Electronic Supplementary Material (ESI) for Chemical Communications.


# β-(1-azulenyl)-L-alanine – a functional probe for determination of $pK_a$ of histidine residudes

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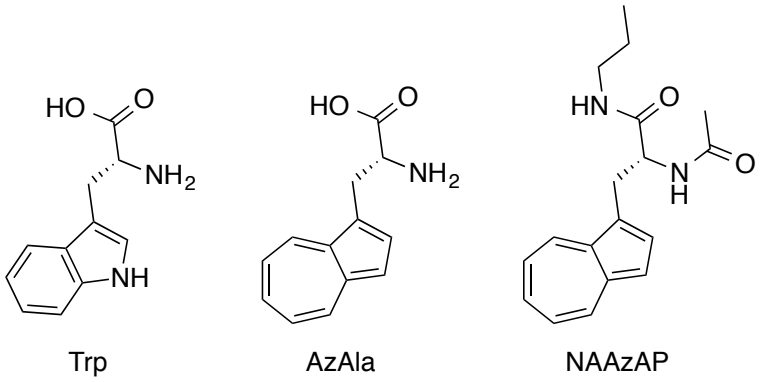
## Supplementary Information




Scheme S1. Chemical structures of tryptophan and tryptophan analogs studied in this work.

**Peptide synthesis and purification.** All peptides were synthesized by manual fluorenylmethyloxycarbonyl (Fmoc) solid phase peptide synthesis strategy. Deprotection of the side chains and cleavage of the peptides from the resin was achieved by subjecting the peptides to a mixture of trifluoroacetic acid (TFA)/$H_2O$/triisopropyl silane (TIS) (95:2.5:2.5, v/v) for 2 hours at room temperature. The solution was then filtered and concentrated to remove excess of TFA using a stream of nitrogen. The crude peptide product was precipitated in cold methyl t-butyl ether (MTBE). The precipitate was washed three times with cold MTBE and dried under nitrogen. The peptides were then purified on a preparative reverse phase high performance liquid chromatography 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). The identities of the purified peptides were confirmed by Bruker Autoflex III Smartbeam MALDI-TOF mass spectrometer. The purity of the peptides was determined on a Shimadzu Prominence UFLC instrument with an analytical Zorbax Eclipse XDB-C18 column (4.6 mm × 150 mm).

**Recombinant expression and purification of M2.** 100 mL of culture containing 100 mg/mL ampicillin was grown overnight from a single colony of *E. coli* BL21 (DE3) pLysS cells containing pET23D at 37 °C. 4 mL of this culture was then added to 1 L of LB containing ampicillin (100 mg/L) and grown at 37 °C until an $OD_{600}$ of 0.6 was reached. The culture was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown overnight at 24 °C. The cells were then pelleted at 7159 *g* for 10 min. The pellets were stored at -80 °C until purification. The cells were lysed by sonication for 20 min (30 s on, 30 s off) on ice in 20 mL of lysis buffer containing 50 mM TRIS (pH 8), 150 mM NaCl, 80 mM Octyl β-D-glucopyranoside (OG), 25 μg/mL lysozyme and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The solution was then

centrifuged at 15,000 *g* for 30 min at 4 °C. The supernatant was kept at 4 °C and the pellets were resuspended in lysis buffer and the procedure was repeated. The two supernatants were then combined and incubated with nickel nitrilotriacetic acid (Ni-NTA) resin in a buffer that contained and 20 mM imidazole at room temperature with gentle shaking for 30 min. The column was then washed with 50 mM TRIS (pH 8), 150 mM NaCl, 80 mM OG, 20% glycerol, then by 50 mM TRIS (pH 8), 150 mM NaCl, 40 mM OG, 20% glycerol, then by 50 mM TRIS (pH 8), 20 mM OG, 20% glycerol, and finally by 50 mM TRIS (pH 8), 4 mM OG, 20% glycerol, 20 mM imidazole. The protein was eluted with buffer containing 50 mM TRIS (pH 8), 4 mM OG, 20% glycerol, 300 mM imidazole. The protein was then applied onto a Bio-Rad 10 DG desalting column and the elution buffer was exchanged with storage buffer containing 50 mM HEPES (pH 8), 20% glycerol, 4 mM OG. The concentration of the protein was measured by UV-vis spectroscopy using $\varepsilon_{280}$ of 8480 $M^{-1}cm^{-1}$.[1]

**Determination of $pK_a$ of imidazole and histidine.** Buffers: 250 mM Citric Acid for pH range 4.5-5.5, 250 mM 2-(N-morpholino)ethanesulfonic acid (MES) for pH range 5.5-6.5, 250 mM 3-(N-morpholino)propanesulfonic acid (MOPS) for pH 6.5, 250 mM 2-[4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) for pH range 7-8, 250 mM 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS) for pH range 7.5-8.5, 250 mM 3-{[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino}propane-1-sulfonic acid (TAPS) for pH range 8.5-9 were used. Equal amounts of MES buffer at pH 6.55 and HEPES buffer at pH 7.02 were mixed for pH 6.84.

