ELECTRONIC SUPPLEMENTARY INFORMATION

A Designed Protein Binding-Pocket to Control Excited-State Intramolecular Proton Transfer Fluorescence

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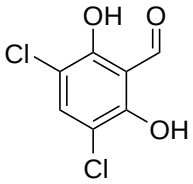
Experimental Section

Materials. Silica gel (40 μm) was purchased from Grace Davison. All solvents used for photophysical experiments were reagent grade. *O*-aminophenol derivatives were purchased from Oakwood chemicals, Hexamethylenetetramine and Sodium Cyanide from Alfa Aesar. All other reagents were purchased from Sigma Aldrich and used without further purification.

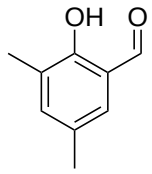
General Method. *NMR Spectroscopy:* ^{1}H and ^{13}C NMR spectra for all compounds were acquired in deuterated solvents (as indicated) on a Bruker Spectrometer at the field strengths reported in the text. The chemical shift data are reported in units of δ (ppm) relative to residual solvent.

General Fluorescence. All fluorescence measurements were taken on a BioTek Synergy M_x multi-mode Microplate Reader.

Synthetic Procedures

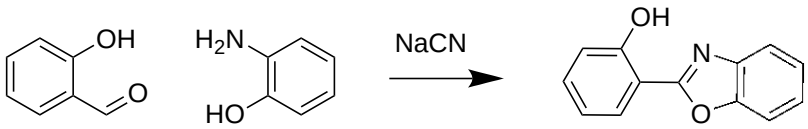


Synthesis of 3,5-dichloro-2,6-dihydroxybenzaldehyde: 4,6-Dichlororesorcinol (1.71 g, 9.57 mmol) was added to a solution of hexamethylenetetramine (2.67 g. 19.1 mmol) in neat trifluoroacetic acid (50.0 mL) under N_2 and heated to 70^{o} C for 12 h. Upon disappearance of starting material, by TLC, 1M HCl (50.0 mL) was added and stirred at 70^{o} C for an additional 5 hours. The reaction was allowed to cool to room temperature and the precipitate was filtered and dried to yield 3,5-dichloro-1,6-dihydroxy benzaldehyde (1.31 g, 65% yield) as a yellow solid. The product was used without further purification. Physical and spectroscopic data were in agreement with literature reports.[1]

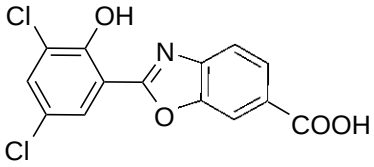


Synthesis of 3,5-dimethyl-2-hydroxybenzaldehyde: 2,4-Dimethylphenol (1.00 mL, 8.28 mmol) was added to a solution of hexamethylenetetramine (2.32 g, 16.52 mmol) in neat trifluoroacetic acid (50.0 mL) under N_2 and heated to 70^{o} C for 12 h. Upon disappearance of starting material, by TLC, 1M HCl (50.0 mL) was added and the reaction stirred for an additional 3 hours at 100^{o} C. Upon completion, the reaction was cooled to room temperature and was extracted with dichloromethane (3x20 mL). The resulting organic fractions were combined, washed with brine, dried over sodium sulfate, and concentrated *in vacuo*. The crude oil was purified via silica gel chromatography (1:9 Ethyl Acetate/Hexanes) to yield 3,5-salicylaldehyde (607 mg, 49%

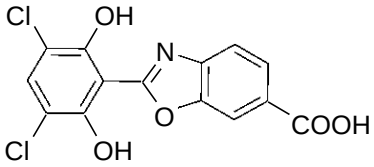
yield) as a yellow liquid. Physical and spectroscopic data were in agreement with literature reports.[2]



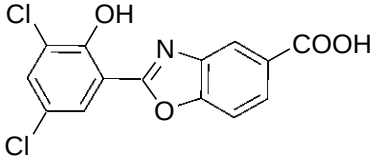
General procedure for the synthesis of 2-(2-hydroxyphenyl)benzoxazole derivatives: To a solution of Salicylaldehyde derivative (1.0 eq.) in DMF (0.1 M) was added 2-Amino Phenol derivative (1.0 eq.) and was stirred at room temperature. Upon disappearance of starting material, sodium cyanide (1.0 eq.) was added and the reaction was stirred for an additional 18 hours exposed to air. Upon reaction completion, the solution was quenched with 0.1M HCl (10x current volume) and the resulting precipitate was collected via vacuum filtration, dried and used without further purification.



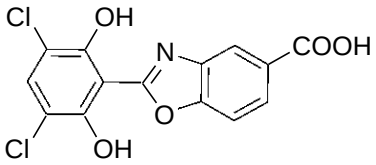
(3): 1.55 mmol scale to yield 2-(3,5-dichloro-2-hydroxyphenyl)benzoxazole-6-carboxylic acid (349 mg, 70% yield). ^{1}H NMR (400 MHz, d$_6$-DMSO) δ 13.3 (s, 1H), 11.78 (s, 1H), 8.32 (dd, *J=0.36, 1.32 Hz*, 1H), 8.09 (dd, *J=1.48, 8.4 Hz,* 1H), 8.00 (d, *J=2.52 Hz,* 1H), 7.98 (dd, *J=0.36, 8.32 Hz,* 1H), 7.91 (d, *J=2.56 Hz,* 1H). ^{13}C (125 MHz, d$_6$-DMSO) δ 166.55, 162.76, 152.59, 148.75, 142.58, 133.44, 128.99, 126.91, 125.75, 123.70, 122.59, 119.39, 112.66, 112.26. HRMS: Calc'd 321.9679, Found 321.9682.



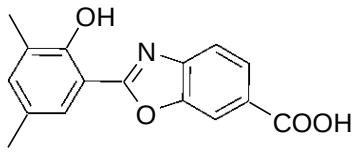
(4): 1.47 mmol scale. The collected precipate was further purified via silica gel chromatography (1:19 Methanol/Dichloromethane) to yield 2-(3,5-dichloro-2,6-dihydroxyphenyl)benzoxazle-6-carboxylic acid as a dark orange solid (135 mg, 27% yield). ^{1}H NMR (600 MHz, d$_6$-DMSO) δ 11.37 (s, 2H), 8.31 (d, *J=1.38 Hz*, 1H), 8.09 (dd *J=1.50, 8.34 Hz,* 1H*),* 7.97 (d, *J=8.34 Hz*, 1H), 7.78 (s, 1H). ^{13}C (125 MHz, d$_6$-DMSO) δ 167.17, 163.13, 153.07, 149.40, 142.65, 122.40, 128.93, 127.03, 119.53, 112.59, 112.55, 104.62. HRMS: Calc'd337.9629, Found 337.9635.



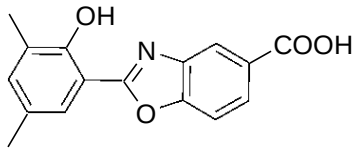
(5): 1.55 mmol scale to yield 2-(3,5-dichloro-2-hydroxyphenyl)benzoazole-5-carboxylic acid as a pale yellow solid (250 mg, 50% yield). ^{1}H NMR (600 MHz, d$_6$-DMSO) δ 11.70 (s, 1H), 8.34 (s, 1H), 8.09 (d, *J*=7.86 Hz, 1H), 7.91 (m, 2H), 7.82 (d, *J*=2.28 Hz, 1H). ^{13}C (125 MHz, d$_6$-DMSO) δ 167.02, 162.12, 152.88, 151.97, 139.55, 133.64, 129.04, 128.38, 125.90, 125.10, 122.95, 121.19, 12.88, 111.77. HRMS: Calc'd 321.9679, Found 321.9686.



