

Supporting Information for:

**Photophysics and Biological Applications of the
Environment Sensitive Fluorophore 6-*N,N*-Dimethylamino-
2,3-Naphthalimide.**

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General Procedures. All peptide synthesis reagents and amino acid derivatives were purchased from Applied Biosystems or Novabiochem. All other chemicals were purchased from Aldrich. Dichloromethane was distilled from calcium hydride under nitrogen, and tetrahydrofuran was distilled from sodium under argon. Analytical thin-layer chromatography (TLC) was carried out on F254 250- μ m silica gel plates, and visualized by UV.

^1H and ^{13}C NMR spectra were acquired on 250 or 400 MHz Bruker spectrometers. NMR samples were prepared in deuteriochloroform (CDCl_3) unless otherwise stated. Chemical shifts are reported in ppm relative to the internal standard (tetramethyl silane for ^1H), and J values are reported in Hz. Electrospray Ionization Mass Spectrometry (ESIMS) was performed on a PerSeptive Biosystems MarinerTM Biospectrometry Workstation (Turbo Ion Source). Fluorescence spectroscopy measurements were made using a Fluoromax-P spectrofluorimeter controlled by the DataMax 2.20 software, and coupled to a NesLab RTE-111 water bath for temperature control. All measurements were made at 20 °C.

Peptide Synthesis. Peptide synthesis was carried using standard Fmoc-based solid phase peptide synthesis (SPPS) protocols on a 0.05 to 0.1 mmol scale using a 0.21 mmol/g loading PAL-PEG-PS solid support. Amino acids were coupled in three-fold excess using a mixture of 0.2 M HBTU/0.2 M HOBt in DMF as activating agents. Each amino acid was activated for two minutes with the HBTU/HOBt mixture (1 eq.) and DIPEA 0.195 M in DMF (1.5 eq.) before being added to the resin. Peptide coupling was

monitored using the 2,4,6-trinitrobenzenesulphonic acid (TNBS) test.¹ Amino acids were used as protected Fmoc-amino acids with the standard side chain protecting groups: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Ile-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH and Fmoc-Val-OH. Phosphotyrosine was introduced as the monobenzyl ester Fmoc-Tyr(PO(OBzl)OH)-OH. High-performance liquid chromatography (HPLC) was performed using a Waters 600E HPLC fitted with a Waters 600 automated control module and a Waters 2487 dual wavelength absorbance detector recording at 228 and 280 nm. For analytical HPLC a Beckman Ultrasphere C₁₈, 5 μ m, 4.6 x 150 mm reverse-phase column was used. For preparative separations a YMC-pack, C₁₈, 250 x 20 mm reversed phase column was used. The standard gradient for analytical and preparative HPLC used was 93:7 to 5:95 over 35 minutes (water:acetonitrile, 0.1% TFA).

5-nitrophenyl-1,3-dioxolane (7). Protection of the aldehyde was performed following reported procedures.^{2,3} 3-Nitrobenzaldehyde (4.0 g, 26.4 mmol) was dissolved in toluene (200 mL). Following this, ethanediol (3.0 mL, 53 mmol, 2.0 eq) and *p*-TsOH (150 mg, 0.8 mmol) were added to the solution and the resulting mixture was refluxed overnight with a Dean-Stark trap. The reflux mixture was then extracted with saturated NaHCO₃ (2 x 75 mL). The organic layer was dried with Na₂SO₄ and concentrated under reduced pressure. The residue was then purified by flash column chromatography (30% EtOAc/hexanes) to afford the desired product as a clear yellow oil that solidified upon standing. (5.09 g, 98%, R_f = 0.7 40% EtOAc/hexanes). ¹H-NMR (400 MHz, CDCl₃, δ): 4.0-4.2 (m, 4H), 5.87 (s, 1H), 7.55 (t, 1H, *J* = 7.93 Hz), 7.79 (d, 1H, *J* = 7.7 Hz), 8.21

(dd, 1H, $J_1 = 1.2$ Hz, $J_2 = 8.2$ Hz), 8.34 (s, 1H). ^{13}C -NMR (100 MHz, CDCl_3 , δ): 148.4, 140.5, 132.8, 129.6, 124.1, 121.8, 102.3, 65.6.

2-(2-Phenylsulfonylmethyl-5-nitrophenyl)-1,3-dioxolane (8). 2-Nitrophenyl-1,3-dioxolane (1.0 g, 5.1 mmol) and chloromethyl-phenyl sulfone (975 mg, 5.1 mmol) were dissolved in dry dimethylsulfoxide. Then, KOH powder (2.00 g) was added to the solution, which immediately turned dark purple. The resulting mixture was stirred overnight at room temperature, then poured over 75 mL 2% aqueous HCl and extracted with EtOAc (3 x 50 mL). The combined organic layers were dried with Na_2SO_4 and concentrated under reduced pressure to give a dark brown oil that was purified by flash column chromatography (30% EtOAc/hexanes). Two regioisomeric products were isolated. The combined yield of the reaction is 1.23 g (35% desired product, $R_f = 0.4$ 40% EtOAc/hexanes). Compounds *ortho*- and *para*- could be identified by the ^1H -NMR signals of the protons *ortho*- to the nitro group. In the case of the desired compound **8**, both signals are present in the NMR spectra. ^1H -NMR (400 MHz, CDCl_3 , δ): 4.01 (s, 4H), 4.73 (s, 2H), 5.93 (s, 1H), 7.32 (d, 1H, $J = 8.3$ Hz), 7.53 (t, 2H, $J = 7.6$ Hz), 7.67 (t, 1H, $J = 7.7$ Hz), 7.72 (d, 2H, $J = 7.6$ Hz), 8.10 (d, 1H, $J = 6.6$ Hz), 8.45 (s, 1H). ^{13}C -NMR (100 MHz, CDCl_3 , δ): 148.1, 140.7, 138.2, 134.5, 133.7, 129.5, 128.6, 123.6, 121.7, 100.2, 65.5, 59.0. HRMS-ESI (m/z): $[\text{M}+\text{H}^+]$ calcd for $\text{C}_{16}\text{H}_{15}\text{NO}_6\text{S}$ 350.0693, found, 350.0677.

Diethyl 6-nitro-2,3-naphthalenedicarboxylate (9). 2-(2-Phenylsulfonylmethyl-5-nitrophenyl)-1,3-dioxolane (400 mg, 1.14 mmol) was dissolved in AcOH (8 mL) and water (2 mL). The solution was refluxed for 5 h and then concentrated under reduced pressure. The resulting solid was confirmed as the desired product by NMR and used in

the next step without further purification. In a 25 mL round bottom flask, 5-nitro-2-phenylsufonylmethylbenzaldehyde (300 mg, 1.00 mmol), diethylmaleate (195 μ L, 207 mg, 1.20 mmol) and 18-crown-6 (13 mg, 0.05 mmol) were dissolved in acetonitrile (10 mL). K_2CO_3 (700 mg) was added and the resulting mixture was stirred for 30 min. The reaction was refluxed for 3 h, then filtered over celite and concentrated under reduced pressure. The resulting dark oily residue was purified by flash chromatography (30% EtOAc/hexanes) to give the desired product (231 mg, 64%, R_f = 0.35 20% EtOAc/hexanes). 1H -NMR (250 MHz, $CDCl_3$, δ): 1.43 (t, 3H, J = 7.1 Hz), 1.44 (t, 3H, J = 7.1 Hz), 4.45 (c, 4H, J = 7.1 Hz), 8.04 (d, 1H, J = 9.0 Hz), 8.27 (s, 1H), 8.35 (dd, 1H, J_1 = 9.0 Hz, J_2 = 2.3 Hz), 8.42 (s, 1H), 8.84 (d, 1H, J = 2.5 Hz). ^{13}C -NMR (62 MHz, $CDCl_3$, δ): 167.1, 166.7, 147.1, 135.9, 132.7, 132.2, 131.8, 130.8, 130.5, 129.6, 125.0, 121.9, 62.3, 62.2, 14.2. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{16}H_{15}NO_6$ 318.0972, found, 318.0986.

Diethyl 6-(dimethylamino)-2,3-naphthalenedicarboxylate (10). Compound **9** (350 mg, 1.11 mmol) was dissolved in MeOH (50 mL). Formalin (7 mL) was added and Pd/C 10% (100 mg) was added to the solution. The resulting mixture was stirred under hydrogen for 2.5 h until TLC showed that the starting material has been consumed. The reaction mixture was then filtered through celite and partly concentrated to approximately 20 mL. Following this, the mixture was poured over 2% aqueous $NaHCO_3$ (150 mL) and extracted (3 x 50 mL EtOAc). The combined organic layers were dried with Na_2SO_4 and concentrated under reduced pressure to give the desired product (295 mg, 84%, R_f = 0.5 30% EtOAc/hexanes). 1H -NMR (250 MHz, $CDCl_3$, δ): 1.39 (t, 3H, J = 7.0 Hz), 1.40 (t, 3H, J = 7.0 Hz), 3.06 (s, 6H), 4.40 (c, 2H, J = 7.1 Hz), 4.42 (c, 2H, J = 7.1 Hz), 6.86 (d,

1H, $J = 2.4$ Hz), 7.17 (dd, 1H, $J_1 = 9.1$ Hz, $J_2 = 2.6$ Hz), 7.7 (d, 1H, $J = 9.1$ Hz), 7.91 (s, 1H), 8.19 (s, 1H). ^{13}C -NMR (62 MHz, CDCl_3 , δ): 169.4, 167.9, 150.5, 136.0, 131.0, 130.7, 130.1, 127.7, 125.9, 123.5, 117.9, 106.1, 61.8, 61.6, 40.7, 14.7, 14.6. HRMS-ESI (m/z): $[\text{M}+\text{H}^+]$ calcd for $\text{C}_{18}\text{H}_{21}\text{NO}_4$ 316.1549, found, 316.1556.