Buffered imidazole solutions were prepared by adding 1 mL of appropriate buffer to 3.13 mL of 0.8 M imidazole stock solution. The pH of the stock solutions was adjusted to desired values; the stock solutions were then diluted to 10 mL. The samples were prepared by adding 10

μL of 415 μM NAAzAP to 590 μL of the buffered imidazole buffer solutions. For blank measurements, 10 μL of water was added instead of NAAzAP, which were then subtracted from the samples. Fluorescence measurements were taken on an Aviv ATF-105 spectrofluorometer at 20 °C. The fluorescence emission spectra were collected for all the samples and blank solutions in the range of 395 nm to 370 nm in a rectangular SpectroSil quartz cuvette (Starna Cells, Inc.) with 0.21 cm excitation path length and 0.21 cm emission path length suitable for sample volumes of 150 μL. The excitation wavelength was set to 357 nm. The samples and the blank solutions were allowed to equilibrate for 5 min at 20 °C before each measurement.

Buffered histidine solutions were prepared by adding 1 mL of the appropriate buffer to 5 mL of 225 mM histidine stock solution. The pH values of these solutions were adjusted to desired values and then diluted to 7.5 mL. The samples were prepared by adding 5 μL of 415 μM of NAAzAP to 295 μL of histidine buffer solutions at different pH. Fluorescence data was collected on an AVIV ATF-105 spectrofluorometer at 20 °C with an equilibration time of 5 min before each measurement. The excitation wavelength was set to 357 nm.

The fluorescence emission spectra were recorded in the range of 395 - 370 nm in a rectangular SpectroSil quartz cuvette (Starna Cells, Inc.) with 0.21 cm excitation path length and 0.21 cm emission path length.

The fluorescence data was then fit to the following equation, where FI is the fluorescence intensity.

$$FI = baseline + \frac{FI_{max1} \times 10^{-pK_{a1}}}{10^{-pH} + 10^{-pK_{a1}}}$$

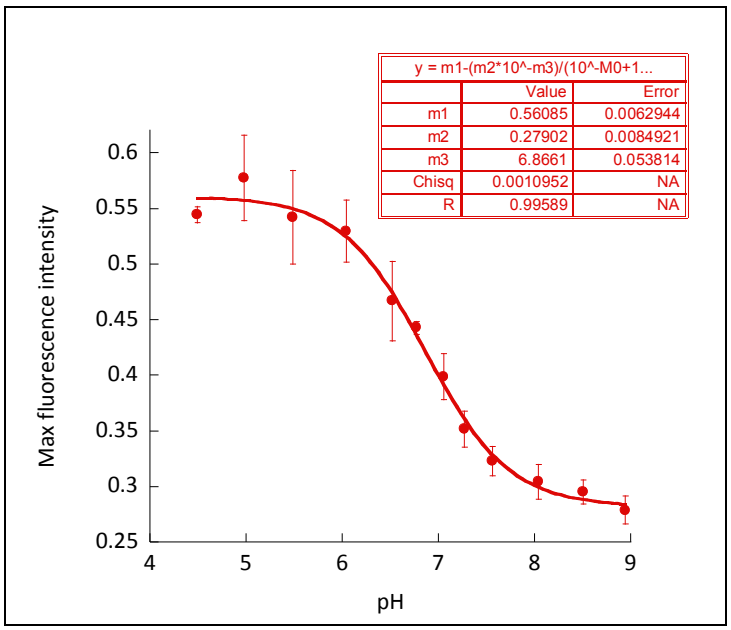



Figure S1: Plot of fluorescence intensity of NAAzAP *vs.* pH in the presence of 0.25 M imidazole.

