Electronic Supplementary Material (ESI) for Chemical Communications

Painting Proteins Blue: ß-(1-Azulenyl)-L-Alanine as a Probe for Studying Protein-Protein Interactions.

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Electronic Supplementary Information

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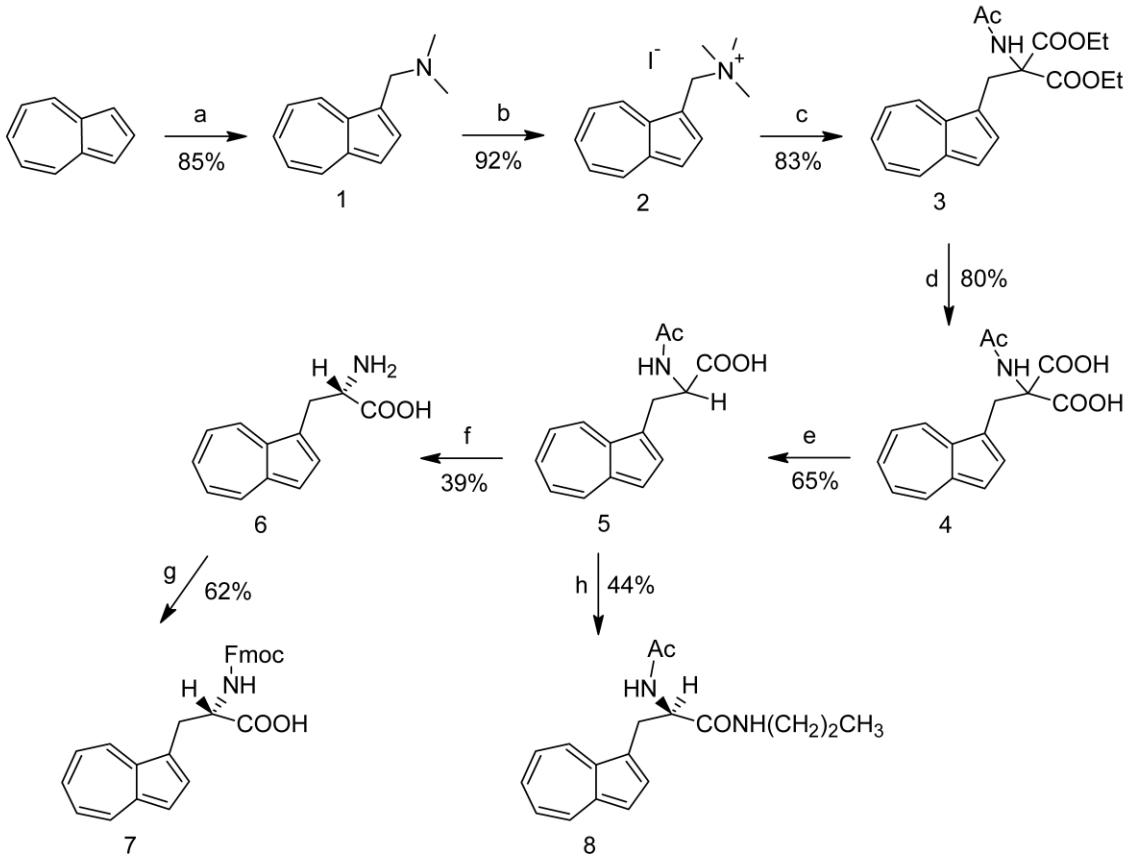
METHODS.

General. ^{1}H NMR spectra of the synthesized compounds were recorded at 25 °C on a Bruker DPX-300 (300 MHz) spectrometer using tetramethylsilane or $HD_2CS(O)CD_3$ as an internal standard. UV/Vis spectra were measured on an Agilent 8453 spectrophotometer. Fmoc-protected amino acids, rink amide-MBHA resin and Wang resin were obtained from GL Biochem Ltd. (Shanghai).

Synthesis of β-(1-Azulenyl)-L-alanine (AzAla) and its Derivatives. Synthesis of AzAla was performed according to the published procedure.[2] We were unable to reproduce some of the separation procedures described in ref.[2] and employed chromatographic separation, where necessary as described in detail below. We have also included previously not reported NMR parameters for all of the synthetic intermediates.

Scheme S1. Schematic representation of synthesis of AzAla and NAAzAP.



1-Dimethylaminomethylazulene (**1**) A mixture of para-formaldehyde (0.12 g; 4 mmol), N,N,N',N'-tetramethyldiaminomethane (0.44 g; 4.4 mmol) and glacial CH_3COOH (8 mL) was heated until a clear solution was obtained. The solution was cooled to 0 °C on and added drop-wise to an ice-cold solution of azulene (1 g; 7.8 mmol) in 10 ml of CH_2Cl_2. The obtained mixture was stirred on ice for 30 min and then at room temperature for 90 min. Then, water (40 mL) and 5% aqueous HCl (20 mL) were added and the aqueous phase was washed four times with CH_2Cl_2 to remove the unreacted azulene. The combined organic layers were re-extracted with 20 mL of water, the aqueous phases were adjusted to pH 12 with 2 M NaOH and extracted twice with di-ethyl ether. The combined ether layers were washed with water, dried over Na_2SO_4 and evapo-rated to give blue oil. Yield: 1.2 g (85%). NMR (DMSO-d_6, ppm): δ = 8.54 (1 H, d, J = 9.6 Hz),

8.36 (1 H, d, *J* = 9.4 Hz), 7.82 (1 H, d, *J* = 3.8 Hz), 7.66 (1 H, dd, *J* = 9.6, 9.6 Hz), 7.34 (1 H, d, *J* = 3.8 Hz), 7.21 (1 H, dd, *J* = 9.6 Hz, 9.6 Hz), 7.18 (1 H, dd, *J* = 9.6, 9.4 Hz), 3.83 (2 H, s), 2.14 (6 H, s).