(6): 1.47 mmol scale to yield 2-(3,5-dichloro-2,6-dihydroxyphenyl)benzoxazole-5-carboxylic acid as a light orange solid (361 mg, 72% yield). ^{1}H NMR (600 MHz, d$_6$-DMSO) δ 11.32 (s, 2H), 8.38 (s, 1H), 8.10 (dd, *J=0.72, 8.52 Hz*, 1H), 7.94 (d, *J=8.52 Hz*, 1H), 7.74 (s, 1H). ^{13}C (125 MHz, d$_6$-DMSO) δ 167.23, 162.06, 152.98, 152.34, 139.19, 122.20, 128.65, 127.82, 120.98, 112.52, 111.66, 104.54. HRMS: Calc'd 337.9629, Found 337.9630.



(7): 1.05 mmol scale to yield 2-(3,5-dimethyl-2-hydroxyphenyl)benzoxazole-6-carboxylic acid as a pale orange solid (213 mg, 71% yield) ^{1}H NMR (400 MHz, d$_6$-DMSO) δ 11.09 (s, 1H), 8.20 (dd, *J=0.48, 1.44 Hz*, 1H), 8.00 (dd, *J=1.48, 8.32 Hz,* 1H), 7.83 (dd, *J=0.44, 8.28 Hz*, 1H), 7.56 (d, *J=2.04 Hz,* 1H), 7.16 (d, *J=2.08 Hz*, 1H), 2.25 (s, 3H), 2.19 (s, 3H). ^{13}C (125 MHz, d$_6$-DMSO) δ 166.65, 164.89, 154.41, 148.32, 142.90, 136.34, 128.27, 128.22, 126.62, 125.67, 124.43, 118.63, 111.83, 108.41, 19.91, 15.53. HRMS: Calc'd 284.0917, Found 284.0909.



(8): 0.88 mmol scale to yield 2-(3,5-dimethyl-2-hydroxyphenyl)benzoxazole-5-carboxylic acid as an yello solid (107 mg, 43% yield) ^{1}H NMR (400 MHz, d$_6$-DMSO) δ 13.12 (s, 1H), 11.01 (s, 1H), 8.22 (d, *J=1.24 Hz*, 1H), 8.00 (dd, *J=1.64, 8.56 Hz,* 1H), 7.81 (d, *J=8.56,* 1H), 7.50 (s, 1H), 7.12 (s, 1H), 2.23 (s, 3H), 2.17 (s, 3H). ^{13}C (125 MHz, d$_6$-DMSO) δ 166.69, 163.85, 154.21, 151.25, 139.44, 136.04, 128.16, 128.15, 127.13, 125.58, 124.26, 120.09, 110.83, 108.37, 19.91, 15.51. HRMS: Calc'd 284.0917, Found 284.0917.

Expression and Purification of Wild-Type TTR and A108G-TTR

Wild-type TTR and A108G-TTR were prepared as previously described[3,4] Briefly, both plasmids (pMMHa) were transformed into competent BL21 cells. Two 1L-flasks of LB medium with ampicillin (100 ∝g/mL) were inoculated with fresh 40 mL overnight cultures. Cells were grown at 37°C with vigorous shaking until reaching an OD_{600nm} of 0.6. At this point, the cells were induced with 1 mM IPTG and left at the same conditions. Six hours post-induction, cells were harvested and resuspended in 10 mM sodium phosphate buffer (pH 7.6), and lysed by one cycle of freeze (-80°C)-thaw, followed by 5 cycles of 3 minutes sonication/3 minutes rest at 4°C. Cell debris was removed by centrifugation. Both variants were purified first using ammonium sulfate precipitation. The supernatant was treated with 40% ammonium sulfate, equilibrated for 30 minutes and again collected following centrifugation. The semi-purified 40% supernatants were then precipitated with a 90% ammonium sulfate treatment. The proteins were then dialyzed overnight into 25 mM Tris pH 8.0, 1 mM EDTA with 3000 MW dialysis tubing (Snakeskin from Pierce Biomedical). Both variants were purified on a Source15Q anion exchange column (Amersham Biosciences) eluting with a 200-350 mM NaCl gradient at room temperature, followed by further purification using a gel filtration column (Superdex 75). Proteins were eluted using 10 mM sodium phosphate (pH 7.6), 100 nM KCl, 1 mM EDTA. Both proteins' purity and mass were confirmed by LC-MS analysis (wild-type TTR: 13892; A108G-TTR: 13878).

NMR SPECTRA FOR CHARACTERIZATION

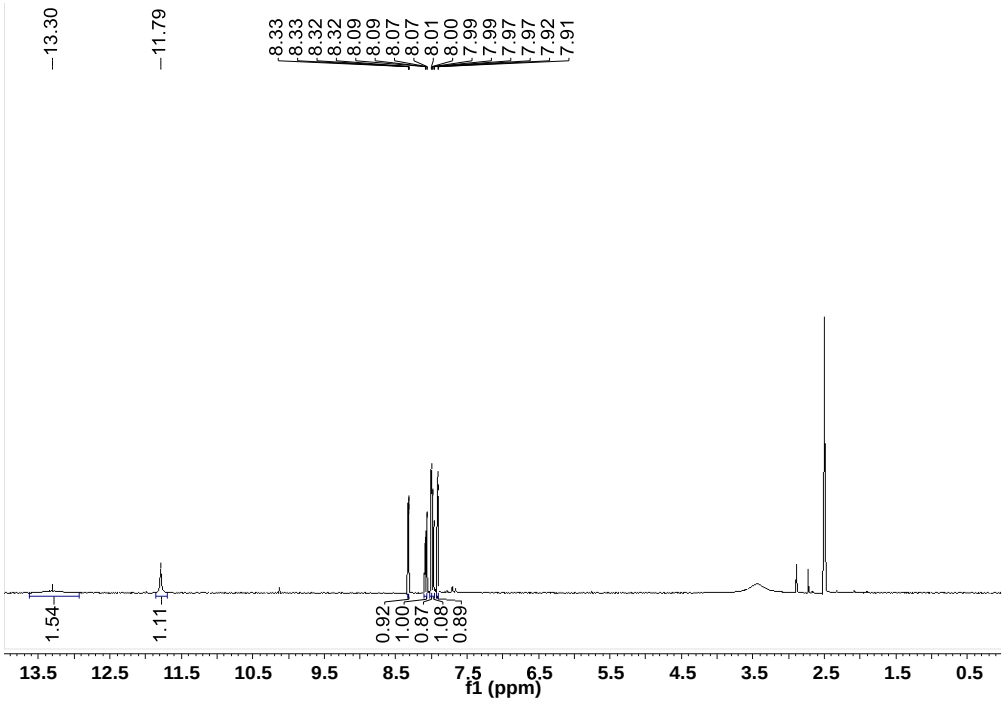


Figure S1: ^{1}H NMR spectra of **3** in d$_6$-DMSO (600 MHz)

