Supporting Information for:

A Versatile Amino Acid Analogue of the Solvatochromic Fluorophore 4-*N*,*N*-Dimethylamino-1,8-naphthalimide: A Powerful Tool for the Study of Dynamic Protein Interactions

*Galen Loving and Barbara Imperiali**

Department of Chemistry and Department of Biology, Massachusetts Institute of Technology,

77 Massachusetts Avenue, Cambridge, MA 02139, USA

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PBS and TBS buffers:

10 × PBS Buffer:

80.0 g NaCl
2.0 g KCl
14.4 g Na_2HPO_4
2.4 g KH_2PO_4

Dissolved in 800 mL of distilled H_2O (pH should be approximately 6.8). Once all the salts are fully dissolved and the pH is checked, then the volume is adjusted to 1 L. Upon diluting the 10× PBS buffer ten-fold with distilled H_2O, the pH should be 7.4. The final 1× PBS concentrations should be 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, and pH 7.4. All references to solutions prepared in PBS buffer pertain to this recipe unless stated otherwise.

10× TBS Buffer:

60.6 g Tris-HCl
87.7 g NaCl

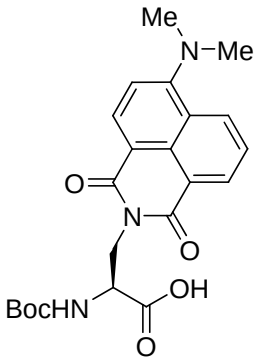
Dissolved in 800 mL of distilled H_2O and pH was adjusted to 7.3 with concentrated HCl. The volume is then adjusted to 1 L. Upon diluting 10× TBS buffer ten fold with distilled H2O, the pH should be 7.4. The final 1× TBS concentrations should be 50 mM Tris-HCl, 150 mM NaCl, and pH 7.4. All references to solutions prepared in TBS buffer pertain to this recipe unless stated otherwise.

Synthesis of 4-*N*,*N*-dimethyl-1,8-naphthalimide derivatives and characterization:

All reagents and solvents were procured from Sigma-Aldrich unless otherwise stated.

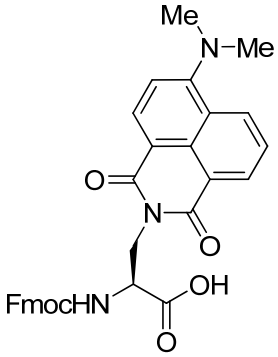
NMR and FT-MS of 4-N,N-dimethyl-1,8-naphthalimide derivatives: ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury 300 MHz NMR and Varian Inova 500 MHz NMR spectrometer. Chemical shifts (δ) for ^{1}H and ^{13}C NMR spectra are reported in parts per million (ppm) and are referenced to residual protium in the deuterated solvent. Coupling constants (*J*) are reported in Hertz (Hz) and multiplicities are abbreviated as singlet (s), doublet (d), triplet (t), and multiplet (m). High-

resolution mass spectra were obtained using a Bruker Daltonics APEXIV 4.7 Tesla Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FT-ICR-MS).



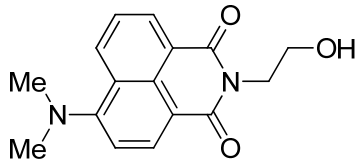
***N*-α-Boc-(4-*N*,*N*-dimethylamino-1,8-naphthalimido)-alanine (6)**. Dissolved (S)-3-amino-2-(Boc-amino)-propionic acid (2.00 g, 9.79 mmol) (Cat. No. A-3220, Bachem) and $NaHCO_3$ (4.11 g, 48.97 mmol) together in dH_2O (49 mL) and transferred to a 60 mL addition funnel. Next, added solid 4-*N*,*N*-dimethylamino naphthalic anhydride[1] (2.60 g, 10.77 mmol) to a 500 mL three-necked round-bottom flask equipped with a magnetic stir bar and reflux condenser. The reaction vessel was evacuated of air by placing under high vacuum, then charging with N_2 gas (3×). Dioxane (245 mL) was then transferred to the reaction vessel via syringe through the rubber septum. The suspension was stirred vigorously as the temperature was raised to reflux. Once at reflux, the aqueous solution of the amino acid was added slowly over 5 min. The reaction was allowed to proceed at reflux for 30 min before allowing cooling to room temperature. The reaction was then concentrated on the rotary evaporator to remove most of the dioxane before diluting to 200 mL with dH_2O and washing with ether to remove unreacted anhydride. The aqueous layer was then acidified with 6 N HCl and extracted with DCM (3 × 100 mL). The organic layers were combined, dried with $MgSO_4$, filtered, and concentrated. The crude was purified by flash column chromatography using ethyl acetate with 0.5% acetic acid as the solvent system. The fractions containing the desired product were combined and azeotroped in toluene (3 × 100 mL) to remove residual acetic acid. The product was isolated as a bright orange solid (2.31 mg, 5.40 mmol, 55% yield, R_f = 0.2 in EtOAc with 0.5% AcOH). ^{1}H-NMR (500 MHz, $CDCl_3$, δ): 1.26 (s, 9H),

3.12 (s, 6H), 4.61 (m, 2H), 4.81 (m, 1H), 5.67 (d, 1H, J = 7.0 Hz), 7.11 (d, 1H, J = 8.5 Hz), 7.65 (apparent triplet, 1H, J = 8.0 Hz), 8.44 (d, 1H, J = 8.0 Hz), 8.48 (d, 1H, J = 8.0 Hz), 8.56 (d, 1H, J = 7.0 Hz). ^{13}C-NMR (300 MHz, $CDCl_3$, δ): 174.0 165.4, 164.9, 157.5, 156.4, 133.6, 132.0, 131.9, 130.8, 125.3, 125.2, 122.8, 114.5, 113.6, 80.6, 53.2, 45.1, 41.0, 28.4. HRMS-ESI (m/z): [M+H$^+$] calcd for $C_{22}H_{25}N_3O_6$ 428.1816, found, 428.1814.



***N*-α-Fmoc-(4-*N*,*N*-dimethylamino-1,8-naphthalimido)-alanine (4).** The solid Boc-4DMNA (2.0 g, 4.68 mmol) was dissolved in dichloromethane (47 mL) and stirred in a 250 mL round-bottom flask as cold TFA (47 mL) was added by addition funnel over 5 min. The reaction was allowed to proceed at ambient temperature for 1.5 hrs before concentrating to dryness and azeotroping with chloroform (3 × 50 mL) to remove residual TFA. The crude was then placed under high vacuum overnight. The crude was redissolved in dH_2O (23 mL) with $NaHCO_3$ (1.97 g, 23.40 mmol). The pH was tested to ensure the solution was basic. A solution of *N*-(9-fluorenylmethoxycarbonyloxy) succinimide (1.74 g, 5.15 mmol) was then prepared in dioxane (117 mL) and slowly added to the stirring solution of the amino acid. The reaction was allowed to proceed for 2 hrs before concentrating to remove most of the dioxane and re-diluting to a total volume of 150 mL in dH_2O. The aqueous layer was washed with diethyl ether (1 × 50 mL) to remove excess Fmoc-OSu. The aqueous layer was acidified with 6 N HCl and the product extracted into dichloromethane (3 × 100 mL). The organic layers were combined, dried with $MgSO_4$, filtered, and concentrated. The product was purified by flash column chromatography using 3:1 ethyl acetate/hexanes with 0.5% acetic acid as the solvent system. The product is an orange solid (2.14 g, 3.89 mmol, 83% yield, R_f = 0.2 in EtOAc with 5% AcOH).