1-Azulenylmethyltrimethylammonium iodide (**2**) Methyl iodide (0.9 g; 6 mmol) was added to 1 g (5.4 mmol) of 1-Dimethylaminomethylazulene in dry EtOH (25 mL) and the obtained mixture was allowed to stand at 4 °C overnight. The blue precipitate that formed was collected and dried. Yield: 1.6 g (92%). NMR (DMSO-d_6, ppm): δ = 8.84 (1 H, d, *J* = 9.6 Hz), 8.61 (1 H, d, *J* = 9.4 Hz), 8.9 (1 H, d, *J* = 3.8 Hz), 7.91 (1 H, dd, *J* = 9.6, 9.6 Hz), 7.54 (1 H, d, *J* = 3.8 Hz), 7.50 (1 H, dd, *J* = 9.6, 9.6 Hz), 7.48 (1 H, dd, *J* = 9.6, 9.4 Hz), 5.02 (2 H, s), 3.05 (9 H, s).

Diethyl acetamido-(1-azulenylmethyl)-malonate (**3**) Diethyl acetaminomalonate (2.2 g; 10.2 mmol) was added to a solution of sodium (0.23 g; 9.9 mmol) in dry EtOH (40 mL). After 15 min, 1-azulenylmethyltrimethylammonium iodide (1 g; 3.1 mmol) was added, the obtained mixture was heated at 85°C for 1 h (the reaction progress was monitored by thin-layer chromatography, R_f(product) = 0.45, CH_2Cl_2:AcOEt; 10:1), diluted with 200 mL of water, adjusted to pH 7 with 5% aqueous HCl and extracted twice with diethyl ether. The reaction time was shortened and the temperature was increased compared to the original protocol to minimize formation of a green byproducts. The combined organic layers were washed with water, dried over Na_2SO_4 and evaporated to give blue slurry. The green byproduct must be removed prior to the next steps. To remove the green byproduct (~10% according to HPLC) flash chromatography was applied using a mixture of CH_2Cl_2 and AcOEt (10:1 v/v) as an eluent. The green byproduct(s) usually stays on the top of the column. The product was crystallized upon standing in a fridge at 8 °C. Yield: 0.92 g (83%). NMR (DMSO-d_6, ppm): δ = 8.34 (1 H,d, *J* = 9.6 Hz), 8.11 (1 H, d, *J* = 10.0 Hz), 8.02 (1 H, s), 7.66 (1 H, dd, *J* = 9.8 Hz), 7.58 (1 H, d, *J* = 3.8 Hz), 7.34 (1 H, d, *J* = 3.8 Hz), 7.20 (1 H, dd, J_1 = 10.0, 9.6 Hz), 7.19 (1 H, dd, *J* = 9.8 Hz, 9.6 Hz), 4.13 (4 H, q, *J* = 7.2 Hz), 3.94 (2 H, s), 1.89 (3 H, s), 1.16 (6 H, t, *J* = 7.2 Hz).

Acetamido-(1-azulenylmethyl)-malonic acid (**4**) 20% aqueous KOH (25 mL) was added to a solution of diethyl acetamino-(1-azulenylmethyl)-malonate (0.9 g; 2.5 mmol) in EtOH (25 mL), and the mixture was heated to 70 °C to minimize formation of the green byproduct. We found this step to be problematic and monitoring the reaction by TLC, CH_2Cl_2:AcOEt; 10:1 is essential to ensure that the reaction mixture is not heated longer than it is necessary. After 1 hour, water

(100 mL) was added and the product was extracted with two times with 20 mL of diethyl ether. The aqueous phase was then adjusted to pH 2 with 6 M HCl, and extracted twice with 20 mL of diethyl ether. The combined organic layers were washed with saturated NaCl solution, dried over Na_2SO_4 and evaporated to dryness to give a blue powder. Yield: 0.7 g (80%). The product was used on the next step without further purification. NMR (DMSO-d_6, ppm): δ = 13.37 (2 H, br s), 8.32 (1 H, d, J = 9.42 Hz), 8.17 (1 H, d, J = 9.80 Hz), 7.7 – 7.6 (3 H, m), 7.33 (1 H, d, J = 3.8 Hz), 7.17 (2 H, m), 3.92 (2 H, s), 1.86 (3 H, s).

α-Acetamido-β-(1-azulenyl)-propanoic acid (5) Acetamino-(1-azulenylmethyl)-malonic acid (0.7 g; 2.3 mmol) was heated at 85-90°C for 7 h in a mixture of THF (15 mL) and 0.2 M HCl (100 mL) under nitrogen atmosphere (the temperature was lowered compared to the original procedure to decrease the amount of green oily byproduct). After 8 hours, the solution was allowed to cool to room temperature and was extracted three times with 20 mL of diethyl ether. The organic layers were combined, washed with water, dried over Na_2SO_4 and evaporated. The crude product was redissolved in ether, filtered to remove the leftover of insoluble green byproduct and evaporated to give a blue powder. Yield: 0.4 g (65%). NMR (DMSO-d_6, ppm): δ = 12.65 (1 H, br s), 8.36 (1 H, d, J = 9.6 Hz), 8.32 (1 H, d, J = 9.4 Hz), 8.23 (1 H, d, J = 7.9 Hz), 7.81 (1 H, d, J = 3.8 Hz), 7.65 (1 H, dd, J = 9.7, 9.7 Hz), 7.33 (1 H, d, J = 3.8 Hz), 7.19 (1 H, dd, J = 9.7, 9.6 Hz), 7.16 (1 H, dd, J = 9.7, 9.4 Hz), 4.52 (1 H, m), 3.51 (1 H, dd, J = 14.8, 5.3 Hz), 3.4 – 3. 25 (1 H + H_2O, m), 1.76 (s, 3 H).

