted into the product *p*-nitroaniline concentration. The reaction rate was calculated by extracting the slope of a linear fit of the first 2 min of the cleavage reaction. The average of reaction rates from three trials were calculated used to construct a Michaelis-Menten plot (**Fig. S7**). These curves were fit to **Eq. S3** in Prism to determine V_{Max} and K_M. Turnover number (k_{cat}) was calculated by dividing V_{Max} by the enzyme concentration.

$$v_0 = V_{Max}\ \frac{[S]}{[S] + /K_M} \qquad \textbf{(Eq. S3)}$$

Table S6. Kinetic Parameters for Trypsin Inhibition by Thioamide Peptides.

Condition	k_{cat} (sec^{-1})	K_M (mM)	k_{cat} / K_M ($mM^{-1}\cdot sec^{-1}$)
No Inhibitor	0.049 ± 0.0028	1.59 ± 0.32	0.031 ± 0.004
100 μM μLLK^{S}AAAμ	0.088 ± 0.0023	2.17 ± 0.62	0.041 ± 0.007
80 μM μLLR^{S}AAAμ	0.045 ± 0.0077	0.65 ± 0.19	0.069 ± 0.014

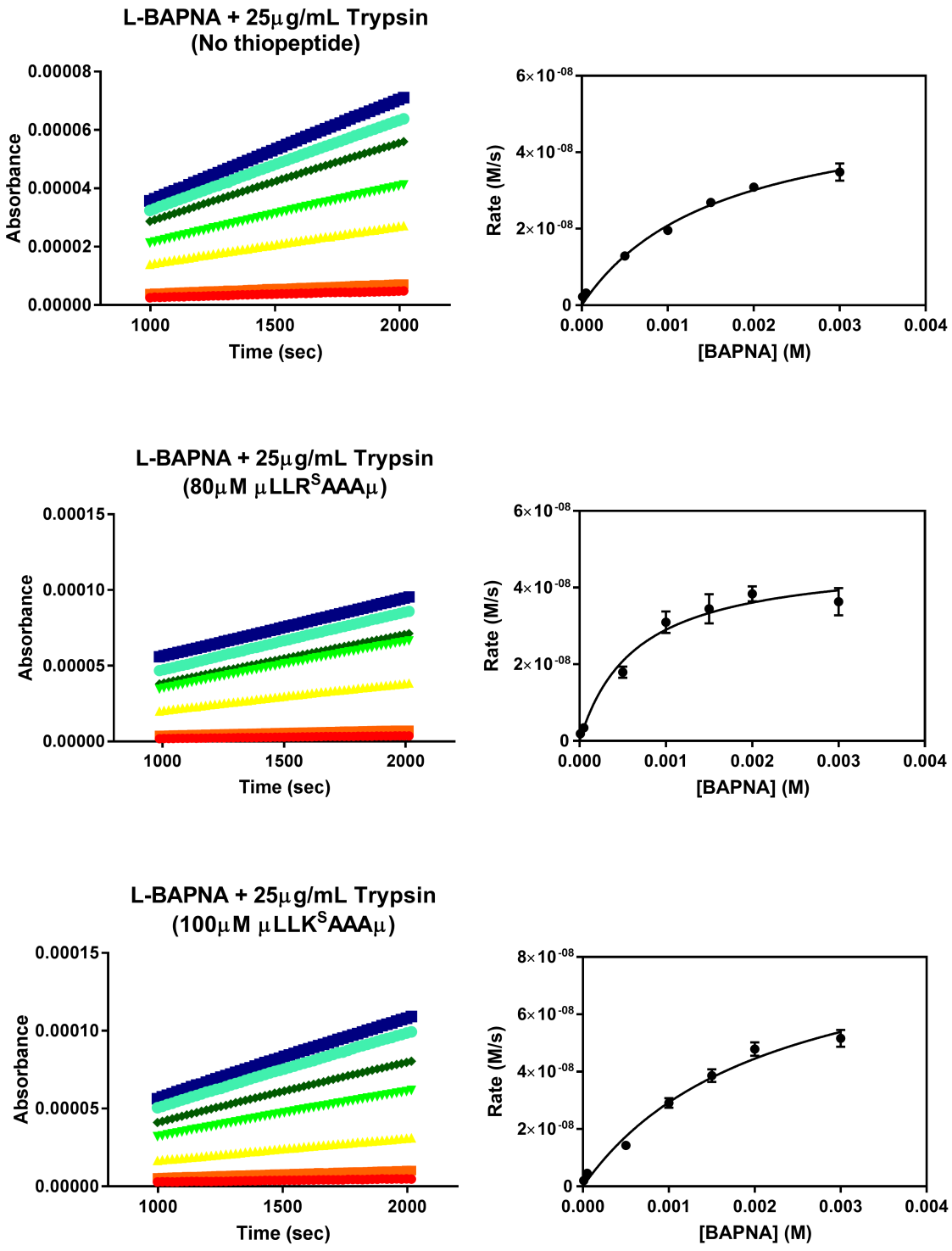


Figure S7. Trypsin Activity Monitored by Colorimetric Substrate BAPNA. Left: A 25 μg/mL trypsin solution was incubated in the presence or absence of thioamide peptides, then mixed with various concentrations of BAPNA. [BAPNA]: 50 μM, 100 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, and 3 mM in red, orange, yellow, light green, dark green, teal, and dark blue, respectively. Right: Michaelis-Menten curves showing fits to Eq. S2.

Computational Modeling

Structure Analysis: Modeling of LLKAAA Peptide Binding to Trypsin. The interaction between the μLLKAAAμ "Oxo" peptide and trypsin was modeled using PyRosetta and the Rosetta Modeling Suite.[5-6] The PDB structure 2PTC was used as a starting structure of trypsin to simulate its docking interactions with the LLKAAA peptide. The native inhibitor peptide was trimmed both N, and C terminally leaving only the VYGGCR 6-mer in the active site of trypsin. This peptide was converted to the LLKAAA peptide, using the mutate residue functionality within the PyRosetta toolbox. After generation of the target peptide in the active site of trypsin, the FlexPepDock refinement protocol within Rosetta was performed on the complex. The FlexPepDock protocol is a Monte-Carlo based simulation which applies gradient-based energy minimization to optimize the peptide's rigid body orientation relative to receptor protein it is docked with. During this optimization, both the peptide and enzyme sidechains and backbones are allowed to flexibly explore torsions.[7] The refine FlexPepDocking protocol was run using the default parameters sampling 1000 decoys with constraints set with a weight of 100 Rosetta Energy Units (REUs).[7] The 10 lowest energy structures, according to the FlexPep score, were visualized in PyMOL.[7-8] As shown in **Figure S8**, all 10 output structures display nearly identical binding interactions spanning the entire peptide from positions P3 to P3'.