6-(dimethylamino)-2,3-naphthalenedicarboxylic anhydride (11). Compound **10** (373 mg, 1.2 mmol), was refluxed in 1:5 MeOH:40% KOH (25 mL) for 3 h. The reaction mixture was then poured over water (150 mL), acidified with 6 M HCl to pH 2 and extracted with EtOAc (5 x 30 mL). The combined organic layers were dried with Na_2SO_4 and concentrated under reduced pressure. The resulting solid residue, corresponding to the diacid intermediate, was used in the next step without purification (228 mg, 85%). The diacid was placed in a sublimation apparatus and heated at 175 °C under vacuum for 4 h. The desired anhydride **11** was recovered after sublimation as a bright yellow solid (213 mg, 77% overall yield, $R_f = 0.6$ 40% EtOAc/hexanes). ^1H -NMR (400 MHz, CDCl_3 , δ): 3.19 (s, 6H), 7.02 (d, 1H, $J = 2.4$ Hz), 7.34 (dd, 1H, $J_1 = 9.2$ Hz, $J_2 = 2.6$ Hz), 7.92 (d, 1H, $J = 9.2$ Hz), 8.20 (s, 1H), 8.30 (s, 1H). HRMS-ESI (m/z): $[\text{M}+\text{H}^+]$ calcd for $\text{C}_{14}\text{H}_{11}\text{NO}_3$ 242.0812, found, 242.0805. For diacid intermediate: HRMS-ESI (m/z): $[\text{M}+\text{H}^+]$ calcd for $\text{C}_{14}\text{H}_{13}\text{NO}_4$ 258.0772, found, 252.0764.

Allyl N- α -Fmoc-N- β -Boc-L-diaminopropionate (13). Fmoc-Dap(Boc)-OH **12** (450 mg, 1.05 mmol) was dissolved in MeOH (15 mL). Cs_2CO_3 (172 mg, 0.53 mmol) was added and the resulting solution was stirred at room temperature for 10 minutes. The reaction mixture was concentrated under reduced pressure and redissolved in DMF (30 mL). Allyl bromide (280 μL , 3.3 mmol) was added and the mixture was stirred for 1 h at room temperature. The reaction was poured over 2% aqueous NaHCO_3 (150 mL) and

extracted with EtOAc (4 x 50 mL). The combined organic layers were dried with Na₂SO₄ and concentrated under reduced pressure. The resulting brown oily residue was purified by flash column chromatography (1% MeOH/CH₂Cl₂, R_f = 0.6 5%MeOH/CH₂Cl₂) to give the desired product **13** as a white powder (516 mg, 78%). R_f = 0.7 (1% MeOH/CH₂Cl₂). ¹H NMR (400 MHz, CDCl₃, δ): 1.34 (s, 9H), 3.48 (br s, 2H), 4.12 (br s, 1H), 4.2-4.4 (m, 2H), 4.55 (br s, 2H), 4.9 (br s, 1H), 5.14 (d, 1H, J = 10.3 Hz), 5.22 (d, 1H, J = 17.1 Hz), 5.8 (br s, 1H), 5.9 (d, 1H, 5.8 Hz), 7.18 (t, 2H, J = 7.3 Hz), 7.27 (t, 2H, J = 7.4 Hz), 7.5 (br s, 2H), 7.65 (d, 2H, J = 7.4 Hz). ¹³C NMR (400 MHz, CDCl₃, δ): 170.4, 156.6, 156.2, 143.8, 141.4, 131.6, 127.8, 127.2, 125.3, 120.9, 119.1, 80.1, 67.3, 66.5, 55.3, 47.2, 42.2, 28.4. HRMS-ESI (m/z): [M+H⁺] calcd for C₂₆H₃₀N₂O₆ 467.2177, found, 467.2177.

Allyl N- α -Fmoc-N- β -[6-(dimethylamino)-2,3-naphthalimido]-L-diaminopropionate (15). Allyl *N*- α -Fmoc-*N*- β -Boc-*L*-diaminopropionate (175 mg, 0.38 mmol) was dissolved in dry dichloromethane (10 mL), and the solution was cooled to 0 °C. Then, TFA (10 mL) was added dropwise and the resulting mixture was stirred at 0 °C for 30 minutes and then at room temperature for 1 h. The reaction was concentrated under reduced pressure, redissolved in toluene and concentrated again. The resulting amine **14** was placed under vacuum for 20 minutes and dissolved in dry DMF (3 mL). DIEA (360 μ L, 2.6 mmol) and anhydride **11** (100 mg, 0.413 mmol) were added, and the mixture was stirred at room temperature for 30 minutes. HOBt/HBTU (2 mL 0.2 M solution in DMF) were added and the reaction was stirred at room temperature for 12 h. HOBt/HBTU (1 mL 0.2 M solution in DMF, 0.5 eq.) was added and the reaction mixture was allowed to stir for another 1 hour. The crude reaction was added to 150 mL 2% aqueous NaHCO₃ and extracted with EtOAc (3 x 30 mL). The combined organic layers were dried with Na₂SO₄ and

concentrated under reduced pressure. The residue was loaded in 20% EtOAc/toluene onto a silica column and purified (gradient 20 to 50% EtOAc/hexanes) to afford a bright yellow oil (220 mg, > 95% yield, $R_f = 0.8$ 10%MeOH/CH₂Cl₂). ¹H-NMR (400 MHz, CDCl₃, δ): 3.12 (s, 6H), 4.1-4.4 (m, 5H), 4.65-4.83 (m, 3H), 5.26 (d, 1H, $J = 10.3$ Hz), 5.38 (d, 1H, $J = 17.1$ Hz), 5.9-6.1 (m, 1H), 7.20 (dd, 1H, $J_1 = 2.0$ Hz, $J_2 = 9.1$ Hz), 7.29 (c, 2H, $J = 7.4$ Hz), 7.29 (td, 2H, $J_1 = 1.7$ Hz, $J_2 = 5.2$ Hz), 7.57 (d, 1H, $J = 7.6$ Hz), 7.60 (d, 1H, $J = 7.5$ Hz), 7.73 (d, 2H, $J = 7.5$ Hz), 7.80 (d, 1H, $J = 9.1$ Hz), 8.03 (s, 1H), 8.10 (s, 1H). ¹³C-NMR (100 MHz, CDCl₃, δ): 169.9, 168.6, 168.5, 156.0, 150.5, 144.1, 143.8, 141.3, 137.7, 131.5, 128.2, 127.7, 127.4, 127.2, 125.2, 125.5, 123.1, 122.5, 120.0, 119.3, 117.9, 107.9, 67.5, 66.8, 53.8, 47.1, 40.4, 39.2. HRMS-ESI (m/z): [M+H⁺] calcd for C₃₅H₃₁N₃O₆ 590.2291, found, 590.2270.

N- α -Fmoc-N- β -[6-(dimethylamino)-2,3-naphthalimido]-L-diaminopropionic acid (5). Fmoc-Dap(6DMN)-OAllyl, **15** (226 mg, 0.383 mmol) was dissolved in dry CH₂Cl₂ (12 mL). Phenyl silane (1.2 mL, 9.57 mmol) and Pd(PPh₃)₄ (20 mg, 0.014 mmol) were added and the reaction mixture was stirred at room temperature for 30 minutes. The reaction mixture was then directly loaded onto a silica flash column and the desired product purified (gradient, 3% to 5% MeOH/CH₂Cl₂). The desired product was isolated as a yellow solid (210 mg, >95%, $R_f = 0.4$ 10%MeOH/CH₂Cl₂). MS-ESI: m/z : 550.1 (MH⁺), 572 (MNa⁺), 1099.3 (M₂H⁺). HRMS-ESI (m/z): [M+H⁺] calcd for C₃₂H₂₇N₃O₆ 550.1973, found, 550.1982.