β-(1-Azulenyl)-L-alanine (**6**) To a solution of α-acetamino-β-(1-azulenyl)-propanoic acid (0.4 g; 1.6 mmol) in 0.1 M phosphate buffer (10 mL; pH 7.5) and 4 M KOH (0.4 mL; 1.6 mmol), acylase I from *Aspergillus melleus* (0.2 g) was added. After 5 h at 37 °C, an aliquot was taken and analyzed by HPLC to prove that reaction is complete. HPLC analysis is critical, as longer reaction times lead to formation of a green byproduct and lower the overall yield of the reaction. The enzyme was then removed by filtration through an AMICON membrane (7 kDa MWCO). The filtrate was acidified to pH 2.5 with 1 M HCl and extracted twice with diethyl ether to remove unreacted α-acetamino-β-(1-azulenyl)-propanoic acid. The aqueous phase was adjusted to pH 7 with 1 M NaOH, and the solvent was evaporated under reduced pressure. The obtained crude product was used without further purification. Yield: 0.12 g (78% for one enantiomer). The yield is based on measured UV/Vis absorbance of aqueous solution at 342 nm using $\varepsilon = 4200 cm^{-1}M^{-1}$. NMR (DMSO-$d_6$, ppm): δ = 8.46 (1 H, d, J = 9.6 Hz), 8.34 (1 H, d, J = 9.2 Hz), 7.90 (1 H, d, J =

4.0 Hz), 7.65 (1 H, dd, *J* = 9.7, 9.6 Hz), 7.35 (1 H, d, *J* = 3.8 Hz), 7.19 (1 H, dd, *J* = 9.6, 9.6 Hz), 7.16 (1 H, dd, *J* = 9.6, 9.2 Hz), 5.7 – 4.3 (br, 3H + H_2O), 3.74 (1 H, dd, *J* = 7.3, 5.1 Hz), 3.62 (1 H, dd, *J* = 14.8, 5.3 Hz), 3.45 (1 H, dd, J_1 = 14.8, 7.3 Hz).

Fmoc-β-(1-Azulenyl)-L-alanine (**7**) To an ice-cold solution of H-Aal-OH (0.12 g; 0.55 mmol) and Na_2CO_3 (0.13 g; 1.2 mmol) in H_2O (2 mL) and dioxane (3.5 mL), Fmoc-OSu (0.18 g; 0.55 mmol) was added in small portions. After 3 h, the mixture was diluted with H_2O and extracted twice with diethyl ether. The aqueous phase was adjusted to pH 3 with 1 M HCl, extracted three times with 15 mL of AcOEt and the combined organic layers were dried over Na_2SO_4 and evaporated. The crude product was precipitated from CH_2Cl_2 with *n*-hexane and used in preparation of peptides without further purification. Yield: 0.15 g (62%). NMR (DMSO-d_6, ppm): δ = 12.7 (1 H, br s), 8.41 (1 H, d, *J* = 9.80 Hz) 8.33 (1 H, d, *J* = 9.2 Hz) 7.9 – 7.8 (3 H, m) 7.78 (1 H, d, *J* = 8.5 Hz) 7.7 – 7.55 (3 H, m), 7.57 - 7.64 (7 H, m) 4.3 (1 H, m) 3.55 (1 H, dd, J_1 = 14.8, J_2 = 5.3 Hz) 3.43 – 3.27 (1 H + H_2O, m).

α-Acetamido-β-(1-azulenyl)-D-propanamide (NAAzAP) (**8**): 2-(6-Chloro-1H-benzotriazole-1- yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) (120 mg, 0.29 mmol) and *n*-propylamine (75 mg, 1.3 mmol) were added to a solution of α-Acetamido-β-(1-Azulenyl)-D-alanine (65.4 mg, 0.25 mmol) in 0.5 mL of dimethylformamide. The reaction mixture was stirred for 30 min at ambient temperature. Then, 25 mL of water was added, pH was raised with saturated sodium bicarbonate solution and the mixture was extracted twice with CH_2Cl_2. The combined organic layers were dried over anhydrous sodium sulfate, filtered and evaporated *in vacuo*. The crude product was purified using flash chromatography (CH_2Cl_2:AcOEt, 1:1). Yield: 33 mg (44%) of blue powder. NMR ($CDCl_3$, ppm): δ = 8.50 (1 H, d, *J* = 9.80 Hz), 8.30 (1 H, d, *J* = 9.42 Hz), 7.78 (1 H, d, *J* = 3.77 Hz), 7.61 (1 H, dd, *J* = 9.60 Hz), 7.33 (1 H, d, *J* = 3.77 Hz), 7.21 (1 H, dd, *J* = 9.80, 9.60 Hz), 7.15 (1 H, dd, *J* = 9.60, 9.40 Hz), 6.46 (2 H, d, *J* = 6.41 Hz), 5.05 (1 H, m), 4.62 (1 H, ddd, *J*=9.56, 7.49, 4.80 Hz), 3.67 (1 H, dd, *J* = 14.13, 4.71 Hz), 3.36 (1 H, dd, *J* = 14.03, 9.51 Hz), 2.97 (2 H, m), 1.16 (2 H, m), 0.63 (3 H, t, *J* = 7.35 Hz).

Analytical HPLC was performed on a Zorbax Eclipse XDB-C18 column (4.6 mm x 150 mm) using a linear gradient of water/acetonitrile with 0.1% TFA added and the following method: 0% acetonitrile (0-1 min), 75% acetonitrile: (13 min), 90% acetonitrile (14-16 min), 0% acetonitrile

(17-20 min) at 1.5 mL/min. The green byproducts appeared in HPLC traces at different retention times of 6.1 and 6.4 min depending on the reaction step.

