

Supporting Information for:

A Highly Selective Fluorescent Probe for Thiol Bioimaging

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General methods

Materials

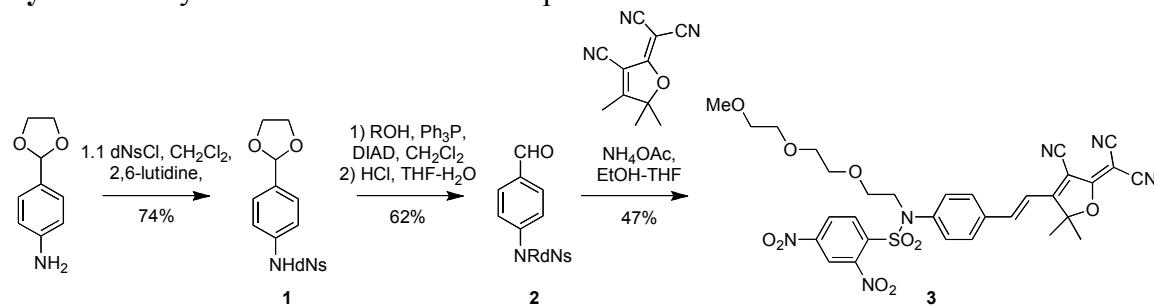
All solvents were of ACS reagent grade or better unless otherwise noted. Anhydrous dichloromethane was dried on a solvent column purification system. Silica gel (40-63 μ m) was obtained from SilliCycle Inc. All other reagents, compounds and chemicals were obtained from commercial suppliers and were used without further purification. Aqueous solutions were freshly prepared with deionized water from a Barnsted Nanopure four cartridge system. Human serum albumin (average molar mass of 69000 g mol⁻¹) was purchased from Sigma. Hydrogen peroxide (H₂O₂), *tert*-butyl hydroperoxide (TBHP),

and sodium hypochlorite (NaOCl) were delivered from 3%, 70%, and 10% aqueous solutions, respectively. Superoxide ($O_2^{-\cdot}$) was added as solid KO_2 .

General methods, instrumentation and measurements

Synthetic manipulations that required an inert atmosphere (where noted) were carried out under nitrogen or argon using standard Schlenk techniques or in an inert-atmosphere glovebox. NMR (^1H and ^{13}C) spectra were recorded on 300 and 500 MHz spectrometers. The chemical shift data for each signal are given in units of δ (ppm) relative to tetramethylsilane (TMS) where δ (TMS) = 0, and referenced to the residual solvent resonances. Splitting patterns are denoted as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). High-resolution mass spectra (HR-MS) were obtained at the MIT Department of Chemistry Instrumentation Facility using electron impact ionization (EI) with a voltage of 70 V or electrospray ionization (ESI). Melting point (m.p.) determination was performed using open capillaries and are reported uncorrected. Absorption spectra were obtained on a Varian Cary 50-Bio UV-visible spectrophotometer (Palo Alto, CA). Fluorescence data were collected on a Horiba Jobin Yvon Fluorolog-3 spectrofluorimeter (Edison, NJ). The spectra in solution were obtained at 25 °C using a quartz cuvette with a path length of 1 cm. The fluorescence quantum yield (Φ_F) of free dye **4** was determined against cresyl violet (Φ_F = 0.54 in methanol).

Synthesis. Synthesis of fluorescent thiol probe 3.



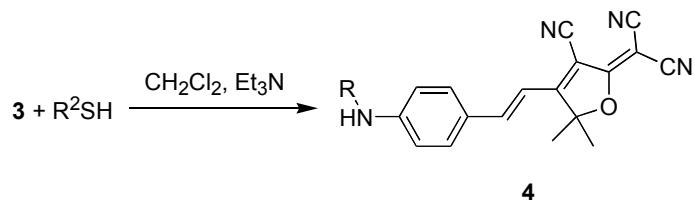
N-(4-(1,3-dioxolan-2-yl)phenyl)-2,4-dinitrobenzenesulfonamide (1). To a stirred solution of 4-(1,3-dioxolan-2-yl)anilineⁱ (165 mg, 1.0 mmol) and 2,6-lutidine (0.15 mL, 1.3 mmol) in dichloromethane (4 mL) at 0°C is added 2,4-dinitrobenzenesulfonyl chloride (294 mg, 1.1 mmol) by portions. The resulting red mixture is allowed to stir overnight as it slowly warms up to room temperature. The reaction is quenched and

neutralized by adding a minimum amount of 2 M HCl, and the product is extracted with dichloromethane. The combined organic layers are washed with anhydrous magnesium sulfate, filtered and the solvent is evaporated under reduced pressure. The crude product is purified by column chromatography on silica gel with 1:1:1 hexanes : dichloromethane : ethyl acetate (v/v/v) to afford **1** as a yellow solid (293 mg, 74%). Analytical samples may be obtained after recrystallization from heptane-PhMe or CHCl₃. m. p. = 140-143°C; ¹H-NMR (500 MHz, (CD₃)₂CO): δ = 9.55 (s, 1H), 8.80 (d, 1H, *J* = 2.1 Hz), 8.60 (dd, 1H, *J* = 8.6, 2.2 Hz), 8.28 (d, 1H, *J* = 8.7 Hz), 7.42 (d, 2H, *J* = 8.5 Hz), 7.36 (d, 2H, *J* = 9.0 Hz), 5.67 (s, 1H), 4.00 (m, 4H); ¹³C-NMR (125 MHz, (CD₃)₂CO): δ = 156.3, 151.5, 149.4, 137.8, 137.4, 133.8, 128.8, 127.9, 122.7, 121.5, 103.8, 66.0; HR-MS (ESI): calcd. for C₁₅H₁₃N₃O₈S [M+H]⁺ 396.0496, found 396.0504.