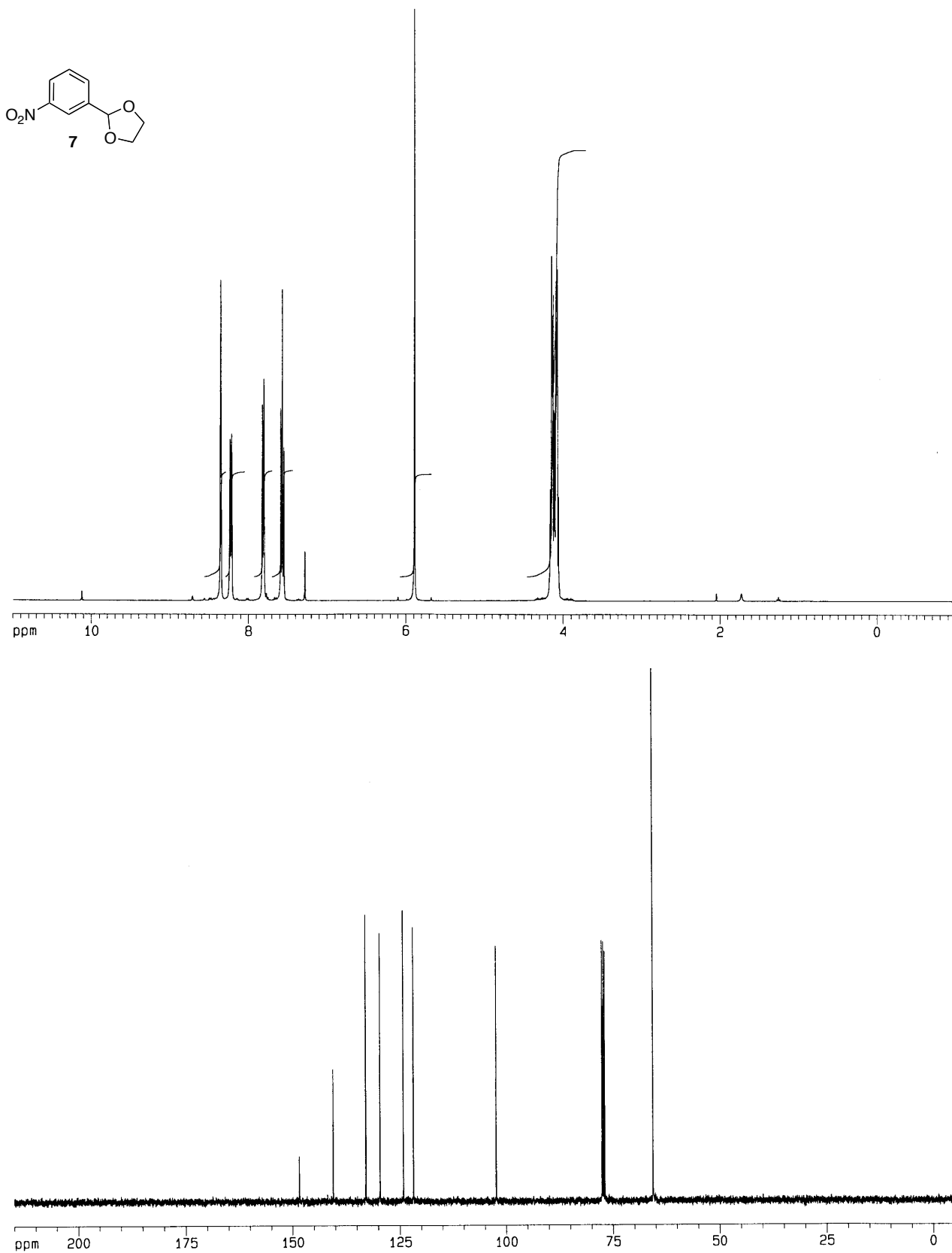
Methyl 2-[6-(dimethylamino)-2,3-naphthalimido] acetate (4). Glycine methyl ester hydrochloride (21 mg, 0.164 mmol) was dissolved in DMF (2 mL), and DIEA (450 μ L, 3.28 mmol, 10 eq.) and anhydride **11** (80 mg, 0.328 mmol) were added. The resulting

mixture was stirred at room temperature for 10 minutes. HOBt/HBTU (1 mL, 0.2 M in DMF) was added and the reaction mixture was stirred for 12 h. Then for an additional 0.5 mL of the HOBt/HBTU mixture were added, and the reaction was stirred for another hour. The reaction was poured over 100 mL 1% aqueous HCl and extracted with EtOAc (2 x 50 mL). The combined organic layers were dried with Na₂SO₄ and concentrated under reduced pressure. The solid residue was purified by flash column chromatography (EtOAc/hexanes 35%) to afford the desired product as a bright yellow solid in quantitative yield ($R_f = 0.5$ 40%EtOAc/hexanes). ¹H-NMR (400 MHz, CDCl₃, δ): 3.12 (s, 6H), 3.76 (s, 3H), 4.47 (s, 2H), 6.96 (d, 1H, $J = 2.0$ Hz), 7.21 (dd, 1H, $J_1 = 9.1$ Hz, $J_2 = 2.5$ Hz), 7.81 (d, 1H, $J = 9.1$ Hz), 8.06 (s, 1H), 8.13 (s, 1H). ¹³C-NMR (100 MHz, CDCl₃, δ): 168.1, 167.9, 167.8, 150.6, 137.8, 131.4, 128.5, 127.5, 125.2, 123.1, 122.8, 117.9, 107.9, 52.8, 40.4, 38.9. HRMS-ESI (m/z): $[M+H^+]$ calcd for C₁₇H₁₆N₂O₄ 313.1188, found, 313.1201. UV: ϵ (388 nm, M⁻¹ cm⁻¹) = 7980.

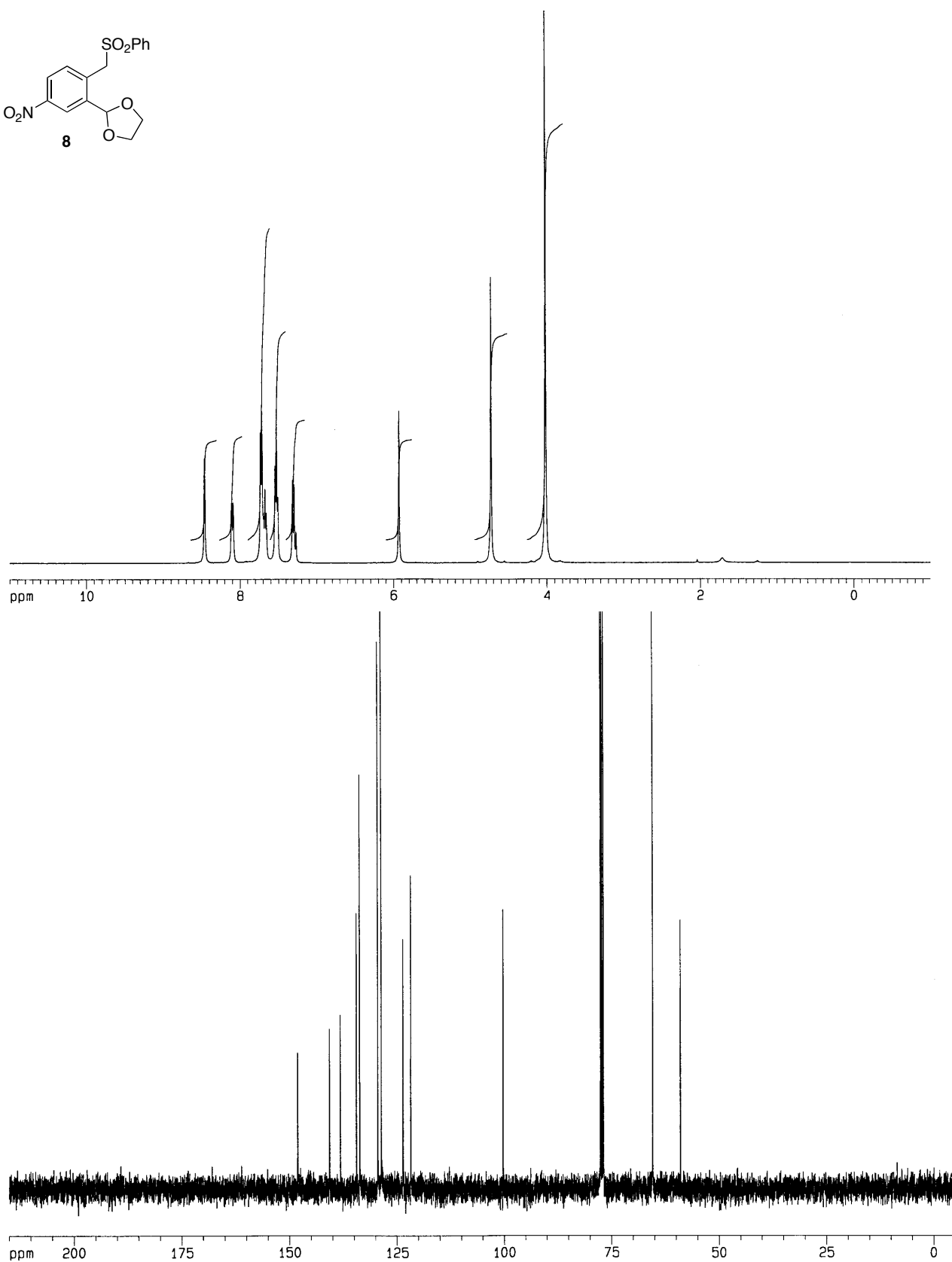
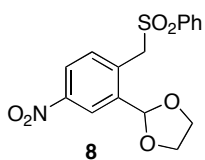
GST-Abl SH2, GST-Src SH2, GST-Crk SH2 and GST-PI3K C-terminal SH2 expression and purification. Src and PI3K SH2 domains were expressed and purified as GST fusion following reported procedures.² In short, plasmids including GST-fusion proteins were transformed into bacteria (E. coli DH5 α chemically competent cells, Invitrogen). Bacteria were grown to mid log phase, induced at 37 °C for 3-4 hours with isopropyl-1-thio- β -D-galactopyranoside, and lysed by treatment with lysozyme (1 mg/mL, 30 minutes) followed by sonication (PBS buffer pH 7.4 with 100 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM DTT, 0.1 mM AEBSF, 30 μ g/mL Leupeptin and 0.5 μ g/mL Pepstatin A). The lysates were clarified by centrifugation, and fusion proteins were purified by binding to glutathione-agarose beads (Amersham Biosciences). Proteins

were eluted from the beads (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8), and finally concentrated by centrifugation through a cellulose membrane with a 10,000 Da cutoff (Millipore). Proteins were quantified using the Micro BCA protein Assay Kit (Pierce) relative to a BSA standard. All fusion proteins were analyzed by Coomassie staining and Western blot analysis with anti-GST antibodies.