Protein Expression and Purification. Recombinant calmodulin (CaM) was expressed in *E. coli* and purified as previously described.[1] After purification the protein was stored in buffer containing 50 mM HEPES (pH 7) and 100 mM NaCl. Purity of CaM was confirmed by SDS-PAGE (Figure S6). The concentration of CaM was determined by UV spectroscopy (ε_{280}(CaM) = 3240 $M^{-1}cm^{-1}$). The protein (stock concentration of 100 μM) was stored at 4 °C and the fluorescence studies were conducted on the day the protein was purified, immediately after confirming the purity by SDS-PAGE.

Synthesis and Purification of Peptides. The peptides described in the current study: smMLCK peptide – RRKWQKTGHAVRAIGRLSS, (AzAla)smMLCK peptide – RRKZQKTGHAVRAIGRLSSS, (AzAla)melittin – GIGAVLKVLTTGLPALISZIKRKRQQ, W3 - $KKWL_9AL_9KK$, Z13 - $KKGL_9ZL_9KK$, W13 - $KKGL_9WL_9KK$, Z3 - $KKZL_9AL_9KK$, D9Z13 – $KKGL_5DL_3ZL_9KK$ (Z – AzAla) were synthesized by manual fluorenylmethyloxycarbonyl (Fmoc) solid phase synthesis utilizing piperazine for deprotection of Fmoc group and HCTU/*N*,*N*-diisopropylethylamine (DIEA) activation procedure. We have used a modification of a previously described protocol optimized for synthesis of very hydrophobic peptides.[3] In short, a four-fold molar excess of Fmoc-protected amino acids was used for coupling, except in the case of Fmoc-AzAla, when 1.2-fold excess was used. The smMLCK and (AzAla)smMLCK peptides were synthesized using rink amide-MBHA resin, and the N-terminal amino group was acylated with a mixture of acidic anhydride and DIEA. Cleavage of the peptides from resin and side-chain deprotection was simultaneously achieved by treatment with a mixture of trifluoroacetic acid (TFA)/H_2O/triisopropyl silane (TIS) (95:2.5:2.5, v/v) for 2 hours at room temperature. After filtration the solution was concentrated ~2 -fold using a stream of nitrogen and a crude product was precipitated by addition of cold diethyl ether (-20 °C). The precipitate was washed with cold ether three times and dried in the stream of nitrogen. The peptides were purified on a preparative reverse phase HPLC system (Varian ProStar 210) with a C4 preparative column (Vydac), using a linear gradient of solvent A (0.1% TFA in Millipore H_2O) and solvent B (90% CH_3CN, 10% H_2O, 0.1% TFA) for smMLCK and (AzAla)smMLCK peptides and B' (60% *i*-PrOH, 30% CH_3CN, 10% H_2O, 0.1% TFA) in case of all other peptides. The identities of the purified peptides were confirmed by

MALDI-TOF mass-spectrometry using a Bruker Autoflex III Smartbeam MALDI-TOF mass spectrometer. Purity of the obtained peptides was assessed using a Shimadzu Prominence UFLC instrument with an analytical Zorbax Eclipse XDB-C18 column (4.6 mm x 150 mm) and a linear A/B or A/B' gradient at 1.75 mL/min flow rate. Final purity of the peptides was 92% or better, the only significant impurities appeared to be peptides without Trp or AzAla, which have no influence on the fluorescence spectra of the studied peptides. Stock solutions in buffer (50 mM HEPES pH 7, 100 mM NaCl) were prepared from the lyophilized powder. Concentrations of the stock solutions of the peptides were determined by UV/Vis absorbance spectroscopy using ε_{280} = 5500 $M^{-1}cm^{-1}$ or ε_{342} = 4212 $M^{-1}cm^{-1}$ for Trp- or AzAla-containing peptides, respectively.

Fluorescence measurements. Fluorescence data were obtained on an ATF 105 spectrofluorometer (Aviv Instruments, Inc) operating in the steady-state mode at 25 °C with emission and excitation band pass set to 2 nm. Measurements were made in rectangular Starna Cells, Inc. SpectroSil® Quartz cuvettes with 0.5 cm excitation path length and 0.5 cm emission path length, for sample volumes of 1.5 mL ; and in rectangular Starna Cells, Inc. SpectroSil® Quartz with 0.15 cm excitation path length and 0.15 cm emission path length for sample volumes of 200 μL.

The samples were allowed to equilibrate for 20 min before measurements were taken. Unless otherwise specified, final concentrations of peptide/NAAzAP were 10 μM. Values of quantum yields (Φ_F) for NAAzAP, smMLCK and (AzAla)smMLCK peptides have been determined using the comparative method of Williams *et al*.,[4] which uses L-Tryptophan as a standard sample with known Φ_F = 0.14.[5] All experiments were carried out using 50 mM HEPES pH 7.2 buffer (uncles explicitly stated) at two different excitation wavelengths (280 nm and 342 nm).

For the lipid containing samples, fluorescence data were obtained on a JY-Horiba fluoromax 2 spectrofluorometer at room temperature. Spectra and individual intensity measurements were taken in quartz cuvettes (0.5 cm excitation path length and 0.2 cm emission path length, sample volume 800 μL) using a 2.5 mm excitation slit width and 5 mm emission slit width for spectra and 5 mm/5 mm slits for single wavelength intensity measurements. The excitation wavelength used to excite AzAla was 342 nm. Fluorescence emission spectra were recorded over the range of 360 nm − 610 nm. Background spectra from samples containing lipid vesicles without peptide were subtracted from the values reported.

Fluorescence lifetime measurements were done on a TM 200 systems (PTI, Inc.) using the LED stroboscopic method. The samples were excited at 280 nm to achieve a stronger signal. A single emission monochromator was used with the slits fully open. Barium sulfate was used as a scatterer to obtain the instrument response function (IRF).