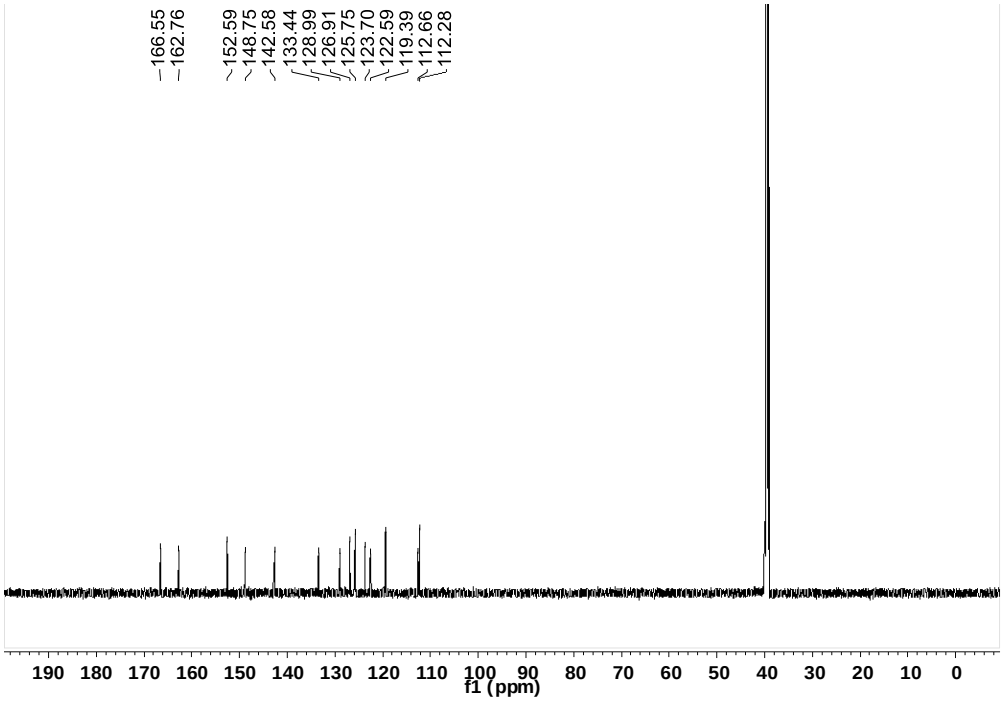


Figure S2: ^{13}C NMR spectra of **3** in d$_6$-DMSO (125 MHz)

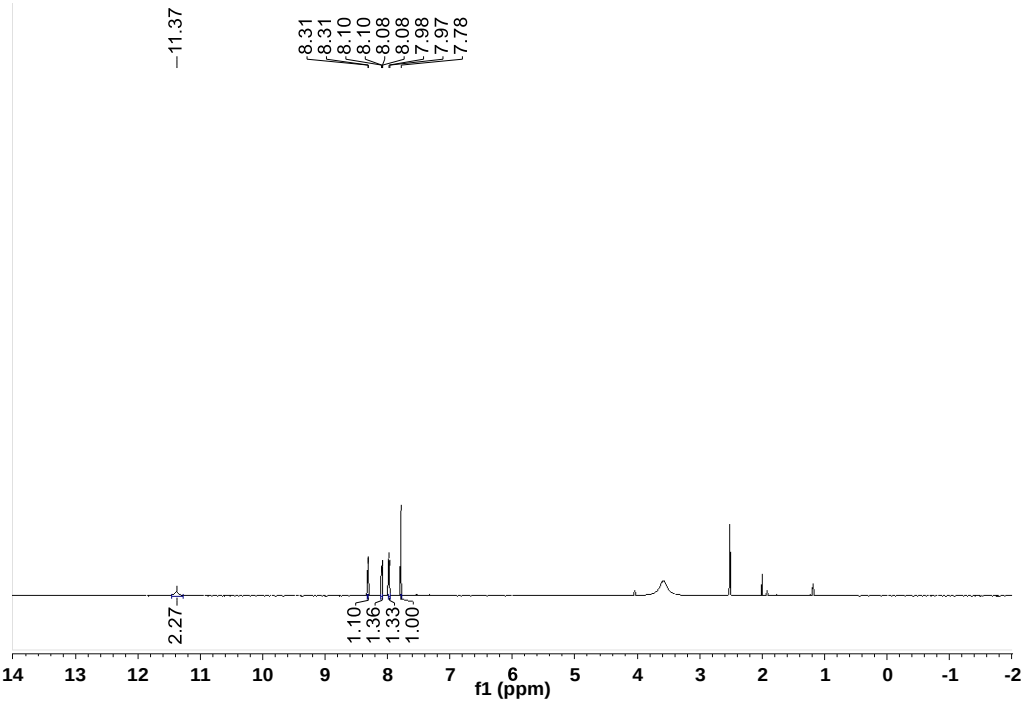


Figure S3: ^{1}H NMR spectra of **4** in d$_6$-DMSO (600 MHz)

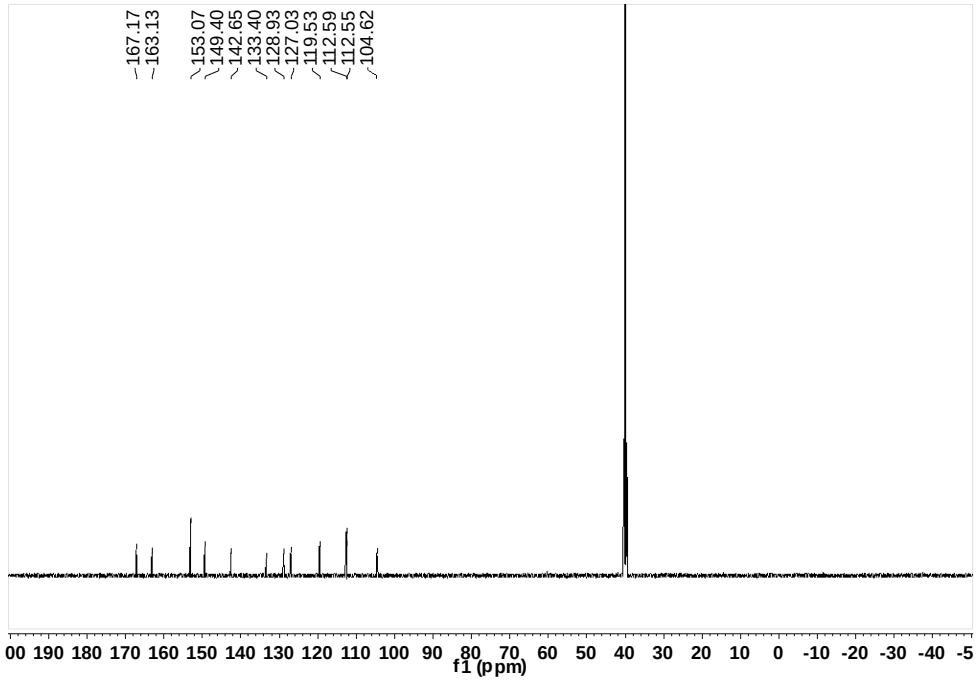


Figure S4: ^{13}C NMR spectra of **4** in d$_6$-DMSO (125 MHz)