5-nitrophenyl)-1,3-dioxolane (7).



2-(2-Phenylsulfonylmethyl-5-nitrophenyl)-1,3-dioxolane (8)



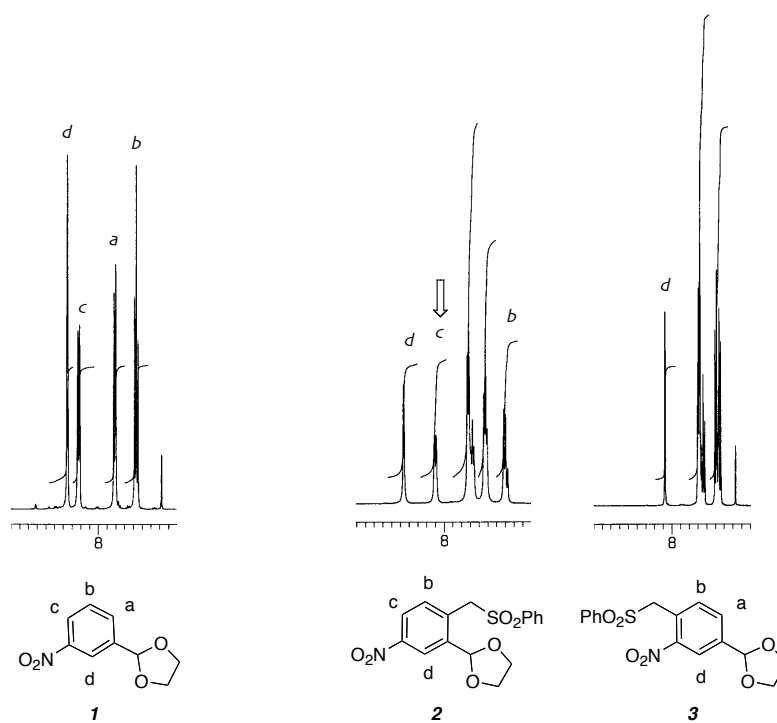
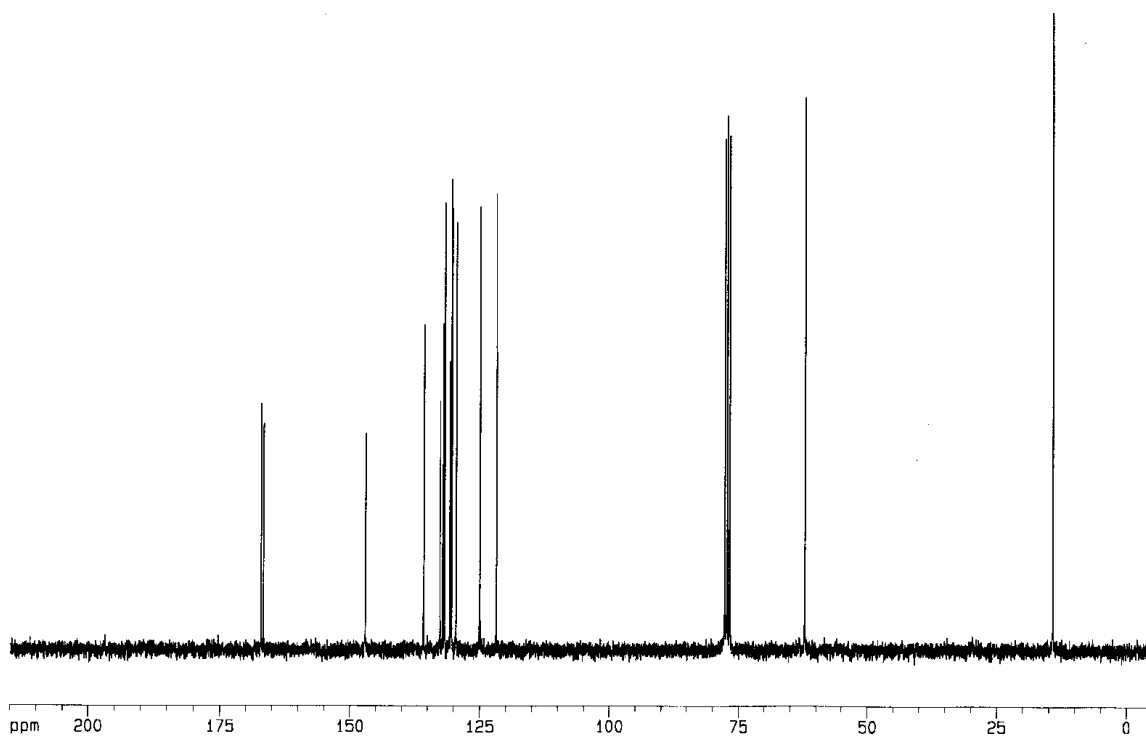
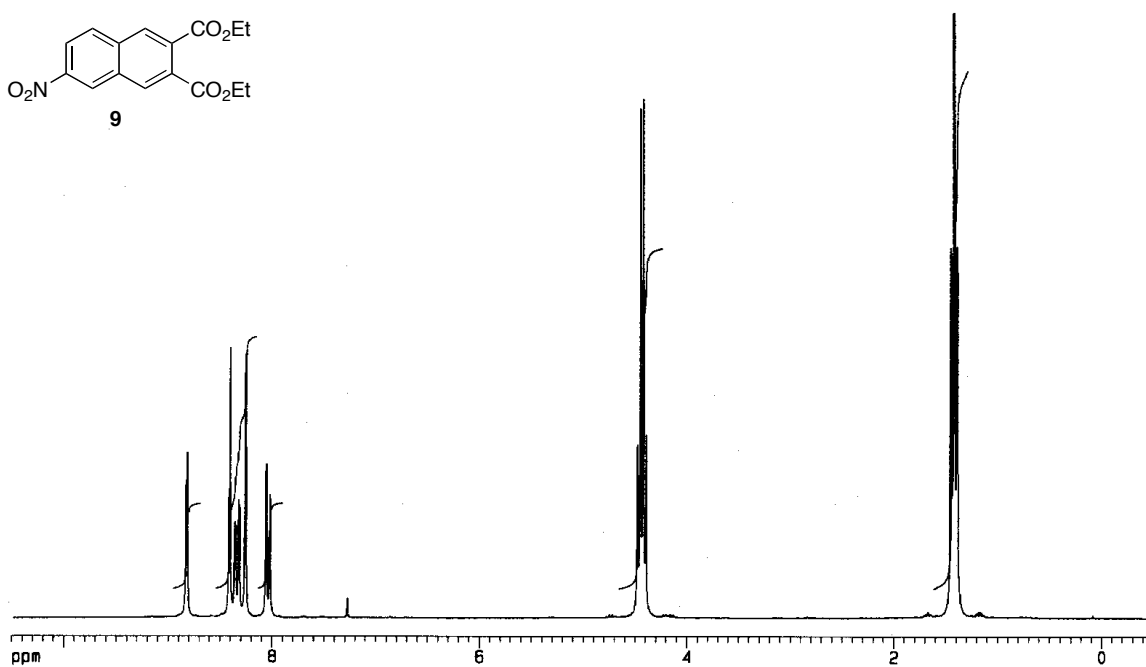
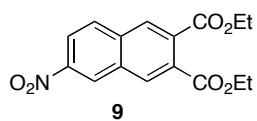
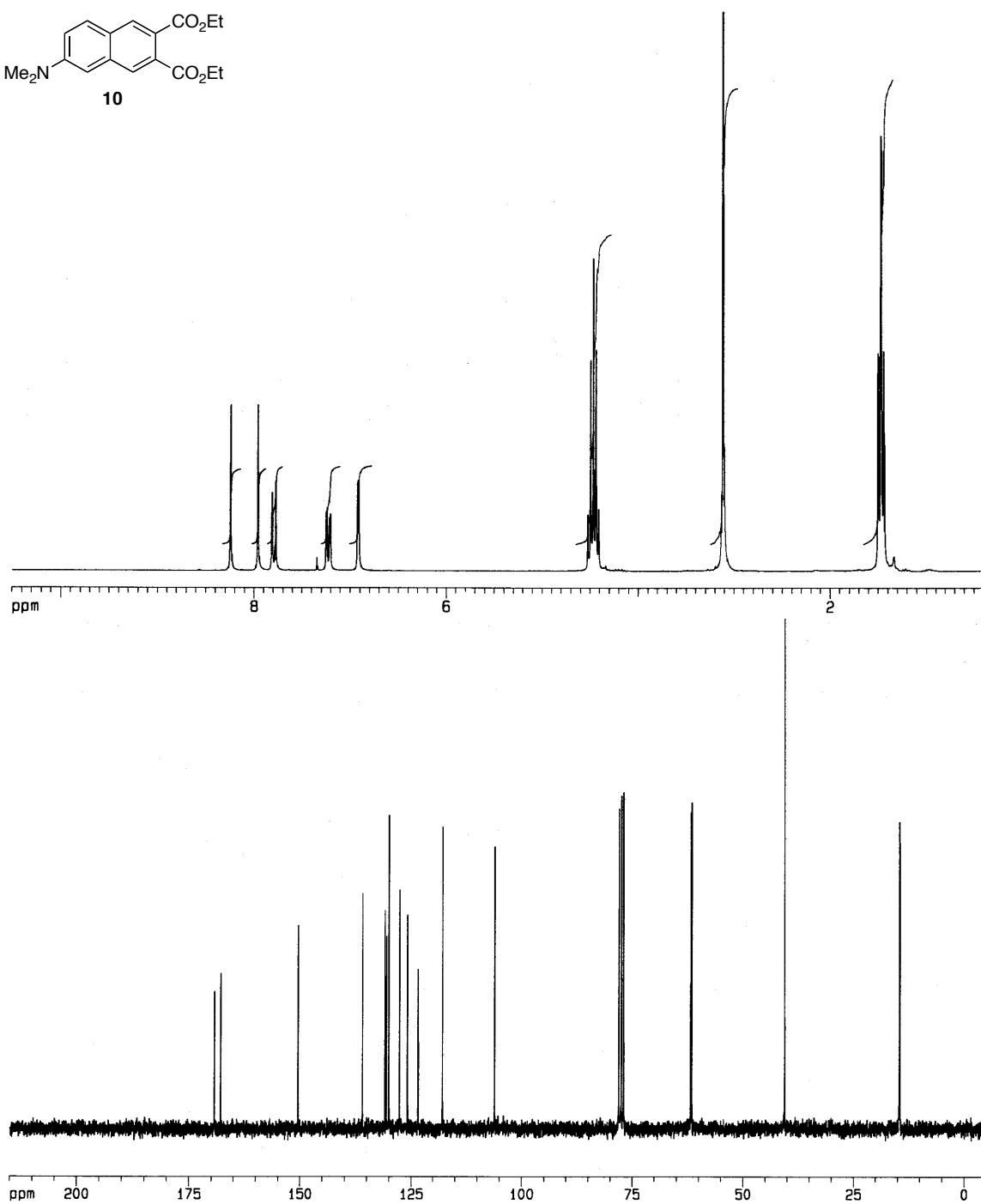