Following the FlexPepDock protocol, the lowest energy structure was subjected to two relax protocols. The Relax algorithm consists of sidechain repacking alongside gradient-based minimization of both backbone and side-chain torsion angles. Initially, the van der Waals repulsion score term is downweighed, but increases in weight with each applied cycle, ultimately returning to its default weight during the final cycle of the Relax.[9] A total of 100 trajectories were sampled, performing 10 FastRelax protocol executions per trajectory.[9] Both protocols were performed using the "ref2015_cart" score function with the dualspace flag set to True. This flag corresponds to the optimization in latter stages of the protocol occurring in cartesian space.[10] Minimizations were performed using the "lbfgs_armigo_nonmonotone" minimization method allowing for minimization of the bond angles. The difference between the two

protocols was that the first included a Flat_Harmonic potential constraint of the active site serine oxygen to the P1 amide carbon with an energy of zero from 2 to 4 Å with a standard deviation of 1 Å dictating the harmonic profile outside of the zero-energy region).[11] The lowest energy structure from the constrained relaxes was then put into an identical relax protocol without the constraint to ensure that the peptide was energetically stable in a cleavable orientation without being forced to be. The ten lowest energy structures observed during the final search were all practically identical in terms of peptide conformation (<0.5 Å RMSD across all peptide atoms). These are shown overlaid in **Figure S9** and are shown separately in **Figure S10**. The energy in REU of each of the structures is plotted as a function of RMSD relative to the lowest energy structure in **Figure S10**. The peptide-enzyme hydrogen bonds observed in the "Relax" outputs were also observed in the FlexPepDock outputs, bolstering our confidence in these simulated docking interactions. The average hydrogen bond distances for all contacts made by the backbone amides in the 10 lowest energy structures are reported in **Figure S10**. The consensus structure from these simulations in shown in **Figure 2** in the main text.