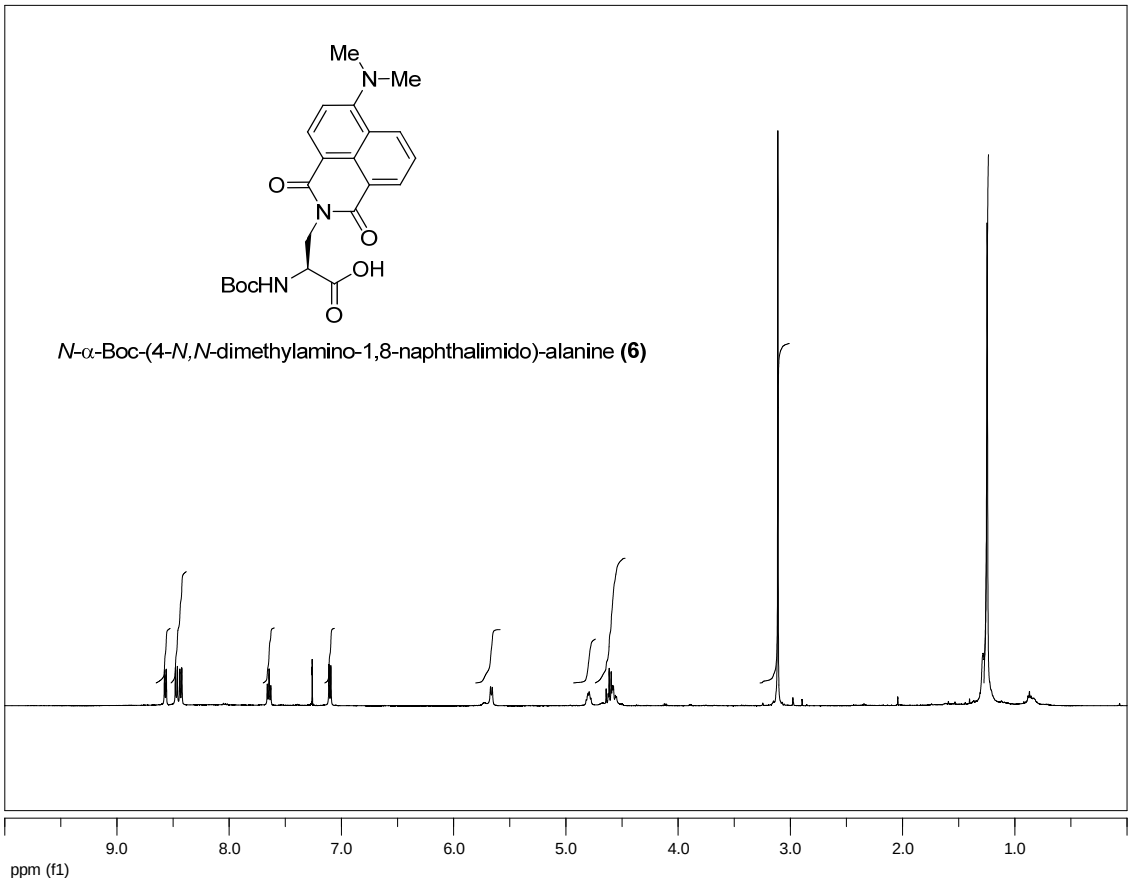
Note: the final product contained a small amount of toluene as a result of azeotroping to ensure the complete removal of residual acetic acid following the purification. ^{1}H-NMR (500 MHz, $CDCl_3$, δ): 3.02 (s, 6H), 3.95 (t, 1H, J = 7.3 Hz), 4.11 (dd, 1H, J_1 = 10.5 Hz, J_2 = 8.0 Hz), 4.21 (dd, 1H, J_1 = 10.8 Hz, J_2 = 7.3 Hz), 4.72 (d, 2H, J = 6.5 Hz), 4.99 (apparent dd, 1H, J_1 = 14.8 Hz, J_2 = 7.3 Hz), 6.17 (d, 1H, J = 8.0 Hz), 6.96 (d, 1H, J = 8.5 Hz), 7.16 (apparent t, 1H, J = 7.5 Hz), 7.21 (apparent t, 1H, J = 8.0 Hz), 7.31 (apparent t, 2H, J = 7.3 Hz), 7.45 (d, 1H, J = 7.5 Hz), 7.5 (d, 1H, J = 7.5 Hz), 7.57 (apparent t, 1H, J = 8.0 Hz), 7.66 (apparent t, 2H, J = 6.5 Hz), 8.34 (d, 1H, J = 8.0 Hz), 8.41 (d, 1H, J = 8.5 Hz), 8.54 (d, 1H, J = 7.5 Hz). ^{13}C-NMR (500 MHz, $CDCl_3$, δ): 173.9, 165.4, 164.9, 157.5, 156.7, 144.3, 144.0, 141.4, 141.3, 133.8, 132.0, 132.0, 130.7, 127.8, 127.3, 127.3, 125.7, 125.5, 125.1, 125.1, 122.6, 120.0, 120.0, 114.0, 113.4, 67.6, 53.6, 47.2, 44.9, 41.0. HRMS-ESI (m/z): [M+H$^+$] calcd for $C_{32}H_{27}N_3O_6$ 550.1973, found, 550.1959. (Both the ^{13}C-NMR and ^{1}H-NMR of this compound exhibit anisochronous resonances for nine of the ten aromatic carbons and all eight of the aromatic protons of the 9-fluorenyl group. A variable temperature experiment showed no coalescence of these aromatic protons at 80 ˚C. The cause of this apparent asymmetry is presumed to be the result of partial overlap of one side to the 9-fluorenyl ring system with that of the fluorescent side-chain of the amino acid. These nuclei would therefore lack chemical equivalence producing the observed spectra).



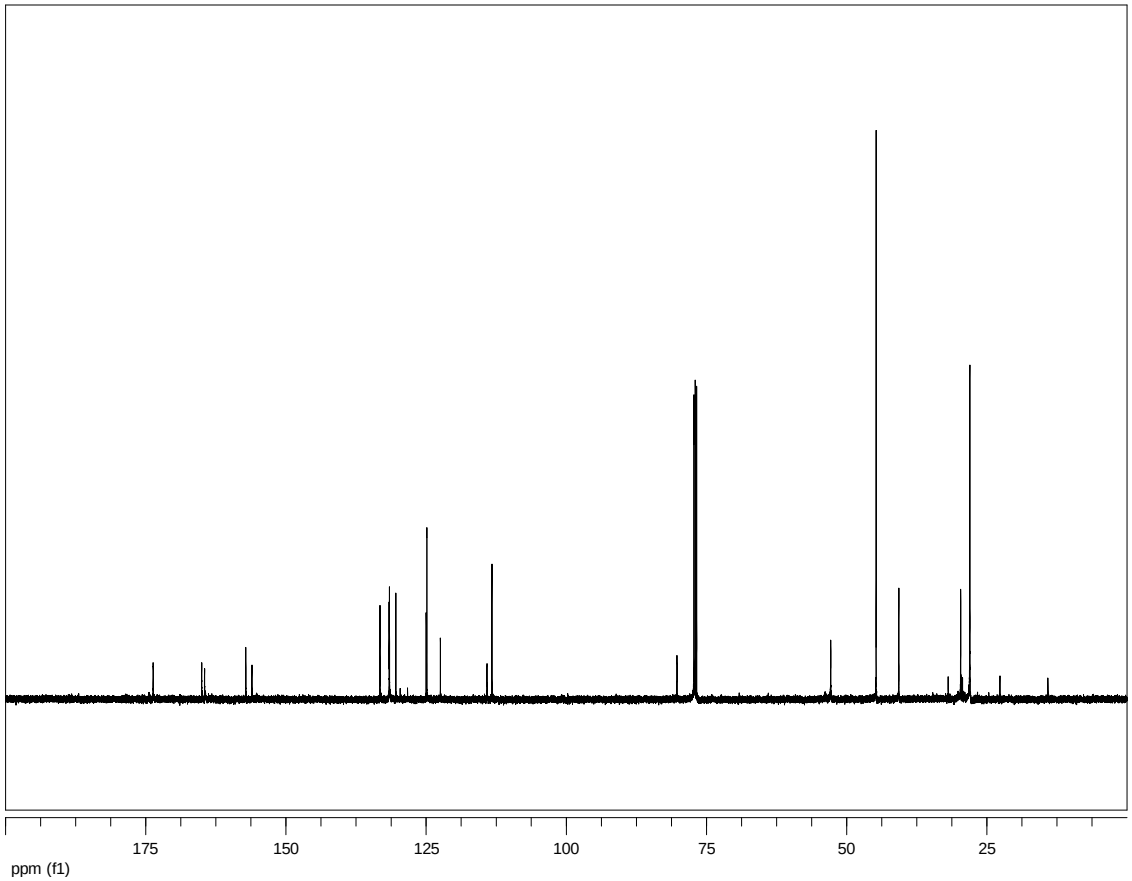
4-*N*,*N*-Dimethylamino-*N*-(2-hydroxy-ethyl)-1,8-naphthalimide (7). Added 4-*N*,*N*-dimethylamino-1,8-naphthalic anhydride (1.00 g, 4.15 mmol) to a 200 mL two-necked round-bottom flask equipped with a reflux condenser, magnetic stir bar, and rubber septum. The air was then evacuated from the reaction vessel by applying vacuum and replacing with N_2 gas (3×). Anhydrous ethanol (42 mL) was then added to the flask by syringe. The suspension was stirred as the temperature was raised to reflux. The anhydride was still present as a suspension at reflux until the addition of