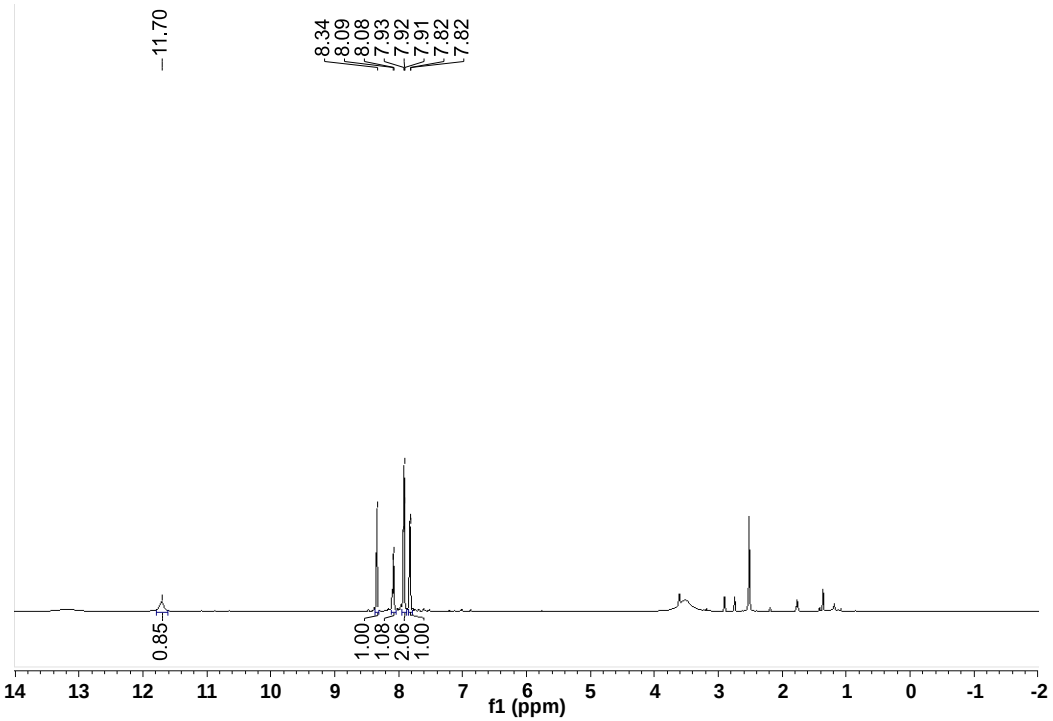


Figure S5: ^{1}H NMR spectra of **5** in d$_6$-DMSO (600 MHz)

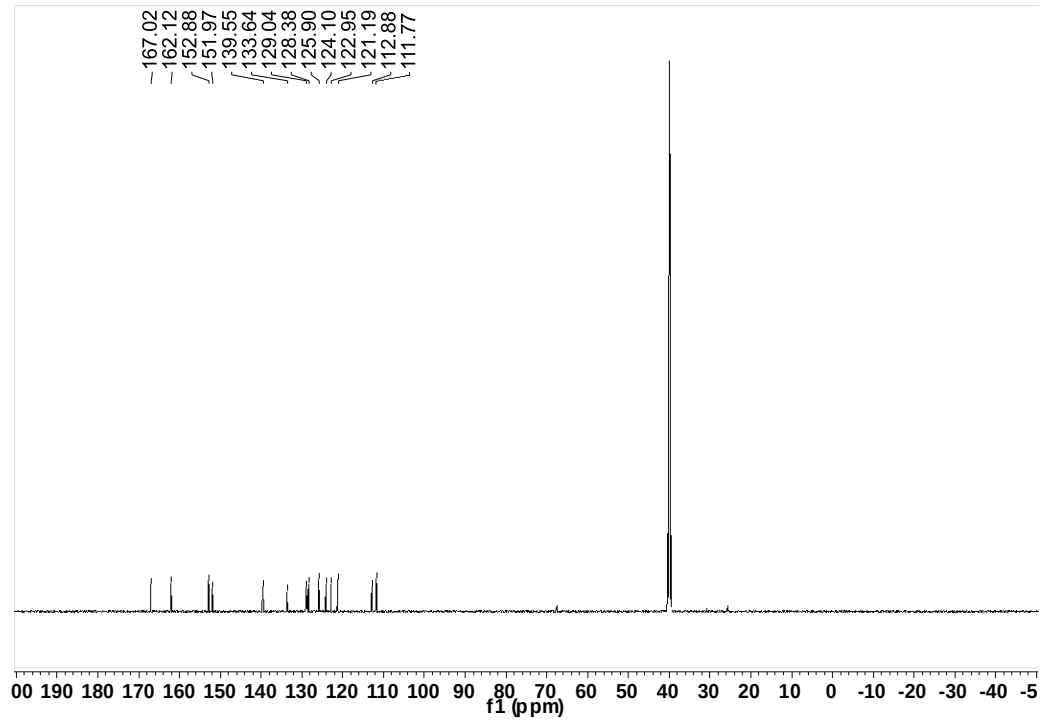


Figure S6: ^{13}C NMR spectra of **5** in d$_6$-DMSO (125 MHz)

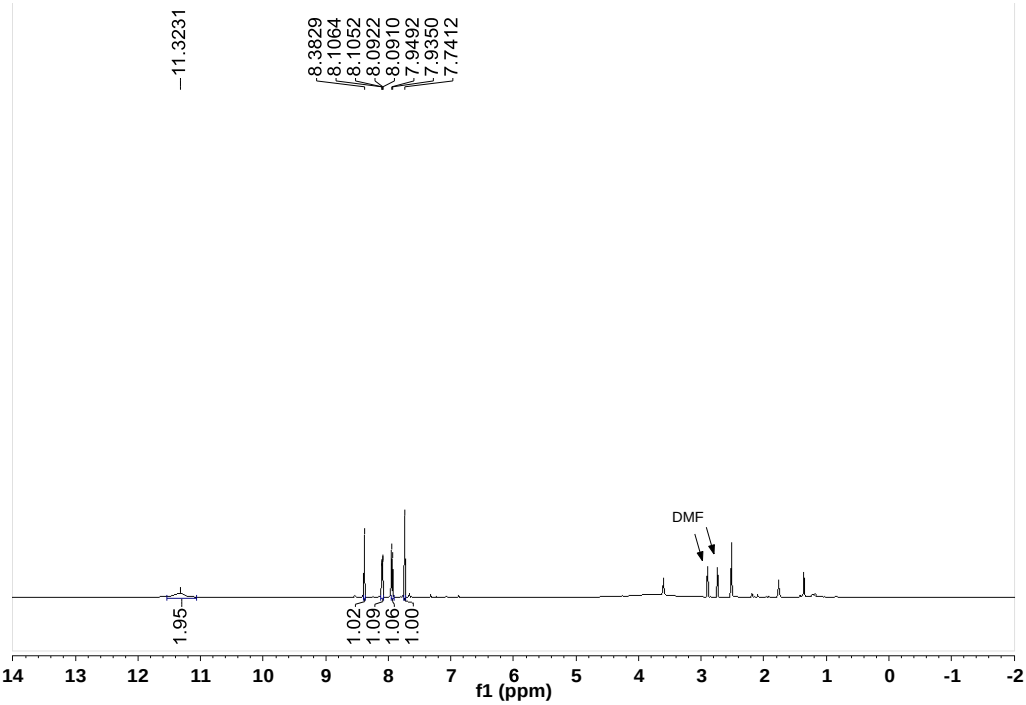


Figure S7: ^{1}H NMR spectra of **6** in d_6-DMSO (600 MHz)