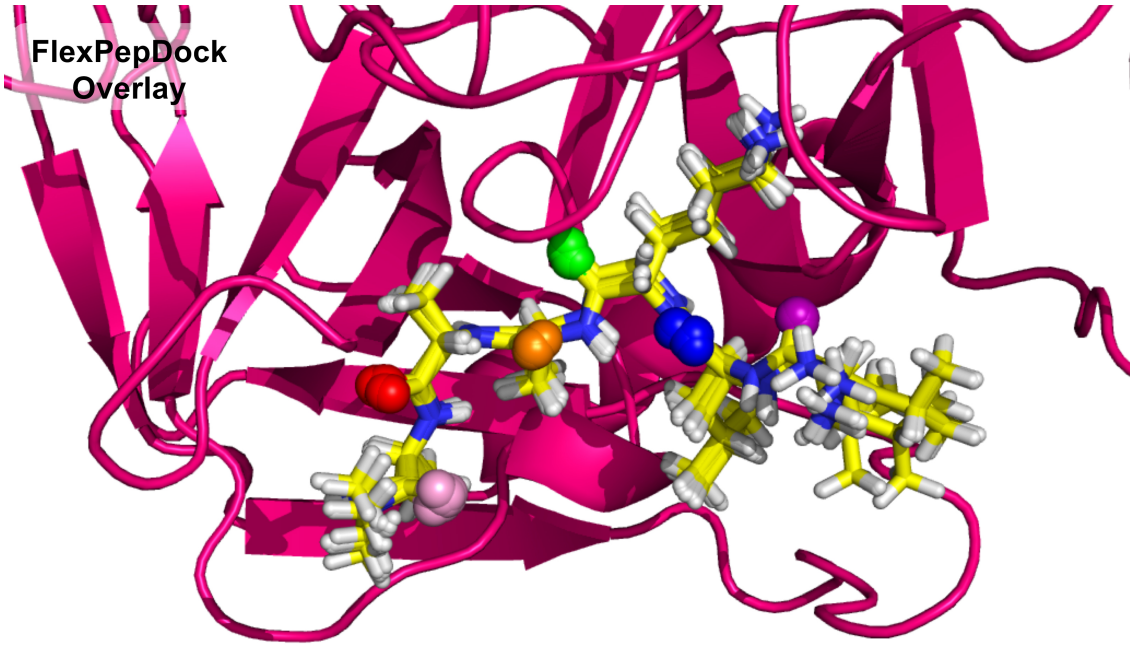


Figure S8. Overlay of Lowest Energy Structures from "FlexPepDock" Simulations of Trypsin. The 10 lowest energy structures of trypsin in complex with docked LLKAAA have minimal variations in carbonyl interactions of the P3 (purple), P2 (blue), P1 (green), P1' (orange), P2' (red), and P3' (pink) residues. Note: Protein backbone structure is static in these simulations.