Figure 1. Detail of the aromatic region of the ^1H -NMR spectra of compounds 1, 2 and 3. Signal corresponding to proton c is not present in the spectra of product 3, which allows the identification of each isomer resulting from the reaction.

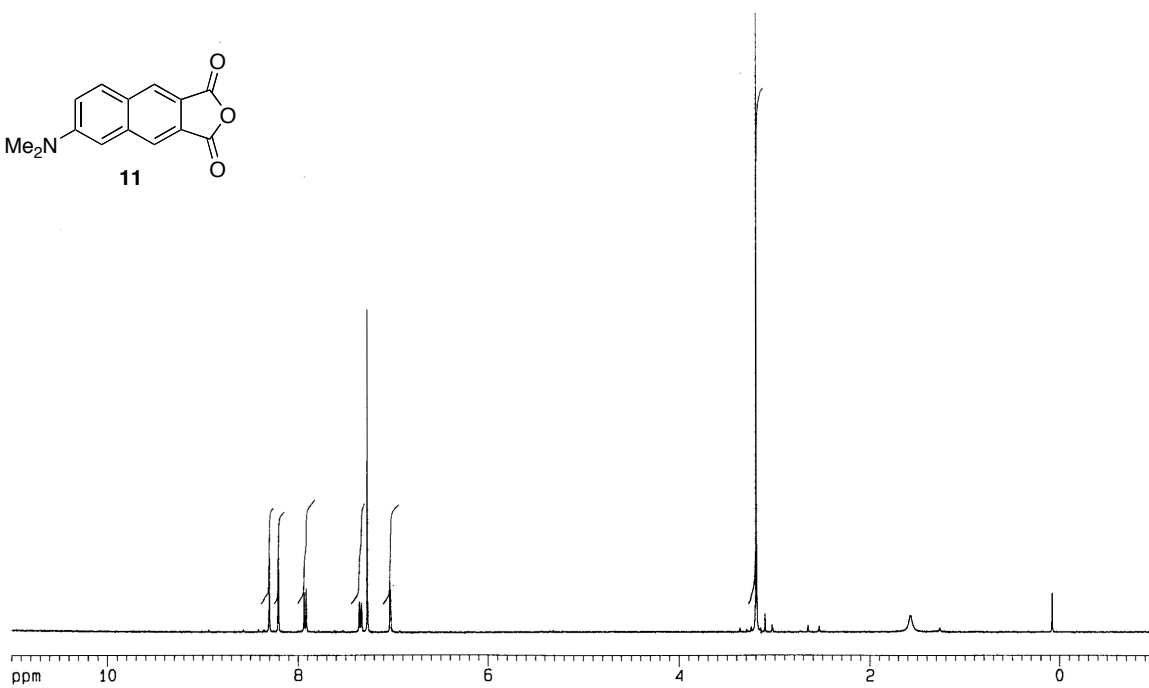
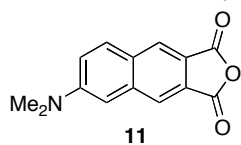
Diethyl 6-nitro-2,3-naphthalenedicarboxylate (9)



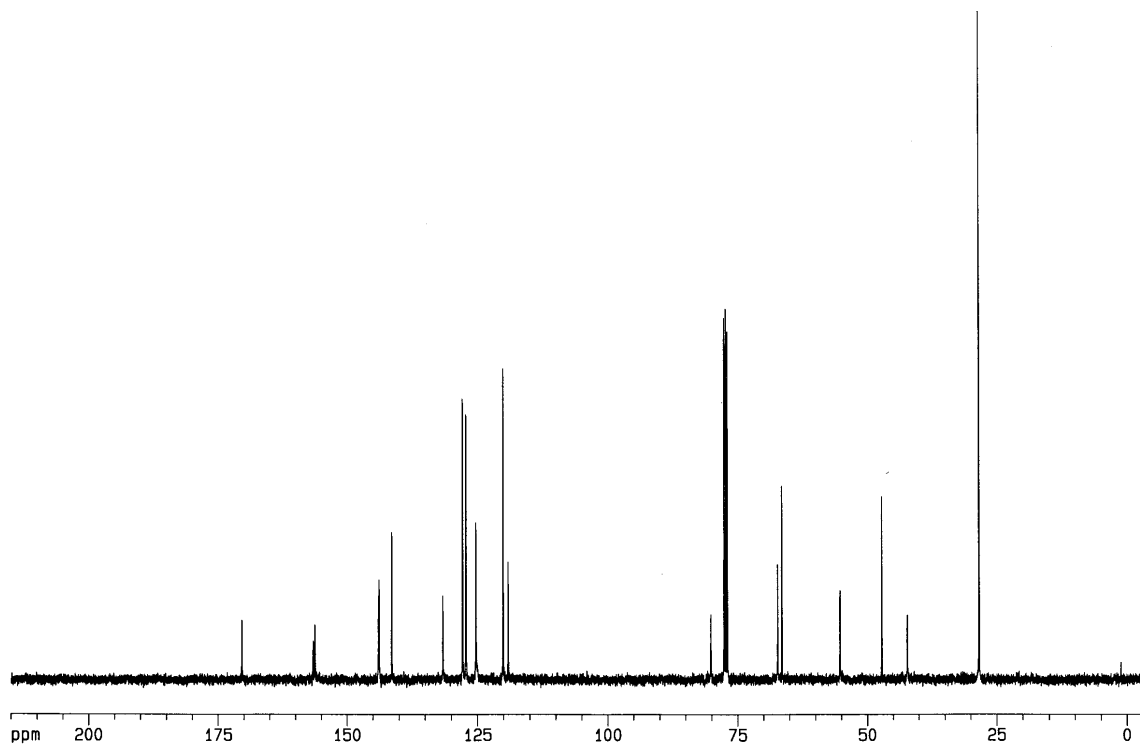
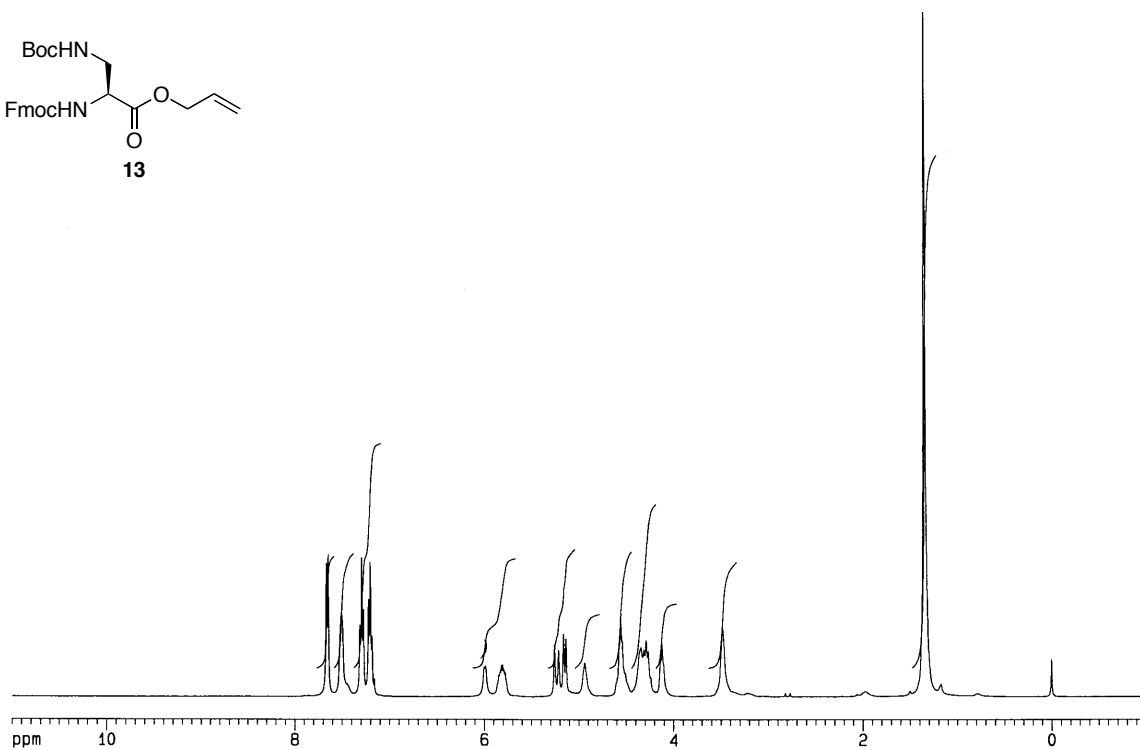
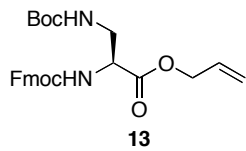