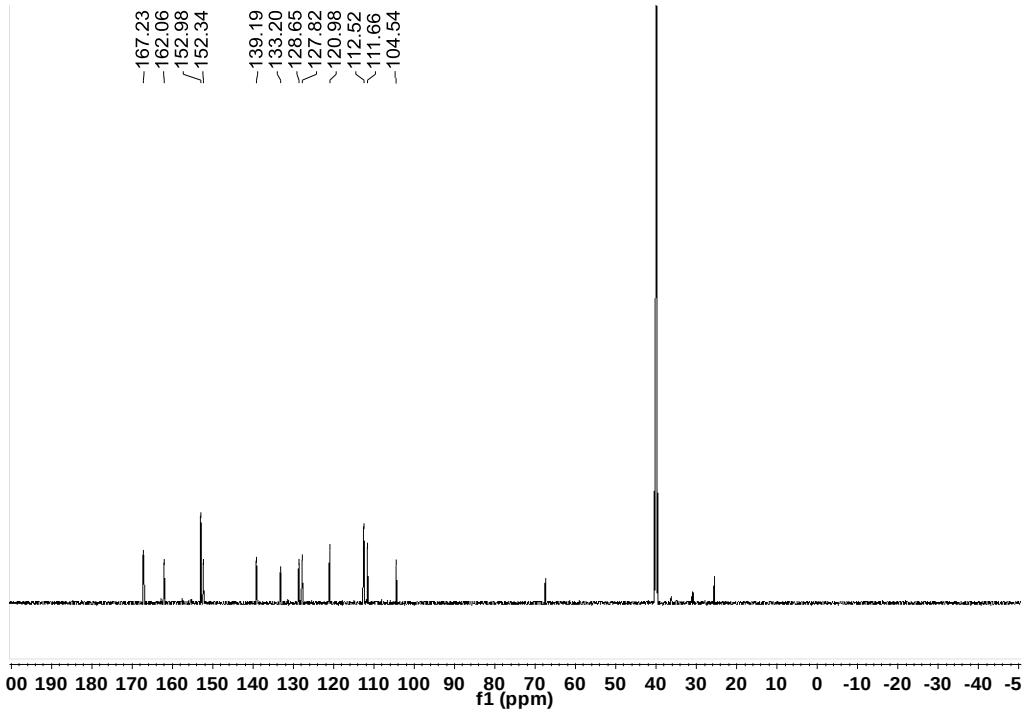


Figure S8: ^{13}C NMR spectra of **6** in d_6-DMSO (125 MHz)

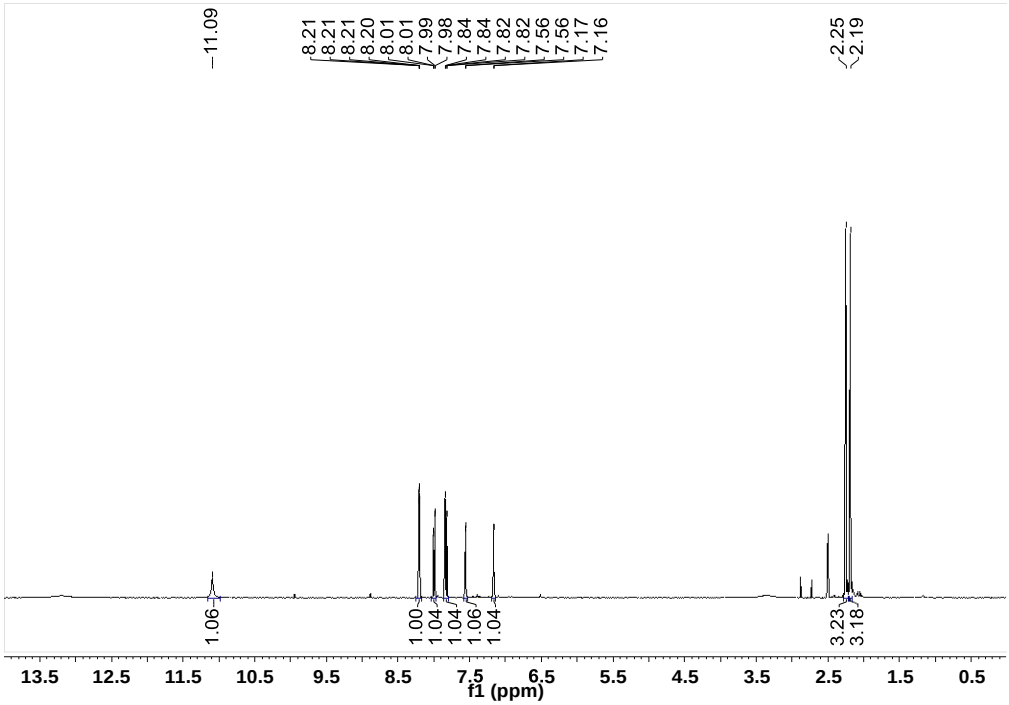


Figure S9: ^{1}H NMR spectra of **7** in d_6-DMSO (400 MHz)

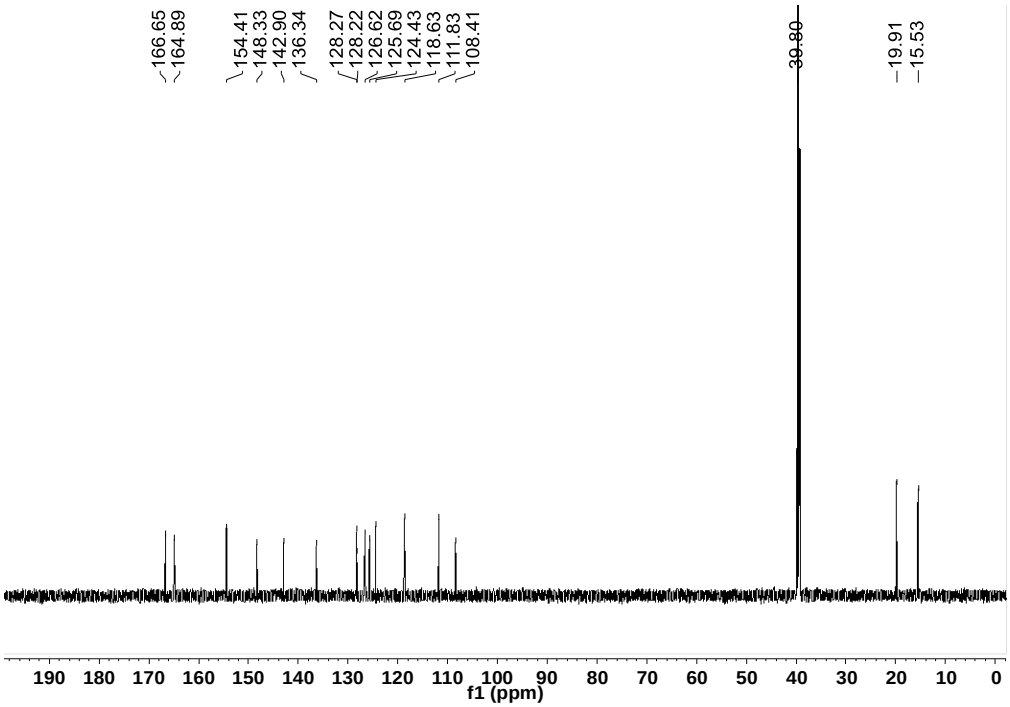


Figure S10: ^{13}C NMR spectra of **7** in d_6-DMSO (125 MHz)