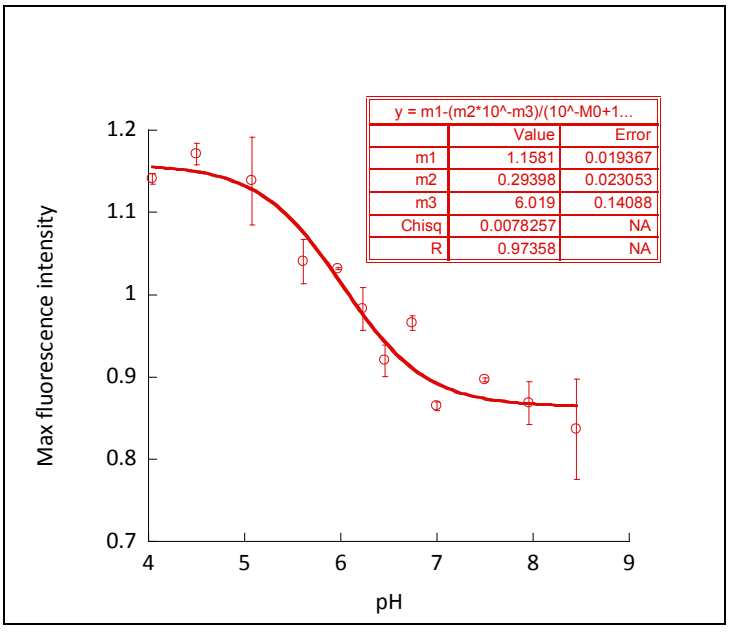



Figure S2: Plot of fluorescence intensity of NAAzAP *vs.* pH in the presence of 0.15 M histidine.

**Universal Buffer.** The fluorescence data was collected for M2 using universal buffer (solution A) as described by Sven Ostling and Pekka Virtama which can be used in the pH range of 2-12.[2] The composition of 0.5 M universal buffer A was 0.25 M sodium hydrogen phosphate dibasic, 0.165 M citric acid, 0.25 M boric acid and 0.24 M sodium hydroxide. Then solution A* was prepared by adding 16 mL of solution A, 1.2 mL of 5 M HCl and 2.8 mL of MilliQ water. Solution B* was prepared by adding 16 mL of solution A and 4 mL of 5 M HCl. The pH measured for solution A* was in the range 10.1-10.25 while the pH of solution B* was in the range of 2.9-3. Calibration curve for resulting pH values was constructed by mixing solutions A* and B* in different proportions (Figure S3). The calibration curve was then used for obtaining the working pH range. For instance, to collect fluorescence data for pH range 4-9.5, solutions A* and B* would be mixed according to Table S1. So to obtain a solution of pH 4 (Solution C*), 0.3 mL of solution A* would be added to 1.7 mL of solution B* and 8 mL of water. Similarly, a solution of pH 9.5 (Solution D*) was prepared by adding 0.2 mL of B* to 1.8 mL of solution A* and 8 mL of water. The resulting solutions C* and D* were then mixed in different proportions to obtain different pH values in that range.

Table S1: Sample preparation for constructing the calibration curve and the measured pH.

| Entry No. | Solution A* | Solution B* | water | Measured pH |
|---|---|---|---|---|
| 1 | 0.1 | 1.9 | 8 | 3.53 |
| 2 | 0.2 | 1.8 | 8 | 3.79 |
| 3 | 0.3 | 1.7 | 8 | 4.22 |
| 4 | 0.4 | 1.6 | 8 | 4.47 |
| 5 | 0.5 | 1.5 | 8 | 4.91 |
| 6 | 0.6 | 1.4 | 8 | 5.22 |
| 7 | 0.7 | 1.3 | 8 | 5.62 |
| 8 | 0.8 | 1.2 | 8 | 5.93 |
| 9 | 0.9 | 1.1 | 8 | 6.26 |
| 10 | 1 | 1 | 8 | 6.48 |
| 11 | 1.1 | 0.9 | 8 | 6.77 |
| 12 | 1.2 | 0.8 | 8 | 7.01 |
| 13 | 1.3 | 0.7 | 8 | 7.44 |
| 14 | 1.4 | 0.6 | 8 | 7.94 |
| 15 | 1.5 | 0.5 | 8 | 8.54 |
| 16 | 1.6 | 0.4 | 8 | 8.87 |
| 17 | 1.7 | 0.3 | 8 | 9.2 |
| 18 | 1.8 | 0.2 | 8 | 9.46 |
| 19 | 1.9 | 0.1 | 8 | 9.8 |

