Supporting Information for:

Thiol-reactive Derivatives of the Solvatochromic 4-*N*,*N*-Dimethylamino-1,8-naphthalimide Fluorophore: A Highly Sensitive Toolset for the Detection of Biomolecular Interactions

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10× TBS Buffer:

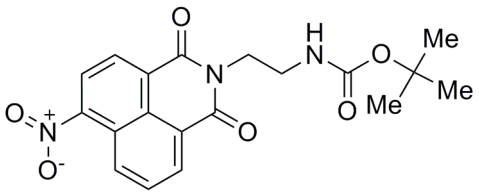
60.6 g Tris-HCl
87.7 g NaCl

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

Synthesis of 4-*N,N*-dimethyl-1,8-naphthalimide cysteine modifying reagents:

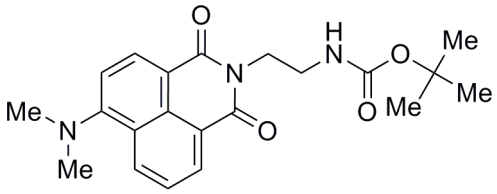
All reagents were procured from Sigma-Aldrich unless otherwise stated.

NMR and FT-MS of the following 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).



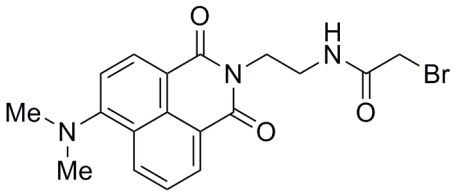
4-Nitro-*N*-(2-*tert*-butoxycarbonylamino-ethyl)-1,8-naphthalimide (10). Dissolved 4-nitro-1,8-naphthalic anhydride, **9** (9.2 g, 37.7 mmol), in 50 mL of DMF, then added DIPEA (17.9 mL, 102.9 mmol). In a separate flask, *N*-Boc-ethylenediamine was dissolved in another 50 mL DMF and added over 4 min to the solution containing the anhydride via an addition funnel. The reaction was allowed to

proceed for 1 hr at ambient temperature before adding the coupling reagents HOBt/HBTU (100 mL, 0.58 M each in DMF) to facilitate closure of the imide ring system. The reaction was allowed to proceed overnight. The next day, the reaction mixture was poured into a large separatory funnel containing 400 mL of diethyl ether and washed with brine (3 × 200 mL) to remove DMF (note- some precipitation of the product from the organic layer may be observed if left for extended periods of time). The organic layer was then dried with $MgSO_4$, filtered, and concentrated to dryness. The crude product was then purified by flash column chromatography using 3:1 hexanes/ethyl acetate to give a light cream colored solid (9.56 g, 24.8 mmol, 72.3% yield, R_f = 0.1 in 3:1 hexanes/EtOAc). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.21 (s, 9H), 3.53 (m, 2H), 4.34 (t, 2H, J = 5.7 Hz), 4.97 (m, 1H), 7.94 (dd, 1H, J_1 = 8.7 Hz, J_2 = 7.5 Hz), 8.36 (d, 1H, J = 8.1 Hz), 8.65 (d, 1H, J = 8.1 Hz), 8.69 (dd, 1H, J_1 = 7.4 Hz, J_2 = 1.1 Hz), 8.77 (dd, 1H, J_1 = 8.7 Hz, J_2 = 0.9 Hz). ^{13}C NMR (300 MHz, $CDCl_3$, δ): 28.4, 39.3, 40.7, 79.5, 123.0, 123.7, 124.1, 127.0, 129.3, 129.5, 130.1, 130.1, 132.7, 149.7, 156.3, 163.0, 163.8. HRMS-ESI (*m/z*): $[M+Na]^+$ calcd for $C_{19}H_{19}N_3O_6$, 408.1166; found, 408.1188.



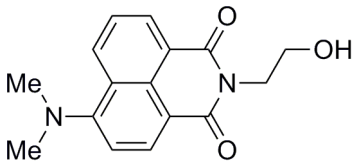
4-*N*,*N*-Dimethylamino-*N*-(2-*tert*-butoxycarbonylamino-ethyl)-1,8-naphthalimide (11). The 4-nitro-*N*-(2-*tert*-butoxycarbonylamino-ethyl)-1,8-naphthalimide, **10** (2.00 g, 5.19 mmol), was added to a 250 mL two-necked round-bottom flask equipped with a reflux condenser, rubber septum, and magnetic stir bar. The assembled reaction vessel was then charged with and inert atmosphere by evacuating the air under reduced pressure and purging with N_2 gas (3×). The flask then received 52 mL of isoamyl alcohol transferred by syringe through the rubber septum. The suspension was stirred as the temperature was raised to 132 ºC. Once the starting material dissolved, 3-dimethylamino-propionitrile (2.35 mL, 20.8 mmol) was added by syringe through the septum of the reaction vessel. The reaction

was refluxed for 22 hrs before stopping by concentrating the reaction mixture to dryness by rotary evaporation. The crude was then purified by flash chromatography using 7:3 hexanes/ethyl acetate to give an orange solid (1.47 g, 3.83 mmol, 74% yield, R_f = 0.25 in 1:1 hexanes/ethyl acetate). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.31 (s, 9H), 3.10 (s, 6H), 3.51 (m, 2H), 4.34 (t, 2H, J = 5.6 Hz), 5.09 (br, 1H), 7.09 (d, 1H, J = 8.1 Hz), 7.64 (dd, 1H, J_1 = 8.6 Hz, J_2 = 7.4 Hz), 8.42 (dd, 1H, J_1 = 8.4 Hz, J_2 = 1.2 Hz), 8.46 (d, 1H, J = 8.1 Hz), 8.56 (dd, 1H, J_1 = 7.4 Hz, J_2 = 1.1 Hz). ^{13}C NMR (500 MHz, $CDCl_3$, δ): 28.2, 39.4, 39.8, 44.7, 78.9, 113.1, 114.5, 122.7, 124.7, 125.1, 130.2, 131.1, 131.2, 132.8, 156.0, 157.0, 164.3, 164.9. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{21}H_{25}N_3O_4$, 384.1923; found, 384.1932.



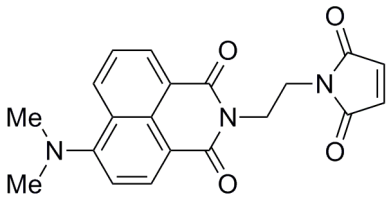
4-*N*,*N*-Dimethylamino-*N*-[2-(2-bromo-acetylamino)-ethyl]-1,8-naphthalimide (2). Dissolved **11** (0.50 g, 1.30 mmol) in dichloromethane (22.0 mL) in a 100 mL round-bottom flask. Cold trifluoroacetic acid (22.0 mL) was then added slowly over 5 min by addition funnel while stirring the reaction. The reaction was allowed to proceed at room temperature for 1.5 hrs before concentrating to dryness by rotary evaporation. The crude solid was redissolved in dichloromethane (100 mL) and washed with 2% $NaHCO_3$ aq. solution (100 mL). The aqueous layer was back-extracted with fresh dichloromethane (2 × 100 mL) and the organic layers were combined (total volume of 300 mL). The organic layer was dried with $MgSO_4$, filtered and concentrated to give the free amine as an orange solid. The free base was then transferred to a 250 mL Schlenk flask under an atmosphere of nitrogen and dissolved in dry dichloromethane (100 mL). The Schlenk flask was cooled to -15 °C in a 1:3 sodium chloride/ice bath. The bromoacetyl bromide (0.40 g, 1.96 mmol) was then added dropwise by syringe followed by *N,N*-diisopropylethylamine (0.25 mL, 1.43 mmol). The reaction was allowed to run for 5 min at -15 ºC before warming to room temperature. After 1.5 hrs, the reaction mixture was diluted to a total volume of 100 mL in dichloromethane and washed with 2% $NaHCO_3$ aq. solution (100 mL)

followed by a second wash with brine (100 mL). The organic layer was dried with $MgSO_4$, filtered, and concentrated by rotary evaporation. The product was purified by flash chromatography using 2:3 hexanes/ethyl acetate to give an orange solid (0.48 g, 1.19 mmol, 91% yield, R_f = 0.10 in 2:3 hexanes/ethyl acetate). The product was stored as solid at -20 ºC. ^{1}H NMR (300 MHz, $CDCl_3$, δ): 3.11 (s, 6H), 3.67 (m, 2H), 3.78 (s, 2H), 4.43 (t, 2H, J = 5.4 Hz), 7.09 (d, 1H, J = 8.1 Hz), 7.27 (br s, 1H), 7.64 (dd, 1H, J_1 = 8.6 Hz, J_2 = 7.4 Hz), 8.43 (dd, 1H, J_1 = 8.7 Hz, J_2 = 1.2 Hz), 8.46 (d, 1H, J = 8.4 Hz), 8.56 (dd, 1H, J_1 = 7.4 Hz, J_2 = 1.1 Hz). ^{13}C NMR (300 MHz, $CDCl_3$, δ): 28.9, 38.5, 40.4, 44.6, 113.1, 114.0, 122.4, 124.7, 124.9, 130.2, 131.2, 131.5, 133.0, 157.2, 164.4, 165.0, 166.0. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{18}H_{18}BrN_3O_3$, 404.0610; found 404.0612.



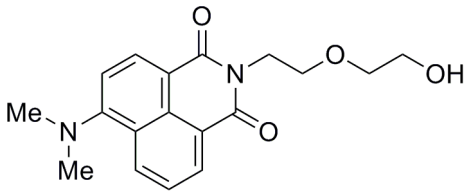
4-*N*,*N*-Dimethylamino-*N*-(2-hydroxy-ethyl)-1,8-naphthalimide (7). Added 4-*N,N*-dimethylamino-1,8-naphthalic anhydride, **5** (1.00 g, 4.15 mmol), to a 200 mL two-necked round-bottom flask equipped with a reflux condenser, magnetic stir bar, and rubber septum. The reaction vessel was then evacuated of air by applying vacuum and flushing 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 solid as the suspension reached reflux until the addition of ethanolamine (0.28 mL, 4.56 mmol) by syringe. The slurry then turned to a clear deep orange solution. The reaction was allowed to proceed at reflux for 1.5 hrs then stopped by cooling to room temperature. The solvent was then removed using rotary evaporation and the crude placed under high vacuum overnight to remove excess ethanolamine. The product obtained was an orange solid requiring no further purification (1.18 g, 4.15 mmol, quantitative yield). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 2.70 (br s, 1H), 3.10 (s, 6H), 3.95 (t, 2H, J = 5.3 Hz), 4.42 (t, 2H, J = 5.3 Hz), 7.06 (d, 1H, J = 8.1 Hz), 7.62 (dd, 1H, J_1

= 8.6 Hz, J_2 = 7.4 Hz), 8.40 (dd, 1H, J_1 = 8.4 Hz, J_2 = 1.2 Hz), 8.42 (d, 1H, J = 8.4 Hz), 8.52 (dd, 1H, J_1 = 7.5 Hz, J_2 = 1.2 Hz). ^{13}C NMR (300 MHz, $CDCl_3$, δ): 42.7, 44.7, 62.1, 113.1, 114.2, 122.6, 124.8, 124.9, 130.2, 131.3, 131.5, 132.9, 157.1, 164.9, 165.4. HRMS-ESI (*m/z*): $[M+Na]^+$ calcd for $C_{16}H_{16}N_2O_3$, 307.1053; found, 307.1059.

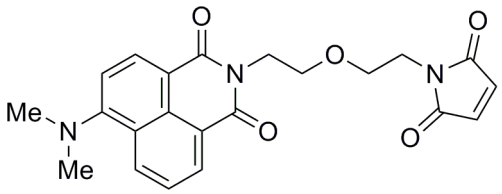


4-*N*,*N*-Dimethylamino-*N*-(2-maleimidyl-ethyl)-1,8-naphthalimide (3). Solid triphenylphosphine (0.42 g, 1.60 mmol) was added to an oven-dried 100 mL Kjeldahl-style Schlenk flask equipped with a magnetic stir bar and rubber septum. The flask was evacuated of air under high vacuum and charged with N_2 gas (3×). The triphenylphosphine was then dissolved in freshly distilled anhydrous THF. The reaction vessel was then cooled to -78 ºC by immersing the Schlenk flask in a dry ice/isopropanol bath. Next, diethyl azodicarboxylate (0.696 mL, 40% solution in toluene, 1.60 mmol) was added dropwise via syringe over 1.5 min. The mixture was allowed to stir for approximately 5 min to allow formation of the betaine. At this point, the reaction mixture was a pale yellow color. The alcohol, **7** (0.50 g, 1.8 mmol), was then added as a solid followed by neopentyl alcohol (0.07 g, 0.8 mmol) to form the oxyphosphonium ion intermediate. Once the two alcohols were fully dissolved, maleimide was added as a solid and the reacting mixture was allowed to warm to room temperature. The reaction was allowed to proceed at room temperature overnight before stopping by concentrating to dryness using a rotary evaporator. TLC indicated that the reaction only preceded 30-40%. The product was isolated by flash column chromatography using toluene with 5% methanol. The product was a bright yellow solid (0.13 g, 0.36 mmol, 22.4% yield, R_f = 0.24 in 1:1 hexanes/ethyl acetate) and was dissolved in DMSO for storage at -80 ºC in 100 mM aliquots. ^{1}H NMR (300 MHz, $CDCl_3$, δ): 3.06 (s, 6H), 3.94 (t, 2H, J = 5.1 Hz), 4.36 (t, 2H, J = 5.1 Hz), 6.58 (s, 2H), 7.04 (d, 1H, J = 8.4 Hz), 7.58 (dd, 1H, J_1 = 8.6 Hz, J_2 = 7.4 Hz), 8.36 (d, 1H, J = 8.1 Hz), 8.38 (dd, 1 H, J_1 = 8.7 Hz, J_2 = 1.2 Hz), 8.46 (dd, 1H, J_1 = 7.4 Hz, J_2 =

1.1 Hz). ^{13}C NMR (300 MHz, $CDCl_3$, δ): 36.2, 38.6, 44.6, 113.1, 114.3, 122.4, 124.7, 125.1, 130.2, 131.0, 131.3, 132.6, 133.9, 156.8, 164.0, 164.6, 170.7. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{20}H_{17}N_3O_4$, 364.1292; found, 364.1308.