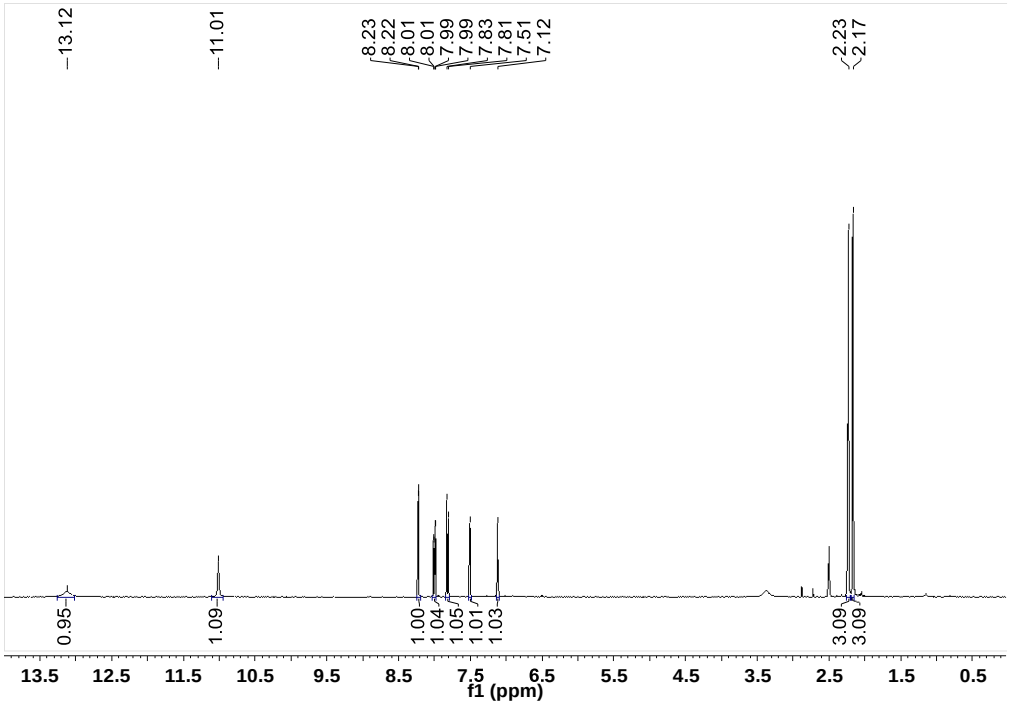


Figure S11: ^{1}H NMR spectra of **8** in d$_6$-DMSO (400 MHz)

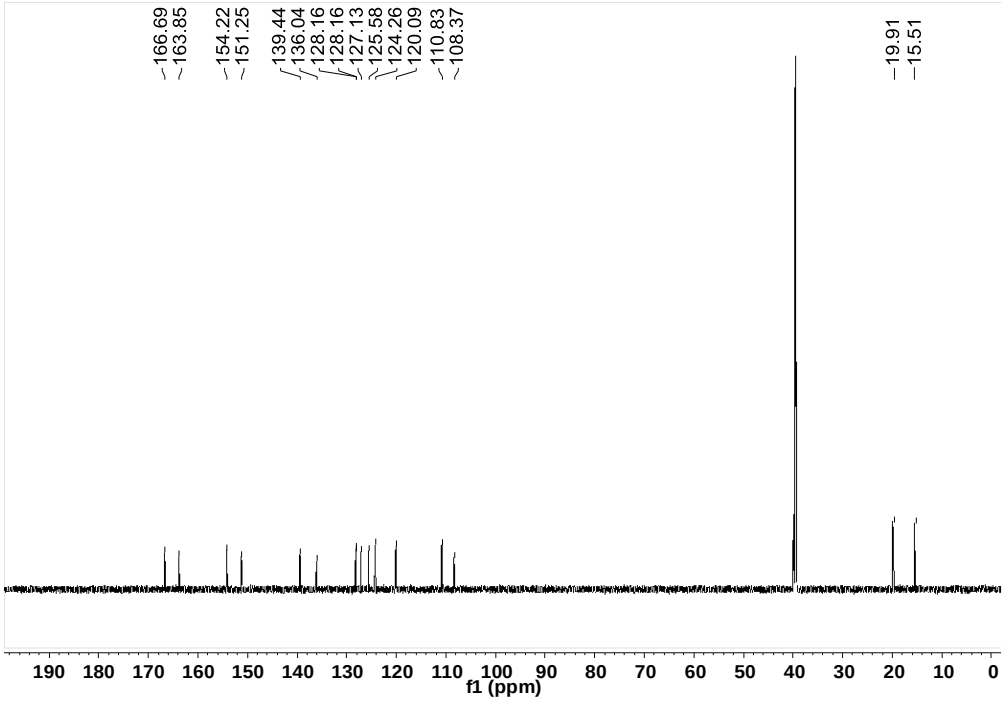


Figure S12: ^{13}C NMR spectra of **8** in d$_6$-DMSO (125 MHz)

Fluorescence binding assay

Method: Stock solutions of **3**-**8** were prepared by diluting solutions of **3**-**8** in DMSO 10-, 20-, and 40-fold in buffer. Each well of a 96-well plate contained 90 µL of 5.55 µM protein solution that was diluted to 100 µL (5.0 µM) with the stock solution of each probe. Each sample was excited at 335 nm at 9.0 mm slit width and the fluorescence was measured from 360-650 nm at 5 nm intervals. Emission traces were cut off at 400 nm for clarity as emission from Tryptophan and light scattering from the sample plate are apparent at 350 nm. All experiments were done in triplicate.

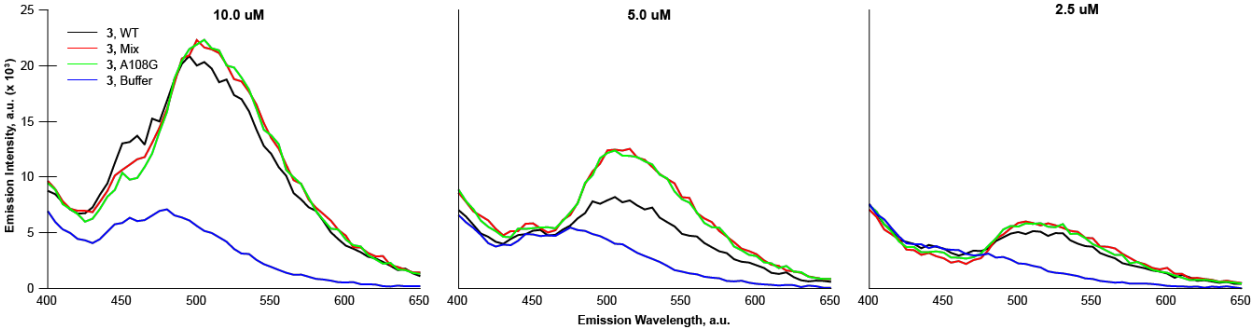


Figure S13: Emission profiles of **3** in 5.0 µM WT, Mix, A108G and buffer solutions.