***N*-(4-formylphenyl)-*N*-(2-(2-methoxyethoxy)ethoxy)ethyl)-2,4-dinitrobenzenesulfonamide (**2**).** To a Schlenk flask are added acetal **1** (198 mg, 0.50 mmol), triphenylphosphine (262 mg, 1.00 mmol) and triethyleneglycol monomethyl ether (0.16 mL / 164 mg, 1.00 mmol). Contents of the Schlenk flask are evacuated and back-filled with argon three times. Dry dichloromethane (3 mL) is added and the resulting solution is cooled to 0°C in an ice-water bath. To the solution is added diisopropyl azodicarboxylate (DIAD, 0.2 mL / 202 mg, 1.00 mmol) dropwise via syringe, and the contents are stirred overnight as they slowly warm up to room temperature. The mixture is then directly subjected to column chromatography on silica gel with 1:1:1 hexanes : dichloromethane : ethyl acetate (v/v/v) as the mobile phase to yield fractions containing the crude *N*-alkylated acetal and triphenylphosphine oxide. Without further purification, after evaporation of the solvent from the chromatography, the crude alkylated intermediate is redissolved in ca. 5 mL of THF and the solution is cooled to 0°C. To the stirred solution is added 1 mL of 2 M HCl and the solution is stirred overnight as it slowly warms up to room temperature. The resulting mixture is neutralized with sat. NaHCO₃, extracted with Et₂O, washed with brine, dried over anhydrous MgSO₄ and the solvents are evaporated under reduced pressure. Purification by column chromatography on silica gel using progressively more polar 9:1 to 7:3 dichloromethane : ethyl acetate (v/v) as the mobile phase affords the aldehyde **2** as a viscous pale yellow oil

(154 mg, 62% over 2 steps). $^1\text{H-NMR}$ (500 MHz, CDCl_3): δ = 10.03 (s, 1H) 8.47 (d, 1H, J = 2.5 Hz). 8.35 (dd, 1H, J = 8.5, 2.5 Hz), 7.93 (d, 1H, J = 8.5 Hz), 7.88 (d, 2H, J = 8.5 Hz), 7.49 (d, 2H, J = 8.5 Hz), 4.06 (t, 2H, J = 5.5 Hz), 3.62-3-57 (m, 8H), 3.55-3.53 (m, 2H), 3.37 (s, 3H); $^{13}\text{C-NMR}$ (125 MHz, CDCl_3): δ = 191.0, 150.0, 143.1, 137.8, 136.2, 133.7, 131.0, 130.3, 126.1, 119.8, 72.1, 70.8, 70.7, 70.5, 68.7, 59.2, 52.4; HR-MS (ESI): calcd. for $\text{C}_{20}\text{H}_{23}\text{N}_3\text{O}_{10}\text{S} [\text{M}+\text{Na}]^+$ 520.0996, found 520.1000.

(E)-N-(4-(2-(4-cyano-5-(dicyanomethylene)-2,2-dimethyl-2,5-dihydrofuran-3-yl)vinyl)phenyl)-N-(2-(2-methoxyethoxy)ethoxyethyl)-2,4-dinitrobenzenesulfonamide (3) Aldehyde **2** (145 mg, 0.29 mmol), 2-(3-cyano-4,5,5-trimethylfuran-2(5H)-ylidene)propanedinitrileⁱⁱ (TCF, 60 mg, 0.30 mmol) and ammonium acetate (23 mg, 0.30 mg) were stirred overnight in the dark at room temperature in a mixture of THF (1.0 mL) and EtOH (0.25 mL). The solution rapidly turns from pale yellow to reddish-orange. The mixture is diluted in water, extracted with ethyl acetate, washed with brine and dried over anh. MgSO_4 . Evaporation of the solvents under reduced pressure yields a crude product that is purified by column chromatography on silica gel using progressively more polar 2:1 to 3:1 ethyl acetate : hexanes as the mobile phase to afford the thiol-reactive probe **3** as an amorphous yellow solid (91 mg, 47%). m. p. = 74-76°C (softens)ⁱⁱⁱ; $^1\text{H-NMR}$ (500 MHz, $(\text{CD}_3)_2\text{CO}$): δ = 8.79 (d, 1H, J = 2.0 Hz), 8.60 (dd, 1H, J = 8.5, 2.0 Hz), 8.22 (d, 1H, J = 8.5 Hz), 8.03 (d, 1H, J = 16.5 Hz), 7.95 (d, 2H, J = 8.5 Hz), 7.51 (d, 2H, J = 8.5 Hz), 7.36 (d, 1H, J = 16.5 Hz), 4.07 (t, 2H, J = 5.5 Hz), 3.57-3.51 (m, 8H), 3.46 (t, 2H, J = 5.5 Hz), 3.27 (s, 3H), 1.92 (s, 6H); $^{13}\text{C-NMR}$ (500 MHz, CDCl_3): δ = 175.1, 173.3, 150.0, 145.3, 141.2, 137.8, 134.2, 133.6, 131.0, 130.6, 130.1, 126.1, 119.8, 116.5, 111.5, 110.8, 119.1, 101.4, 98.0, 72.1, 70.74, 70.67, 70.5, 68.6, 59.2, 52.4, 26.6; HR-MS (ESI): calcd. for $\text{C}_{31}\text{H}_{30}\text{N}_6\text{O}_{10}\text{S} [\text{M}+\text{Na}]^+$ 701.1636, found 701.1610.



(E)-2-(3-cyano-4-(4-(2-(2-methoxyethoxy)ethoxy)ethylamino)styryl)-5,5-dimethylfuran-2(5H)-ylidene)propanedinitrile (4) To a stirred solution of sulfonamide **3** (30 mg, 0.044 mmol) in CH_2Cl_2 (2 ml) were added $\text{HSCH}_2\text{CO}_2\text{H}$ (5.3 mg, 0.057 mmol) and Et_3N (8.4 mg, 0.088 mmol). Stirring was continued at room temperature for 30 min. Following removal of the solvent under reduced pressure, the residue was dissolved in ether (50 mL) and washed with an aqueous NaHCO_3 solution (20 mL X 3). The organic layer was dried over anhydrous MgSO_4 , filtered, and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel using 2:1 ethyl acetate : hexanes as the mobile phase to give free dye **4** (16.2 mg, 82%). m. p. = 133-135°C; $^1\text{H-NMR}$ (500 MHz, CDCl_3): δ = 7.60 (d, 1H, J = 16.0 Hz), 7.51 (d, 2H, J = 8.5 Hz), 6.75 (d, 1H, J = 15.5 Hz), 6.65 (d, 2H, J = 8.5 Hz), 5.37 (br, 1H), 3.76 (t, 2H, J = 5.5 Hz), 3.68 (m, 6H), 3.59 (t, 2H, J = 5.5 Hz), 3.44 (q, 2H, J = 5.5 Hz), 3.41 (s, 3H), 1.76 (s, 6H); $^{13}\text{C-NMR}$ (500 MHz, CDCl_3): δ = 176.4, 174.6, 153.3, 148.7, 132.6, 123.1, 113.3, 112.8, 112.0, 111.5, 109.2, 97.0, 95.0, 72.1, 70.8, 70.7, 70.5, 69.1, 59.2, 43.1, 30.5, 29.9, 27.0; HR-MS (ESI): calcd. for $\text{C}_{25}\text{H}_{28}\text{N}_4\text{O}_4$ $[\text{M}+\text{H}]^+$ 449.2183, found 449.2184

Absorbance and Fluorescence Studies.