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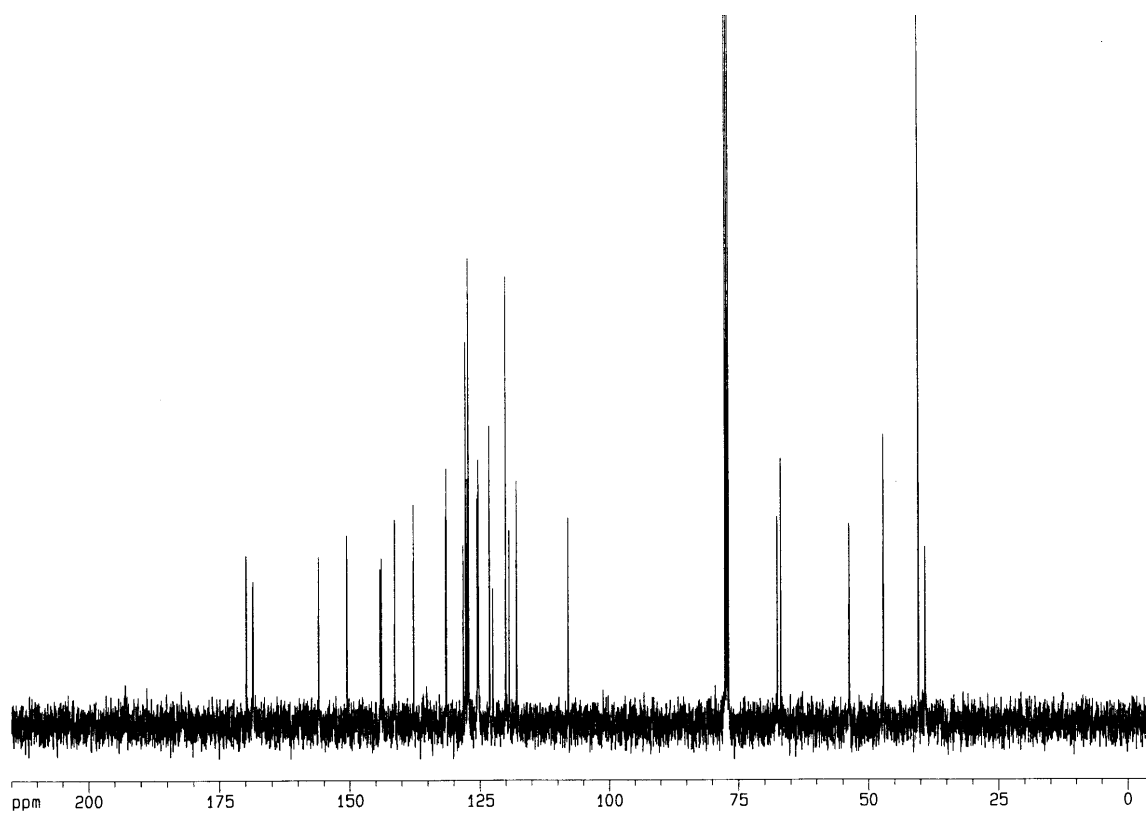
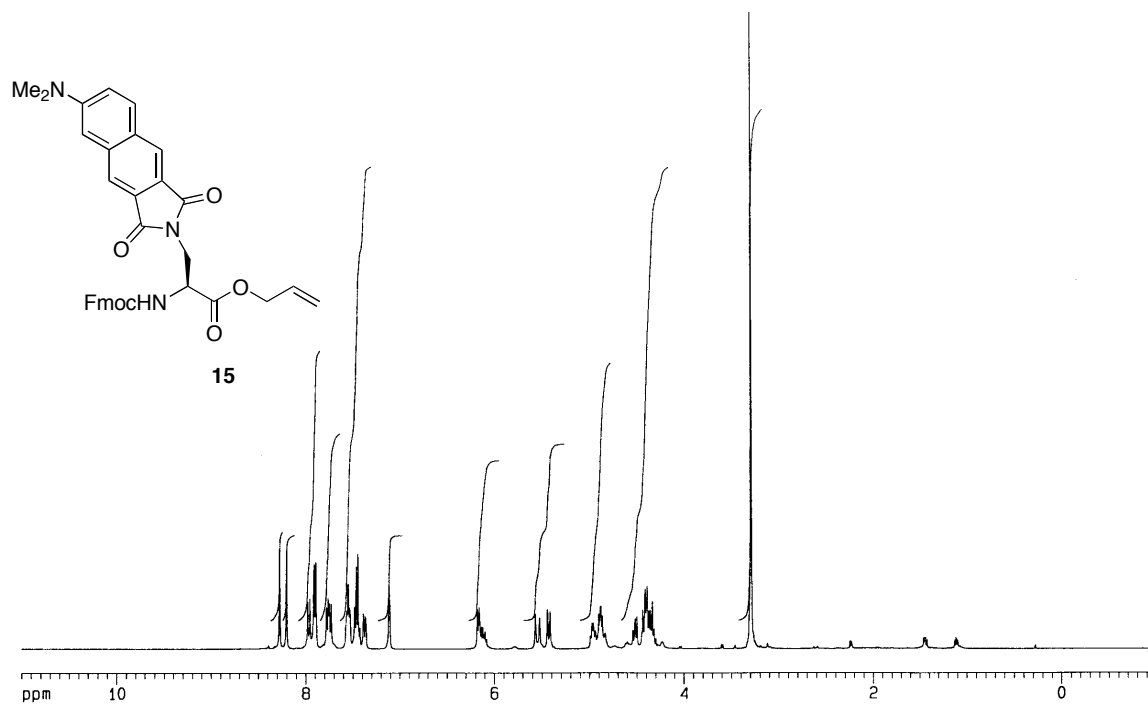
6-(dimethylamino)-2,3-naphthalenedicarboxylic anhydride (11)



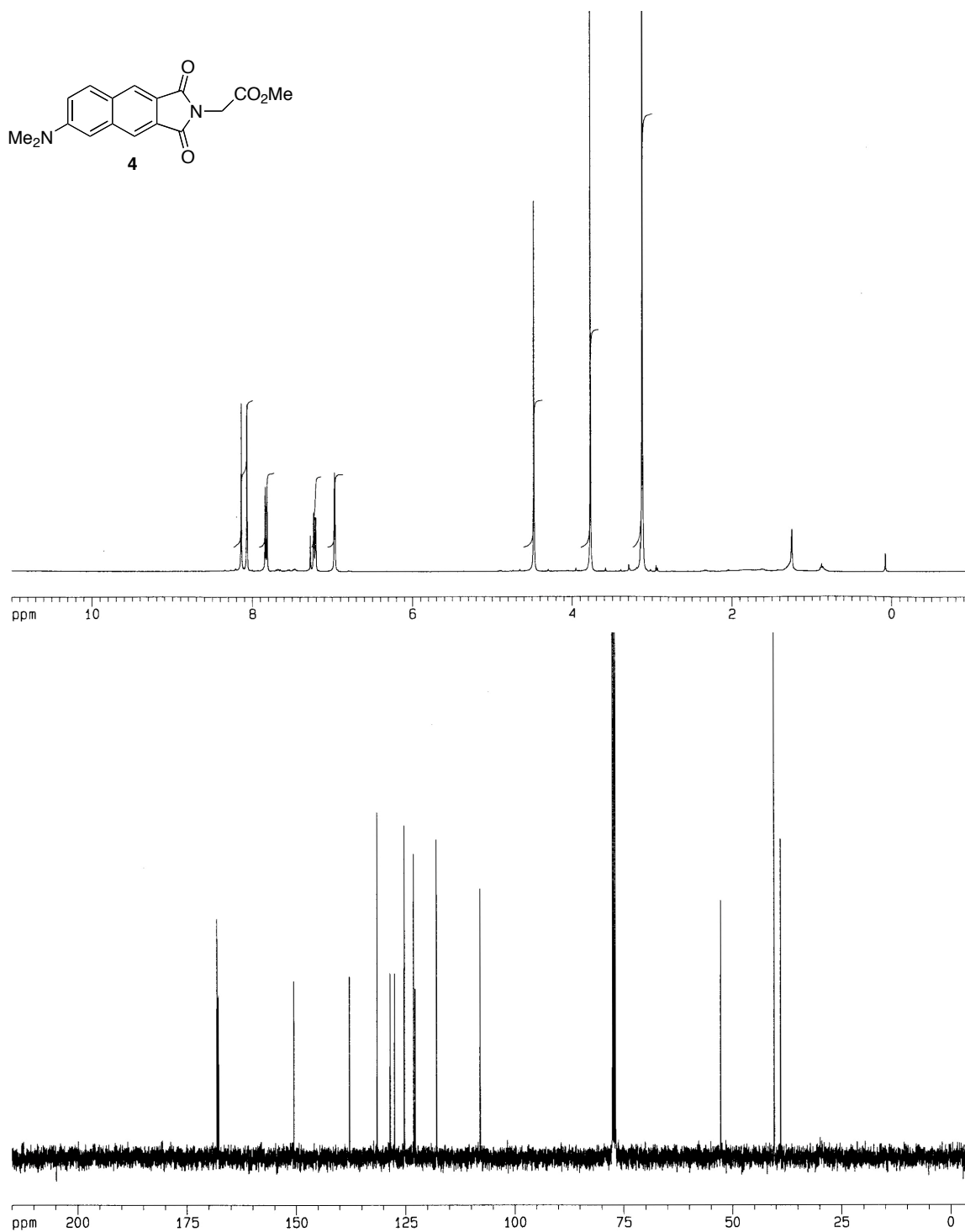
allyl N- α -Fmoc-N- β -Boc-L-diaminopropionate (13).



allyl N- α -Fmoc-N- β -[6-(dimethylamino)-2,3-naphthalimido]-L-diaminopropionate (15).