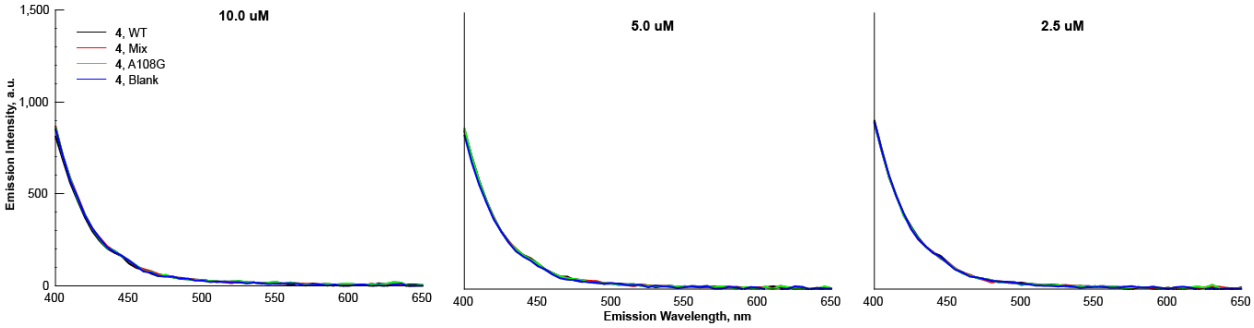


Figure S14: Emission profiles of **4** in 5.0 µM WT, Mix, A108G and buffer solutions.

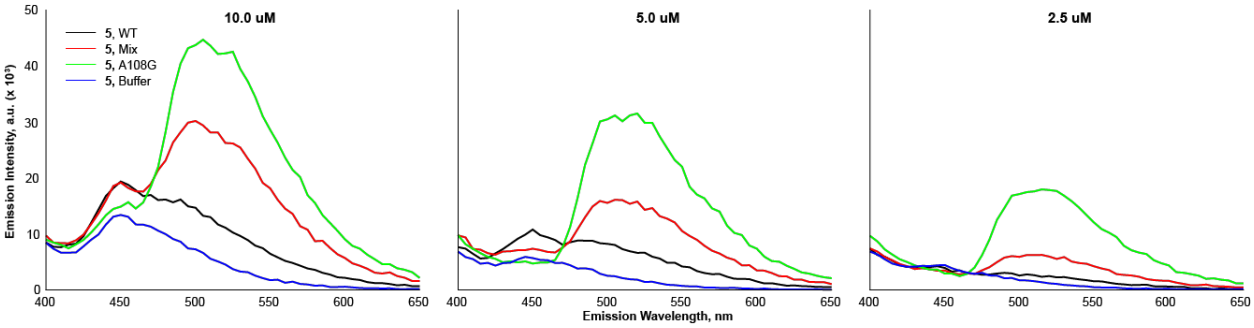


Figure S15: Emission profiles of **5** in 5.0 µM WT, Mix, A108G and buffer solutions.

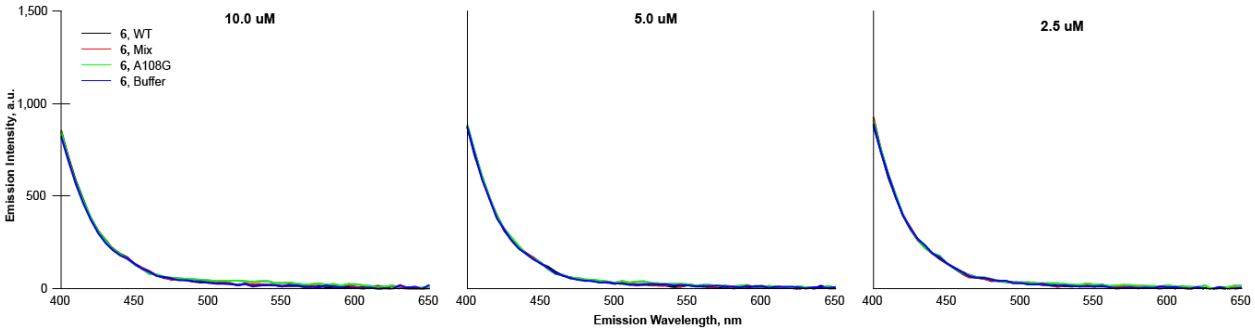


Figure S16: Emission profiles of **6** in 5.0 μM WT, Mix, A108G and buffer solutions.

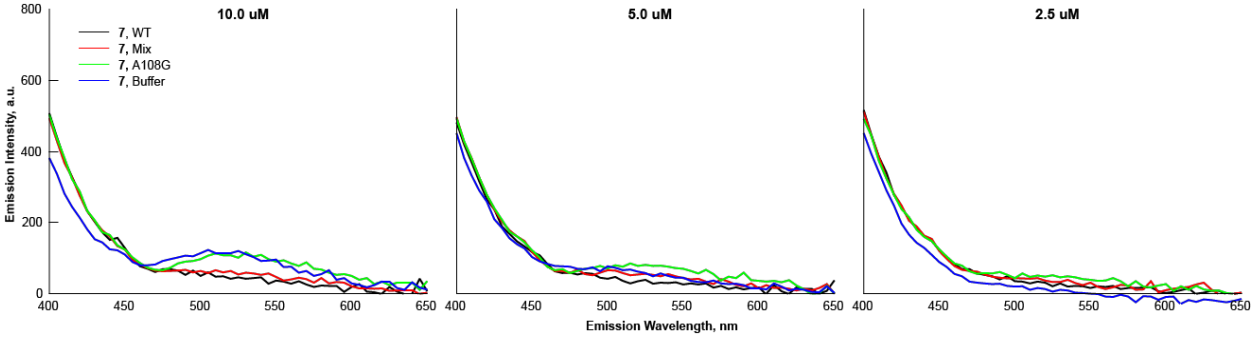


Figure S17: Emission profiles of **7** in 5.0 μM WT, Mix, A108G and buffer solutions.

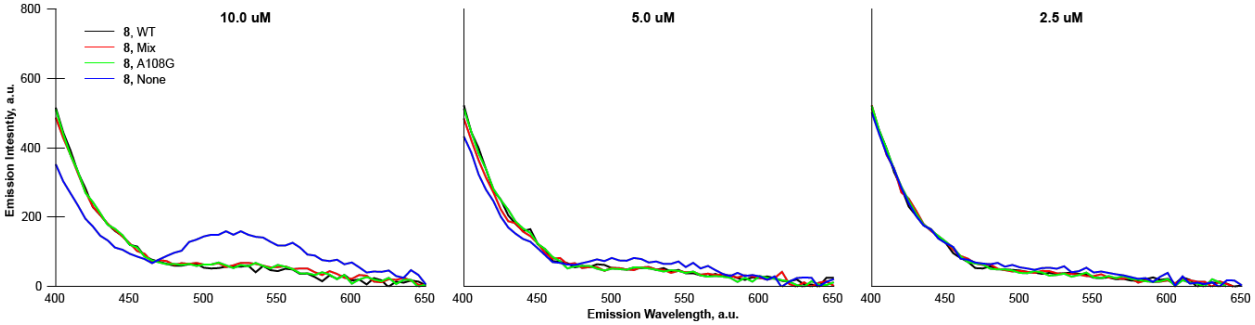


Figure S18: Emission profiles of **8** in 5.0 μM WT, Mix, A108G and buffer solutions.