Table S1. Photophysical properties of probe **3** and free-dye **4**.^a

	λ_{max} (nm)	λ_{em} (nm)	ϵ_{max} ($\text{M}^{-1}\text{cm}^{-1}$)	Φ_F^b
Probe 3	405	560	21 200	0.0008
Free-dye 4	563 (587)	623 (627)	35 100 (40 400)	0.01 (0.24)

^a All measurements were done in HEPES buffer (10 mM, pH 7.4). Probe **3** and free dye **4** were excited at 400 nm and 560 nm, respectively. Values in parenthesis () were measured in the presence of 1% human serum albumin (HSA) in HEPES buffer (10 mM, pH 7.4).

^b Quantum yields vs. quinine sulfate (0.54, 0.1 M H_2SO_4) for **3** and vs. cresyl violet (0.54, MeOH) for **4**.

*Absorption and emission spectra of probe **3** and free-dye **4** as a function of pH*

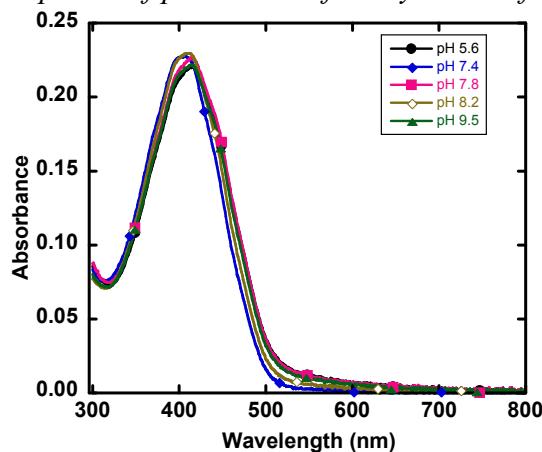


Figure S1. Absorption spectra of probe **3** in different pH buffers. 0.1 M acetate buffer for pH 5.6; 10 mM HEPES buffer for pH 7.4; 0.1 M phosphate buffer for pH 7.8; 0.1 M Tris buffer for pH 8.2; 0.1 M carbonate buffer for pH 9.5.

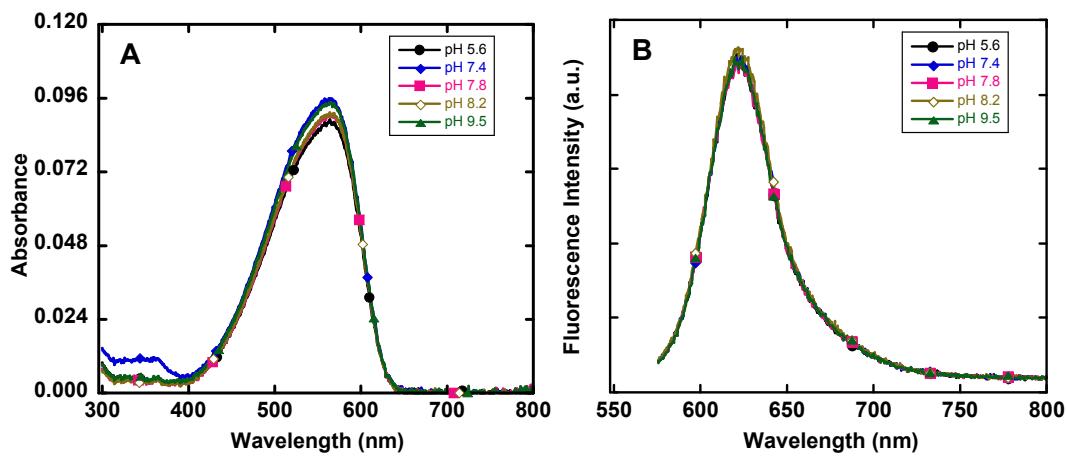


Figure S2. (A) Absorption and (B) emission spectra ($\lambda_{\text{ex}} = 560$ nm) of free-dye **4** in different pH buffer systems. 0.1 M acetate buffer for pH 5.6; 10 mM HEPES buffer for pH 7.4; 0.1 M phosphate buffer for pH 7.8; 0.1 M Tris buffer for pH 8.2; 0.1 M carbonate buffer for pH 9.5.

Absorption and emission spectra of free-dye 4 in different media

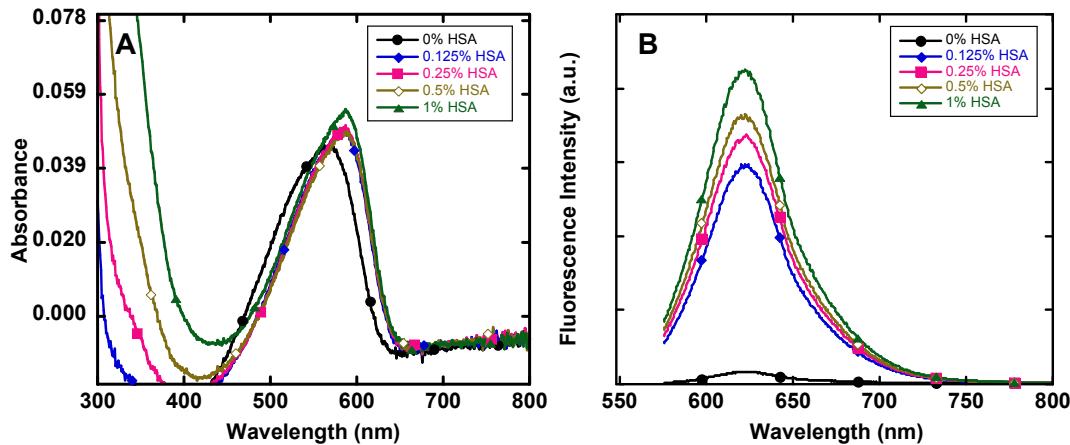


Figure S3. (A) Absorption and (B) fluorescence spectra ($\lambda_{\text{exc}} = 560$ nm) of dye **4** (3.3×10^{-6} M) in different concentrations of human serum albumin solutions in HEPES buffer solution (10 mM, pH 7.4). The signal below 400 nm observed in Figure A is due to absorption from HSA.