Lipid Vesicle Preparation. Small unilamellar vesicles (SUVs) were prepared using the ethanol dilution method.[6-8] Peptides dissolved in 1:1 ethanol/water and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) lipids dissolved in chloroform were mixed in appropriate ratios, dried under a stream of N_2, and subsequently dried under high vacuum for 60 min. The vesicles were formed by adding 10 μL of 100% ethanol to the lipid-peptide film to dissolve. Upon dissolution, 890 μL of HBS (100 mM HEPES and 150 mM NaCl, pH 7.1) was pipetted directly into the ethanolic lipid/peptide mixture while vortexing. The final concentrations were 2 μM peptide and 250 μM lipid.

Quenching Measurements. Fluorescence emission intensity was recorded using the excitation wavelength of 342 nm and the emission wavelength of 382 nm. Corrections were made for sample dilution. To determine the quenchability of membrane associated peptides by acrylamide, fluorescence was measured on samples containing vesicles with peptides before and after the stepwise additions of acrylamide from a 4 M aqueous stock solution. To determine the quenchability of membrane-associated AzAla-labeled peptides by 10-DN, the emission intensity of samples containing 10-DN was compared to that in its absence. Samples containing vesicle-incorporated peptides were prepared as above except that for the samples containing 10-DN, a variable molar percentage of the lipid was replaced by an equivalent amount of 10-DN.

Surface Plasmon Resonance (SPR) measurements. All SPR measurements were performed on a Biacore® 3000 and the data were evaluated using the Biaevaluation software. A SA chip (Biacore, GE Healthcare) was loaded with approximately 1000 RUs of biotin labeled CaM. All experiments were performed using 50 mM HEPES 100 mM NaCl 5 mM $CaCl_2$ pH 7.0 as running buffer. Injections of 50 μL were followed by 3 minutes of dissociation at a flow rate of 50 μL/min. The surface was regenerated with an injection of 50 μL of 0.5 M EDTA solution at pH 8. Peptide concentration ranged from 0 to 1000 nM, and each peptide dilution series was repeated three times. Binding kinetics was determined by fitting the data to a Langmuir 1:1 model.

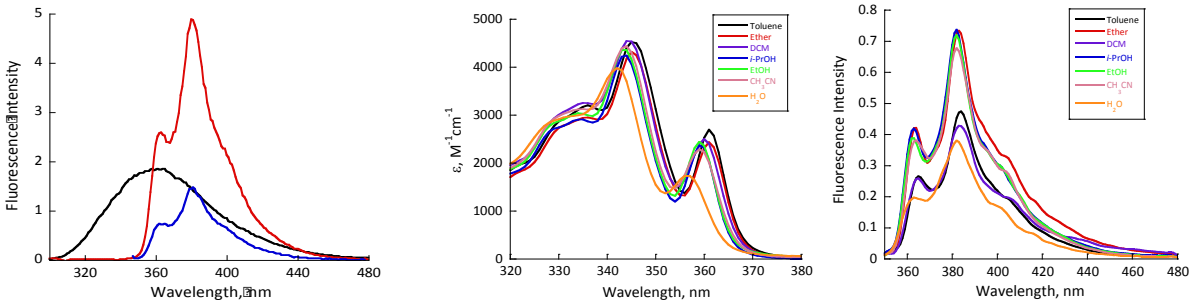


Figure S1. a) Fluorescence emission spectra of Trp (λ_{ex} 280 nm, black) and NAAzAP (λ_{ex} 280 nm, blue; λ_{ex} 342 nm, red; 10 µM Trp or NAAzAP, 50 mM HEPES, pH 7; standard cuvette). b) UV/Vis spectra of NAAzAP (0.25 mM) measured in different solvents. c) Fluorescence spectra of NAAzAP (5 µM) measured in different solvents (λ_{ex} = 342 nm, micro cuvette). Samples were prepared by a dilution of NAAzAP (0.5 mM solution CH_3CN) with the appropriate solvent.

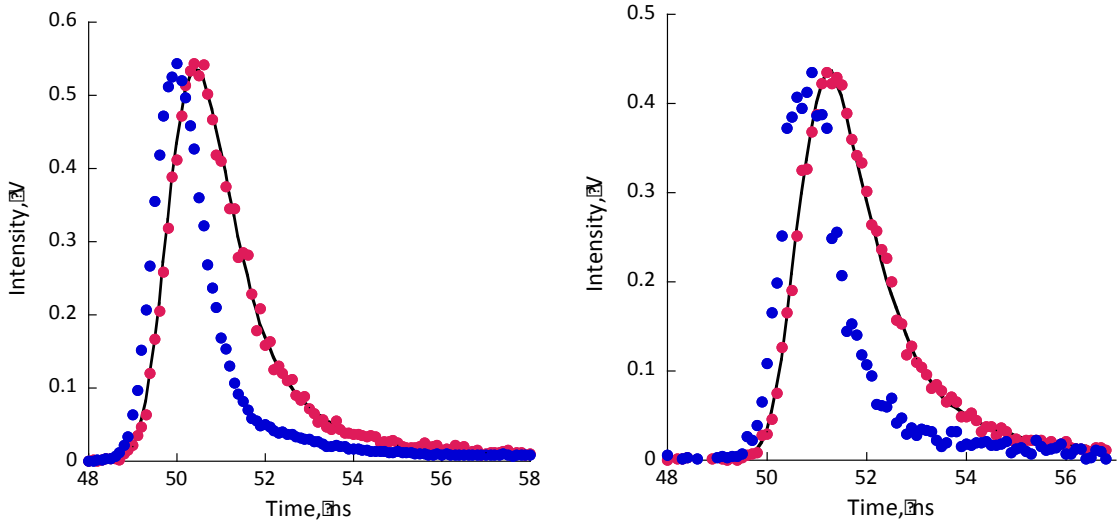


Figure S2. Fluorescence lifetime measurements of NAAzAP (left) and (AzAla)smMLCK peptide (right). The samples were excited at 280 nm. The data is shown in red and the instrument response function (IRF) is shown in blue. The data was fit to a single exponential function to give lifetimes of 0.64 ns (χ^2=1.35) for 0.64 ns (χ^2=1.12) for (AzAla)smMLCK peptide (right).