Methyl 2-[6-(dimethylamino)-2,3-naphthalimido] acetate (4).



UV spectroscopy.

Absorption spectra did not show a strong sensitivity to solvent polarity. Increasing the polarity from toluene (max. absorption 372 nm) to methanol (max. absorption 380 nm) only induced a small red shift of 8 nm. Solvents of intermediate polarity exhibited absorption maxima within that region (i.e. *N,N*-dimethyl formamide 380 nm, dichloromethane 376 nm, tetrahydrofuran 370 nm).

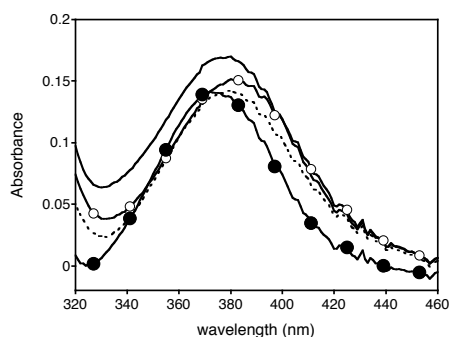


Figure 2. Representative UV spectra of compound 4 in different solvents: methanol (dashed line), dichloromethane, *N,N*-dimethyl formamide (open circles) and toluene (closed circles).

Quantum yield measurements.

Quantum yields (Φ) were measured by comparison with a standard, which emits in a similar region to the test sample. From the available standard compounds we quinine sulfate in 0.1 M H_2SO_4 ($\Phi = 0.54$) was selected.³ Absorbance of the measured solutions did not exceed 0.1 at and above the excitation wavelength to avoid non-linear effects.

UV absorbance at the excitation wavelength (375 nm), and fluorescence emission spectra of a series of solutions of the standard and compound x were recorded. Solutions were prepared with different absorbances, ranging from 0 (solvent blank) to 0.1. A series

of graphs of integrated fluorescence vs absorbance were obtained. The data were fitted to a linear equation with gradient m , and intercept = 0. See Figure 3 as an example of the plots obtained.

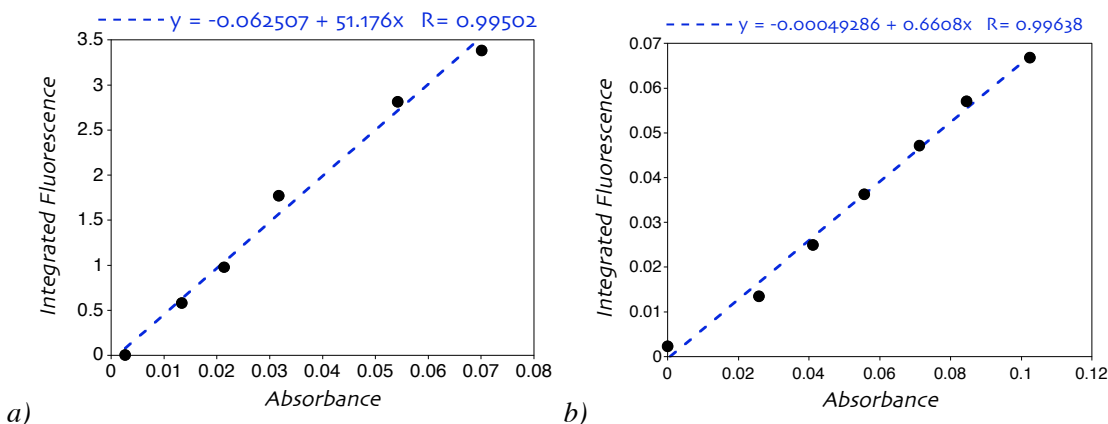


Figure 3. a) Plot of integrated fluorescence vs absorbance at 375 nm for quinine sulfate in 0.1 M H₂SO₄. The gradient m is proportional to the quantum yield of the standard. b) Plot of integrated fluorescence vs absorbance at 375 nm of 6-N,N-dimethylamino Naphthalene-containing peptide (Abl-bp) in 10 mM phosphate buffer, pH 7.5. The gradient m is proportional to the quantum yield.

The gradients of graphs in Figure 3 are proportional to the quantum yield of the different samples. Absolute values were calculated using the standard sample, which has a fixed and known fluorescence quantum yield value, according to the following equation:

$$\Phi_x = \Phi_{std} \left(\frac{grad_x}{grad_{std}} \right) \left(\frac{\eta_{std}^2}{\eta_x^2} \right) \quad (1)$$

Where the subscripts *std* and *x* denote standard and test respectively. The value Φ is the fluorescence quantum yield, *grad* is the gradient m from the plot of integrated fluorescence intensity vs absorbance, and η the refractive index of the solvent.

Fluorescence titrations.

Previous experiments using DANA as reporter amino acid in different SH2-binding peptides showed that the (-)2 position, relative to the phosphotyrosine, was the only position where some fluorescence emission intensity changes were observed. Introduction of DANA in -1, +2 or +6 did not induce any fluorescence changes upon binding to the corresponding SH2 domains (Table 1).

peptide	-5	-4	-3	-2	-1	pTyr	+1	+2	+3	+4	+5	+6	+7	change
FerTyrK/1	K	I	L	K	Q	pY	D	H	P	N	I	Φ	I	NO
FerTyrK/2	K	I	L	K	N	pY	D	Φ	P	N	I	Y	I	NO
S. Abl					Φ	pY	E	N	V					NO
S. Crk					Φ	pY	D	H	P					NO
Pax118-1	E	E	E	Φ	V	pY	S	F	P	N				YES
Pax118-2	E	E	E	H	V	pY	S	Φ	P	N				NO
Pax31-1	E	E	E	T	P	pY	S	Φ	P	N				NO
Pax31-2	T	E	E	Φ	P	pY	S	Y	P	T				YES

Table 1. Peptide sequences tested for fluorescence emission increase upon SH2 binding. FerTyrK corresponds to peptides with Fer Tyrosine Kinase binding sequences, S. Abl and S. Crk are the minimal consensus sequences obtained by combinatorial library screening by Songyang et. al. for Abl and Crk binding. Pax118 and Pax31 correspond to peptides derived from the 118 and 31 phosphorylation sites in the protein Paxillin. Only Pax118-1 and Pax31-2 containing DANA (Φ) at position (-)2 showed an increase in fluorescence upon SH2 complexation. All experiments were performed by adding the corresponding SH2 to 5 μM peptide solutions.

Saturation of Pax118-1 with Abl or Crk SH2 induced a modest increase in the emission intensity (about two-fold), and a very small blue shift of about 10 nm with almost no selectivity in the signal (Figure 4a). Pax31-2 showed some selectivity against Abl, but in

this case the fluorescence increase and the emission band shift was even smaller (Figure 4b).

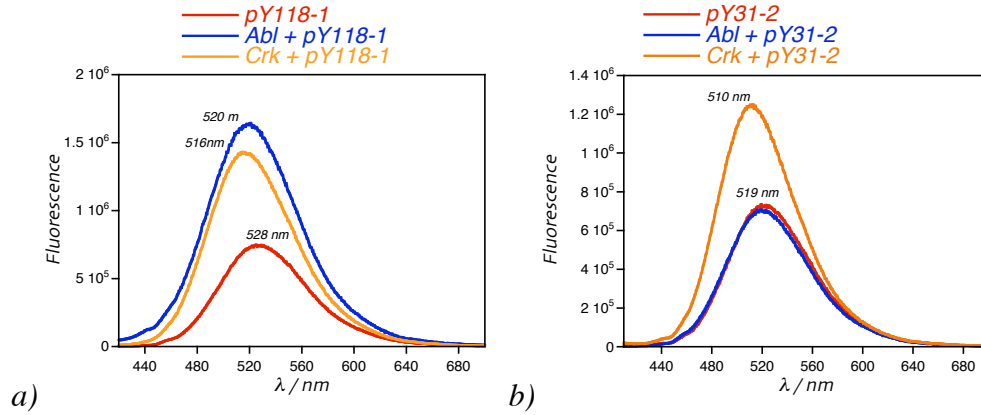


Figure 4. Fluorescence emission spectra of DANA peptides in the presence of Abl SH2 and Crk SH2. a) Peptide Pax118-1, b) Peptide Pax31-2 (See table 1 for sequences). Peptides were 5 μ M in phosphate buffer pH 7.5.