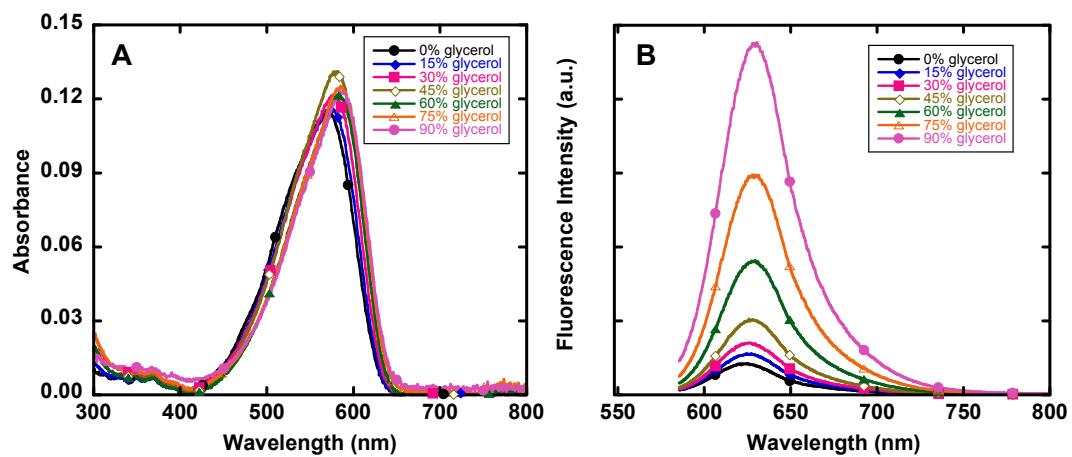


Figure S4. (A) Absorption and (B) fluorescence spectra ($\lambda_{\text{ex}} = 570$ nm) of dye 4 (3.3 \times 10^{-6} M) in glycerol/methanol solutions with increasing viscosity.

Response of probe 3 to different analytes

A solution of probe **3** (0.125 mM in CH₃CN, 20 μ L) was diluted with HEPES buffer (180 μ L, 10 mM, pH 7.4). Esterase (Porcine liver, 27 unit/mg) was prepared to 1.4 unit/mL in HEPES buffer (10 mM, pH 7.4). All other analytes were prepared as concentration of 0.5 mM. Analytes (50 μ L) were added to solutions probe **3** in 96-well flat bottom microtiter plates, and the reactions were monitored at 37 °C for 15 min. The fluorescence signal for each well was measured at 627 nm ($\lambda_{\text{ex}} = 565$ nm) (**Figure S5**).

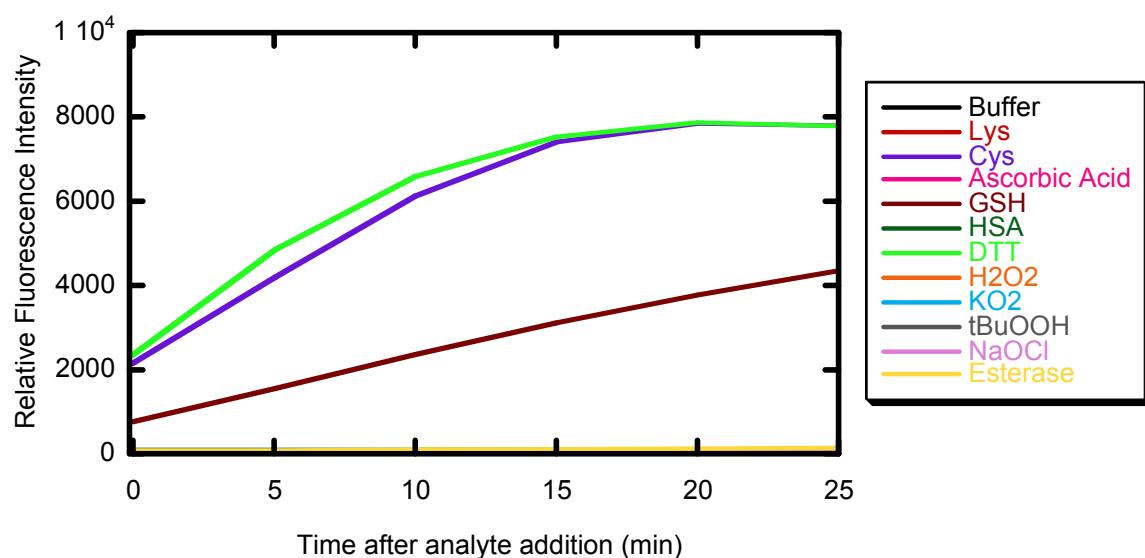


Figure S5. Relative fluorescence intensity ($\lambda_{\text{ex}} = 565$ nm, $\lambda_{\text{em}} = 627$ nm) response of probe **3** (10 μ M) with different analytes (100 μ M) in HEPES buffer solution (10 mM, pH 7.4) as a function of elapsed time at 37 °C. Porcine liver esterase was added to a concentration of 1.4 unit/mL.