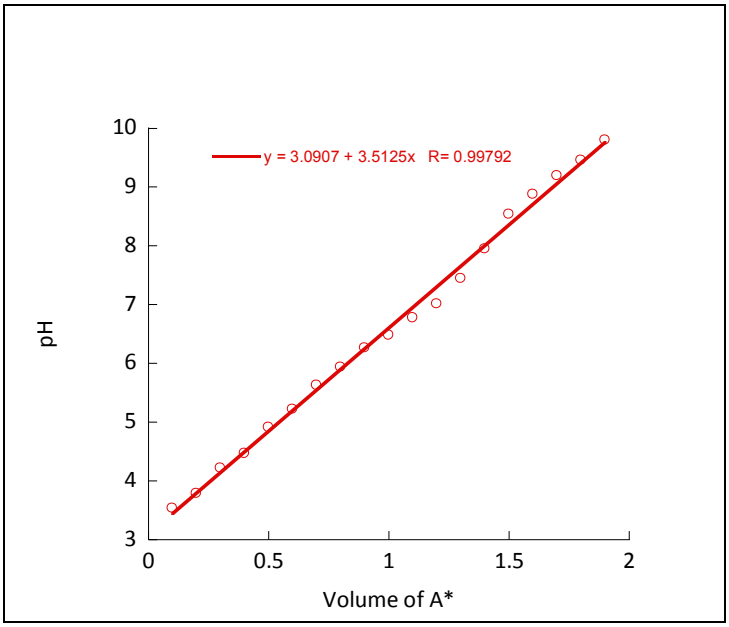



Figure S3: Calibration curve of pH *vs.* volume of solution A*.

**Preparation and reconstitution of M2TM in vesicles.** 25 mM stock solution of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (POPG) and 50 mM stock solution of 1-palmitoyl-2-oleolyl-*sn*-glycero-3-phosphocholine (POPC) in chloroform were made. 50 mM cholesterol stock solution in chloroform was freshly made each time. Lipid films were made by using these stock solutions in the ratio 4:1:2 POPC:POPG:cholesterol (8 mM total lipid concentration). Chloroform was evaporated under a stream of nitrogen and the resulting films were further dried under vacuum for 3-4 hours. The stock solutions of M2TM and M2TM W2AzAla were prepared in ethanol. The concentrations of these solutions were determined by UV-vis spectroscopy using $\varepsilon_{280}$= 5500 $M^{-1}$ $cm^{-1}$ and $\varepsilon_{342}$= 4212 $M^{-1}$ $cm^{-1}$ for Trp and AzAla containing peptides, respectively. The ethanolic stock solutions of M2TM and M2TM W2AzAla (3:1) were added to the lipid film to obtain a target peptide:lipid ratio of 1:100 monomer:lipid. The solution was vortexed to dissolve the film and the solvent was again evaporated under a stream of nitrogen. The film was immediately hydrated with 1980 μL of water and 220 μL of buffer of pH 8 formed by mixing solutions C* and D* in appropriate proportion. The sample was vortexed for 2 min and then the vesicles were sized to a target diameter of 100 nm by 33 passes through a 100 nm polycarbonate filter membranes in a mini extruder. The pH 8 M2 vesicle solution was then divided into two equal parts. Equal volume of C* solution was added to one part and D* solution to another. The resulting solutions were then mixed in appropriate proportions to obtain pH values in that range.

**$pK_a$ determination of His37 in M2TM in vesicles.** Fluorescence data was collected on an AVIV ATF-105 spectrofluorometer in a rectangular SpectroSil quartz cuvette (Starna Cells, Inc.) with 0.21 cm excitation path length and 0.21 cm emission path length. The AzAla in the M2 tetramer was excited at 342 nm and the fluorescence emission spectra were recorded in the range

of 375-400 nm at the end of the equilibration time of 5 min at 21°C for each measurement. The fluorescence data was fit to the following equation, where FI stands for fluorescence intensity.3

$$FI = baseline + \frac{FI_{max1} \times 10^{-pK_{a1}}}{10^{-pH} + 10^{-pK_{a1}}} + \frac{FI_{max2} \times 10^{-pK_{a2}}}{10^{-pH} + 10^{-pK_{a2}}}$$

**Proteoliposome Flux Assay.**

**Preparation of lipid film.** 25 mM chloroform stock solution of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (POPG) and 50 mM stock solution of 1-palmitoyl-2-oleolyl-*sn*-glycero-3-phosphocholine (POPC) in chloroform were made. 50 mM cholesterol stock solution in chloroform was freshly made each time. Lipid films were made by using these stock solutions in chloroform in the ratio 4:1:2 POPC:POPG:cholesterol (25 mM total lipid concentration). The chloroform was evaporated under a stream of nitrogen and the resulting films were further dried under vacuum for 3-4 hours.