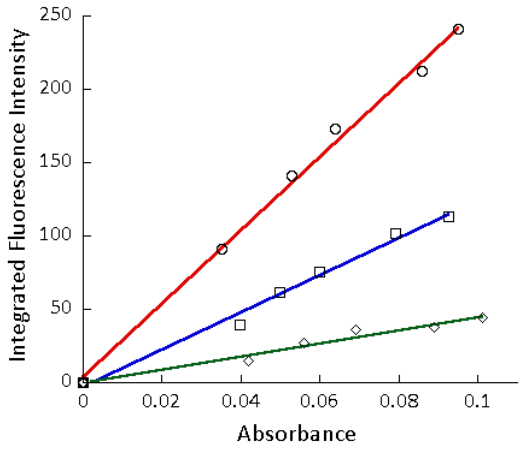


Figure S3. Determination of quantum yields of NAAzAP (L-tryptophan (red, λ_{ex} = 280 nm), NAAzAP (blue, λ_{ex} = 342 nm), NAAzAP (green, λ_{ex} = 280 nm)).

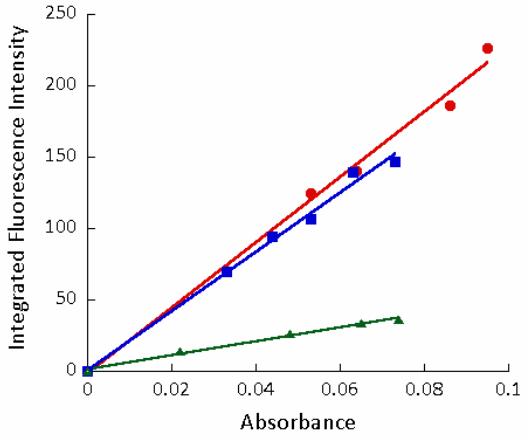


Figure S4. Determination of quantum yields of smMLCK and (AzAla)smMLCK peptides (L-tryptophan (red, λ_{ex} = 280 nm), smMLCK (blue, λ_{ex} = 280 nm), (AzAla)smMLCK (green, λ_{ex} = 280 nm)).

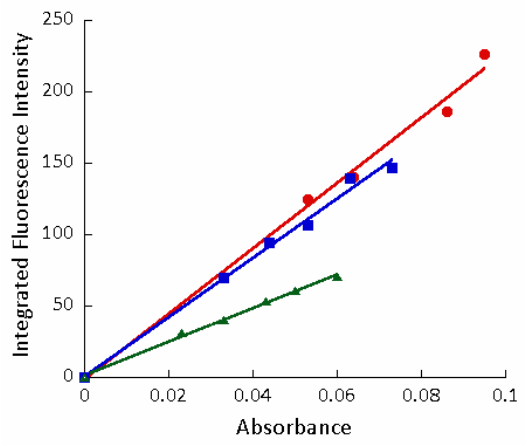


Figure S5. Determination of quantum yields of smMLCK and (AzAla)smMLCK peptides (L-tryptophan (red, λ_{ex} = 280 nm), smMLCK (blue, λ_{ex} = 280 nm), (AzAla)smMLCK (green, λ_{ex} = 342 nm)).

Table S1. Determined values of quantum yields for NAAzAP, smMLCK and (AzAla)smMLCK peptides

	Φ_F, λ_{ex} = 280 nm	Φ_F, λ_{ex} = 342 nm
smMLCK	0.127	N/A
NAAzAP	0.025	0.071
(AzAla)smMLCK	0.030	0.072

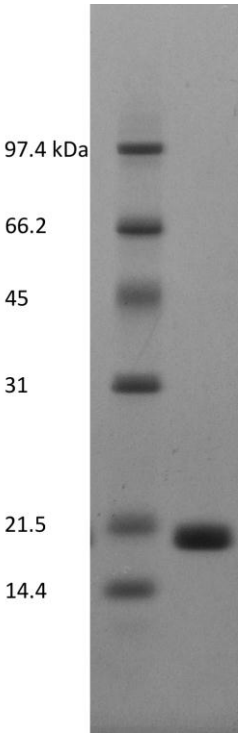


Figure S6. SDS-PAGE gel (10 %) of CaM protein used in this study.

MALDI-TOF data.

Table S2. Calculated and observed m/z values in MALDI-TOF mass spectra of the synthesized peptides.

Peptide	Observed Species	Calculated mass, Da	Found, Da
smMLCK	$[M+H]^+$	2335.32	2335.04
(AzAla)smMLCK	$[M+H]^+$	2346.43	2346.18
(AzAla)melittin	$[M+H]^+$	2898.75	2898.48
D9Z13	$[M+H]^+$	2821.95	2821.75
Z13	$[M+K]^+$	2858.01	2858.09
Z3	$[M+Na]^+$	2855.68	2856.03
W13	$[M+Na]^+$	2831.01	2831.56
W3	$[M+H]^+$	2823.02	2822.83

Study of Fluorescence Properties of Peptides Incorporated in Lipids.