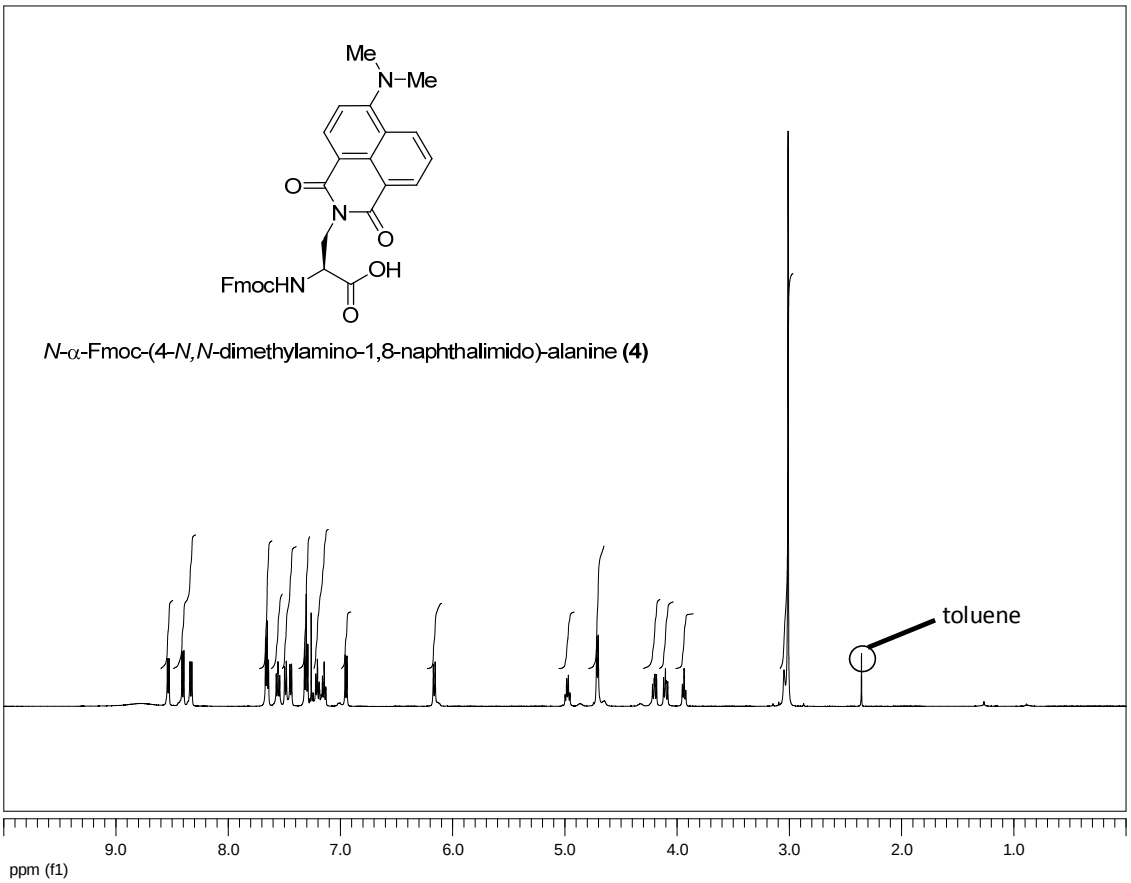
ethanolamine (0.28 mL, 4.56 mmol) by syringe. At this point, the slurry becomes a clear deep orange solution. The reaction is allowed to proceed at reflux for 1.5 hrs the stopped by removing the heat source and allowing to cool to room temperature. The solvent is then removed using a rotavap and the crude placed on the high vacuum line overnight to remove excess ethanolamine. The product obtained was an orange solid that required no further purification (1.18 g, 4.15 mmol, quantitative yield). ^{1}H-NMR (300 MHz, $CDCl_3$, δ): 3.11 (s, 6H), 3.96 (t, 2H, J = 5.3 Hz), 4.43 (t, 2H, J = 5.1 Hz), 7.07 (d, 1H, J = 8.1 Hz), 7.63 (dd, 1H, J_1 = 8.6 Hz, J_2 = 7.4 Hz), 8.41 (dd, 2H, J_1 = 8.4 Hz, J_2 = 1.2 Hz), 8.43 (d, 1H, J = 8.4 Hz), 8.53 (dd, 1H, J_1 = 7.5 Hz, J_2 = 1.2 Hz). ^{13}C-NMR (300 MHz, $CDCl_3$, δ): 165.8, 165.3, 157.5, 133.3, 131.8, 131.6, 130.6, 125.3, 125.1, 123.0, 114.6, 113.5, 62.4, 45.1, 43.0. HRMS-ESI (m/z): [M+Na^+] calcd for $C_{16}H_{16}N_2O_3$ 307.1053, found, 307.1059.

NMR of 4-N,N-dimethyl-1,8-naphthalimide derivatives:

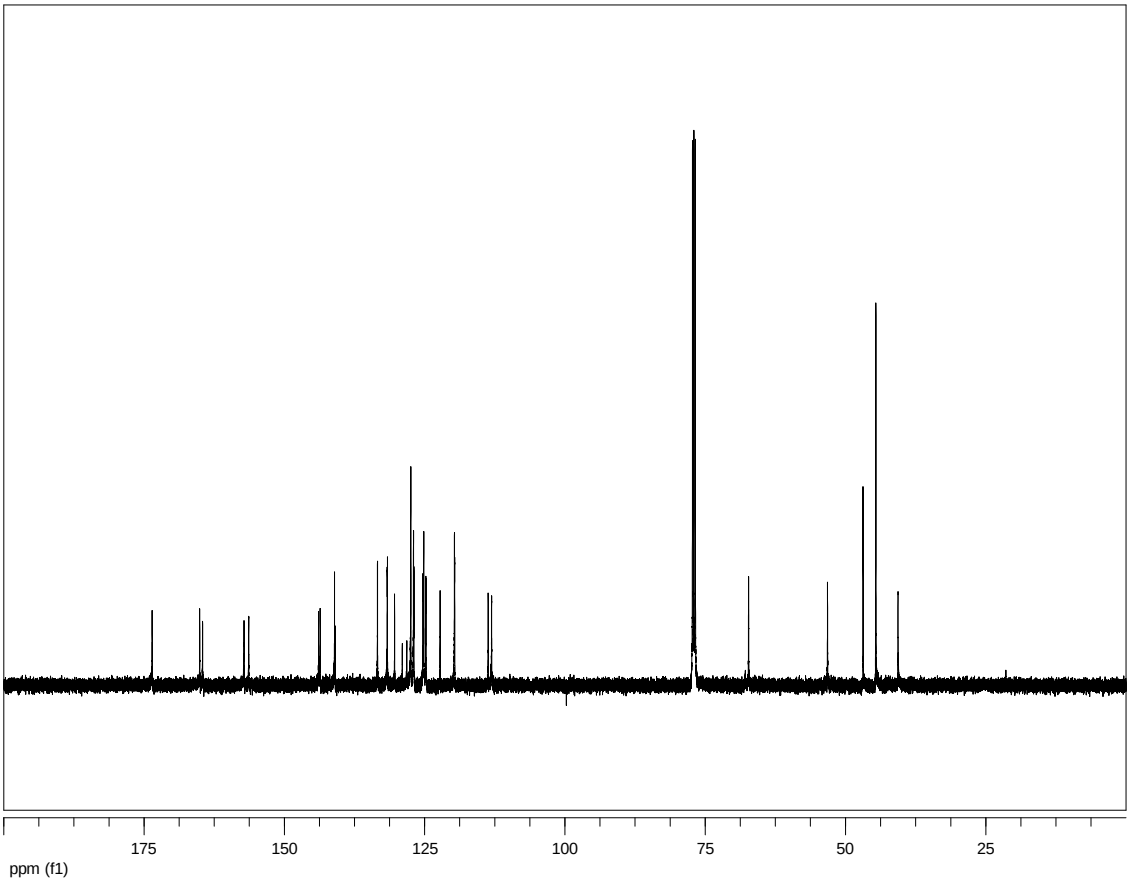


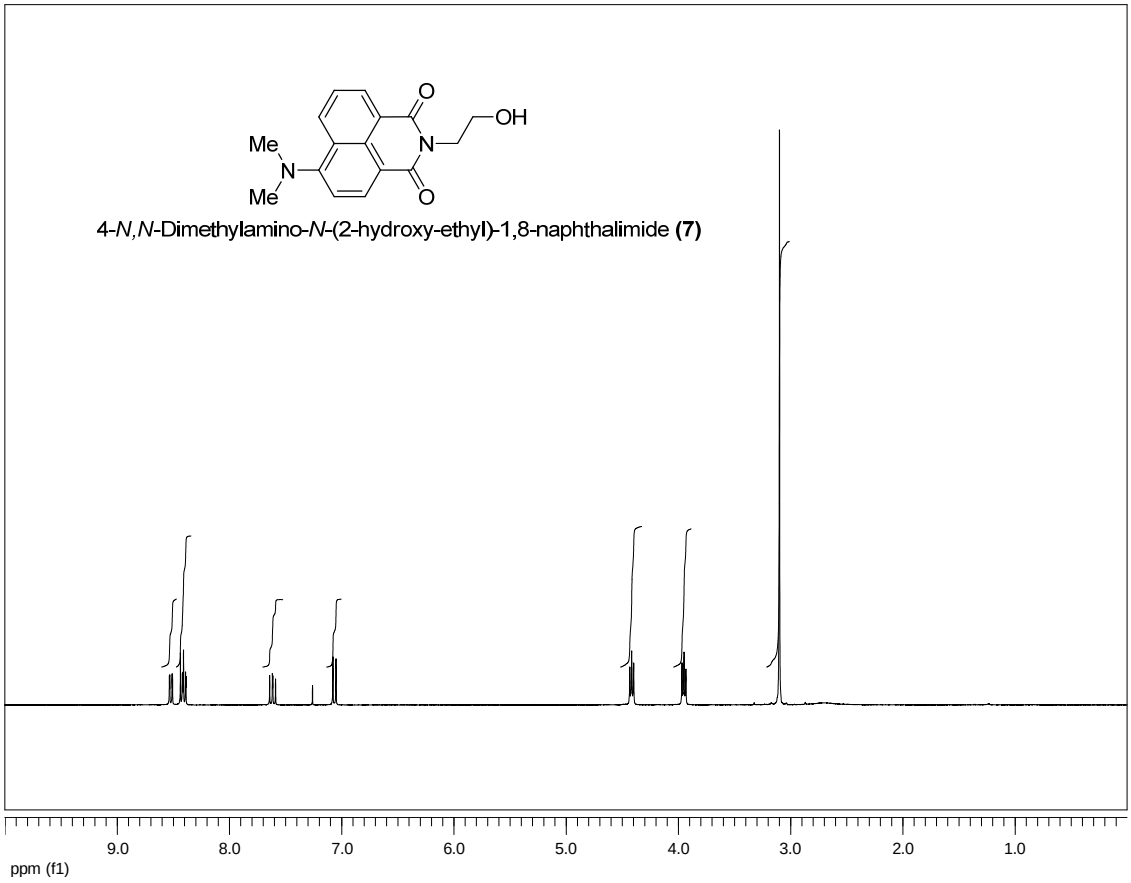
N-α-Boc-(4-*N,N*-dimethylamino-1,8-naphthalimido)-alanine **(6)**