**Sample preparation for M2 protein.** The lipid film was resuspended in 269 µL of $K^+$ buffer (10 mM $K_2HPO_4$, 50 mM $K_2SO_4$ and 5 mM MOPS, pH 7.4) and vortexed for 2 min. Then 92 µL of 0.218 mM protein was added to obtain 1: 5 x $10^{-4}$ lipid:protein tetramer ratio. For M2TM peptides, the ethanolic stock solutions of peptides were added in the same ratio as the protein and the film was dissolved by vortexing. For AzAla M2TM, the wild type peptide and the AzAla modified M2TM was added in 3:1 ratio. The solvent was evaporated under a stream of nitrogen and the resulting film was immediately hydrated with $K^+$ buffer. The sample was vortexed for 2 min and frozen. 40 µL of 10 mM pH indicator dye hydroxypyrene trisulfonic acid trisodium salt (HPTS or pyranine) was added upon thawing. After vortexing for few minutes, the sample was subjected to 10-15 freeze-thaw cycles (dry ice /ethanol and 42 °C water bath) to obtain dye containing liposomes. The resulting liposomes were then sized to a target diameter of 100 nm by

33 passes through a 100 nm polycarbonate filter membranes in a mini extruder. The liposomes were then dialyzed against 1.2 L $K^+$ buffer for 48 hrs at 4 °C in a 7K MWCO Slide-A-Lyzer mini dialysis devices. 1 g of Amberlite XAD-4 resin was added to the buffer to bind the unincorporated dye.

**Proton flux assay.** The assay was performed on an AVIV ATF-105 spectrofluorometer in arectangular SpectroSil quartz cuvettes (Starna Cells, Inc.) with 0.5 cm excitation path length and 0.5 cm emission path length. The excitation and emission bandwidth were set to 1 nm. Pyranine/HPTS, a pH indicator dye was used to determine the intraliposomal pH. The assay was calibrated by using the ratio of fluorescent signal of deprotonated form to the pH independent isosbestic point as a function of pH. The emission and the excitation wavelength for deprotonated form are 515 nm and 460 nm while that for isosbestic point are 515 nm and 417 nm respectively. 37.5 μL of 1 M p-xylene bis-pyridinium bromide (DPX) and 30 μL of liposomes were added to 2945 μL of $Na^+$ buffer (10 mM $Na_2HPO_4$, 50 mM $Na_2SO_4$, and 5 mM MOPS, pH 7.4) in the cell. Then 15 μL of 5 μM valinomycin was added to the sample in the cuvette holder and the sample was stirred continuously while performing the kinetic measurements. In order to evaluate the background $K^+$ leakage, the sample was also run by adding ethanol instead of valinomycin. . Kinetic data was monitored at excitation wavelength of 460 nm for 300 s at 1 s interval immediately after addition. . Then the excitation wavelength was changed to 417 nm and kinetic data was collected for the isosbestic point. Experiments were performed in triplicate. Data points for the first 3 s were not considered, data points between 3 s and 23 s were used for flux calculations, a representative graph is shown in Figure S4.

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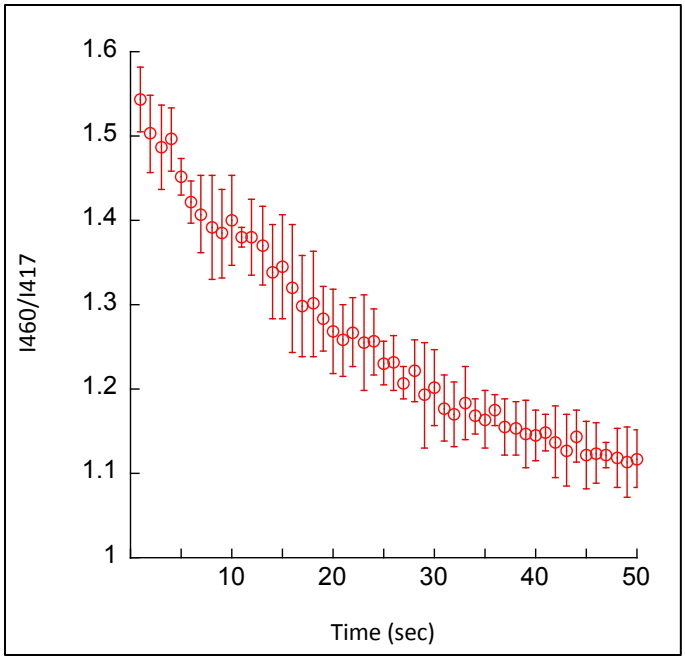


Figure S4. The ratio of fluorescence intensity (460 nm:417 nm) of pyranine as a function of time upon addition of valinomycin to vesicles containing the full length M2 protein.