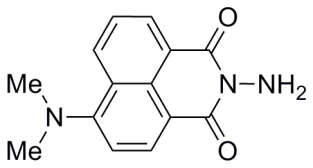


4-*N*,*N*-Dimethylamino-*N*-[2-(2-hydroxy-ethoxy)-ethyl]-1,8-naphthalimide (8). Added 4-*N,N*-dimethylamino-1,8-naphthalic anhydride, **5** (1.00 g, 4.15 mmol), to a 200 mL two-necked round-bottom flask equipped with a reflux condenser, magnetic stir bar, and rubber septum. The reaction vessel was then evacuated of air by applying vacuum and flushing 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 solid as the suspension reached reflux until the addition of 2-(2-aminoethoxy)-ethanol (0.454 mL, 4.56 mmol) by syringe. The slurry then turned to a clear deep orange solution. The reaction was allowed to proceed at reflux for 1.5 hrs then stopped by cooling to room temperature. The solvent was removed by rotary evaporation and the crude placed under high vacuum overnight to remove excess 2-(2-aminoethoxy)-ethanol. The product was isolated by flash column chromatography using a gradient of 1:3 to 1:4 hexanes/ethyl acetate. The product was a dark orange oil (1.33 g, 4.05 mmol, 97.7% yield, R_f = 0.1 in 1:3 hexanes/ethyl acetate). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 2.76 (br s, 1H), 3.07 (s, 6H), 3.66 (m, 4H), 3.82 (t, 2H, J = 5.6 Hz), 4.39 (t, 2H, J = 5.7 Hz), 7.05 (d, 1H, J = 8.4 Hz), 7.60 (dd, 1H, J_1 = 8.3 Hz, J_2 = 7.4 Hz), 8.38 (dd, 1H, J_1 = 8.6 Hz, J_2 = 1.1 Hz), 8.42 (d, 1H, J = 8.4 Hz), 8.51 (dd, 1H, J_1 = 7.4 Hz, J_2 = 1.1 Hz). ^{13}C NMR (300 MHz, $CDCl_3$, δ): 39.2, 44.6, 61.7, 68.4, 72.1, 113.1, 114.4, 122.7, 124.7, 125.0, 130.2, 131.1, 131.3, 132.8, 157.0, 164.2, 164.8. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{18}H_{20}N_2O_4$, 329.1496; found, 329.1506.

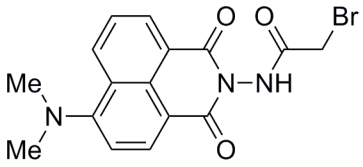


4-*N*,*N*-Dimethylamino-*N*-[2-(2-maleimidyl-ethoxy)-ethyl]-1,8-naphthalimide (4). Prior to assembling the Mitsunobu reaction, the alcohol, **8** (0.50 g, 1.51 mmol), was transferred to a 50 mL pear-shaped flask equipped with rubber stopper and placed under high vacuum overnight to remove trace water that may inhibit the reaction. An oven-dried 100 mL Kjeldahl-style Schlenk flask equipped with a magnetic stir bar and rubber septum was charged with solid triphenylphosphine (0.36 g, 1.37 mmol). The Schlenk flask was then evacuated of air by placing under high vacuum and charged with N_2 gas (3×). Freshly distilled dry THF (4 mL) was then transferred to the Schlenk flask by syringe and the triphenylphosphine stirred at ambient temperature until completely dissolved. This solution was then lowered to -78 ºC by immersing in a dry ice/isopropanol bath. Next, diethyl azodicarboxylate (0.597 mL, 40% solution in toluene, 1.37 mmol) was added dropwise via syringe over 2 min. The mixture was allowed to stir for approximately 5 min to allow formation of the betaine. At this point, the reaction mixture was a pale yellow color. Meanwhile, a solution of the alcohol, **8**, was prepared by dissolving in 6 mL of freshly distilled dry THF. This solution was then transferred very slowly to the reaction vessel by syringe to avoid raising the temperature. Once the transfer was complete, neopentyl alcohol (0.06 g, 0.69 mmol) was added as a solid. The reaction was allowed to stir approximately 5 min to allow formation of the oxyphosphonium ion intermediate. Solid maleimide (0.13 g, 1.37 mmol) was then added and the reaction was allowed to warm to room temperature and stirred overnight. The reaction was stopped by concentrating to dryness on the rotary evaporator and the product was isolated by flash column chromatography using 1:2 hexanes/ethyl acetate. The product was a bright yellow solid (0.15 g, 0.37 mmol, 24.4 % yield, R_f = 0.22 in 1:2 hexanes/ethyl acetate) and was dissolved in DMSO for storage at -80 ºC in 100 mM aliquots. ^{1}H NMR (300 MHz, $CDCl_3$, δ): 3.09 (s, 6H), 3.65 (m, 4H), 3.74 (t, 2H, J = 6.0 Hz), 4.34 (t, 2H, J = 5.9 Hz), 6.50 (s, 2H), 7.09 (d, 1H, J = 8.1 Hz), 7.63 (dd, 1H, J_1 = 8.4

Hz, J_2 = 7.2 Hz), 8.42 (apparent d, 2H, J = 8.1 Hz), 8.51 (dd, 1H, J_1 = 7.5 Hz, J_2 = 1.2 Hz). ^{13}C NMR (300 MHz, $CDCl_3$, δ): 37.2, 38.8, 44.7, 67.2, 67.6, 113.2, 114.9, 122.9, 124.8, 125.1, 130.2, 131.0, 131.1, 132.6, 133.8, 156.6, 163.9, 164.4, 170.5. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{22}H_{21}N_3O_5$, 408.1554; found, 408.1567.

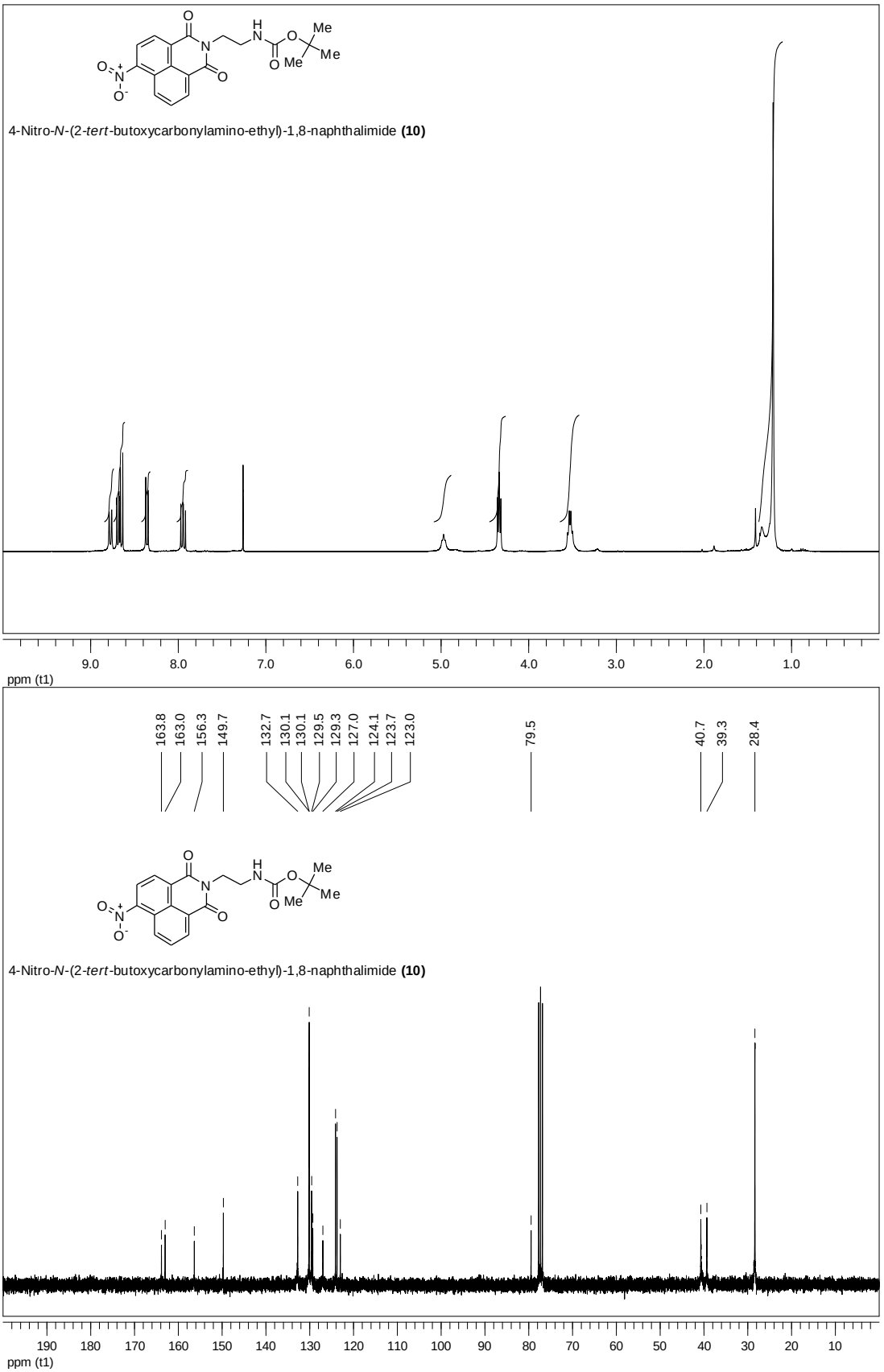


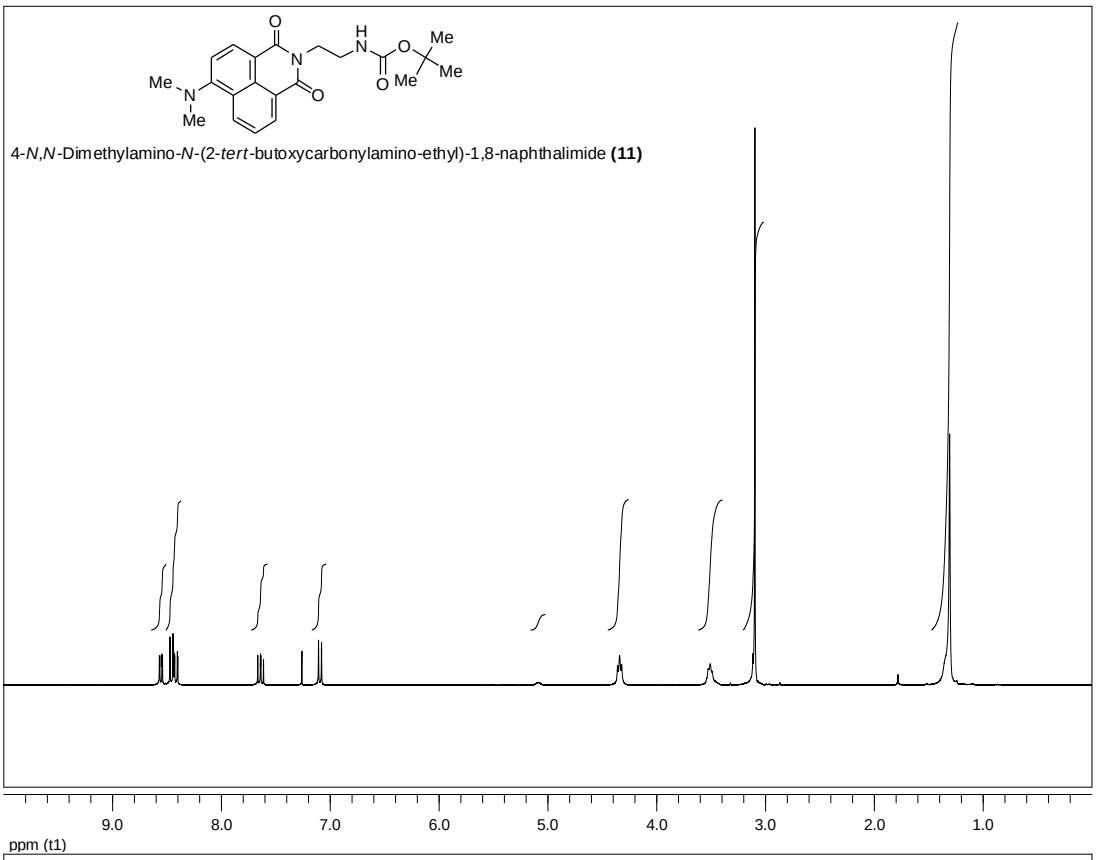
***N*-Amino-4-*N*,*N*-dimethylamino-1,8-naphthalimide (6)**. Added 4-*N,N*-dimethylamino-1,8-naphthalic anhydride, **5** (1.00 g, 4.14 mmol), to a 200 mL two-necked round-bottom flask equipped with a reflux condenser, magnetic stir bar, and rubber septum. The reaction vessel was then evacuated of air by applying vacuum and flushing 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 solid as the suspension reached reflux until the addition of hydrazine monohydrate (0.402 mL, 8.29 mmol) by syringe. The slurry then turned to a clear deep orange solution. The reaction was allowed to proceed at reflux for 45 min then stopped by cooling to room temperature. The reaction mixture was diluted to a final volume of 200 mL with cold water to precipitate the product, which was then filtered and purified by flash chromatography using 1:3 hexanes/ethyl acetate. The product was a bright yellow solid (0.925 g, 3.624 mmol, 87.4 % yield, R_f = 0.23 in 1:3 hexanes/ethyl acetate). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 3.09 (s, 6H), 5.26 (br s, 2H), 7.02 (d, 1H, J = 8.4 Hz), 7.58 (dd, 1H, J_1 = 8.4 Hz, J_2 = 7.5 Hz), 8.37 (dd, 1H, J_1 = 8.6 Hz, J_2 = 0.8 Hz), 8.38 (d, 1H, J = 8.4 Hz), 8.49 (dd, 1H, J_1 = 7.4 Hz, J_2 = 0.8 Hz). ^{13}C NMR (300 MHz, $CDCl_3$, δ): 44.8, 113.0, 113.4, 121.9, 124.6, 124.9, 128.7, 131.0, 131.5, 132.8, 157.3, 160.7, 161.0. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{14}H_{13}N_3O_2$, 256.1081; found, 256.1083.