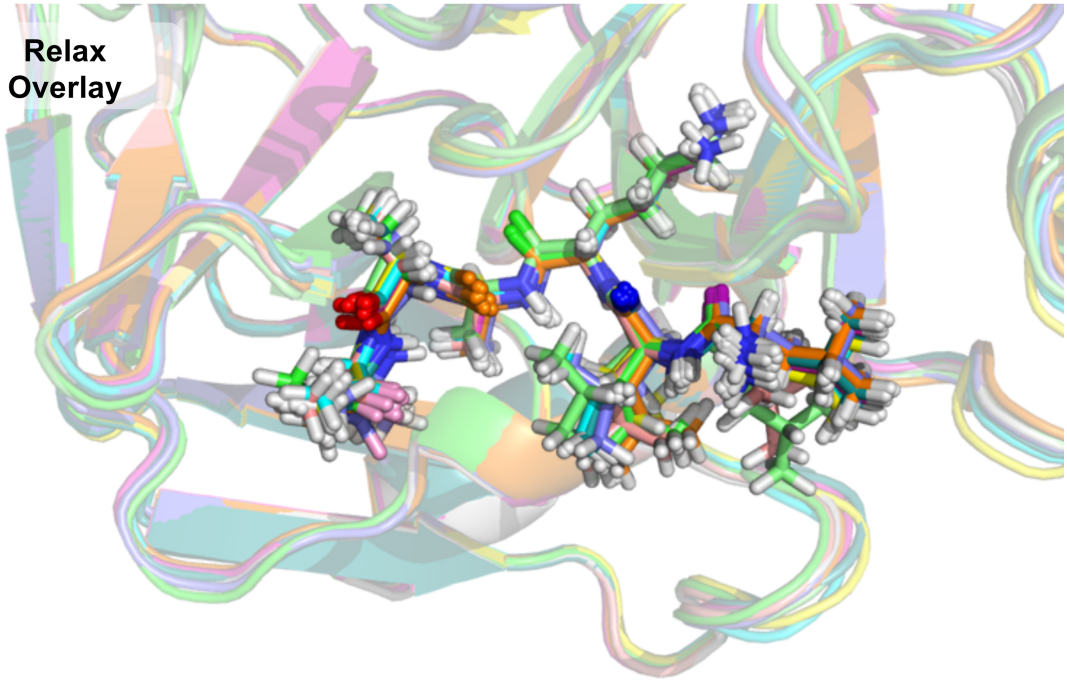
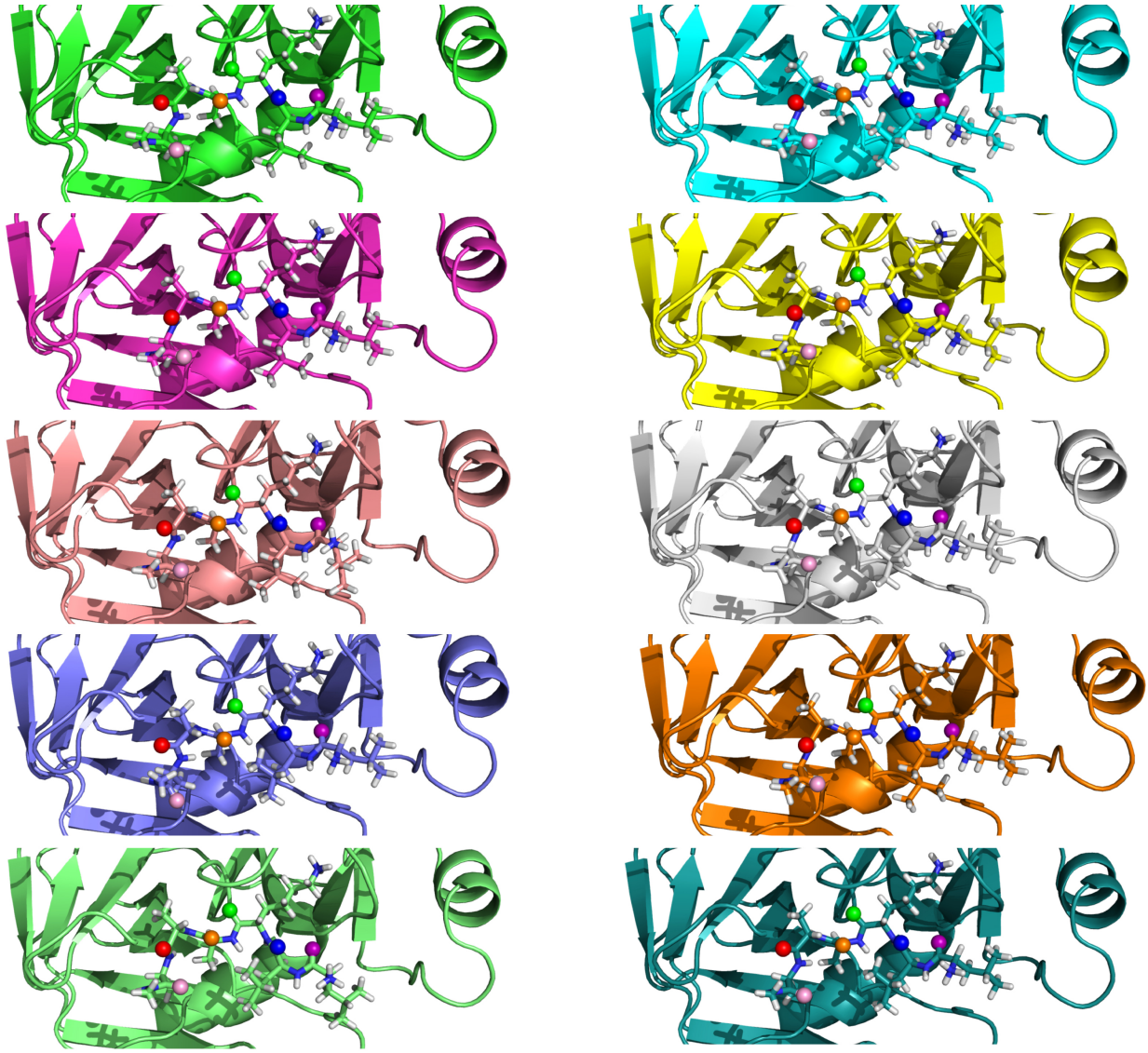


Figure S9. Overlay of Lowest Energy Structures from "Relax" Simulations of Trypsin. The 10 lowest energy structures of trypsin in complex with docked LLKAAA have minimal variations in carbonyl interactions of the P3 (purple), P2 (blue), P1 (green), P1' (orange), P2' (red), and P3' (pink) residues. In spite of extensive protein dynamics, the positions of the carbonyl atoms remain relatively fixed.