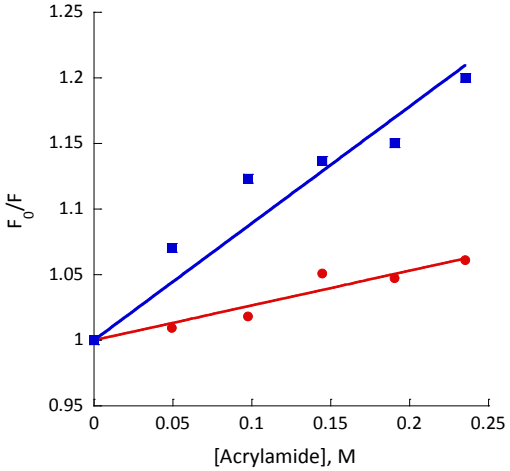


Figure S7. Stern-Volmer plot of the Acrylamide quenching experiment for Z3 (blue, K_{SV} = 0.89 M^{-1}) and Z13 (red, K_{SV} = 0.24 M^{-1}) peptides inserted in lipids.

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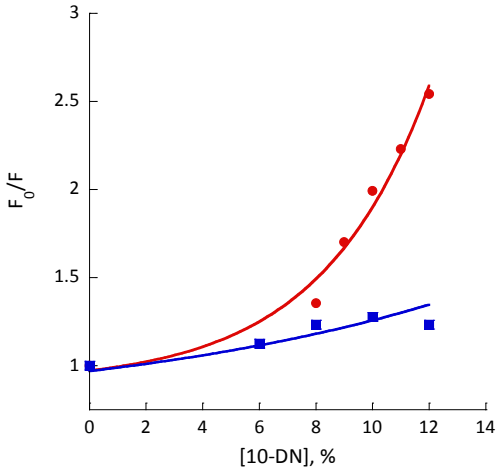

Figure S8. 10-DN quenching of Trp fluorescence in Z3 (blue) and Z13 (red) peptides with variation in the AzAla position.

Steady-state Fluorescence Study of Quenching of NAAzAP and the Synthesized AzAla- and Trp-Containing Peptides in the Presence of Different Quenchers.

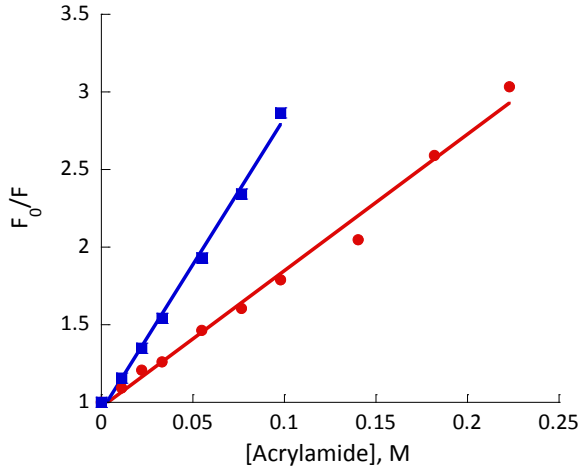

Figure S9. Stern-Volmer plot of the Acrylamide quenching experiment for NAAzAP ([amide] = 10 μM; λ_{ex} = 342 nm, K_{SV} = 8.8 M^{-1} (red); λ_{ex} = 280 nm, K_{SV} = 18.8 M^{-1} (blue)).

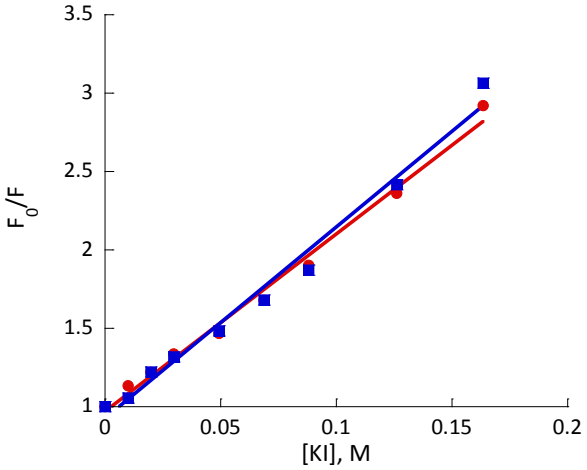

Figure S10. Stern-Volmer plot of the KI quenching experiment for NAAzAP ([amide] = 10 μM; λ_{ex} = 342 nm, K_{SV} = 11.3 M^{-1} (red); λ_{ex} = 290 nm, K_{SV} = 12.2 M^{-1} (blue)).

Steady-state Fluorescence Study of Quenching of NAAzAP in the Presence of Methionine or Imidazole.

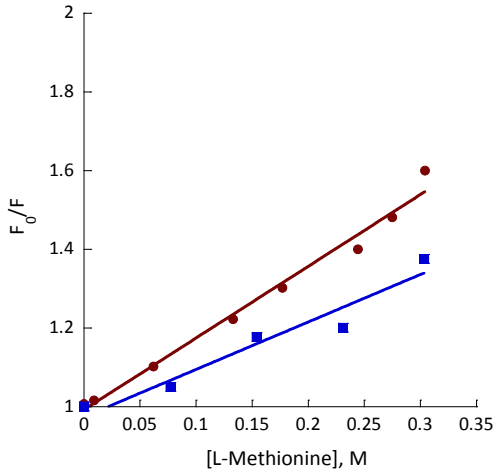

Figure S11. Stern-Volmer plot of the L-methionine quenching experiment for NAAzAP (brown, K_{SV} = 1.8 M^{-1}) and (AzAla)smMLCK (blue, K_{SV} = 1.2 M^{-1}) (5 μM in 50 mM HEPES, pH 7, λ_{ex} = 342 nm).