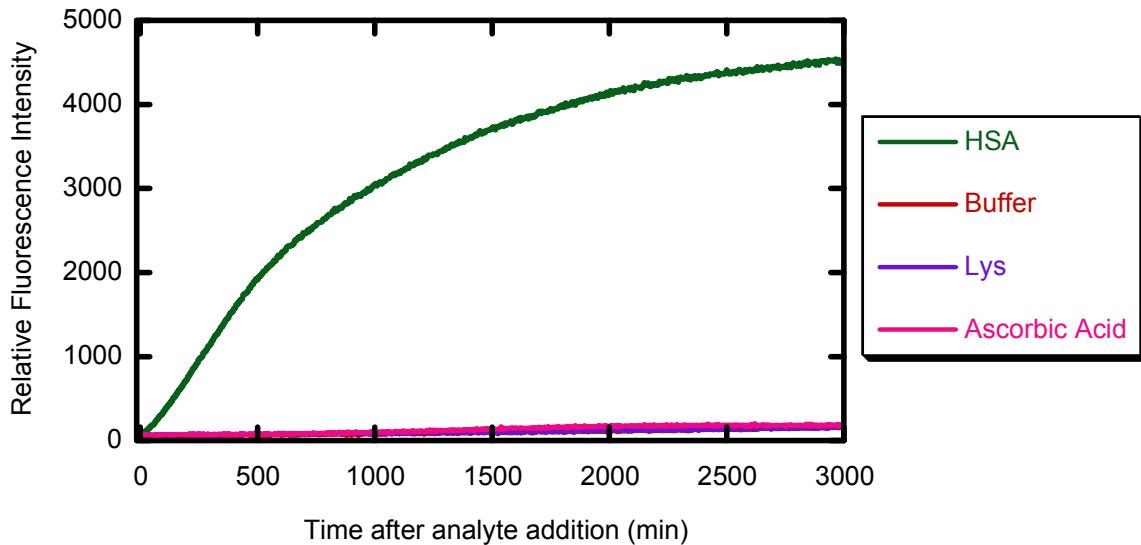


Figure S6. Relative fluorescence intensity ($\lambda_{\text{ex}} = 565$ nm, $\lambda_{\text{em}} = 627$ nm) response of probe **3** (10 μM) with human serum albumin (100 μM) in HEPES buffer solution (10 mM, pH 7.4) as a function of elapsed time at 37 °C. The relatively sluggish response is attributed to the fact that the globular protein HSA possesses only 1 free thiol (Cys 34) on its surface that is not involved in disulfide linkages, and that it is found in a sterically hindered location.^{iv}

Pseudo-first Order Kinetics

Rate constants were determined for the reaction between probe **3** and thiol analytes (Cys, DTT, GSH) under the conditions described above (**Figure S5, S6**). Since $[\text{Analyte}] = 10$ [**3**], the fluorescence data from **Figure S5, S6** was modeled assuming pseudo-first order kinetics. Linear calibration of the fluorescence signal against the concentration of fluorophore **4** produced from the reaction of probe **3** with thiol analytes was carried under the assumption that probe **3** is completely converted to fluorophore **4** in the presence of a large excess of thiols, and therefore the fluorescence signal at saturation corresponds to $[\mathbf{4}] = 10$ μM . Fluorescence data were modeled (least squares curve fitting) against eq. 1 (see below) where the parameter a is added to account for the delay between the addition of the reagents and the time at which the first measurement can be acquired due to experimental limitations (**Figure S7, S8**). The pseudo-first order rate constants (k_{obs}) obtained are of 2.1×10^{-3} s^{-1} , 2.4×10^{-3} s^{-1} , 0.8×10^{-3} s^{-1} and 2.0×10^{-5} s^{-1} for Cys, DTT, GSH and HSA, respectively. These correspond to second order rate constants (k) of 21 M^{-1} s^{-1} , 24 M^{-1} s^{-1} , 8 M^{-1} s^{-1} , and 0.2 M^{-1} s^{-1} after division by the concentration of analyte (100 μM).

$$f(t) = 10\mu M [1 - \exp(-k_{obs}(t + a))] \quad \text{eq. 1}$$

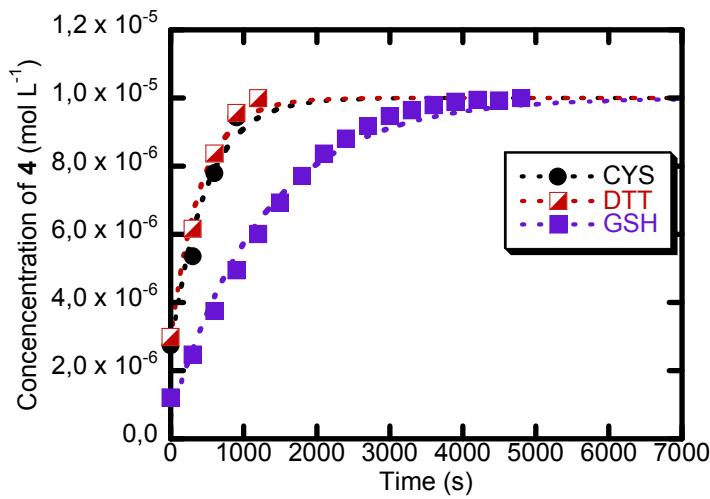


Figure S7. Pseudo-first order kinetics for the fluorescence ($\lambda_{\text{ex}} = 565 \text{ nm}$, $\lambda_{\text{em}} = 627 \text{ nm}$) response of probe **3** (10 μM) with different analytes (100 μM) in HEPES buffer solution (10 mM, pH 7.4) as a function of elapsed time at 37 °C.