**Proton flux assay data analysis.** The first 2-3 s were discarded from the analysis to allow full mixing of the sample. The data was analyzed by dividing the deprotonated HPTS signal ($I_{460}$) by the averaged isosbestic signal ($I_{417}$) to obtain a signal ratio ($I_{460}/I_{417}$) as a function of time. This signal ratio was converted to intraliposomal pH based on the calibration curve for HPTS. The intraliposomal pH was then converted to $[H^+]$ by assuming an intraliposomal phosphate concentration at 10 mM and MOPS concentration at 5 mM. The intraliposomal proton concentration was converted to number of protons by multiplying with Avogadro's number and intraliposomal volume. The resulting proton count was then divided by number of functional tetramers per liposomes, which was obtained by assuming 50:50 functional tetramers and a single phospholipid surface area of $6.3 \times 10^{-19}$ $m^2$.

**Calibration curve for pyranine.** Buffers containing 10 mM $Na_2HPO_4$, 50 mM $Na_2SO_4$ and 5 mM MOPS were prepared at different pH (6.38, 6.59, 6.79, 7.02, 7.19, 7.40, 7.62 and 7.8). Samples were made by adding 75 µL of 10 µM pyranine to 2997 µL of buffer with different pH. The signal for deprotonated form of dye and pH insensitive isosbestic point was monitored. The excitation wavelengths for deprotonated form and isosbestic point were set to 460 nm and 417 nm respectively. The emission spectrum was collected in the range of 530-490 nm on an AVIV ATF-105 spectrofluorometer in rectangular,SpectroSil quartz cuvettes (Starna Cells, Inc.) with 0.5 cm excitation path length and 0.5 cm emission path length. The calibration curve was obtained by plotting the ratio of the signal of deprotonated form to that of isosbestic point as a function of pH.

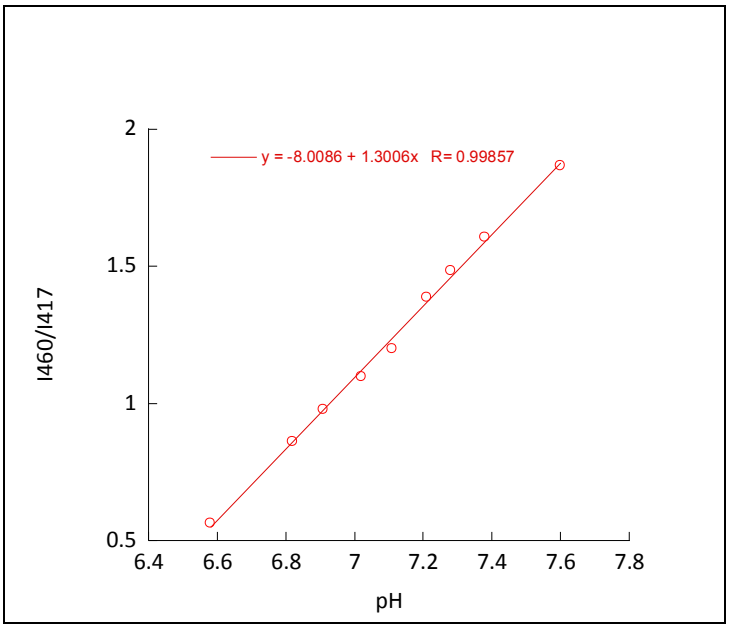


Figure S5: Calibration curve of pyranine dye. The ratio of signal of deprotonated form of dye to the signal at isosbestic point ($I_{460}/I_{417}$) as a function of pH.

**References:**

**1.** T. Leiding, J. Wang, J. Martinsson, W. F. DeGrado and S. P. Arskold, *Proc. Natl. Acad. Sci. U.S.A.*, 2010, **107**, 15409.

**2.** Ostling, S.; Virtama, P. *Acta Physiologica*, 1946, **11**, 289–293.