4-*N*,*N*-Dimethylamino-*N*-[2-bromoacetamido]-1,8-naphthalimide (1). The solid *N*-amino-4-*N,N*-dimethylamino-1,8-naphthalimide, **6** (0.50 g, 1.96 mmol), was transferred to an oven-dried 100 mL Schlenk flask along with a magnetic stir bar. The flask was then capped with a rubber septum and evacuated of air by placing under high vacuum followed by charging with N_2 gas (3×). The starting material was then partially dissolved in dry dichloromethane (39 mL) and stirred as *N,N*-diisopropylethylamine (0.357 mL, 2.15 mmol) was added through the septum via syringe. The Schlenk flask was then cooled to -15 ºC in an ice bath of 3:1 ice/NaCl. Once cooled, bromoacetyl bromide (0.255 mL, 2.94 mmol) was added to the suspension dropwise via syringe and stirred for 5 min before bringing the reaction vessel back to room temperature. A white solid, presumed to be a bromide salt, was observed precipitating from the reaction mixture over time. After allowing the reaction to run overnight, the mixture was diluted to a total volume of 100 mL in dichloromethane and washed with $NaHCO_3$ (3 × 50 mL). The organic layer was dried with $MgSO_4$, filtered, and concentrated on the rotary evaporator. The desired product was isolated by flash chromatography using 1:1 hexanes/ethyl acetate. The reaction yielded two products: the desired monoacylated product, **1** (0.24 g, 0.93 mmol, 47.6% yield, R_f = 0.10 in 1:1 hexanes/ethyl acetate), and a diacylated byproduct in nearly a 1:1 ratio. The desired product was stored as a solid at -20 ºC. ^{1}H NMR (300 MHz, $CDCl_3$, δ): 3.13 (s, 6H), 4.31 (s, 2H), 7.08 (d, 1H, J = 8.4Hz), 7.64 (dd, 1H, J_1 = 8.6 Hz, J_2 = 6.9 Hz), 8.44 (dd, 1H, J_1 = 8.4 Hz, J_2 = 1.2 Hz), 8.46 (d, 1H, J = 8.1 Hz), 8.57 (dd, 1H, J_1 = 7.5 Hz, J_2 = 1.2 Hz), 8.62 (br s, 1H). ^{13}C NMR (500 MHz, $CDCl_3$, δ): 26.5, 44.7, 113.2, 113.5, 122.3, 124.8, 125.1, 130.4, 132.0, 132.3, 133.7, 157.8, 161.6, 162.2, 164.5. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{16}H_{14}BrN_3O_3$, 376.0291; found, 376.0304.

NMR spectra of synthesized derivatives:




4-N,N-Dimethylamino-N-(2-tert-butoxycarbonylamino-ethyl)-1,8-naphthalimide (11)
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6.0
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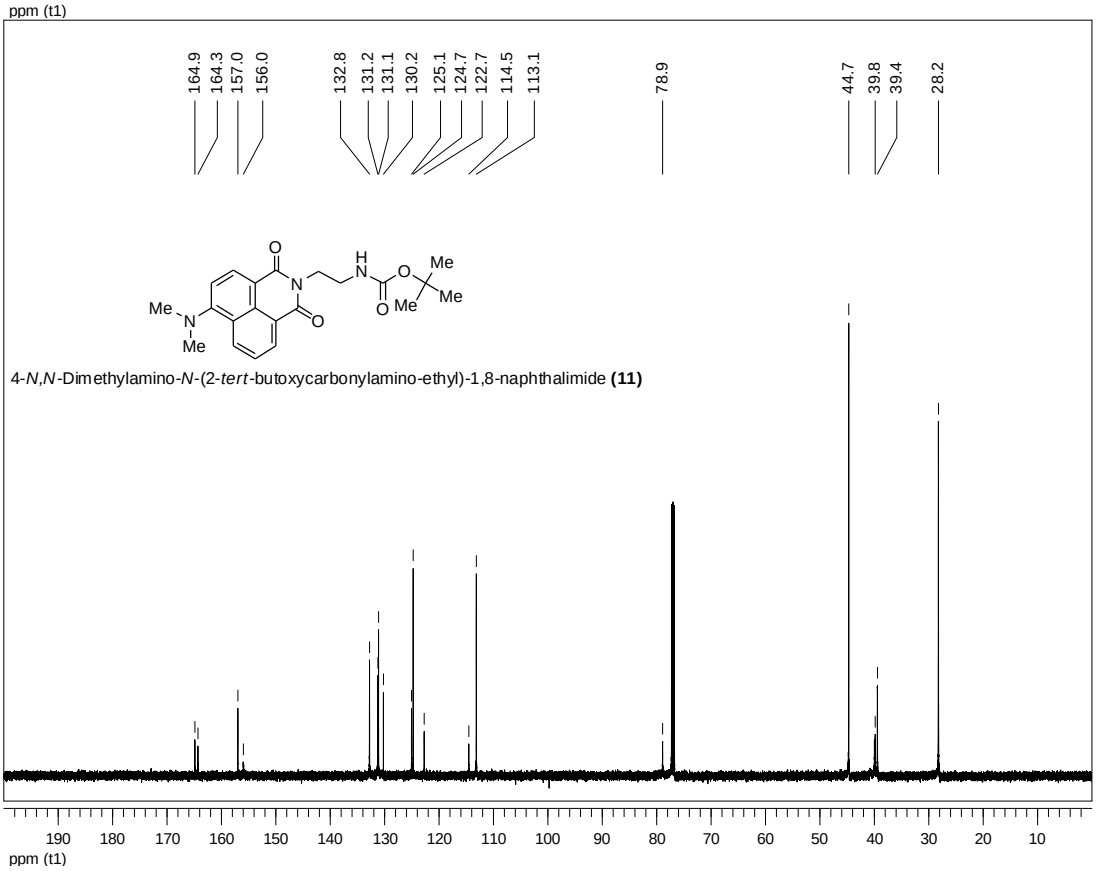
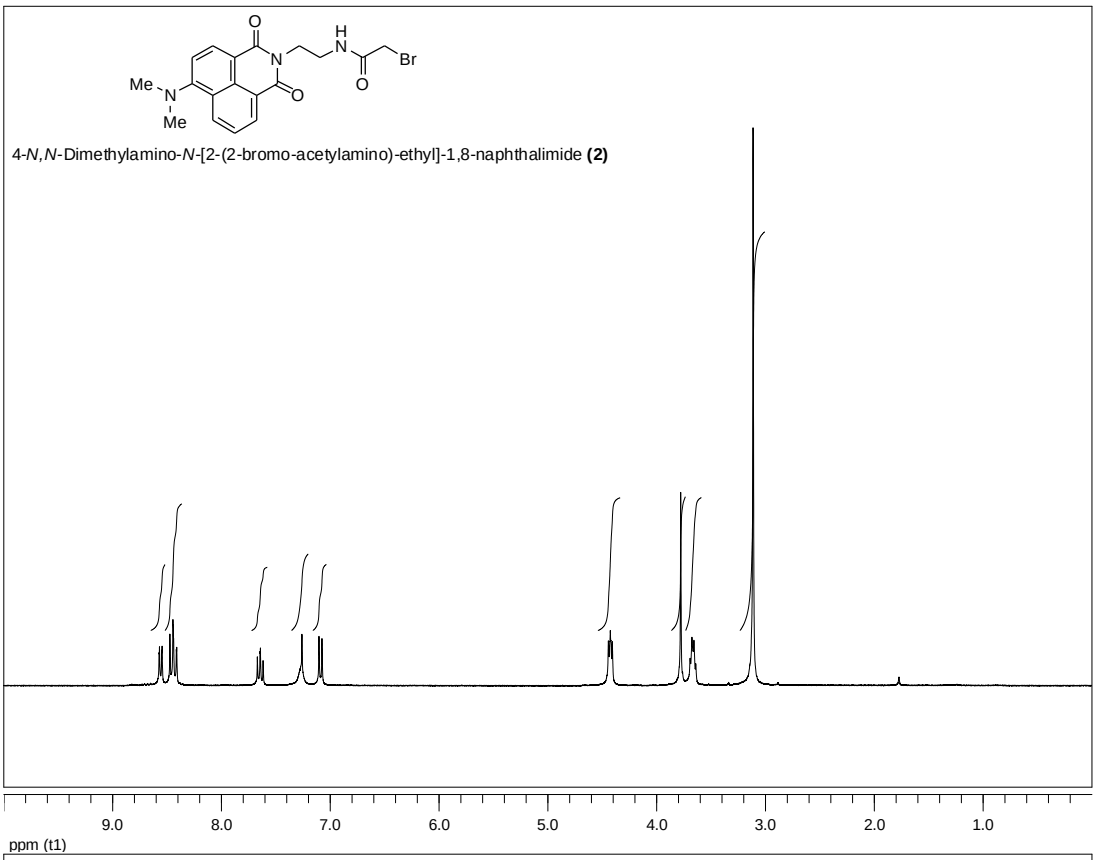
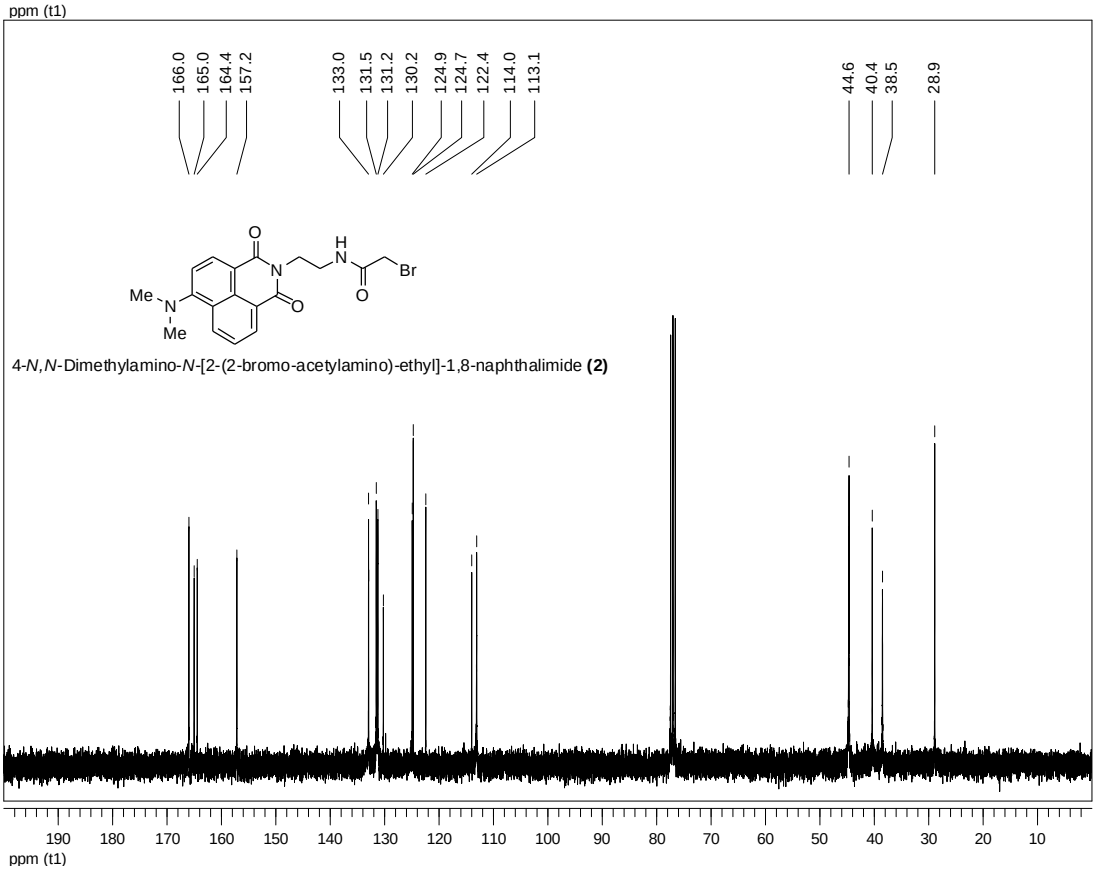
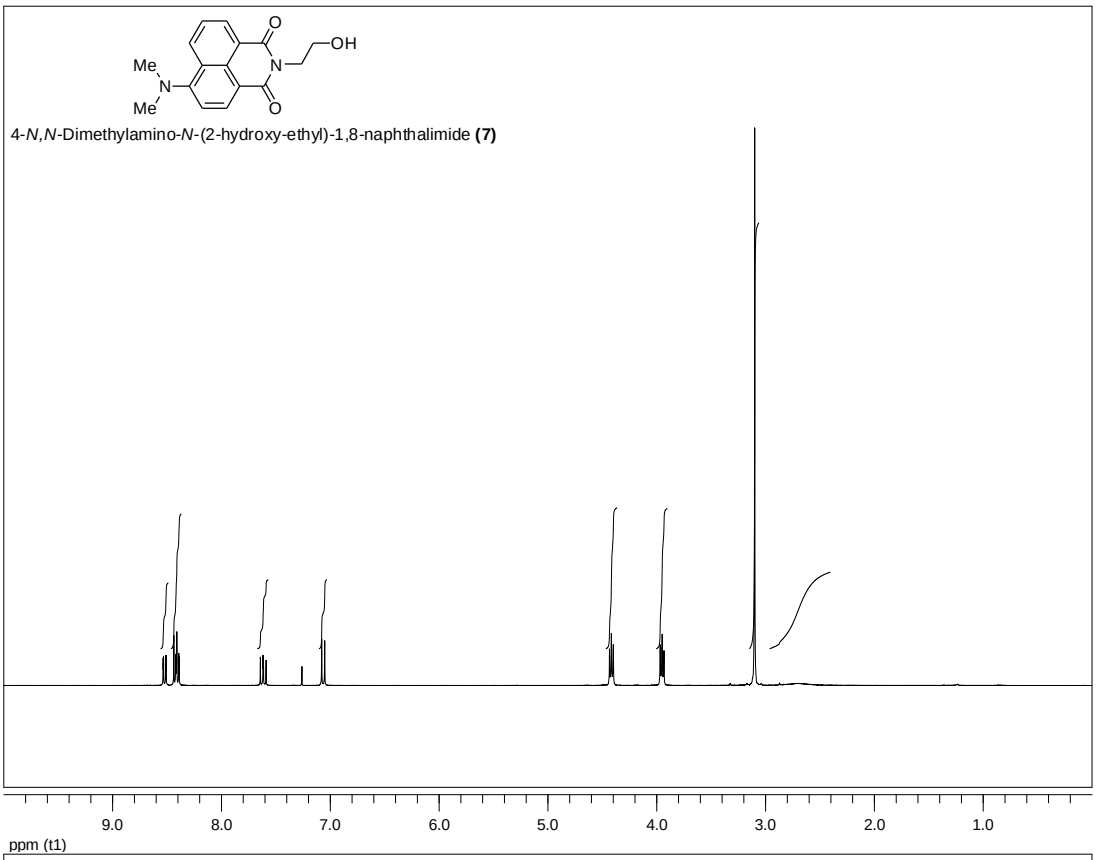
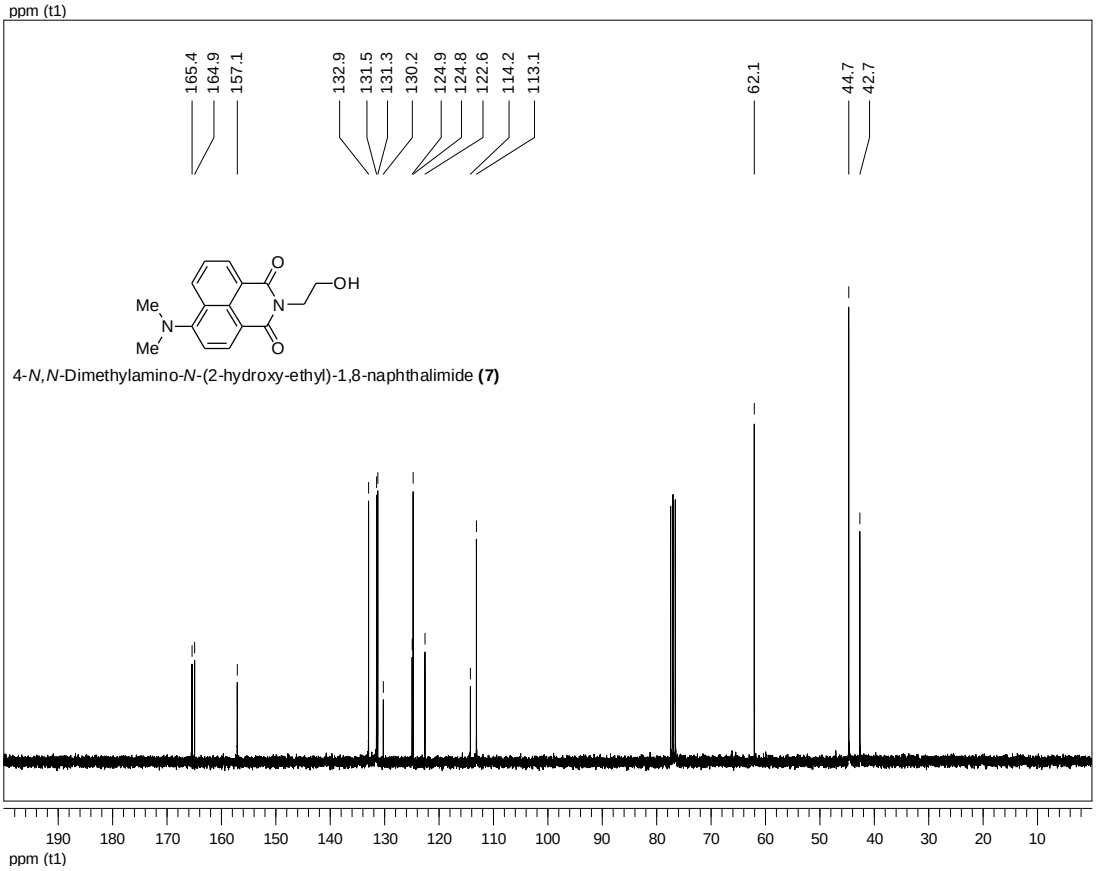
4.0
3.0
2.0
1.0
ppm (t1)