	N-H • • • O=C[a]	C=O • • • H-N[a]
P3	1.94 ± 0.11 Å	N/A
P2	N/A	N/A[d]
P1	1.74 ± 0.02 Å[b] 2.47 ± 0.19 Å[c]	N/A N/A
P1'	2.03 ± 0.05 Å	2.92 ± 0.23 Å
P2'	N/A	1.93 ± 0.05 Å
P3'	N/A	1.91 ± 0.10 Å

[a]Protein group • • • Peptide group
[b]Hydrogen bond to Gly_{193} backbone N-H.
[c]Hydrogen bond to Ser_{195} backbone N-H.
[d]Hydrogen bond to Gln_{192} sidechain before relax.

Figure S10. Lowest Energy Structures from "Relax" Simulations of Trypsin. Top: The 10 lowest energy structures of trypsin in complex with docked LLKAAA have minimal variations in carbonyl interactions of the P3 (purple), P2 (blue), P1 (green), P1' (orange), P2' (red), and P3' (pink) residues. Bottom Left: A plot of the energy (in Rosetta Energy Units, REU) as a function of the RMSD of each structure relative to the lowest energy structure from the Relax simulations. Bottom Right: Average O-to-H distances for amide hydrogen bonds from the 10 structures shown.

Imaging Peptide Studies

Serum Stability Assay. A 25 μL solution of 50 μM TB1 or TB1-R^S_6 in sterile Mill-Q water was incubated at 37 °C in the presence of 25 μL mouse serum (Sigma Aldrich M5905). After incubating for the desired time, the serum proteins were precipitated by adding 50 μL methanol, and chilled at -20 °C for 10 min. After precipitation, samples were pelleted using an Eppendorf 5415R centrifuge at 13,000 RPM for 10 min at 4 °C. Next, 80 μL of supernatant was diluted to 200 μL with Milli-Q water and analyzed by HPLC, after the addition of 0.8 μL of a 1.2 mM 5,6-carboxyfluorescein internal standard solution. A Phenomenex Luna C8(2) analytical column (Torrance, CA, USA) was used to analyze all samples using the gradients shown in **Table S7**. MALDI MS was used to check peptide identities (**Table S8**). The amount of intact peptide was quantified based on peak areas in HPLC chromatograms (**Fig. S11).** All peptides were monitored at 484 nm. The internal standard was used to normalize the amount of intact peptide in each sample. For each time point, samples were run in triplicate, and the amount of intact peptide was determined to be the average from three separate runs. To determine percent intact peptide, the average ratio of intact peptide to internal standard was compared to the ratio at t = 0 for all samples. Percent intact peptide over two time points is shown in **Figure S11** for TB1 peptides.

Table S7. HPLC Gradient for Serum Stability Assay*.

Time (min)	%B
0:00	2.0
5:00	2.0
15:00	30.0
35:00	50.0
37:00	100.0
39:00	100.0
41:00	2.0

*Solvent A: 0.1 % TFA in water; Solvent B: 0.1 % TFA in acetonitrile

Table S8. Retention Times and Masses of TB1 Peptides.

Peptide	Retention Time (min)	Observed Mass (m/z)
TB1	26.2	1795.00
TB1-R^S_6	27.5	1810.75