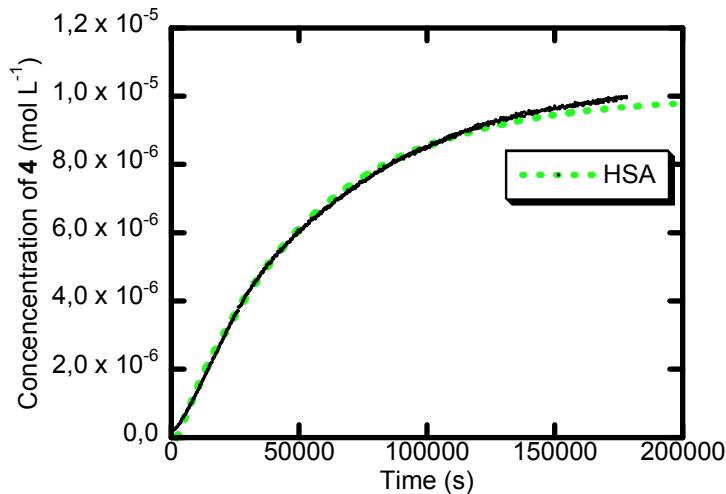
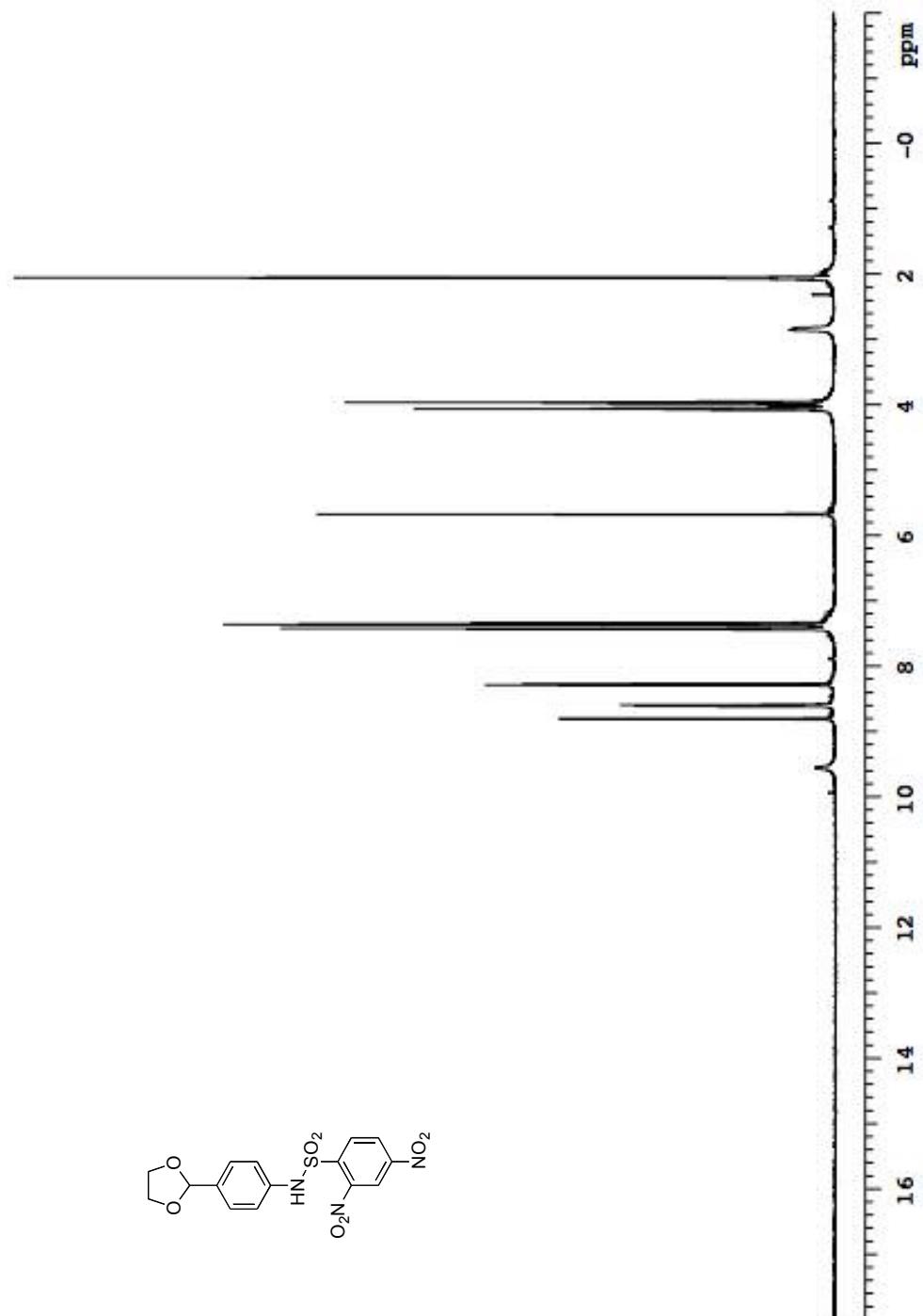


Figure S8. Pseudo-first order kinetics for the fluorescence ($\lambda_{\text{ex}} = 565 \text{ nm}$, $\lambda_{\text{em}} = 627 \text{ nm}$) response of probe **3** (10 μM) with human serum albumin (100 μM) in HEPES buffer solution (10 mM, pH 7.4) as a function of elapsed time at 37 °C. The relatively sluggish response is attributed to the fact that the globular protein HSA possesses only 1 free thiol (Cys 34) on its surface that is not involved in disulfide linkages, and that it is found in a sterically hindered location.⁴