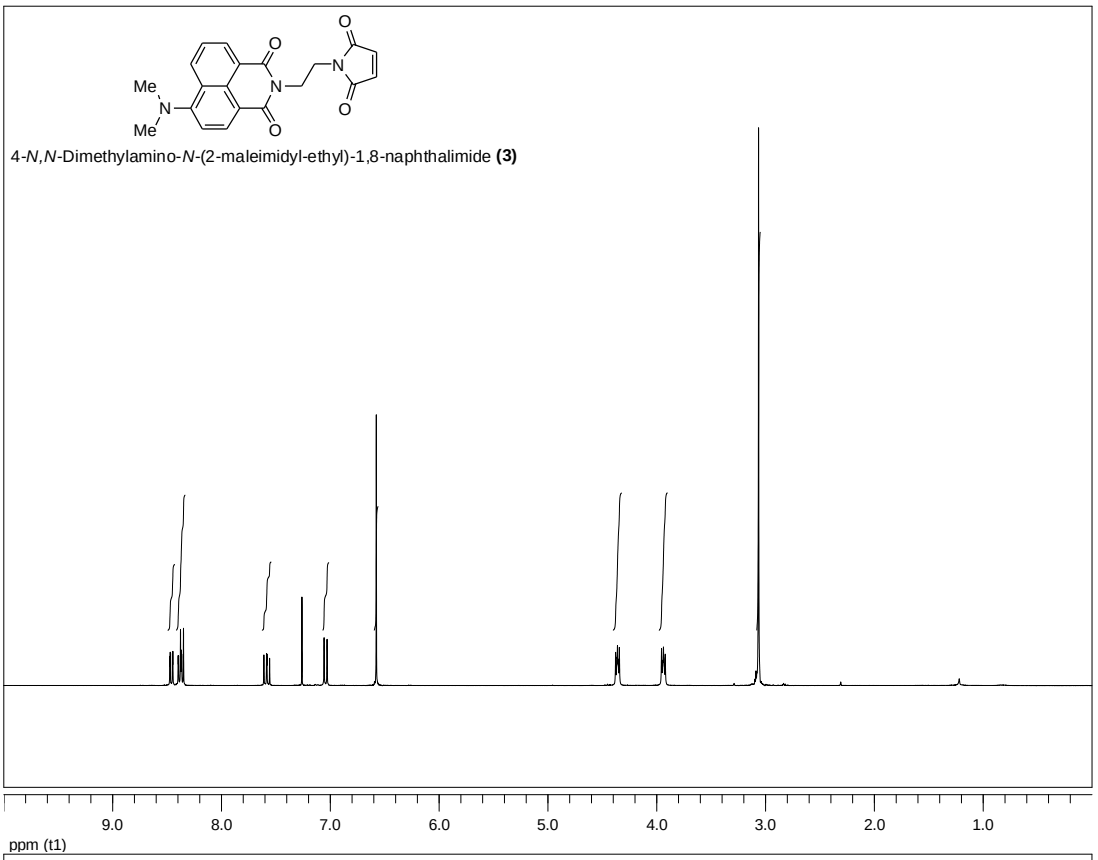

164.9
164.3
157.0
156.0
132.8
131.2
131.1
130.2
125.1
124.7
122.7
114.5
113.1
78.9
44.7
39.8
39.4
28.2
4-N,N-Dimethylamino-N-(2-tert-butoxycarbonylamino-ethyl)-1,8-naphthalimide (11)
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm (t1)

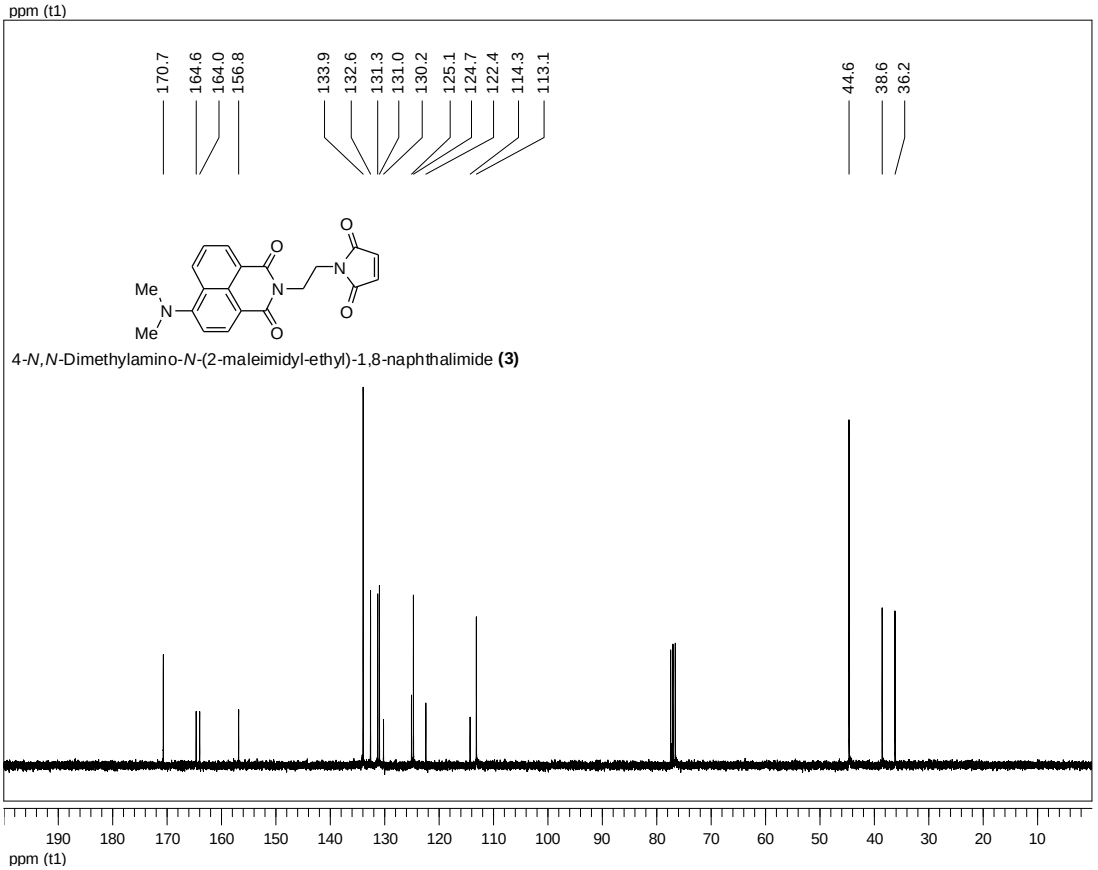

4-*N*,*N*-Dimethylamino-*N*-[2-(2-bromo-acetylamino)-ethyl]-1,8-naphthalimide **(2)**
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm (t1)