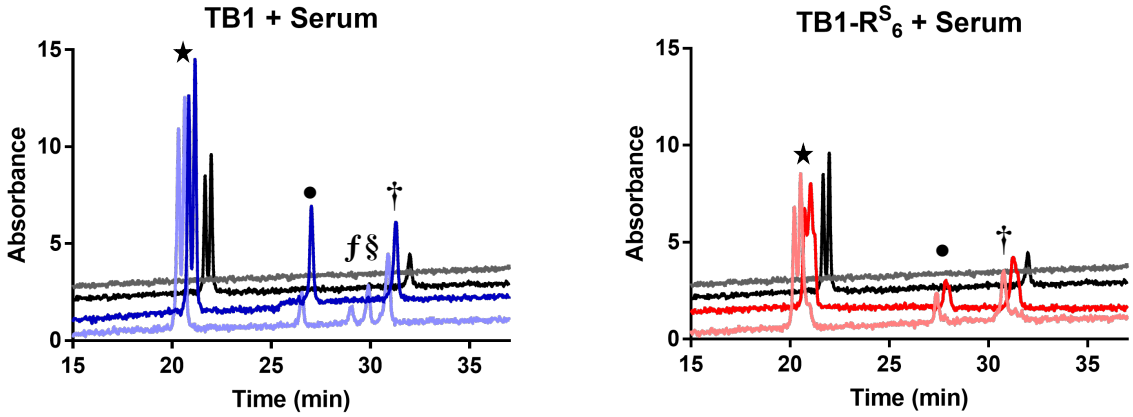


Figure S11. Serum Stability Assay. Representative HPLC chromatograms. Absorbance was monitored at 484 nm. Left: Dark blue curve is at zero min, light blue curve is at 90 min, black curve is internal standard alone, and gray curve is serum alone; * indicates 5,6-carboxyfluorescein internal standard, • indicates intact peptide (observed mass: 1795.9 m/z), § indicates cleavage of $TB1_{1\text{-}5}$ (observed mass: 1477.4 m/z, theoretical mass: 1475.7 m/z), and † indicates internal standard bound to serum proteins (other cleavage product indicated *ƒ* by could not be identified by MALDI MS). Right: Dark red curve is at zero h, light red curve is at 8 h, black curve is internal standard (5,6-carboxyfluorescein) alone, and gray curve is serum alone; * indicates 5,6-carboxyfluorescein internal standard, • indicates intact peptide (observed mass: 1810.6 m/z), and † indicates internal standard bound to serum proteins.

Neuropeptide Y Y1R Activation Assay. To determine the K_i of TB1 and TB1-R^S_6, a DiscoverX PathHunter® eXpress NPY1R CHO-K1 β-Arrestin GPCR Assay was employed. Provided cells were thawed and plated onto a 96 well plate and stored in a 37 °C incubator with 5% CO_2 for 48 h. Solutions of both peptides in DPBS buffer at varying concentrations were plated into a separate 96 well plate to be transferred to the assay plate. Cells were treated with 5 µL of TB1 or TB1-R^S_6 antagonist for 30 min in the incubator (37 °C with 5% CO_2), followed by treatment with 5 µL of 50 nM (final working concentration) peptide YY (PYY) for 1.5 h in in the incubator (37 °C with 5% CO_2). Next, cells were treated with the detection solution provided in the assay kit and allowed to incubate at room temperature for 1 h. Finally, the assay plate luminescence was read on a Tecan M1000pro in luminescence mode (no filter) with an integration time of 100 ms after incubation at 1 h and 3 h post-treatment**.** Within each trial, luminescence signal was normalized to the maximum (treatment with 50 nM PYY) and the minimum (treatment with buffer). The normalized data were then analyzed by averaging data across three independent biological replicates (different batches of cells) on different days. A few data sets or data points were dropped because outlier data points at the high or low concentrations made it infeasible to normalize the data in a sensible way. The final data set consisted of 7-8 data points at each concentration for both TB1 and TB1-R^S_6, with at least two trials in every biological replicate. These averaged plots are shown in **Figure 4** in the main text. These averaged data were fit to **Eq. S4** in Kaleidagraph (to determine an IC_{50} for competition with PYY.

$$\text{Norm. Luminescence} = 1 - \frac{[\text{L}]}{[\text{L}] + \text{IC}_{50}} \qquad \textbf{(Eq. S4)}$$

The IC_{50} values determined for TB1 and TB1-R^S_6 (719 ± 143 nM and 1363 ± 323 nM, respectively) were then used to calculate K_I values using **Eq. S5**, where [L] is 50 nM PYY and the

EC_{50} for PYY is 4.1 nM, according to the assay manufacturer, DiscoverX (https://www.discoverx.com/getmedia/782b3f48-40b1-45ae-a6c2-f6827c9d8f5f/93-0397E2CP0M.aspx).

$$K_I = \frac{IC_{50}}{1 + [L]/EC_{50}} \quad \textbf{(Eq. S5)}$$

K_I values of 53.0 ± 10.6 nM and 101 ± 23.9 nM were determined for TB1 and TB1-R^S_6, respectively, with error propagated according to NIST Handbook guidelines (https://www.itl.nist.gov/div898/handbook/mpc/section5/mpc552.htm).