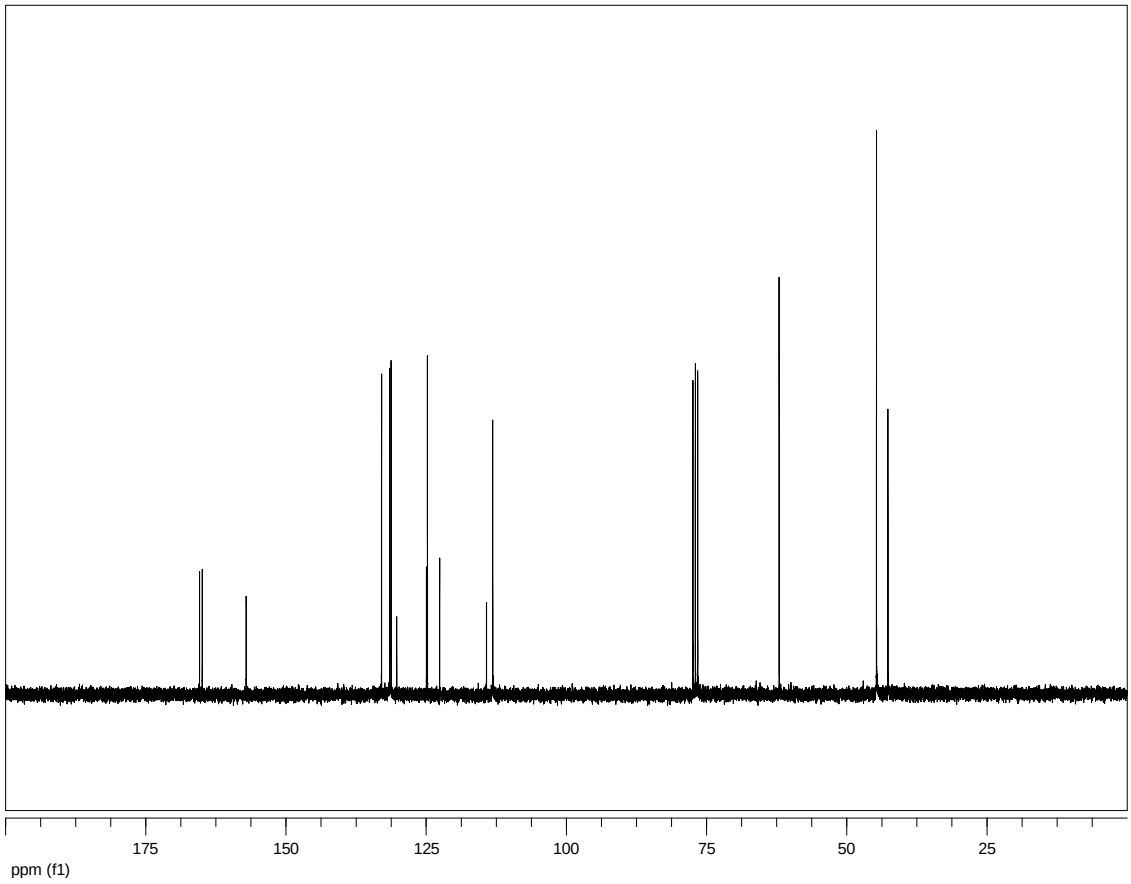


N-α-Fmoc-(4-*N*,*N*-dimethylamino-1,8-naphthalimido)-alanine **(4)**





4-*N*,*N*-Dimethylamino-*N*-(2-hydroxy-ethyl)-1,8-naphthalimide **(7)**



Synthesis of the KR peptide series:

The KR peptide series was prepared using standard Fmoc-based solid-phase peptide synthesis techniques (SPPS).[2] Each peptide was prepared manually using 0.21 mmol/g loading Fmoc-PAL-PEG-PS resin (Cat. No. GEN913383, Applied Biosystems) in a Poly-Prep® chromatography column (Cat. No. 731-1550, Bio-Rad). Fmoc-protected amino acids with the standard side-chain protecting groups were used: Fmoc-Arg(Pbf)-OH and Fmoc-Lys(Boc)-OH. A Boc-Lys(Boc)-OH residue was incorporated at the N-terminus of each member of the series in order to yield the free α-amino group following cleavage of the peptides from the solid support. The KR peptides of 4-DMAP, 6-DMN, Dansyl, and NBD were all prepared from the parent $KR_{Dap(Aloc)}$ peptide while KR_{BADAN} was prepared from the $KR_{Cys(Mmt)}$ peptide. The (S)-2-(Fmoc-amino)-3-(Aloc-amino)-propionic acid (Fmoc-Dap(Aloc)-OH) and the Fmoc-Cys(Trt)-OH residues were both incorporated at position 4 within the $KR_{Dap(Aloc)}$ and $KR_{Cys(Mmt)}$ peptides, respectively. Synthesis of the $KR_{Dap(Aloc)}$ peptide was performed on a 105 μmole scale and the $KR_{Cys(Mmt)}$ peptide was prepared on a 21 μmole scale. Couplings were executed using 6 equiv of each amino acid with exception to position 4. Here, only 3 equiv of the Fmoc-Dap(Aloc)-OH and Fmoc-Cys(Mmt)-OH residues were used. All of the amino acids were dissolved to a final concentration of 50 mM in DMF containing a 1:1 mixture of HOBt/HBTU (50 mM each) with *N*,*N*-diisopropylethylamine (100 mM, 12 equiv). Each coupling reaction was allowed to proceed at room temperature for 30-45 min. The coupling was monitored by a colormetric assay for detecting free primary amines known as the 2,4,6-trinitrobenzenesulphonic acid (TNBS) test.[3] Removal of the Fmoc group prior to each coupling step was performed using a 20% 4-methylpiperidine solution[4] in DMF (3 × 5 min). Once the syntheses of the $KR_{Dap(Aloc)}$ and $KR_{Cys(Mmt)}$ peptides were complete, the Dap and Cys residues were selectively deprotected in order to append the desired fluorophores. Removal of the Aloc group was performed according to the previously described protocol.[5] The Mmt group was removed using a 1% trifluoroacetic acid (TFA) solution in dichloromethane with 5% triisopropylsilane added as a cation scavenger (4 × 15 min). The side-chains of the Dap and Cys residues were then alkylated with the appropriate fluorophores. The KR_{BADAN} peptide was prepared by

first dissolving 6-bromoacetyl-2-dimethylaminonaphthalene (BADAN, AnaSpec) (3.3 equiv) in *N*-methylpyrrolidone with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (1 equiv). The solution was then added directly to the resin-bound KR_{Cys} peptide. The coupling reaction was allowed to proceed overnight in the dark at room temperature. The KR_{4DMAP} and KR_{6DMN} peptides were prepared by coupling the anhydride precursors of 4-DMAP and 6-DMN to the side-chain of Dap according to the previously described protocol.[5] The KR_{dansyl} and KR_{NBD} peptides were prepared by treating the resin-bound KR_{Dap} peptide with a 50 mM solution of 5-(dimethylamino)-naphthalene-1-sulfonyl chloride (dansyl chloride, 2 equiv) and 4-floro-7-nitrobenzofurazan (NBD fluoride, 2 equiv) dissolved in DMF with *N*,*N*-diisopropylethylamine (2 equiv). The reactions were allowed to proceed at room temperature overnight in the dark. Preparation of the KR_{4DMN} peptide was unique among the series in that it was prepared using the Fmoc-4DMNA building block (**4**) instead of coupling the fluorophore to the fully constructed peptide. The KR peptides were cleaved from the resin using a solution of 96:2:2 TFA/H_2O/EDT for 3 hrs. The cleavage cocktail was then filtered and evaporated. The crude peptides were triturated with cold diethyl ether (3 × 10 mL) and purified by high-performance liquid chromatography (HPLC) using a Waters 600E HPLC with a Waters 600 automated control module and Waters 2487 dual wavelength absorbance detector set at 228 and 280 nm. The separations were performed using a preparative YMC-pack, C_{18}, 20 × 250 mm reverse-phase column. A YMC-pack, C_{18}, 4.6 ×150 mm reverse-phase column was used for analytical HPLC. The following analytical HPLC method was used to verify purity of the peptides:

Time (min)	Flow Rate (mL/min)	H_20 % (with 0.1% TFA)	MeCN % (with 0.1% TFA)	Gradient
initial	1.00	95	5	linear
5.0	1.00	95	5	linear
40.0	1.00	55	45	linear

Table S1: Characterization of purified KR peptide series.