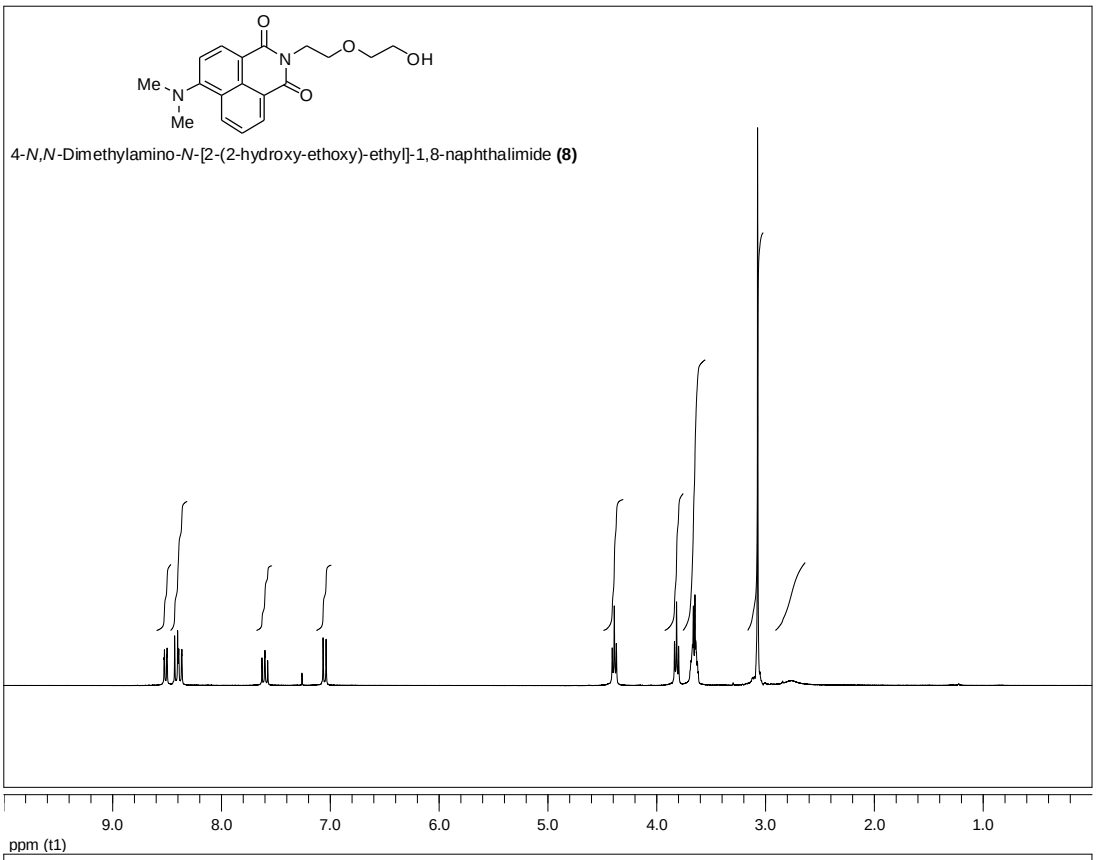

166.0
165.0
164.4
157.2
133.0
131.5
131.2
130.2
124.9
124.7
122.4
114.0
113.1
44.6
40.4
38.5
28.9
4-*N*,*N*-Dimethylamino-*N*-[2-(2-bromo-acetylamino)-ethyl]-1,8-naphthalimide **(2)**
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm (t1)

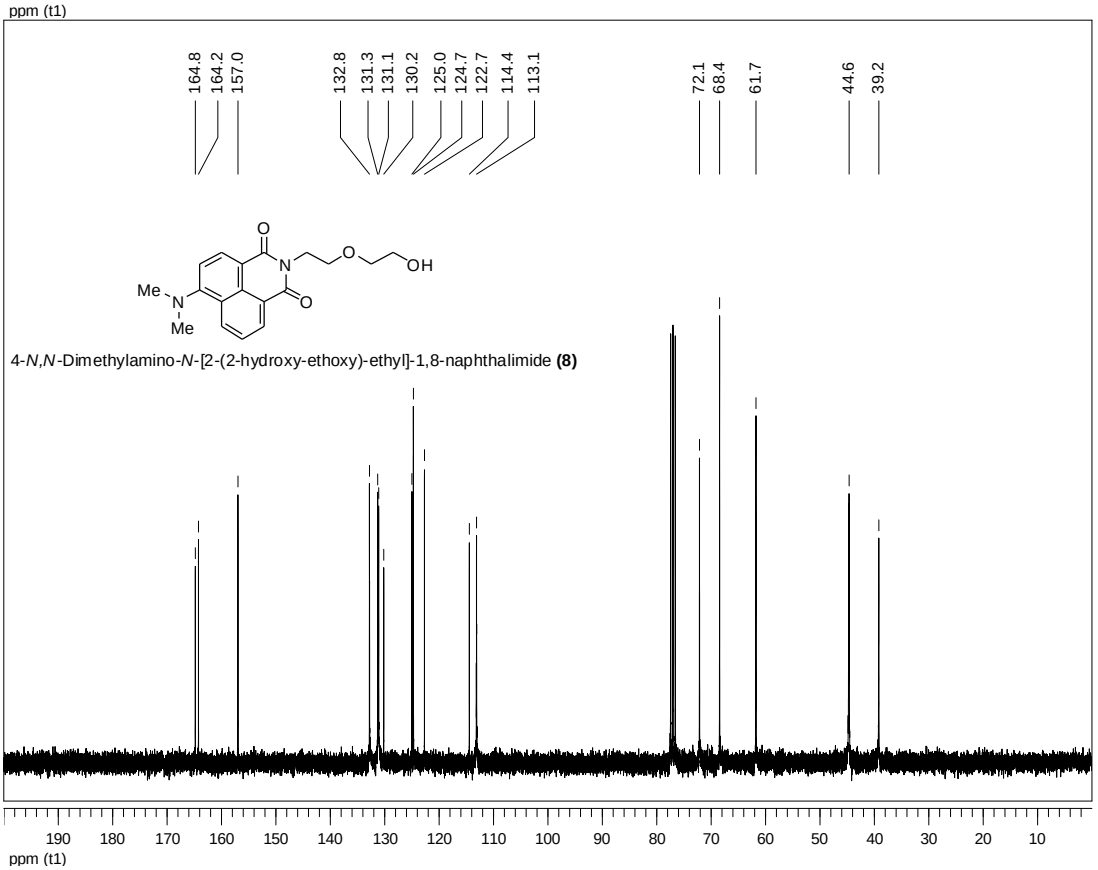

Me
N
Me
O
N
OH
O
4-*N*,*N*-Dimethylamino-*N*-(2-hydroxy-ethyl)-1,8-naphthalimide **(7)**
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm (t1)

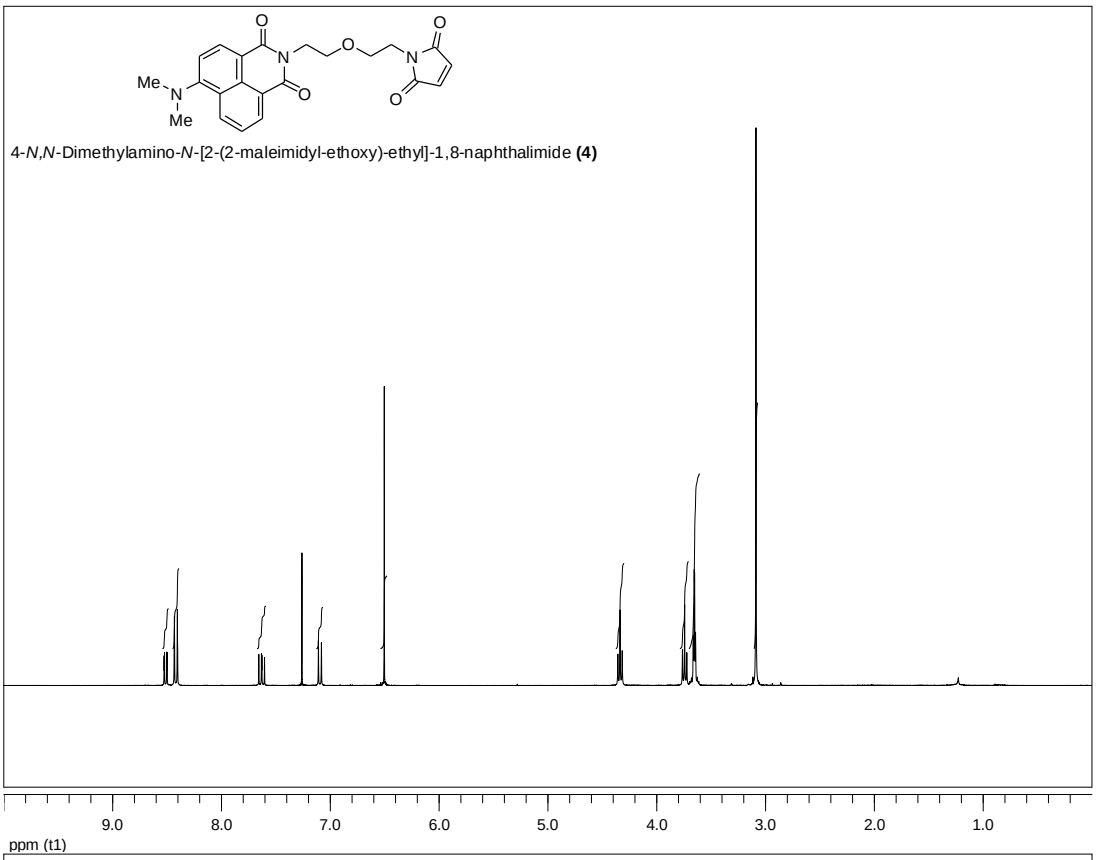

165.4
164.9
157.1
132.9
131.5
131.3
130.2
124.9
124.8
122.6
114.2
113.1
62.1
44.7
42.7
Me
N
Me
O
N
OH
O
4-*N*,*N*-Dimethylamino-*N*-(2-hydroxy-ethyl)-1,8-naphthalimide **(7)**
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm (t1)

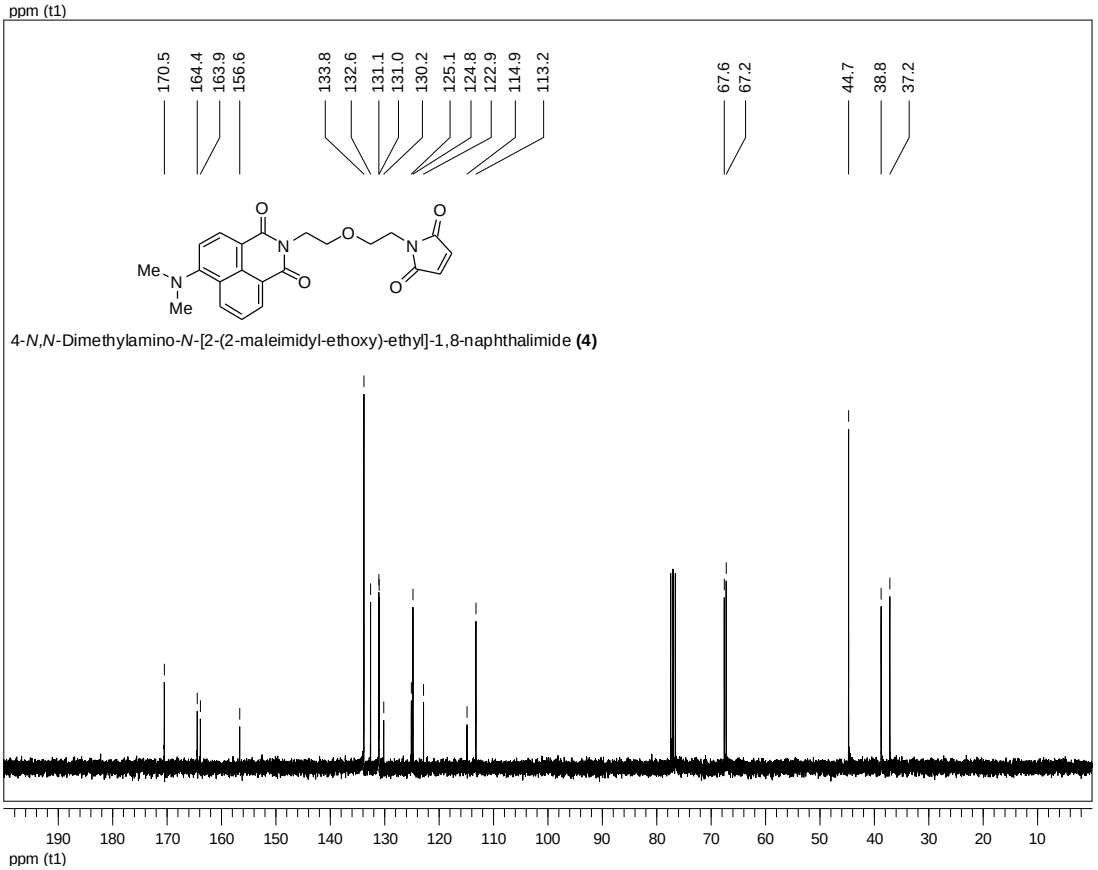

4-*N*,*N*-Dimethylamino-*N*-(2-maleimidyl-ethyl)-1,8-naphthalimide **(3)**
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm (t1)