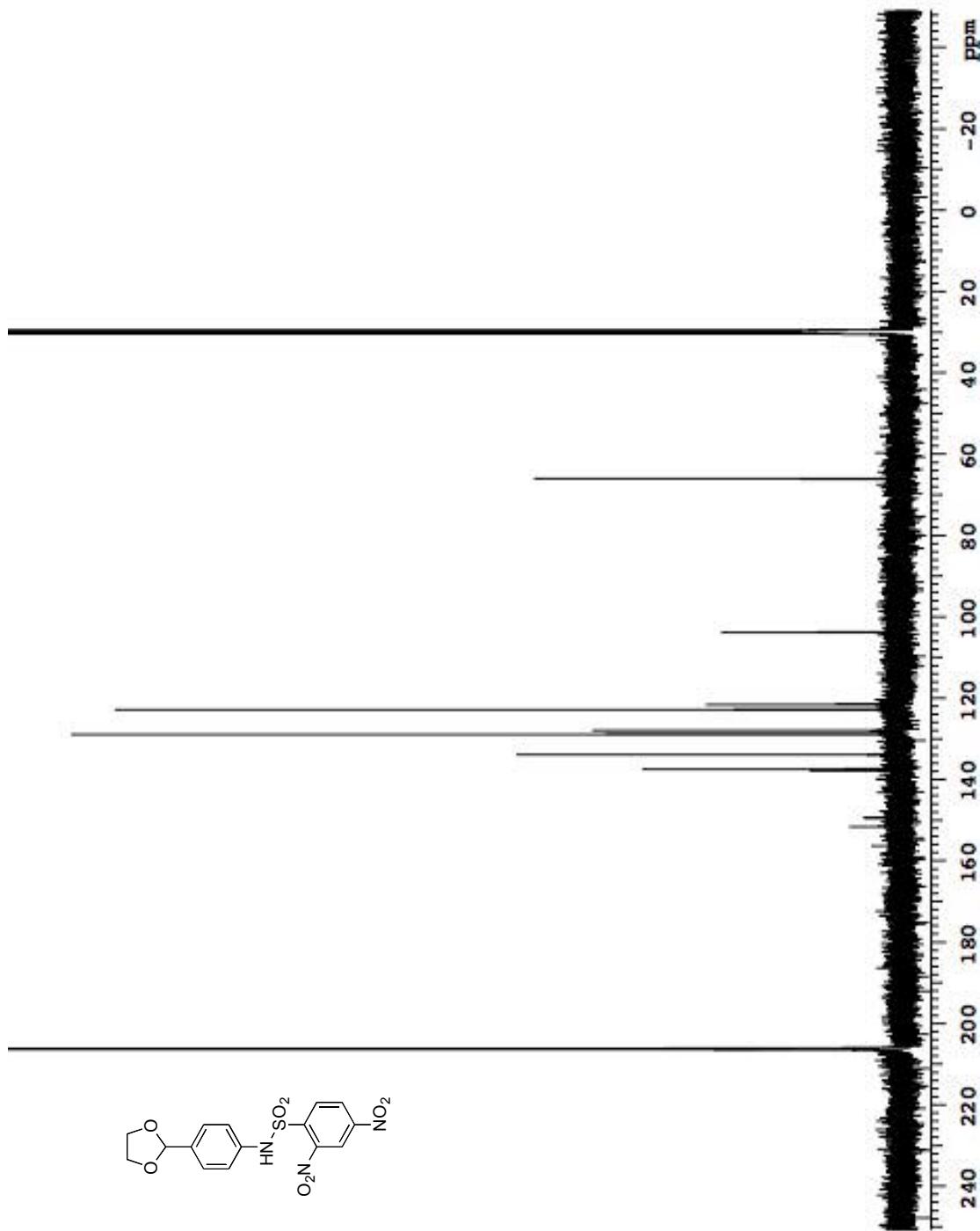
Fluorescence Microscopy and Cell-Uptake Studies.

For live cell imaging studies, fibroblast 3T3 cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) in an atmosphere of 5% CO₂, 95% air at 37 °C. Cells were plated on 24-well plate and allowed to adhere for 24 hr. After 24 h, the cells were washed with DPBS (Dulbecco's phosphate buffered saline) and then incubated at 37 °C in the presence of 25 μM probe **3** (1:100 DMSO/DPBS, v/v, pH 7) for 10 min. For the control experiment, living 3T3 cells were pretreated with 100 μM *N*-methylmaleimide in DMSO- DPBS solution (1:100, v/v, pH 7) for 1 hr at 37 °C. After washing the cells three times with DPBS buffer, maleimide pre-treated cells were incubated in the presence of 25 μM probe **3** (1:100 DMSO/DPBS, v/v, pH 7) for 10 min at 37 °C. For both sets of cells, fluorescence imaging was performed after washing the cells three times with DPBS buffer, fixing with 4% paraformaldehyde and staining of the cell nuclei with Hoechst 33342. The fluorescence images were obtained using an Nikon Eclipse TE2000-S fluorescence microscope with 100 x objective equipped with a Photometrics Cascade 512B CCD camera using excitation and emission filters from Chroma Technology. The Hoechst 33342 (ex: 365/10 nm; em: 400nm LP) and Cy3 (ex: 546/10 nm; em: 590nm LP) filter sets were used for collecting the nuclear stain and probe **3**'s images, respectively.

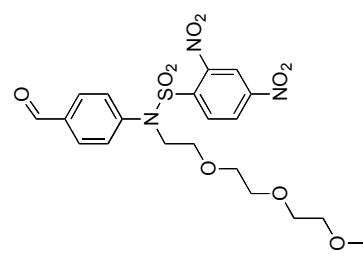
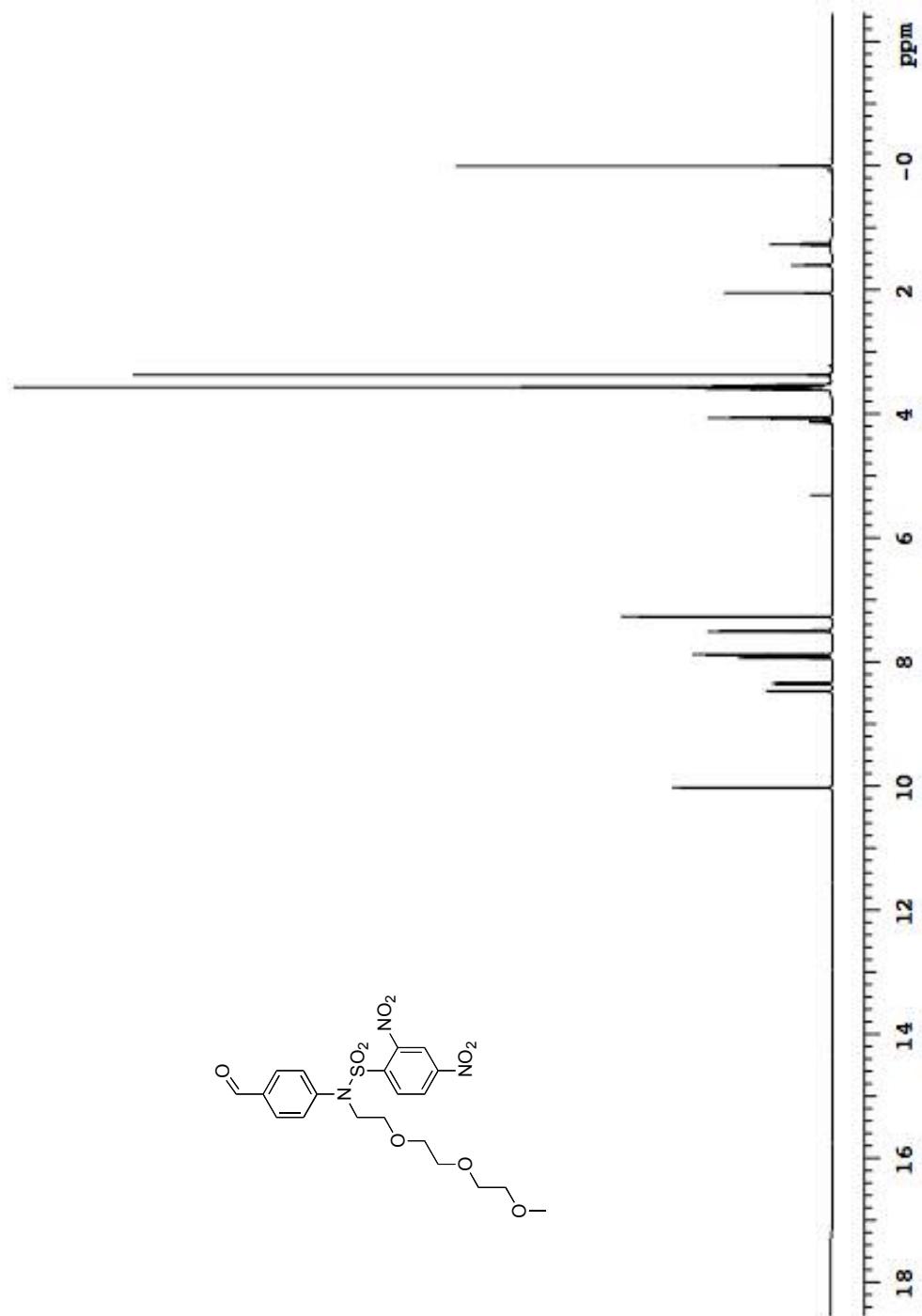
¹H-NMR Spectrum of **1** in (CD₃)₂CO (500 MHz):