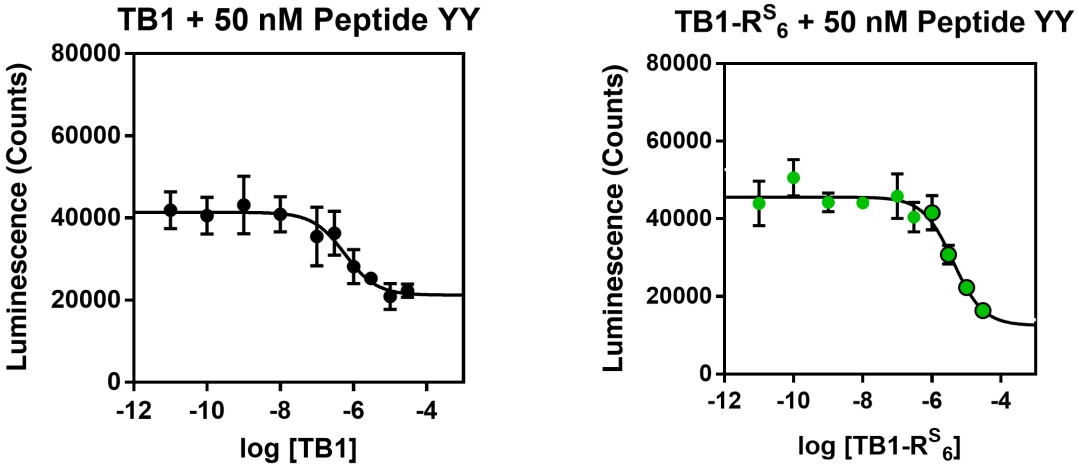


Figure S12. Representative TB1 and TB1-R^S_6 Binding Trials. Luminescence signal after treatment of cells with 50 nM peptide YY followed by various concentrations of TB1 or TB1-R^S_6. Traces shown are averages of three technical replicates from one biological replicate of the DiscoverX PathHunter® eXpress NPY1R CHO-K1 β-Arrestin GPCR Assay. Open symbols indicate that only two trials were included in final set of 7-8 data sets across all three biological replicates. Black curve: TB1, Green curve: TB1-R^S_6.

Cell Culture and Imaging. The following media were used in cell culture: DMEM (Corning), 10% FBS (Atlanta Biologicals), 1% Glutamine Supplement (Gibco), 1% Penicillin/Streptomycin (Gibco). MCF-7 and QBI-293 cultures were synchronized prior to experiments. For cellular imaging, cells were plated onto 35mm MatTek dishes, pre-treated with poly-D-lysine containing a 10 mm diameter glass insert 2 days prior to imaging at 37 °C in 5% CO_2. Immediately prior to the experiment, medium was removed by aspiration and wells were washed 1X with 1 mL Hanks Balanced Salt Solution (HBSS, Gibco). Treatments were performed on MCF-7 and QBI-293 cells in parallel. All studies were conducted at room temperature. For imaging of the peptide alone, 500 µL of HBSS containing Hoechst 33342 was added to the plate, followed by 500 µL of 500 nM TB1 or TB1-R^S_6 in HBSS for 15 min (final concentration of 250 nM peptide). For competition binding studies, 1 µM NPY in HBSS was added directly to the plate after HBSS wash and incubated for 30 min at room temperature before treatment using the conditions above. At the completion of each experiment, wells were washed 3X with HBSS and immediately imaged using a VT-iSIM confocal microscope (Hoechst excitation = 405 nm; Fluorescein excitation = 488 nm). Images were compiled using ImageJ (NIH). For each Z-stack, the first 10 stacks were combined into a composite image. All brightness, contrast, etc. settings for the images in the main text and in **Figure S13** are normalized to the same values and smoothed.