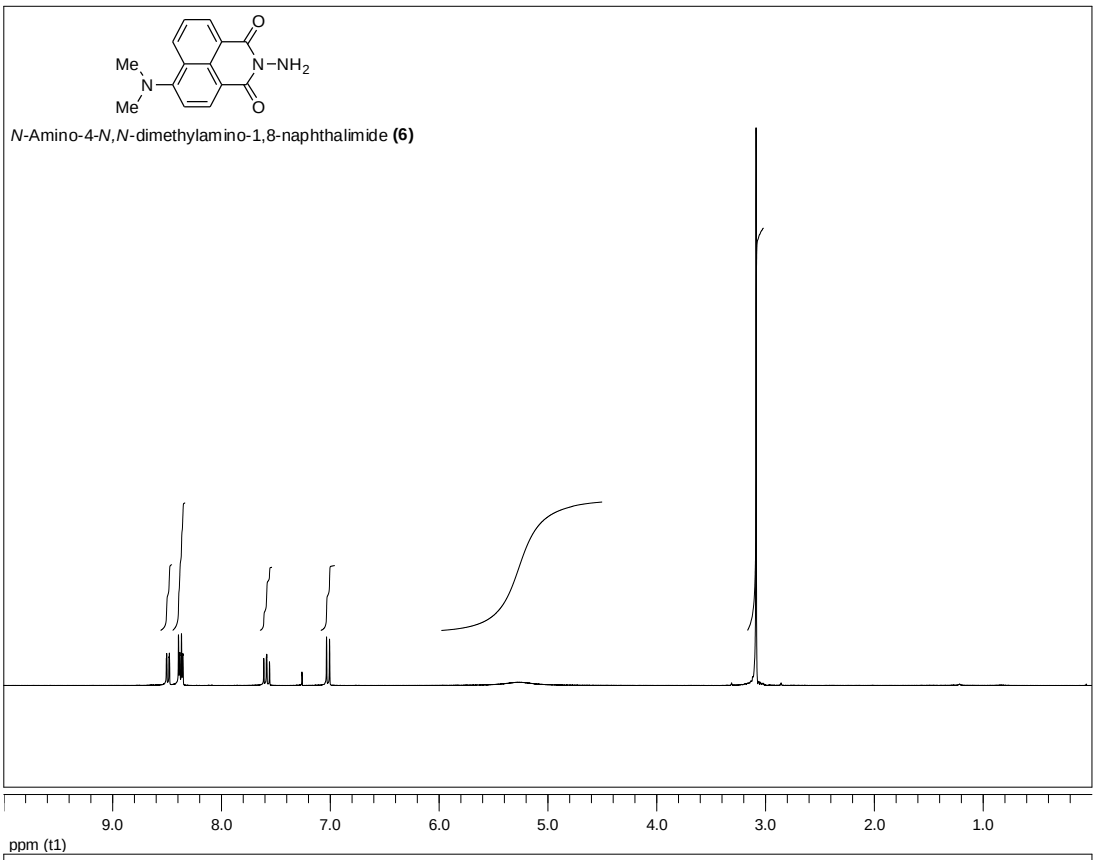

170.7
164.6
164.0
156.8
133.9
132.6
131.3
131.0
130.2
125.1
124.7
122.4
114.3
113.1
44.6
38.6
36.2
4-*N*,*N*-Dimethylamino-*N*-(2-maleimidyl-ethyl)-1,8-naphthalimide **(3)**
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm (t1)

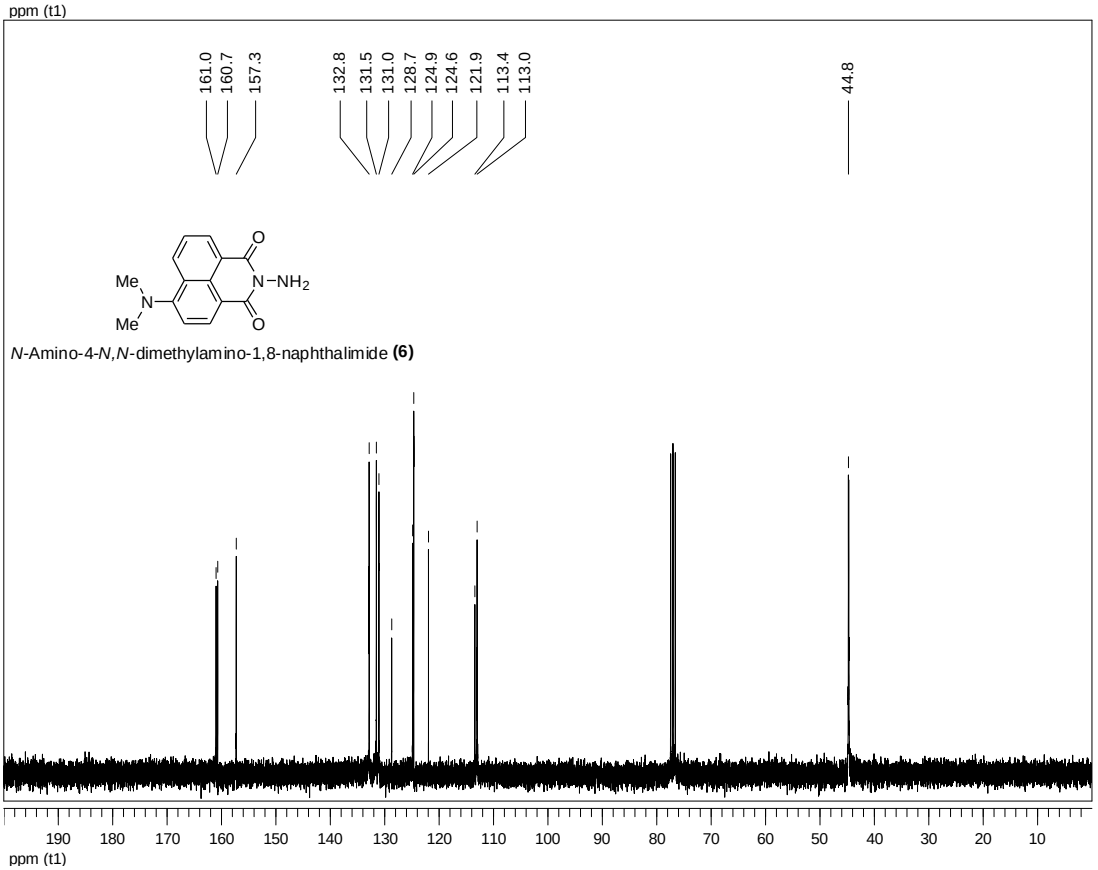

4-*N*,*N*-Dimethylamino-*N*-[2-(2-hydroxy-ethoxy)-ethyl]-1,8-naphthalimide **(8)**
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm (t1)

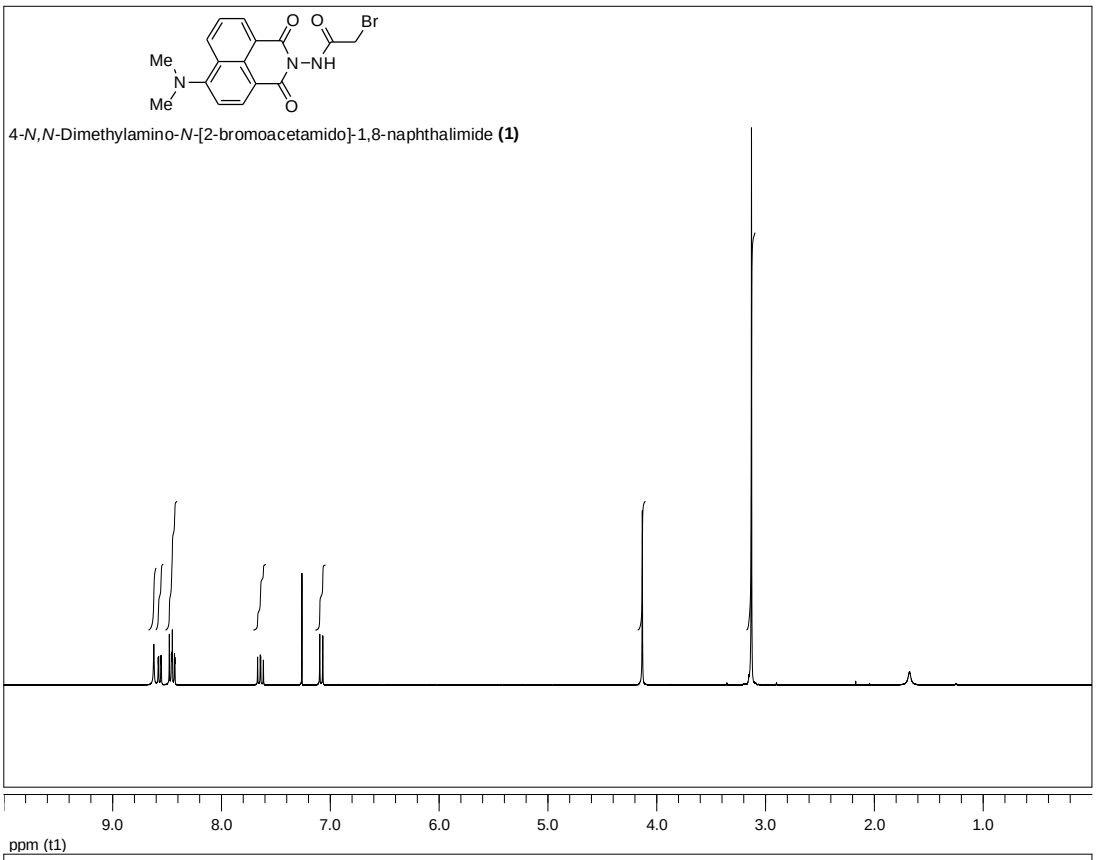

164.8
164.2
157.0
132.8
131.3
131.1
130.2
125.0
124.7
122.7
114.4
113.1
72.1
68.4
61.7
44.6
39.2
4-*N*,*N*-Dimethylamino-*N*-[2-(2-hydroxy-ethoxy)-ethyl]-1,8-naphthalimide **(8)**
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm (t1)

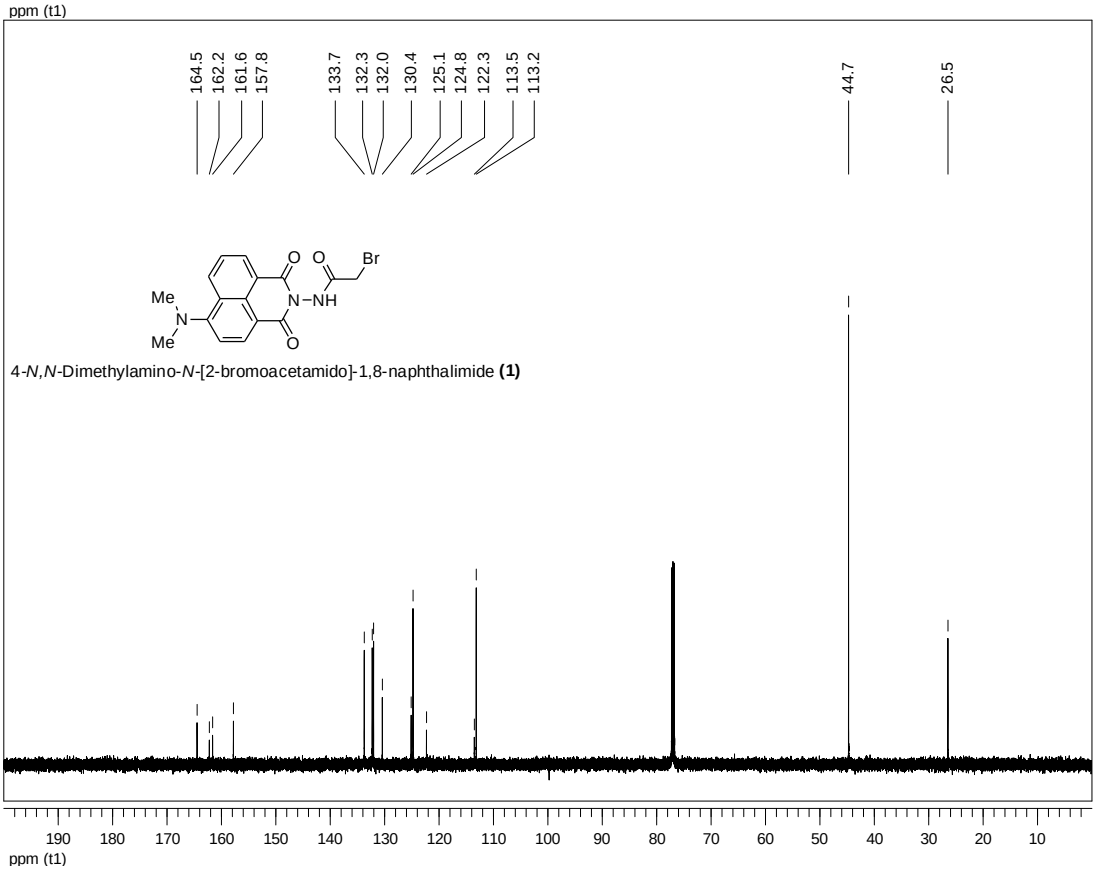

4-*N*,*N*-Dimethylamino-*N*-[2-(2-maleimidyl-ethoxy)-ethyl]-1,8-naphthalimide **(4)**
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm (t1)