KR peptides	Peptide Sequence								HPLC Ret. Time (min)	$[M+xH]^{x+}$ Calc.	$[M+xH]^{x+}$ found
	N-term	1	2	3	4	5	6	C-term			
KR_{4DMAP}	H_2N-	K	R	R	**Dap(4DMAP)**	K	K	-$CONH_2$	21.2	974.0 (1+)	973.7 (1+)
KR_{4DMN}	H_2N-	K	R	R	**Dap(4DMN)**	K	K	-$CONH_2$	23.4	1024.1 (1+)	1023.6 (1+)
KR_{6DMN}	H_2N-	K	R	R	**Dap(6DMN)**	K	K	-$CONH_2$	24.0	1024.1 (1+)	1023.7 (1+)
KR_{BADAN}	H_2N-	K	R	R	**Cys(BADAN)**	K	K	-$CONH_2$	26.9	1029.1 (1+)	1029.7 (1+)
KR_{Dansyl}	H_2N-	K	R	R	**Dap(dansyl)**	K	K	-$CONH_2$	20.2	1034.0 (1+)	1034.2 (1+)
KR_{NBD}	H_2N-	K	R	R	**Dap(NBD)**	K	K	-$CONH_2$	16.1	482.5 (2+) 322.0 (3+)	482.4 (2+)[α] 321.9 (3+)[α]

[α] Peptide mass determined by ESI-MS on a Mariner electrospray mass spectrometer (PerSpective Biosystems). All other KR peptides in this series were confirmed by MALDI mass spectroscopy on a PerSeptive Biosystems Voyager MALDI-TOF instrument using a 2,5-dihydroxybenzoic acid matrix.

Synthesis of the M13 mutant peptide series:

The M13 mutant peptide series was prepared using standard Fmoc-based solid-phase peptide synthesis techniques (SPPS).[2] The peptides were prepared using 0.17 mmol/g loading Fmoc-NovaPEG Rink Amide resin LL (Cat. No. 01-64-0483, Novabiochem®). Fmoc-protected amino acids with the standard side-chain protecting groups were used: Fmoc-Ala-OH, Fmoc-Phe-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Val-OH, and Fmoc-Trp(Boc)-OH. The M13 peptide mutants containing 4-DMAP, 6-DMN, Dansyl, and NBD were all prepared from the parent $M13_{Dap(Aloc)}$ peptide while $M13_{BADAN}$ was prepared from the $M13_{Cys(Mmt)}$ peptide. Synthesis of all the peptides of this series began by coupling the first seven residues (positions 13-19) with an ABI 431A peptide synthesizer (Applied Biosystems). The synthesis was performed on the 40 μmol scale using 4 equiv of the Fmoc amino acids in each cycle. It had previously been discovered from earlier attempts at making these peptides that the coupling efficiency was extremely low for residues incorporated after the alanine at position 10 in the M13 sequence. This problem was overcome by using a pseudoproline dipeptide, Fmoc-Val-Ser($\Psi^{Me,Me}$pro)-OH (Cat. No. 05-20-1001, Novabiochem), at positions 11-12 in place of Fmoc-Val-OH and Fmoc-Ser(tBu)-OH. The remaining residues were all coupled manually using 6 equiv of the Fmoc amino acids. All of the building blocks

were dissolved to a final concentration of 50 mM in DMF containing a 1:1 mixture of HOBt/HBTU (50 mM each) with *N*,*N*-diisopropylethylamine (100 mM, 12 equiv). Each coupling reaction was allowed to proceed at room temperature for 30-45 min. The coupling efficiency was monitored using the TNBS test.[3] Removal of the Fmoc group prior to each coupling step was performed using a 20% 4-methylpiperidine solution[4] in DMF (3 × 5 min). After the incorporation of Fmoc-Ile-OH at position 9, the resin was split into three aliquots. The Fmoc-Dap(Aloc)-OH, Fmoc-Cys(Mmt)-OH, and Fmoc-4DMNA building blocks were then coupled at position 8. A Boc-Arg(Pbf)-OH residue was incorporated at the N-terminus of the $M13_{Dap(Aloc)}$, $M13_{Cys(Mmt)}$, and $M13_{4DMN}$ peptides in order to yield the free α-amino group following cleavage from the solid support. Once the syntheses of the $M13_{Dap(Aloc)}$ and $M13_{Cys(Mmt)}$ peptides were complete, the Dap and Cys residues were selectively deprotected in order to append the desired fluorophores. Removal of the Aloc group was performed according to the previously described protocol.[5] The Mmt group was removed using a 1% TFA solution in dichloromethane with 5% triisopropylsilane added as a cation scavenger (4 × 15 min). The side-chains of the Dap and Cys residues were then alkylated with the appropriate fluorophores. The $M13_{BADAN}$ peptide was prepared by first dissolving BADAN (3.3 equiv) in *N*-methylpyrrolidone with 1,8-DBU (1 equiv). The solution was then added directly to the resin-bound $M13_{Cys}$ peptide. The coupling reaction was allowed to proceed overnight in the dark at room temperature. The $M13_{4DMAP}$ and $M13_{6DMN}$ peptides were prepared by coupling the anhydride precursors of 4-DMAP and 6-DMN to the side-chain of Dap according to the previously described protocol.[5] The $M13_{dansyl}$ and $M13_{NBD}$ peptides were prepared by treating the $M13_{Dap}$ peptide with a 50 mM solution of dansyl chloride (2 equiv) and NBD fluoride (2 equiv) dissolved in DMF with *N*,*N*-diisopropylethylamine (2 equiv). The reactions were allowed to proceed at room temperature overnight in the dark. Preparation of the $M13_{4DMN}$ peptide was unique among the series in that it was prepared using the Fmoc-4DMNA building block instead of coupling the fluorophore to the fully constructed peptide. The M13 mutant peptides were cleaved from the resin using a solution of 96:2:2 TFA/H_2O/EDT for 3 hrs. The cleavage cocktail was then filtered and evaporated. The crude peptides were triturated with cold diethyl ether (3 × 10

mL) and purified by HPLC using a Waters 600E HPLC with a Waters 600 automated control module and Waters 2487 dual wavelength absorbance detector set at 228 and 280 nm. The separations were performed using a preparative YMC-pack, C_{18}, 20 × 250 mm reverse-phase column. A YMC-pack, C_{18}, 4.6 ×150 mm reverse-phase column was used for analytical HPLC. The following analytical HPLC method was used to characterize the peptides:

Time (min)	Flow Rate (mL/min)	H_20 % (with 0.1% TFA)	MeCN % (with 0.1% TFA)	Gradient
initial	1.00	95	5	linear
5.0	1.00	95	5	linear
40.0	1.00	55	45	linear

Table S2: Characterization of purified M13 mutant peptide series.

M13 peptide mutants	Peptide Sequence																					HPLC Ret. Time (min)	$[M+xH]^{x+}$ Calc.	$[M+xH]^{x+}$ found
	N-term	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	C-term				
$M13_{4DMAP}$	H_2N-	R	R	W	K	K	N	Dap(4DMAP)	I	A	V	S	A	A	N	R	F	K	K	-$CONH_2$	34.8	2332.6 (1+)	2332.2 (1+)
$M13_{4DMN}$	H_2N-	R	R	W	K	K	N	Dap(4DMN)	I	A	V	S	A	A	N	R	F	K	K	-$CONH_2$	36.0	2382.6 (1+)	2383.1 (1+)
$M13_{6DMN}$	H_2N-	R	R	W	K	K	N	Dap(6DMN)	I	A	V	S	A	A	N	R	F	K	K	-$CONH_2$	37.0	2382.6 (1+)	2382.3 (1+)
$M13_{BADAN}$	H_2N-	R	R	W	K	K	N	Cys(BADAN)	I	A	V	S	A	A	N	R	F	K	K	-$CONH_2$	33.0	2387.6 (1+)	2388.6 (1+)
$M13_{Dansyl}$	H_2N-	R	R	W	K	K	N	Dap(dansyl)	I	A	V	S	A	A	N	R	F	K	K	-$CONH_2$	38.1	2392.6 (1+)	2393.6 (1+)
$M13_{NBD}$	H_2N-	R	R	W	K	K	N	Dap(NBD)	I	A	V	S	A	A	N	R	F	K	K	-$CONH_2$	34.0	774.9 (3+) 581.4 (4+) 465.3 (5+)	774.4 (3+)$^{\alpha}$ 581.1 (4+)$^{\alpha}$ 465.1 (5+)$^{\alpha}$

$^{\alpha}$ Peptide mass determined by ESI-MS on a Mariner electrospray mass spectrometer (PerSpective Biosystems). All other M13 peptide mutants in this series were confirmed by MALDI mass spectroscopy on a PerSeptive Biosystems Voyager MALDI-TOF instrument using a 2,5-dihydroxybenzoic acid matrix.