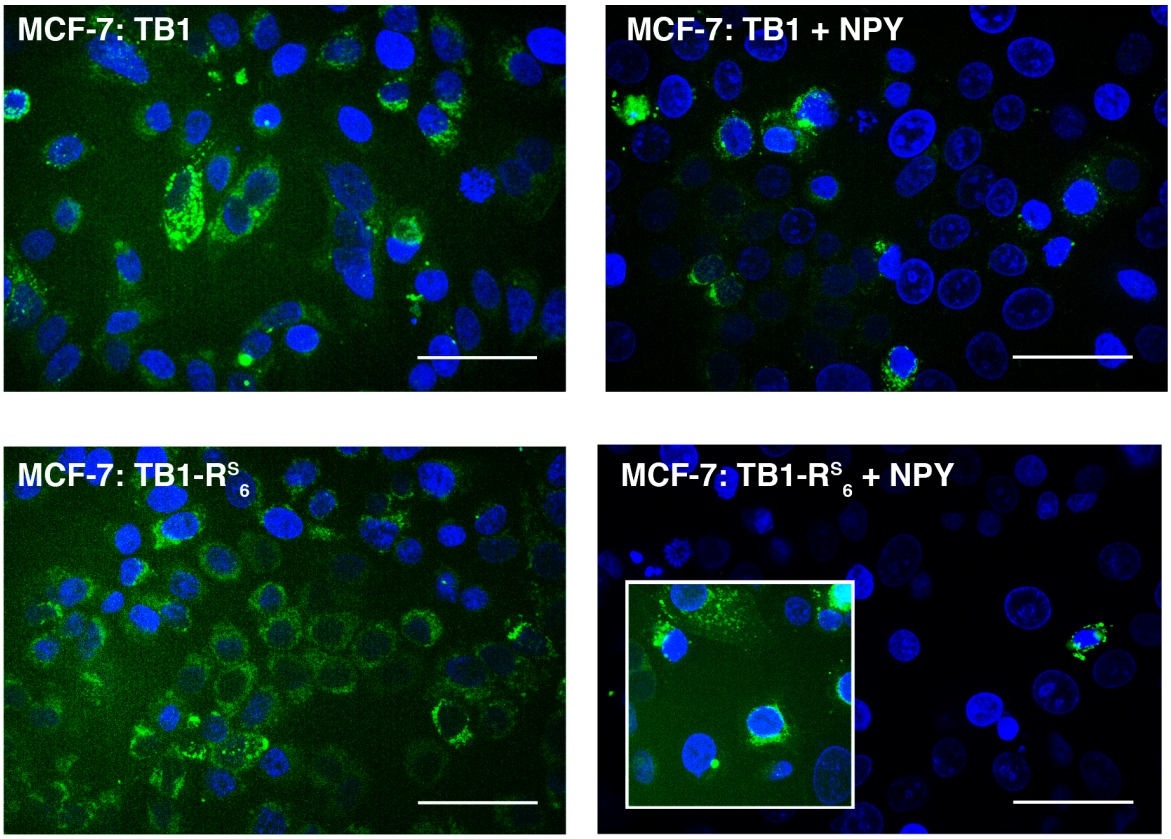


Figure S13. Additional Representative Images of TB1 and TB1-R^S_6 Cell Binding. MCF-7 cells were incubated with 250 nM TB1 or TB1-R^S_6 for 15 min before imaging. For + NPY conditions, cells were preincubated with 1 μM NPY. Inset: In some cases, signal from TB1 or TB1-R^S_6 was observed in + NPY conditions, possibly due to insolubility or unblockable internalization. Scale bar indicates 50 μm.

Fluorescence Lifetime Analysis. TCSPC measurements of fluorescence lifetime decays were collected using a pulsed LED with a maximum emission at 486 nm. Fluorescence was collected at 515 nm with the slit widths adjusted for each measurement to keep the ACD value between 1 - 3 % of the SYNC value. The instrument response function (IRF) was collected for each slit width used for collection. For these experiments labeled TB1 or TB1-R^S_6 were diluted to 175 μM in water. Lifetime data were fit using PowerFit10 distributed by PTI. Each decay was fit to a single exponential decay where the time regime was selected to minimize the chi-squared values and the residuals.

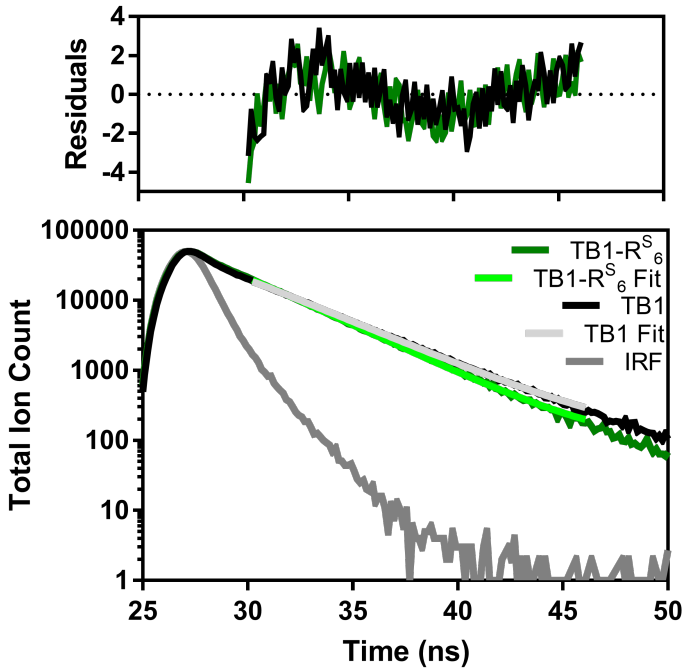


Figure S14. Fluorescence Lifetime Measurements of TB1 and TB1-R^S_6 Peptides. TB1 (black) and TB1-R^S_6 (green) fluorescence decays for 175 μM solutions in water. The instrument response function (IRF) is shown in grey. IRF-corrected single exponential fits to the decay data provide lifetime values of 3.452 ± 0.010 for TB1 and 3.076 ± 0.007 for TB1-R^S_6.

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