170.5
164.4
163.9
156.6
133.8
132.6
131.1
131.0
130.2
125.1
124.8
122.9
114.9
113.2
67.6
67.2
44.7
38.8
37.2
4-*N*,*N*-Dimethylamino-*N*-[2-(2-maleimidyl-ethoxy)-ethyl]-1,8-naphthalimide **(4)**
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm (t1)


Me
N
Me
O
N–NH2
O
N-Amino-4-N,N-dimethylamino-1,8-naphthalimide (6)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm (t1)


161.0
160.7
157.3
132.8
131.5
131.0
128.7
124.9
124.6
121.9
113.4
113.0
44.8
Me
N
Me
O
N–NH2
O
N-Amino-4-N,N-dimethylamino-1,8-naphthalimide (6)
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm (t1)


Me
N
Me
O
O
N–NH
O
O
Br
4-*N*,*N*-Dimethylamino-*N*-[2-bromoacetamido]-1,8-naphthalimide **(1)**
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm (t1)


164.5
162.2
161.6
157.8
133.7
132.3
132.0
130.4
125.1
124.8
122.3
113.5
113.2
44.7
26.5
Me
N
Me
O
O
N–NH
O
O
Br
4-*N*,*N*-Dimethylamino-*N*-[2-bromoacetamido]-1,8-naphthalimide **(1)**
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm (t1)

Synthesis of the M13 peptide:

The peptide used in this study represents a truncated form of the originally described M13 peptide discovered by Blumenthal et al. (1). The sequence encompasses only the minimum residues required to bind the Ca^{2+}-CaM activated complex. The residue numbering refers to that of the original fragment.

M13 Peptide

Residue #	N-term	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	C-term
Sequence	H_2N-	R	R	W	K	K	N	F	I	A	V	S	A	A	N	R	F	K	K	-$CONH_2$

The M13 peptide was prepared using standard Fmoc-based solid-phase peptide synthesis techniques (SPPS) (*2*) with 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. Synthesis of the peptide 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 been discovered from earlier attempts at making the M13 peptide that the coupling efficiency was extremely low for residues incorporated after the alanine at position 10 in the sequence. This problem was overcome by using a pseudoproline dipeptide, Fmoc-Val-Ser($\Psi^{Me,Me}$pro)-OH (Cat. No. 05-20-1001, Novabiochem), for positions 11-12 in place of Fmoc-Val-OH and Fmoc-Ser(tBu)-OH. The remaining residues were coupled manually using 6 equiv of the Fmoc amino acids. 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). A Boc-Arg(Pbf)-OH residue was incorporated at the N-terminus of the peptide in order to yield the free α-amino group following cleavage from the solid support. The M13 mutant peptide was 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 peptide was 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 separation was 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 peptide:

Time (min)	Flow Rate (mL/min)	H_2O % (with 0.1% TFA)	MeCN % (with 0.1% TFA)	Gradient
initial	1.00	93	7	linear
5.0	1.00	93	7	linear
40.0	1.00	0	100	linear

The peptide mass was determined by ESI-MS on a Mariner electrospray mass spectrometer (PerSeptive Biosystems).

Retention time (min)	ESI-MS (m/z) $[M+xH]^{x+}$ calcd	ESI-MS (m/z) $[M+xH]^{x+}$ found
21.6	740.9 [M+3]	740.6 [M+3]
	555.9 [M+4]	555.7 [M+4]
	444.9 [M+5]	444.8 [M+5]

Subcloning of the synthetic CaM gene into the pET-14b vector:

Sequence of codon-optimized CaM gene.

```
ATGGCAGATCAACTGACTGAAGAACAGATTGCGGAATTTAAAGAAGCATTCAGCCTGTTC
GACAAAGATGGCGATGGCACCATTACGACCAAGGAGCTGGGTACGGTGATGCGTTCTCTG
GGTCAGAACCCAACTGAGGCAGAACTGCAGGATATGATCAACGAGGTTGATGCTGACGGT
AATGGCACCATCGACTTCCCGGAATTCCTGACCATGATGGCCCGTAAAATGAAAGACACC
GATTCCGAAGAAGAAATCCGTGAGGCTTTCCGTGTATTCGACAAAGACGGTAACGGCTAC
ATTTCTGCGGCGGAACTGCGCCATGTGATGACCAACCTGGGCGAAAAACTGACCGACGAA
GAAGTTGACGAGATGATCCGCGAAGCTGATATCGACGGTGATGGTCAGGTCAACTATGAA
GAATTTGTTCAGATGATGACTGCGAAGCACCACCACCACCACCAC
```

Translation of codon-optimized CaM gene.

```
MADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADG
NGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDE
EVDEMIREADIDGDGQVNYEEFVQMMTAKHHHHHH
```

Subcloning of codon-optimized CaM gene. The synthetic CaM construct ordered from Bio Basic Inc. was designed for optimal expression in *E. coli.* The gene was inserted into a high-copy pUC-57 vector 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(CaM) 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	XhoI	1.0 μL	XhoI
1.0 μL	NcoI	1.0 μL	NcoI
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 CaM 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 the NEB 2-Log DNA ladder.

Two ligation reactions were assembled: one containing both the CaM 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	CaM 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 were 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 reactions were 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. Three colonies from the non-control plate were selected and amplified for sequencing. All selected colonies were determined to be the desired construct. The new vector, pET-14b[CaM], was transformed into BL21-Gold(DE3) competent cells from Stratagene for protein expression.

Cysteine mutagenesis of wild-type CaM gene:

These seven cysteine mutants of the CaM construct were prepared by site-directed mutagenesis using the following set of PAGE-purified primers along with the corresponding reverse compliments ordered on the 50 nmole scale from Operon:

E11C-
CTGACTGAAGAACAGATTGCGTGCTTTAAAGAAGCATTCAGCCTG

S38C-
GGGTACGGTGATGCGTTGTCTGGGTCAGAACCC

M76C-
CCTGACCATGATGGCCCGTAAATGCAAAGACACCGATTCCG

E87C-
CCGATTCCGAAGAAGAAATCCGTTGCGCTTTCCGTGTATTCGAC

N111C-
CTGCGCCATGTGATGACCTGCCTGGGCGAAAAACTGACC

E114C-
GTGATGACCAACCTGGGCTGCAAACTGACCGACGAAGAAG

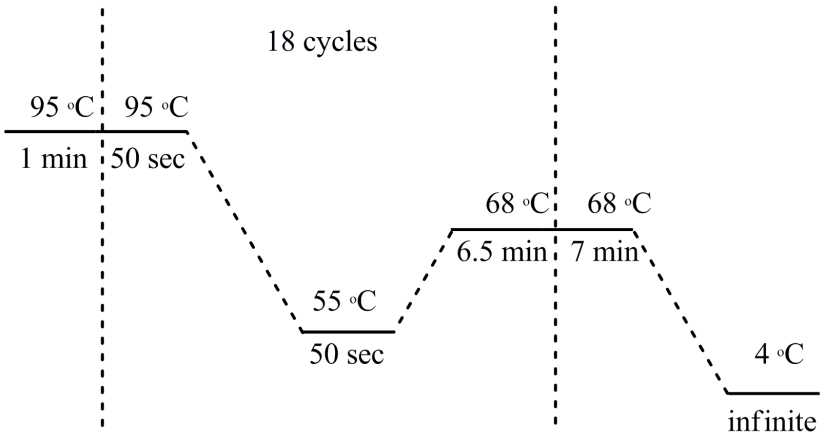
M145C-
CTATGAAGAATTTGTTCAGATGTGCACTGCGAAGCACCACCACC

Upon receiving the primers, stock solutions were prepared in sterile water (125 ng/µl). The reactions were assembled accordingly:

5.0 µL	10× Pfu Turbo rxn buffer.
0.5 µL	pET14b vector [CaM] (20 ng/µL)
1.0 µL	fwd primer (125 ng/µL)
1.0 µL	rev primer (125 ng/µL)
2.5 µL	dNTP's (10 mM each)
40.0 µL	dH_2O (sterile)
50.0 µL	total volume

A control reaction was also performed in which the primers were excluded from the reaction mixture. Next, 1 µL of Pfu Turbo (2.5 U/µL, Stratagene) was added to each reaction. The reaction solutions were mixed thoroughly by gentle pipetting, centrifuged briefly and loaded into an MJ-mini PCR machine from Bio-Rad. The PCR method employed was as follows:

PCR method:



After the PCR reaction was complete the parent vector was digested by adding 1 μL of DpnI (20 U/μL) to each reaction mixture. The reactions were then mixed with gentle pipetting and incubated at 37 ºC for 1.5 hrs each. The reaction mixtures were then used to transform XL1-blue DH5α cells obtained from Stratagene. Colonies from each transformation were selected and submitted for sequencing to confirm the desired constructs were obtained. The DNA from each of the chosen colonies was isolated using a Qiagen Miniprep kit.

Expression and purification of wild-type CaM and cysteine mutants:

The calmodulin constructs were designed with a C-terminal hexahistidine tag for easy purification of the expressed product. The CaM-His_6 mutants were transformed in BL21-Gold(DE3) competent cells from which 20% glycerol stocks were prepared for subsequent protein expression. The stocks were used to inoculate 5 mL LB-broth starter cultures containing carbenicillin (50 μg/mL). The starter cultures were allowed to grow overnight in a 37 ºC shaker at 225 rpm. The next day, the starter cultures were used to inoculate 1 L LB-broth cultures containing carbenicillin (50 μg/mL) and grown at 37 ºC with agitation at 225 rpm. The cultures were allowed to grow to an $OD_{600nm} \approx 0.5$ after which the 1 L shaker flasks were 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 pellets were then resuspended in a 0.9% NaCl aqueous solution (30 mL) and transferred to 50 mL conical tubes to be repelleted for storage at -80 ºC.

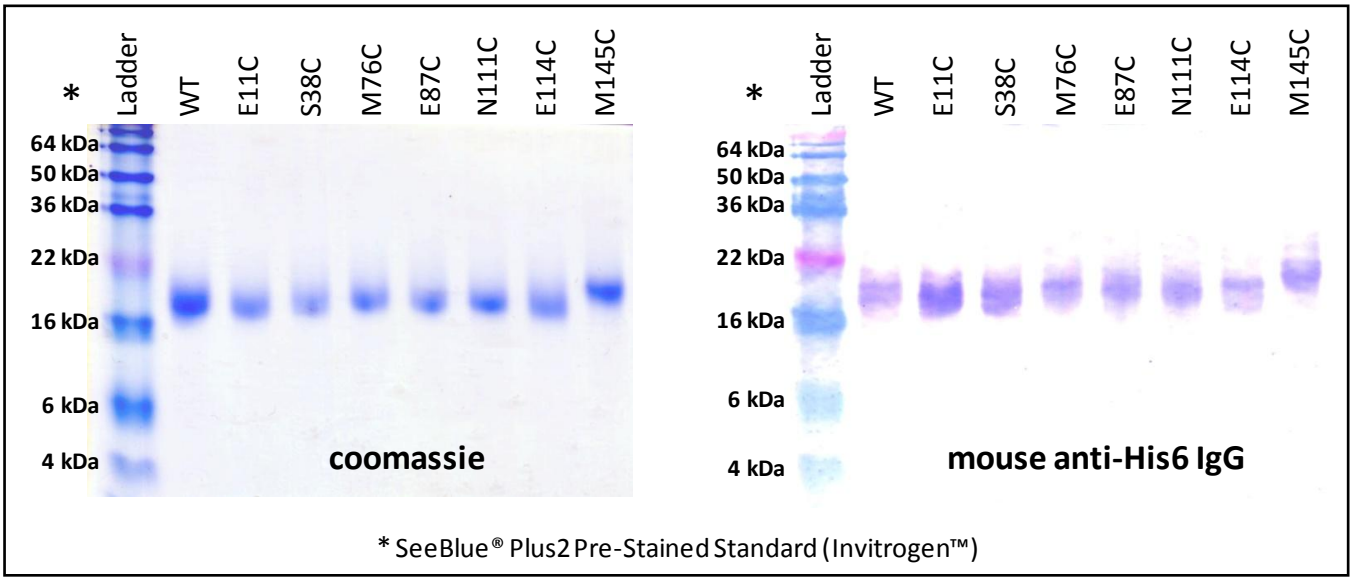
On the day of the protein preparation, the pellets were thawed at room temperature and resuspended in the following lysis buffer:

4 mL	10× TBS 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 pellets were 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 lysates were then transferred to 45 mL polycarbonate bottles 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 TBS buffer for each construct. After the spin, the clarified supernatants were transferred to clean 50 mL conical tubes and mixed with the pre-equilibrated Ni-NTA agarose resin. The samples were tumbled gently on a rotisserie at 4 ºC for 1 hr to allow binding of the His-tagged proteins. The resin was then recovered by passing the lysates through 20 mL spin columns obtained from Bio-Rad. The resin was washed first with 4 mL of TBS buffer, then 4 mL of 10 mM imidazole in TBS buffer and then with 4 mL of 20 mM imidazole in TBS buffer at 4 ºC. The protein was then eluted in 6 × 1 mL fractions of 250 mM imidazole in TBS 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 solutions were dialyzed against 3 × 2 L changes of TBS buffer. The protein concentrations were then quantified using the Bio-Rad protein assay solution. Protein yields were ~80 mg/L for the purified CaM mutants.