Preparation of calmodulin construct:

Subcloning of eCaM gene into pET-14b vector. The gene for the CaM-His6 construct was ordered from Bio Basic Inc. and codon-optimized for expression in *E. coli*. The peptide sequence was based on the calmodulin homolog found in *Homo sapiens*. In order to denote that the synthetic gene was not obtained from the genomic DNA, the construct is referred to as eCaM. The gene was delivered in a pUC-57 vector, which has a high-copy-number for ease of amplification. The product was delivered as a lyophilized powder and also as a stab of transformed DH5α cells in LB agar with carbenicillin. A sterile loop was dipped in the stab and swabbed over a fresh LB agar plate containing

carbenicillin (50 µg/mL). The plate was incubated overnight at 37 ºC. The next day, a colony was selected and amplified in order to isolate the vector using a Qiagen Plasmid Miniprep kit. The DNA was then quantified by Abs at 260 nm (0.11 µg/µl).

A double digestion of the amplified pUC-57(eCaM) vector was performed simultaneously with the double digestion of the pET-14b vector. The reaction conditions were as follows:

pET-14b vector:		pUC-57 vector:	
1.28 µL	pET-14b (2.34 µg/µL)	27.0 µL	pUC-57 (0.11 µg/µL)
41.2 µL	dH_2O (sterile)	15.5 µL	dH_2O (sterile)
5.0 µL	10× NEB buffer 2	5.0 µL	10× NEB buffer 2
0.5 µL	100× BSA	0.5 µL	100× BSA
1.0 µL	*Xho*I	1.0 µL	*Xho*I
1.0 µL	*Nco*I	1.0 µL	*Nco*I
50.0 µL	total volume	50.0 µL	total volume

The reactions were allowed to proceed for 1 hr at 37 ºC before stopping with the addition of 12.5 µL of 5× DNA loading buffer. The both reaction mixtures were then loaded onto a 1% agarose gel and resolved at 110 V. The desired restriction products (the eCaM insert of the pUC-57 vector and the linearized pET-14b expression vector) were cut from the gel and isolated using a QIAquick™ Gel Extraction kit. The subcloning vector and insert were then quantified on 1% agarose gel by comparing band intensities to those of a 2-Log DNA ladder (New England BioLabs®).

Two ligation reactions were assembled: one containing both the eCaM insert and the pET-14b vector, and the other with only the pET-14b vector as a negative control. The reaction conditions were as follows:

vector (control)		vector + insert	
0.3 µL	pET-14b (120 ng/µL)	0.3 µL	pET-14b (120 ng/µL)
3.2 µL	dH_2O (sterile)	1.0 µL	eCaM insert (15 ng/µL)
1.0 µL	T4 DNA ligase	2.2 µL	dH_2O (sterile)
0.5 µL	10× T4 ligase buffer	1.0 µL	T4 DNA ligase
		0.5 µL	10× T4 ligase buffer
5.0 µL	total volume	5.0 µL	total volume

Note- There was 36 µg of pET-14b vector in each reaction and 15 µg of the insert in the ligation. The insert is 480 bp and the vector is 4500 bp. This corresponds to a 4:1 molar ratio of insert to vector.

The reaction was allowed to proceed for 16 hrs at 16 ºC. The reactions were then used to transform two 30 µL batches of DH5α cells plated on LB/agar with 50 µg/mL carbenicillin. The next day, no colonies were observed for the negative control. Colonies from the non-control plate were amplified and submitted for sequencing using the standard T7 primer (TAA TAC GAC TCA CTA TAG GG). All selected colonies were determined to contain the desired construct. The new vector, pET-14b(eCaM), was transformed into BL21-Gold(DE3) competent cells from Stratagene for expression.

eCaM DNA sequence:

CCATGGCAGATCAACTGACTGAAGAACAGATTGCGGAATTTAAAGAAGCATT
CAGCCTGTTCGACAAAGATGGCGATGGCACCATTACGACCAAGGAGCTGGGT
ACGGTGATGCGTTCTCTGGGTCAGAACCCAACTGAGGCAGAACTGCAGGATA
TGATCAACGAGGTTGATGCTGACGGTAATGGCACCATCGACTTCCCGGAATT
CCTGACCATGATGGCCCGTAAAATGAAAGACACCGATTCCGAAGAAGAAATC
CGTGAGGCTTTCCGTGTATTCGACAAAGACGGTAACGGCTACATTTCTGCGGC
GGAACTGCGCCATGTGATGACCAACCTGGGCGAAAAACTGACCGACGAAGA
AGTTGACGAGATGATCCGCGAAGCTGATATCGACGGTGATGGTCAGGTCAAC
TATGAAGAATTTGTTCAGATGATGACTGCGAAGCACCACCACCACCACCACT
AACTCGAG

Expression and purification of CaM-His6. The CaM-His6 construct was transformed in BL21-Gold(DE3) competent cells from which a 20% glycerol stock was prepared for subsequent protein expression. The stock was used to inoculate a 5 mL LB-broth starter culture containing carbenicillin (50 µg/mL). The starter culture was allowed to grow overnight in a 37 ºC shaker at 225 rpm. The next day, the starter culture was used to inoculate a 1 L LB-broth culture containing carbenicillin (50 µg/mL) and grown at 37 ºC with agitation at 225 rpm. The culture was allowed to grow to an $OD_{600nm} \approx 0.5$ after which the 1 L shaker flask was transferred to a 25 ºC shaker (225 rpm) for induction. The cells were induced with 0.1 mM IPTG and shaken for 4 hrs before harvesting at 5000 × g (4 ºC). The pellet was then resuspended in 0.9% NaCl aqueous solution (30 mL) and transferred to a 50 mL conical tube and repelleted for storage at -80 ºC.

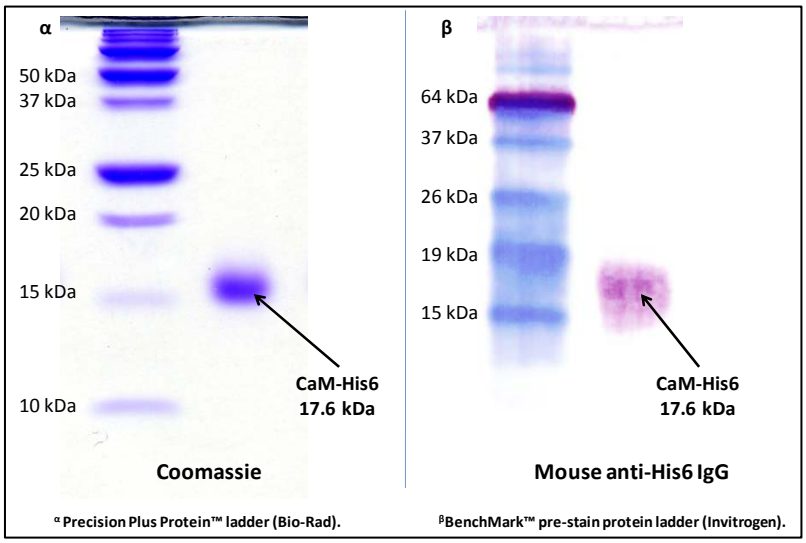
On the day of the protein preparation, the pellet was thawed at room temperature and resuspended in the following lysis buffer:

4 mL	10× PBS buffer
400 µL	NP-40 Alternative
4 mL	glycerol
40 mg	lysozyme
6 mg	dithiothreitol
40 µL	Protease Cocktail III (Calbiochem)
40 mL	final vol. after diluting with dH_2O

Once the pellet was fully resuspended, the cells were sonicated using a Branson Sonifier 450 at 50% power with a 30% duty cycle for 2 min at 4 ºC. The lysate was then transferred to 45 mL polycarbonate bottle and spun at 100,000 × g in a Type 28 rotor from Beckman Coulter to pellet DNA and other cellular debris. Meanwhile, a 5 mL bed of Ni-NTA agarose resin obtained from Qiagen was pre-equilibrated in PBS buffer. After the spin, the clarified supernatant was transferred to a clean 50 mL conical tube where it received the pre-equilibrated Ni-NTA agarose resin and was tumbled gently on a rotisserie at 4 ºC for 1 hr. The resin was then recovered by passing the lysate through 20 mL spin column obtained from Bio-Rad. The resin was washed first with 4 mL of PBS buffer, then 4 mL of 10 mM imidazole in PBS buffer and then with 4 mL of 20 mM imidazole in PBS buffer at 4 ºC. The protein was then eluted in 6 × 1.5 mL fractions of 250 mM imidazole in PBS buffer at 4 ºC. The fractions were assayed for protein and combined appropriately. The imidazole was removed by dialysis using a 3.5 kDa MWCO Slide-A-Lyzer dialysis cassette from Pierce. The protein solution was dialyzed against 3 × 2 L changes of TBS buffer. The protein was quantified using the Bio-Rad protein assay solution. Protein yield was ~150 mg/L for the purified CaM mutant.