Fluorescence titration of **Crk-bp**, **Crk-bp2** and **Abl-bp** peptides with non-target SH2 domains showed only small changes in fluorescence intensity in most cases (see article for discussion).

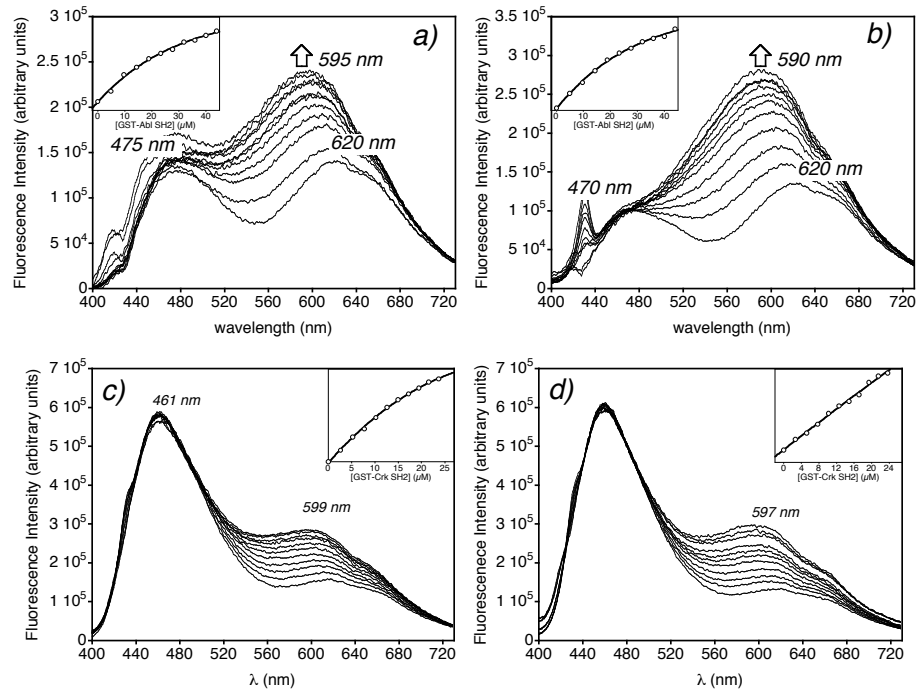


Figure 5. Fluorescence titrations of peptides with non-target SH2 domains. a) *Crk-bp* + GST-AblSH2, b) *Crk-bp2* + GST-Abl SH2, c) *Abl-bp* + GST-Crk SH2, d) *Abl-bp2* + GST-Crk SH2.

Expression of GST-Crk SH2.

Human Crk SH2 domain was expressed as a GST fusion protein transformed into *E. coli* NB42 strain. Expression of SH2 domains was carried out following reported conditions:

- The culture was inoculated into prewarmed LB media with carbenicilin 50 $\mu\text{g/mL}$.
- The sample was then shaken at 37 °C for at least 2 h (passed semi-logarithmic growth $A_{600} = 0.5$),
- IPTG was added to 0.1 mM.
- The sample was shaken at 25 °C for four hours. Using lower temperature than previously reported conditions minimizes inclusion bodies formation.
- Then the sample was centrifuged (5000 g for 30 min at 4 °C) and the pellet was resuspended in ice-cold *lysis buffer* (see below).
- The slurry was then sonicated until the cells were disrupted (20 sec sonication and 30 seconds in ice for 4 times). Note: it was observed that treatment of the resuspended bacteria with lysozyme (1 mg/mL) for 30 min at 4 °C improved the lysis process and resulted in higher yields.

Lysis buffer: PBS with 100 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM DTT, AEBSF, leupeptin and PepA. (add 100 μL AEBSF + 25 μL Leupeptin + 12.5 μL Pep A + to 10 mL total buffer).

Purification:

- The sample was recentrifuged to remove particulate debris (5000 g for 30 min at 4 °C). Then the supernatant was incubated for at least 1 hour with 10 mL of glutathione Sepharose 4 Fast Flow (Amersham Biosciences)
- The column was equilibrated with 5 volumes of binding buffer at 4 °C.
- The sample was then loaded using a syringe and low flow rate (0.2 to 0.5 mL/min) at 4 °C.
- The column was then washed with 5 volumes of binding buffer (higher rate of 1 mL/min) room temperature.
- The GST fusion protein was eluted with elution buffer at room temperature.

Binding buffer: PBS pH 7.4 (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM NaHPO₄).

Elution buffer: 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.

The pooled fractions were then collected and analyzed using gel electrophoresis to confirm purity using 10% SDS-PAGE.

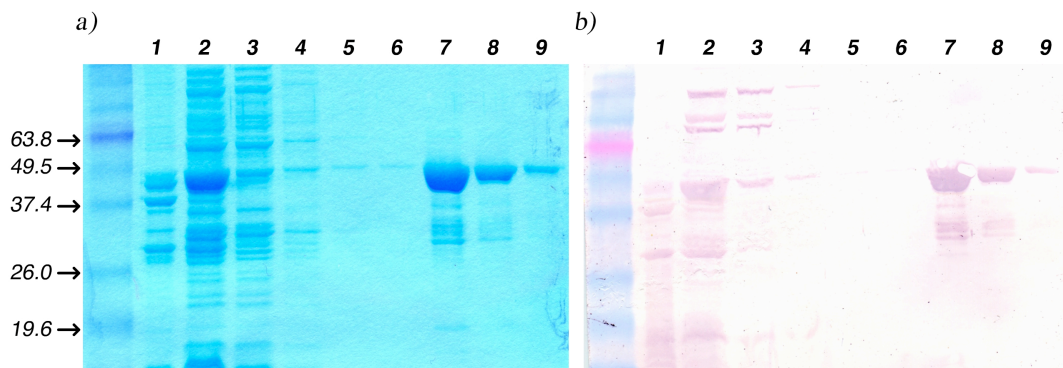


Figure 6: 10% SDS-PAGE of GST-Crk SH2 fusion protein expression. a) Coomassie staining of the gel, b) Corresponding Western Blot using anti-GST antibody. Lane 1: cell pellet; 2: supernatant after lysis; 3: column flow through; 4 to 6 column washes with PBS pH 7.4; 7 to 9 protein elution using reduced glutathione.

Western Blot:

The gel was transferred to a nitrocellulose membrane (1h, 100 V), and probed using the standard procedure:

- Block overnight with TBST + powder milk.
- Wash with TBST (2 x 10 mL x 10 min.).
- Incubate with primary antibody (anti-GST, dilution 1:10,000 in TBST) for 30 min.
- Wash with TBST (3 x 10 mL x 10 min.).
- Incubate for 30 min with secondary antibody (anti-goat conjugated with alkaline phosphatase, 1:1,000 dilution in TBS).
- Wash with TBST (2 x 10 mL x 10 min.). Wash with TBS (1 x 10 mL x 10 min.).
- Add One Step NBT/BCIP and incubate until the bands appear. Quench the reaction with ddH₂O.

Once the protein was isolated, it was separated from the reduced glutathione using the Ultrafree-15 centrifugal filter (Biomax-5). The stock solution of Crk-SH2-GST was concentrated at 2000 g until the volume was approximately 2 mL. Phosphate buffer (10 mM phosphate, pH 7.5 100 mM NaCl) was added to a final volume of 15 mL and centrifuged again to a final volume of 2 mL. This procedure was repeated three times to ensure that the concentration of the contaminant solutes was minimal. The final volume was 1.2 mL.

Bio-Rad Protein Assay was used to quantify the protein isolated. Followed the standard procedure:

Prepare dye reagent diluting 1 part Dye Reagent Concentrate with 4 parts distilled water.

Prepare stock BSA dilutions corresponding to 0.1, 0.2, 0.3, 0.4, 0.5 0.6, 0.7, 0.8 and 0.9 mg/mL

Add 20 μ L of each stock dilution to 980 μ L dye reagent

Incubate at room temperature for 5 min and measure absorbance at 595 nm (use blank of 20 μ L of distilled water and 980 μ L dye reagent prepared at the same time as the samples)

Plot the observed absorbance against BSA concentration:

Three samples of increasing concentration of Crk-SH2-GST stock solution were prepared. Then, 10, 15 and 20 μ L of stock solution with water up to 20 μ L as necessary were added to 980 μ L dye reagent. Absorbance measurements of these solutions in comparison with the standard curve afforded a final concentration of 11 mg/mL of Crk-SH2-GST.

Expression of GST-Abl SH2, GST-Src SH2 and GST-PI3K SH2

Abl-SH2 was received as a GST fusion in a pGEX-KT vector, amp-resistant, IPTG-inducible. The SH2 domain was cloned using BamH1 (5') EcoR1 (3') sites.

The expression of GST-Abl SH2 GST-Src SH2 and GST-PI3K SH2 was carried out using the same protocol and conditions as the GST-Crk SH2 mutant, but for these constructs it was found that expression at 37 °C resulted in increased yields of protein with no inclusion body formation.

¹ Hancock, W. S.; Battersby, J. E. *Anal. Biochem.* **1976**, *71*, 260-264.

² Smith, D. B.; Johnson, K. S. *Gene*, **1988**, *67*, 31-40.

³ Melhuish, W. B. *J. Phys. Chem.*, **1961**, *65*, 229-235