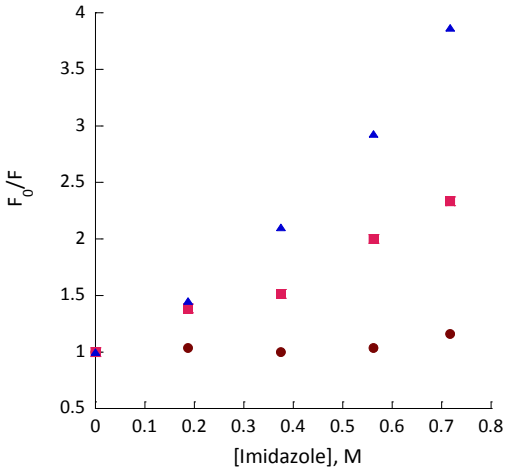

Figure S12. Stern-Volmer plot of the imidazole pH-dependent quenching experiment for NAAzAP. pH value of imidazole solution was adjusted before each experiment. Background fluorescence of imidazole was subtracted. (10 μM amide, 50 mM citrate, pH 5 (brown), 50 mM HEPES, pH 7 (red), 50 mM TAPS, pH 9; λ_{ex} = 357 nm,).

Surface Plasmon Resonance (SPR) Study of smMLCK Peptides Binding to CaM.

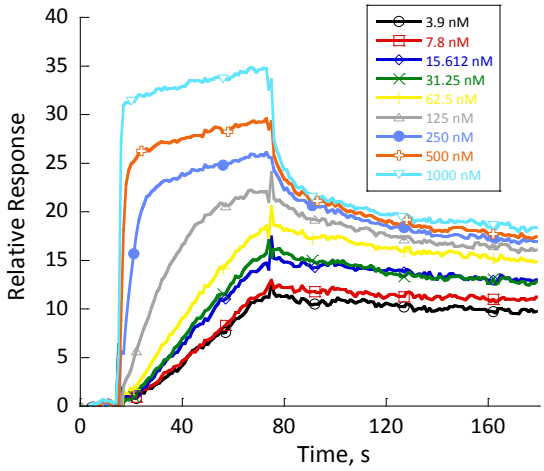

Figure S13. Overlay plot showing SPR binding curves for various concentrations of (AzAla)smMLCK in the presence of a fixed concentration of CaM.

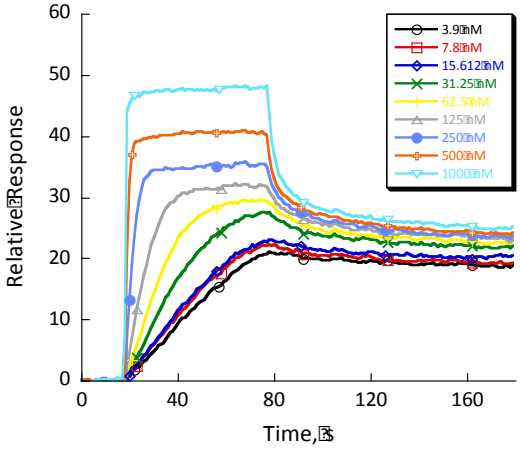

Figure S14. Overlay plot showing SPR binding curves for various concentrations of (AzAla)smMLCK in the presence of a fixed concentration of CaM.

Table S3. Parameters of binding kinetics determined by SPR

Peptide	k_{on}, $s^{-1}M^{-1}$	k_{off}, s^{-1}	K_d, M
smMLCK	$4.44\pm0.77\times10^{5}$	$3.85\pm0.63\times10^{-3}$	$8.65\pm0.31\times10^{-9}$
(AzAla)smMLCK	$6.63\pm0.13\times10^{5}$	$8.75\pm1.48\times10^{-4}$	$1.32\pm0.21\times10^{-9}$

Fluorescence Polarization Study of Binding of (AzAla)smMLCK Peptide to CaM.

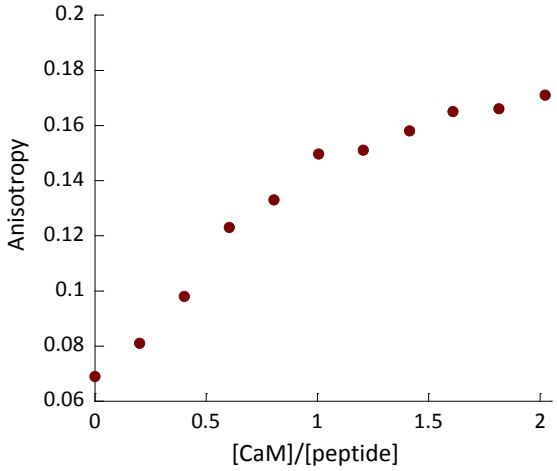

Figure S15. Steady-state fluorescence anisotropy of (AzAla)smMLCK peptide titrated with CaM. The excitation was set at 295 nm (band pass 2 nm), and the emission was observed at 384 nm (band pass 4 nm).

Study of AzAla-analog of melittin binding to CaM.

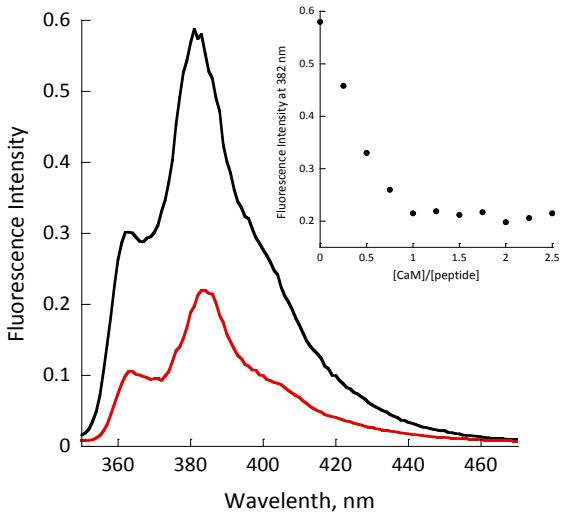

Figure S16. Fluorescence emission spectra of (AzAla)melittin (10 □M peptide, 50 mM HEPES, pH 7, 100 mM NaCl) before (black), after (red) addition of 12 □M of CaM. The inset shows the corresponding titration curve. λ_{ex} is 342 nm in all cases.

Reference.

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