SDS PAGE (15% polyacrylamide) analysis was performed on the protein to confirm purity by Coomassie staining. Western blotting of the gel also confirmed the presence of the poly-histidine tag when probed with the mouse anti-his IgG. After transferring to nitrocellulose for 1 hr at 4 ºC at 100 V, the nitrocellulose was blocked with nonfat dry milk (3 g) in TBS buffer (30 mL) for approximately 4 hrs. The milk solution was then poured off and washed with TBST buffer (3 × 30 mL for 5 min each wash). Next, a 1:10,000 dilution in TBST (15 mL) of the mouse anti-his IgG was added to the

nitrocellulose and incubated for 1 hr. The antibody solution was poured off and the nitrocellulose sheet was again washed with TBST buffer (3 × 30 mL) for 5 min each wash). A 1:10,000 dilution of the goat anti-mouse IgG(H+L) alkaline phosphatase conjugate in TBST (15 mL) was added to the nitrocellulose and incubated for 1 hr. The antibody solution was poured off and the nitrocellulose sheet was again washed with TBST buffer (3 × 30 mL) for 5 min each wash). Last, 10 mL of 1-Step™ NBT/BCIP from Pierce was added and incubated with the nitrocellulose until the band was visible. The nitrocellulose was then washed with dH_2O and blotted dry.



Fluorescence experiments:

General procedures. All fluorescence spectra were recorded using a Fluoromax 3 instrument (Horiba Jobin Yvon) at 25 ˚C. The samples were each excited at the wavelength appropriate for the fluorescent peptide. The slit widths were set to 3 nm for excitation and 6 nm for emission. The data points were collected at 0.5 nm increments with a 0.1 s integration period. All spectra were corrected for intensity using the manufacturer-supplied correction factors and corrected for background fluorescence by subtracting a blank scan of the buffer system.

KR peptide study in dioxane/water. Concentrated stocks of each of the purified peptides in the series were prepared in deionized H_2O and quantified by UV-visible spectrometry using the known or measured extinction coefficients of the corresponding fluorophores. Samples of each peptide were

then prepared to a final concentration of 5 μM in either TBS buffer or dioxane containing 18-crown-6 (5 mM) as an additive to enhance solubility. This was done using a 10 mL volumetric flask. The samples were then transferred to a 100 μL quartz cuvette for measurement. This process was repeated in triplicate for both solvent systems. The fold-increase measured for each fluorescent peptide at the wavelength of maximum emission in dioxane was calculated by integrating the area under the curve spanning a 5 nm window centered on the wavelength of interest. The area measured in dioxane was then divided by the area measured in TBS buffer. The results of three trials were then averaged and the error calculated from the standard deviation and the Student's t-value for a 90% confidence interval.

Ca^{2+}-CaM-M13 mutant study. Concentrated stocks of each of the purified peptides in the series were prepared in deionized H_2O and quantified by UV-visible spectrometry using the known or measured extinction coefficients of the corresponding fluorophores. A concentrated stock solution of the CaM-His6 construct was also prepared in TBS buffer and quantified by measuring the absorbance in 6 M guanidinium chloride using the predicted extinction coefficient ($\varepsilon_{280\ nm}$ = 2980 cm^{-1} M^{-1}) based on residue composition.[6] Additionally, stocks of $CaCl_2$ (2 mM) and ethylene diamine tetraacetic acid (EDTA, 5 mM) were prepared. From these stock solutions, four experiments were conducted in triplicate as shown in Table S3.

Table S3: Experimental conditions for Ca^{2+}-CaM-M13 mutant study.

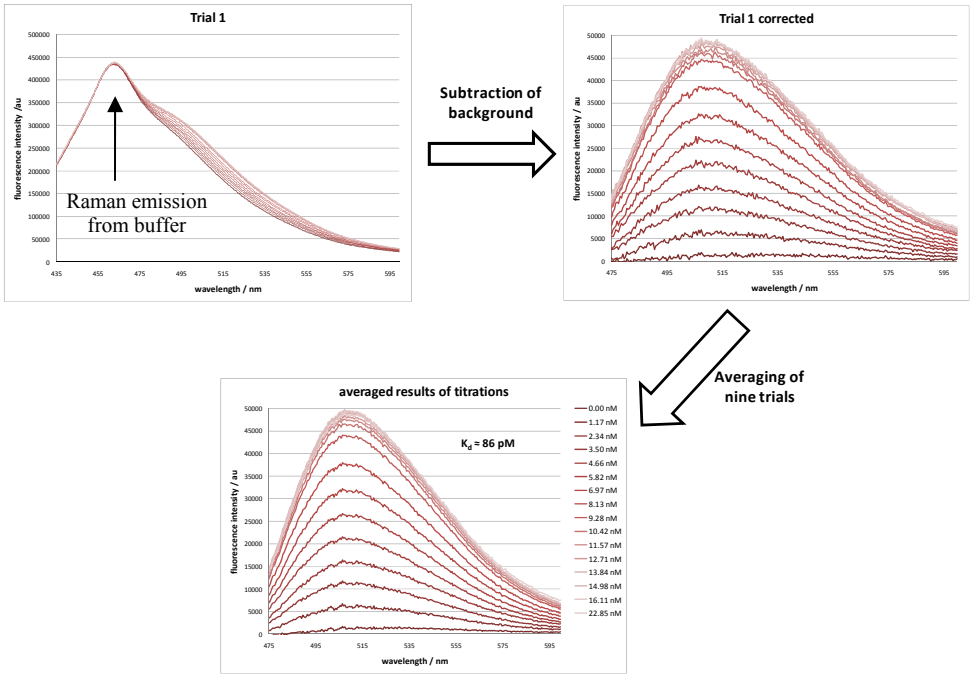
Experiment	M13 (10 μM)	EDTA (40 μM)	CaM-His6 (15 μM)	$CaCl_2$ (200 μ)
●	✓	✓		
△	✓	✓	✓	
○	✓	✓	✓	✓
□	✓	✓		✓

The fold-increase measured for each fluorescent peptide at the wavelength of maximum emission under condition '○' was calculated by integrating the area under the curve spanning a 5 nm window centered on the wavelength of interest. The area measured for condition '○' was then divided by the area measured for conditions '●' and '△' to calculate the two reported ratios. The results of

three trials were averaged and the reported errors were calculated from the standard deviations and the Student's t-value for a 90% confidence interval.

Titration of $M13_{4DMN}$ peptide with Ca^{2+}-CaM. This experiment was conducted at 25 ˚C in TBS buffer (pH 7.4) containing 200 μM $CaCl_2$ and 0.1% BSA. The BSA was added to prevent nonspecific adsorption of the CaM construct or the mutant M13 peptide to the walls of the quartz cuvette or pipette tips. The $M13_{4DMN}$ peptide was dissolved in the buffer system to an initial concentration of 10.2 nM and was gradually diluted to 9.9 nM as the titrant was added. Due to the low signal-to-noise ratio obtained using our instrumentation, lower concentrations of the fluorescent M13 peptide gave less reliable results. The titrant was a stock solution of the CaM construct (920 μM). The total CaM concentration was varied from 0 nM to 22.9 nM as fluorescence spectra were collect at each increment. Due to the low concentration of the fluorescent peptide, the excitation slit width was widened to 10 nm and the integration period extended to 0.2 s. The data from nine replicate titrations was imported into SPECFIT/32™ Global Analysis System for Windows (version 3.0.39) to calculate the K_d of the interaction. The results of the nine trials were then averaged and the error calculated from the standard deviation and the Student's t-value for a 95% confidence interval. The data processing of a typical titration under these conditions is depicted in Figure S1.

Figure S1: Titration of the $M13_{4DMN}$ peptide with Ca^{2+}-CaM.