Binding Studies of 3 and 5 in A108G Protein

Procedure: 1.0 mL of 5.0 μM solution of A108G protein was titrated with sub 1.0 μL injections of stock solutions in DMSO of either **3** or **5**, to which the total added DMSO did not exceed 2% of the total solution volume. 1:1 binding isotherms were fit using the Thordson fitting program using. Despite the two binding sites of Transthyretin, Tafamidis binds with negative cooperativity and only one binding isotherm was observed in our studies. Therefore, we found a 1:1 binding isotherm valid for this model. The K_a of **3** into A108G was found to be 9793 M^{-1} and the K_a of **5** into A108G was found to be 11338 M^{-1}. To verify that variations in absorptions were due to binding and not dilution, **5** was titrated into buffered solution and the change in absorption was found to be linear. Thus, we believe observed isotherms are due to binding.

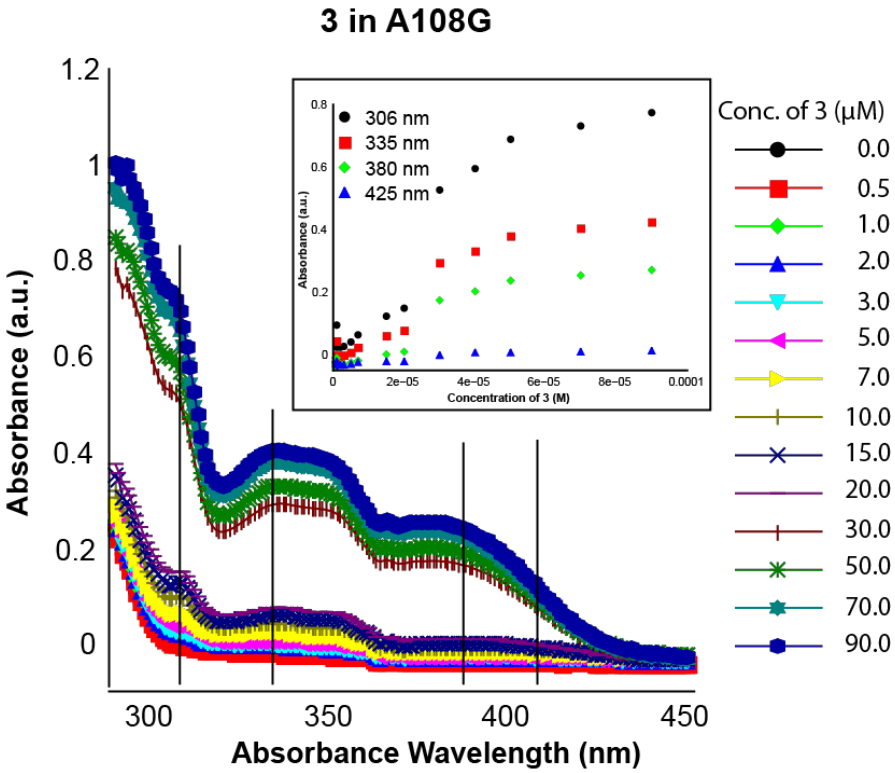


Figure S19. Binding of 3 into 5 μM A108G solution. Inset: Absorbances at four wavelengths.

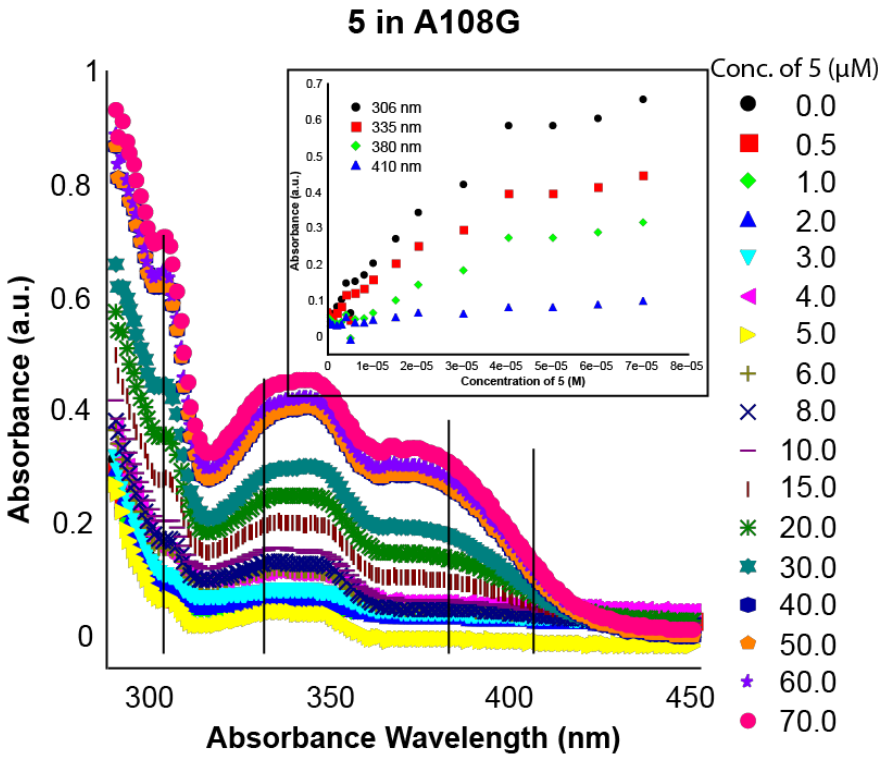


Figure S20. Binding of 5 into 5 μM A108G solution. Inset: Absorbances at four wavelengths.

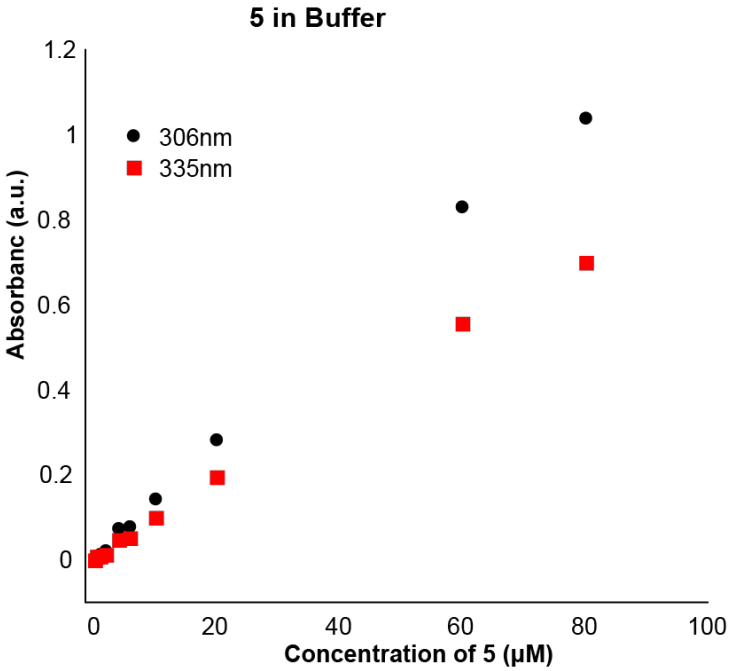


Figure S21: Absorbances of **5** in buffer solution at two wavelengths.

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