SDS PAGE (15% polyacrylamide) analysis was performed on the proteins 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 under 100 V, the nitrocellulose membrane 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 the membrane 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 membrane 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 membrane 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 dried.



ESI-MS of expressed CaM constructs:

The cysteine mutants of the CaM-His_6 protein were also analyzed by mass spectrometry to confirm the identity of the isolated protein products. All eight of the expressed products matched the calculated molecular weights determine from the primary structures minus the N-terminal methionine due to methionine aminopeptidase activity in *E. coli*.

CaM-His6 construct	ESI-MS (m/z) $[M+xH]^{x+}$ calcd	ESI-MS (m/z) $[M+xH]^{x+}$ found
Wild-type	1948.7 [M+9]	1948.1 [M+9]
	1753.9 [M+10]	1755.2 [M+10]
	1594.6 [M+11]	1593.8 [M+11]
E11C	1945.8 [M+9]	1945.1 [M+9]
	1751.3 [M+10]	1750.5 [M+10]
	1592.2 [M+11]	1591.4 [M+11]
S38C	1950.5 [M+9]	1950.2 [M+9]
	1755.5 [M+10]	1754.7 [M+10]
	1596.0 [M+11]	1595.3 [M+11]
M76C	1945.6 [M+9]	1944.9 [M+9]
	1751.1 [M+10]	1750.3 [M+10]
	1592.0 [M+11]	1591.3 [M+11]

CaM-His6 construct	ESI-MS (m/z) $[M+xH]^{x+}$ calcd	ESI-MS (m/z) $[M+xH]^{x+}$ found
E87C	1945.8 [M+9]	1945.3 [M+9]
	1751.3 [M+10]	1750.6 [M+10]
	1592.2 [M+11]	1593.4 [M+11]
N111C	1947.5 [M+9]	1946.7[M+9]
	1752.8 [M+10]	1752.2 [M+10]
	1593.6 [M+11]	1592.9 [M+11]
E114C	1945.8 [M+9]	1944.9 [M+9]
	1751.3 [M+10]	1752.7 [M+10]
	1592.2 [M+11]	1591.2 [M+11]
M145C	1945.6 [M+9]	1945.1 [M+9]
	1751.1 [M+10]	1751.5 [M+10]
	1592.0 [M+11]	1591.4 [M+11]

The protein masses were determined by ESI-MS on a Mariner electrospray mass spectrometer (PerSeptive Biosystems).

Labeling efficiencies:

The extent of labeling of each construct was determined by measuring the absorbance of the attached dye and dividing that value by the known extinction coefficient to calculate the dye concentration (Table S1). This value was then compared to the predetermined concentration of the protein construct to yield the ratios shown below.

Table S1

			Labeling ratio of CaM Mutants:							
Fluorophore:	**Abs_{max} (nm)**	**ε (cm^{-1} M^{-1})[a]**	**Wild-type**	**E11C**	**S38C**	**M76C**	**E87C**	**N111C**	**E114C**	**M145C**
1	440	8800	0.01	0.64	0.16	0.47	0.62	0.62	0.84	0.55
2	440	8800	0.04	0.85	0.41	0.61	0.70	0.78	0.84	0.50
3	440	8800	0.00	0.81	0.63	0.60	0.62	0.69	0.84	0.53
4	440	8800	0.00	0.72	0.67	0.60	0.60	0.65	0.79	0.67
MDCC	419	50000	0.00	0.42	0.35	0.30	0.28	0.25	0.31	0.21
IANBD	478	25000	0.01	0.56	0.18	0.46	0.47	0.43	0.52	0.31
BADAN	387	21000	0.01	0.53	0.31	0.43	0.43	0.42	0.48	0.31
IAEDANS	336	5700	0.00	0.30	0.25	0.27	0.39	0.32	0.52	0.30
PyMPO	412	23000	0.02	0.60	0.52	0.45	0.47	0.43	0.50	0.41

[a]The extinction coefficients of the commercial dyes were obtained from the suppliers (AnaSpec, Inc. and Molecular Probes®).

Emission and Excitation settings for SPECTRAmax GEMINI XS plate reader:

Table S2

Fluorophore	exc (nm)	em (nm)
1-4	405	440-660
MDCC	400	430-650
IANBD	455	490-710
BADAN	365	400-620
IAEDANS	336	440-660
PyMPO	412	440-660

Denaturing of fluorescent CaM constructs to determine background emission:

There exist two primary sources of background fluorescence directly associated with a solvatochromic fluorophore when used to develop a fluorescent protein probe. The first involves the intrinsic

fluorescence exhibited by the fluorophore when fully solvated by water. The second is a ramification of appending the fluorophore to the surface of a globular protein. This frequently results in a change in the photophysical properties of the fluorophore that would be expected if it were participating in some type of non-specific interaction with the local topology of the attached protein. By denaturing the protein in a concentrated solution of guanidine hydrochloride, the secondary and tertiary structures that may contribute to this effect are disrupted permitting one to determine the degree of background emission generated (Figure S1).

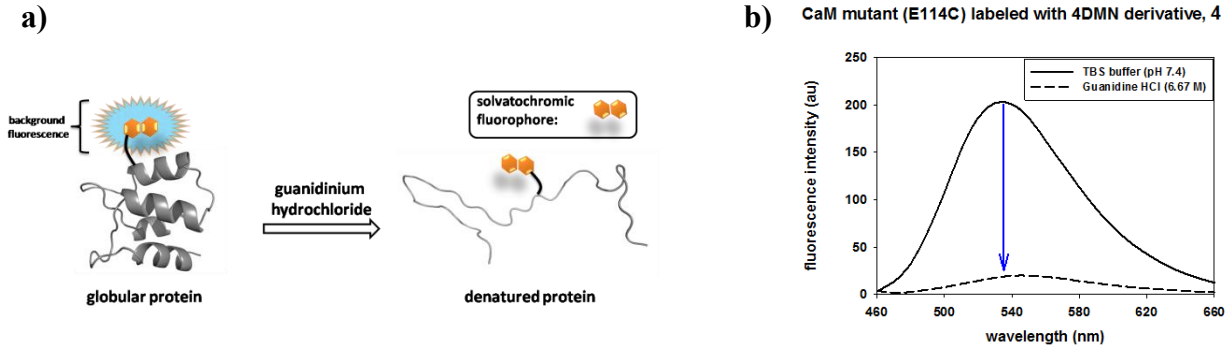


Figure S1. (a) Graphic depicting the tendency of solvatochromic fluorophore to exhibit background fluorescence when appended to a globular protein until denatured, thus fully exposing the fluorophore to the aqueous environment. (b) Example of a typical fluorescent response of a labeled CaM mutant under both natured and denatured conditions (TBS buffer versus guanidine hydrochloride solution at pH 7.4)

A series of experiments were conducted on each of the sixty-three fluorescent CaM constructs prepared in this study in which the degree of background fluorescence associated with the appending of the fluorophore to the protein was determine by measuring the emission spectra of the constructs in TBS buffer (pH 7.4) and guanidine hydrochloride (6.67 M, pH 7.4) and calculating the ratio of intensity at the wavelength of maximum emission in the natured state. The results of this work are displayed in Figure S2.

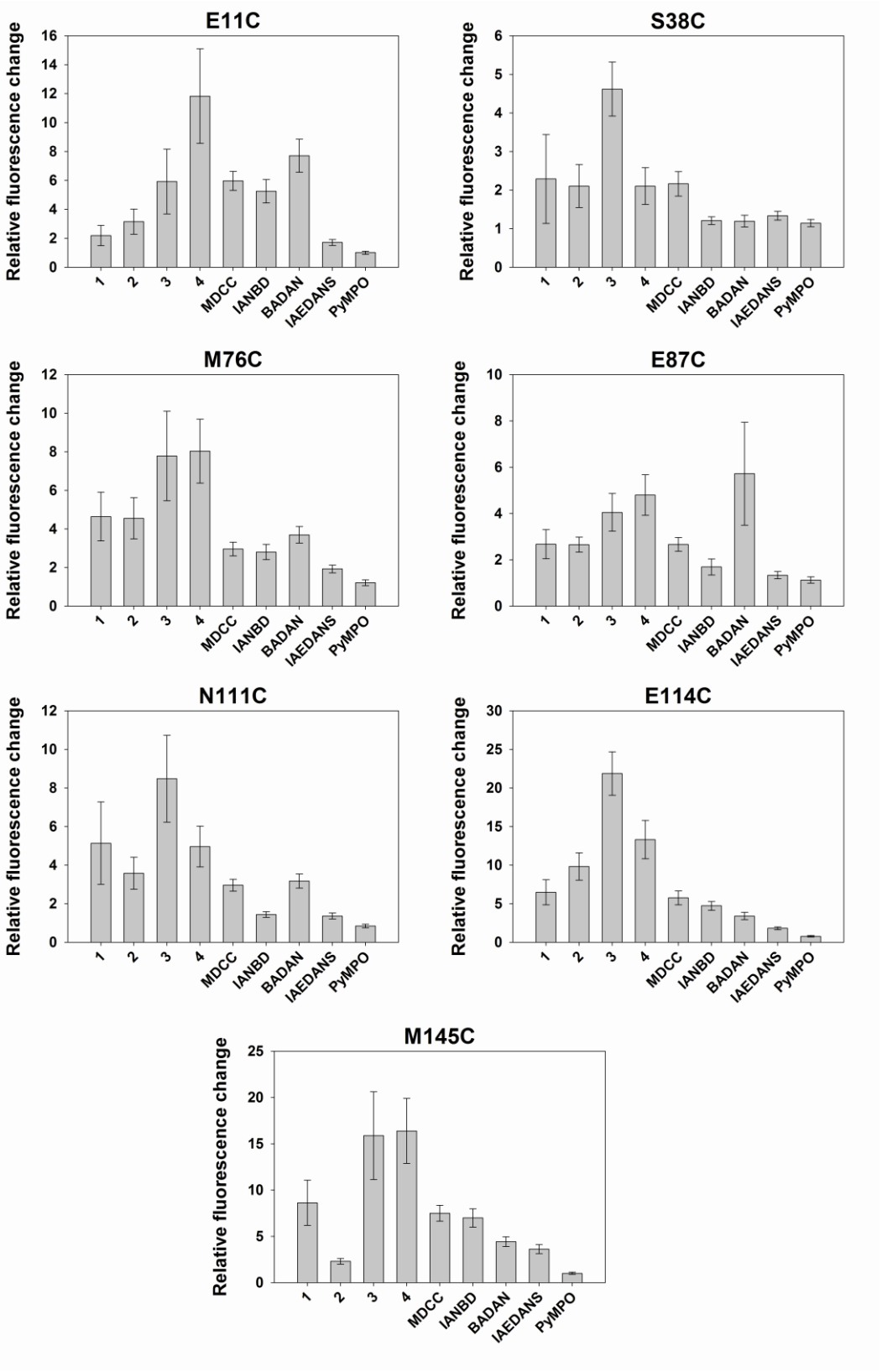


Figure S2. The fluorescent constructs were measured at a concentration of 5 μM in TBS buffer (pH 7.4) and guanidine hydrochloride (6.67 M, pH 7.4). The vertical bars represent the magnitude of the ratio of fluorescence in TBS buffer over that of the construct in the guanidine solution. A greater ratio indicates higher initial background fluorescence due to non-specific interactions of fluorophore with

local features of the attached protein. Indicated errors represent the 90% confidence interval for three trials.

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