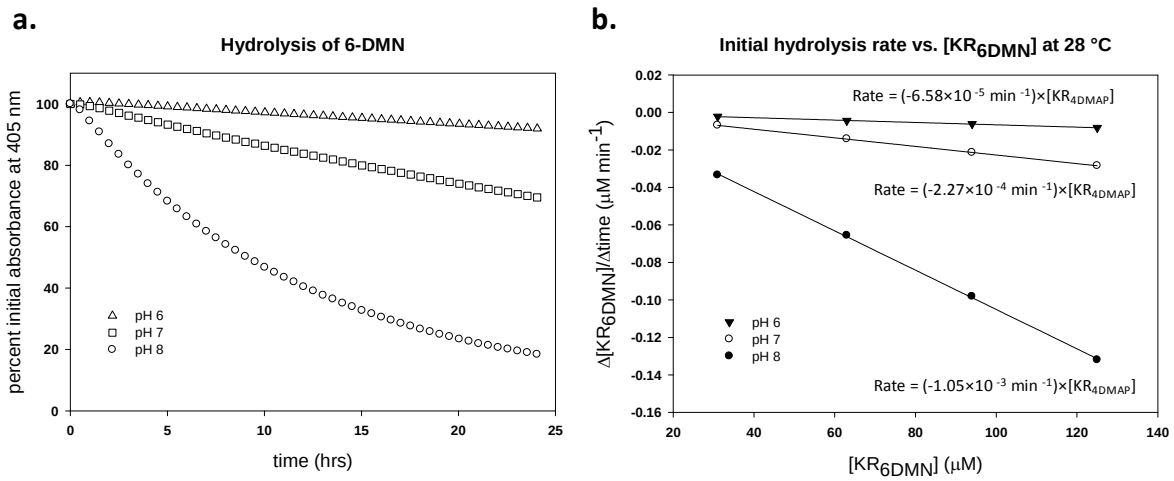
Kinetic measurements of 4-DMAP and 6-DMN hydrolysis:

The KR_{4DMAP} peptide was screened at four concentrations (10 μM, 20 μM, 30 μM, and 40 μM) and three different pH levels (pH 6, 7, and 8) in PBS buffer at 28 ˚C. Since 4-DMAP undergoes a loss of fluorescence upon hydrolysis, the percent conversion of the fluorophore can be defined as the percent loss of fluorescence from time zero. These same conditions were also applied to the KR_{6DMN} and KR_{4DMN} peptides. The experiments were performed using a PerkinElmer® HTS 7000 plate reader with NUNC™ 96-well black PolySorb plates for both the KR_{4DMAP} and KR_{4DMN} peptides and a Falcon™ 96-well black/clear bottom Optilux™ plate for the KR_{6DMN} peptide. The plates were assembled such that each condition was repeated a total of four times. A series of blank samples (PBS buffer without peptide added) were also added to measure the background, which would later be subtracted from the fluorescence/absorbance measurements. The wells were loaded with 200 μL of each sample and then topped with 80 μL of white, light, mineral oil (Mallinckrodt Chemicals) to prevent evaporation. After assembly, the plates were placed in the plate reader and monitored at 30 min intervals over the course of 24 hrs. The 4-DMAP and 4-DMN fluorophores were both excited at 405 nm using a 10 nm band-pass filter. The fluorescence emission of 4-DMAP and 4-DMN was measured using a 595 nm filter and 535 nm filter, respectively. Since the byproduct of 6-DMN hydrolysis is highly fluorescent, the reaction rates were determined by measuring the absorbance at 405 nm (see Figure S2 and S3 for results). At this wavelength, the ring-closed product absorbs to an appreciable extent while the ring-opened product does not absorb at all. Four concentrations for the KR_{6DMN} peptide were screened (31 μM, 63 μM, 94 μM, and 125 μM).

After the data was recorded, it was plotted as a function of time. Trend lines were fit to the initial time points using the least-squares method and used to compute the initial rates. The initial rates were then plotted as a function of peptide concentration. Again, a trend line was fit to these plots for each pH level to compute the pseudo first-order rate constants. The trend lines gave excellent fits ($R^2 >$ 0.99) when the Y-intercept was set at zero. While the 4-DMAP and 6-DMN fluorophores showed

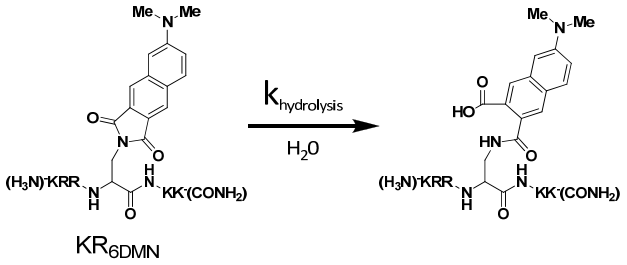
significant signs of hydrolysis over 24 hrs, no change was observed for 4-DMN. (Note – This experiment was conducted using potassium phosphate buffers containing 0.2 M phosphate).

Figure S2: Hydrolysis data for the 6-DMN fluorophore.



Panel (a) depicts the absorbance of 6-DMN at 405 nm over time at three pH levels. The decrease is due to conversion of the fluorescent species to the ring-opened byproduct. Panel (b) indicates the rate dependence of the hydrolysis reaction on the initial fluorophore concentration.

Figure S3: Measured rate constants of 6-DMN hydrolysis.



KR_{6DMN}

$$Rate = k_{hydrolysis} \times [KR_{4DMAP}]$$

PBS buffer	$k_{hydrolysis}$ (min^{-1})	90% confidence interval (min^{-1})
pH 6	6.58×10^{-5}	$\pm 0.09 \times 10^{-5}$
pH 7	2.27×10^{-4}	$\pm 0.03 \times 10^{-4}$
pH 8	1.05×10^{-3}	$\pm 0.02 \times 10^{-3}$

Treatment of KR peptide series with 4-methylpiperidine:

Approximately 20 mg of Pal-PEG resin for each of the three KR peptides was transferred to a small 2 mL spin column (Bio-Rad) and treated with a 20% solution of 4-methylpiperidine in DMF (3 × 5 min) to simulate the conditions of a typical Fmoc deprotection step in SPPS. Afterward, the resin was

washed thoroughly with fresh DMF and finally with DCM before cleaving the peptides from the resin using a solution of 96:2:2 TFA/H_2O/EDT for 2.5 hrs. The resin was then filtered off and the TFA removed by evaporation. The crude products were then dissolved in 200 μL of dH_2O and analyzed by MALDI-MS, HPLC, and ESI-MS. Due to the highly polar nature of KR peptides, a slow gradient (95:5 to 55:45 H_2O/MeCN with 0.1% TFA over 35 mins.) was used to resolve the starting material from the 4-methylpiperidine adducts. The HPLC method monitored the absorbance at 228 nm and 280 nm. The isolated products were then further analyzed by ESI-MS to confirm their identity.

Table S4: Conformation of 4-methylpiperidine adducts to 4-DMAP and 6-DMN

4-methylpiperidine adducts of KR peptides	$[M+xH]^{x+}$ Calc.	MALDI MS $[M+H]^{1+}$ found	ESI MS $[M+xH]^{x+}$ found
KR_{4DMAP}	1073.1 (1+)	1073.5 (1+)	
	537.1 (2+)		536.9 (2+)
	358.4 (3+)		358.3 (3+)
KR_{6DMN}	1123.2 (1+)	1123.6 (1+)	
	562.1 (2+)		562.0 (2+)
	375.1 (3+)		375.0 (3+)
KR_{4DMN}	No adducts observed		

Determination of 4-DMN extinction coefficient (in TBS buffer pH 7.4):

For the purpose of the studies described in this account, the extinction coefficient of the 4-DMN chromophore was determined in TBS buffer (pH 7.4). The derivative used for this measurement was 4-*N*,*N*-Dimethylamino-*N*-(2-hydroxy-ethyl)-1,8-naphthalimide **(7)**, which is fully soluble in water at the concentration required for this measurement. A series of three Beer-Lambert plots were conducted. For each trial, the dry solid (~25 mg) was weighed and dissolved in 1 mL of DMSO to facilitate solubility. The concentrated DMSO solution was then transferred to a 1 L volumetric flask and diluted in TBS buffer (pH 7.4). Four additional dilutions of the fluorophore were prepared from the 1 L stock and the absorption spectra of each were recorded. The peak of maximum absorption was measured at 440 nm. The absorbance measurements made at this wavelength were plotted as a function of the chromophore

concentration. The slope of the least-squares linear fit to the data points was averaged with that of two other trials to give the calculated extinction coefficient at 440 nm ($\varepsilon_{440\ nm} = 8.8 \times 10^3\ cm^{-1}\ M^{-1}$).

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(1) Kollar, J.; Hrdlovic, P.; Chmela, S.; Sarakha, M.; Guyot, G. *J. Photochem. Photobiol., A* **2005**, *170*, 151-159.

(2) Chan, W. C.; White, P. D. *Fmoc solid phase peptide synthesis: a practical approach*; Oxford University Press: New York, 2000.

(3) Hancock, W. S.; Battersby, J. E. *Analytical Biochemistry* **1976**, *71*, 260-264.

(4) Hachmann, J.; Lebl, M. *J. Comb. Chem.* **2006**, *8*, 149.

(5) Sainlos, M.; Imperiali, B. *Nat. Protoc.* **2007**, *2*, 3201-3209.

(6) Gasteiger, E.; Gattiker, A.; Hoogland, C.; Ivanyi, I.; Appel, R. D.; Bairoch, A. *Nucleic Acids Res.* **2003**, *31*, 3784-3788.