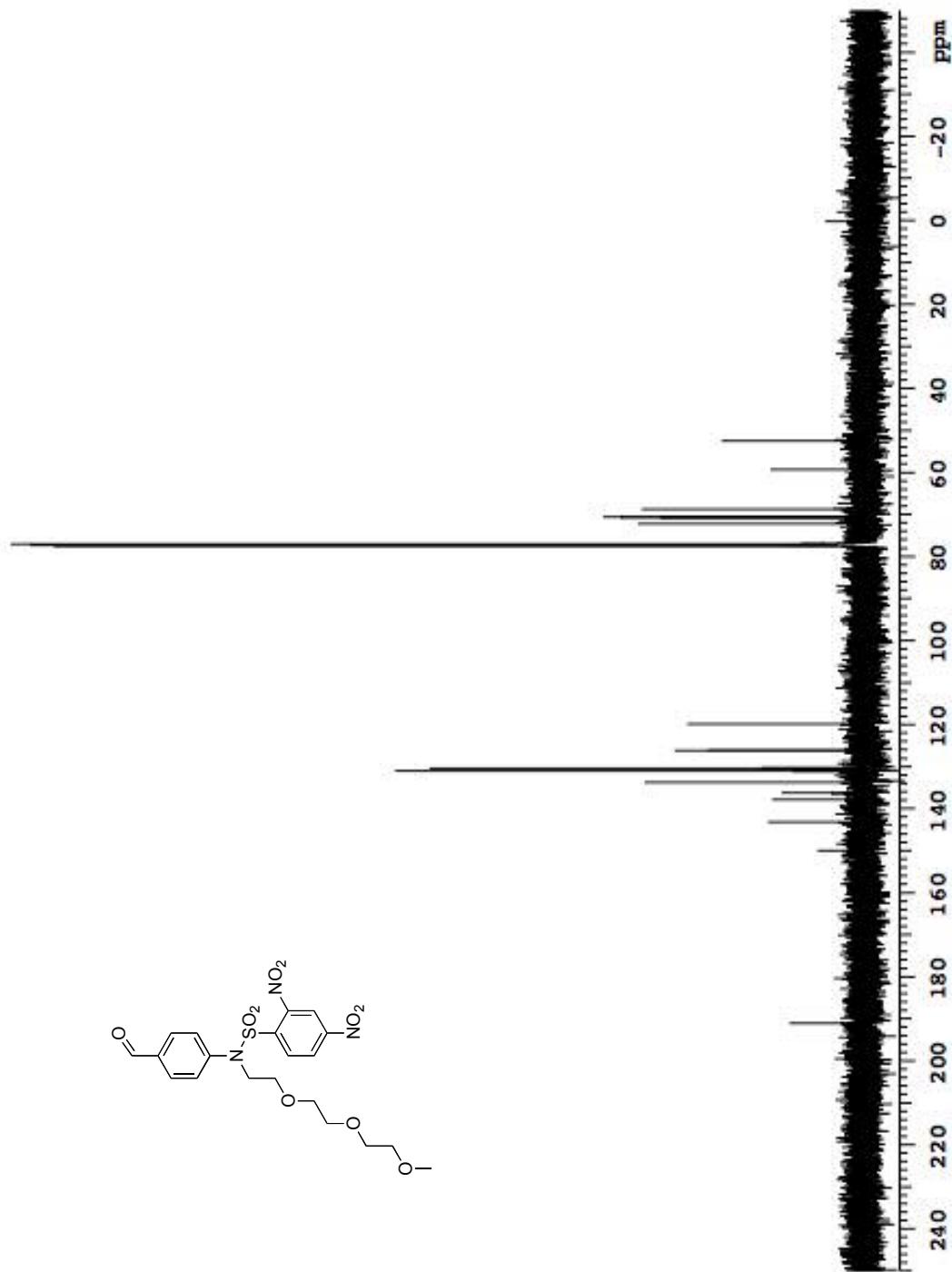
^{13}C -NMR Spectrum of **1** in $(\text{CD}_3)_2\text{CO}$ (125 MHz):



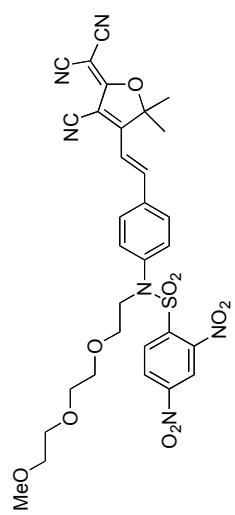
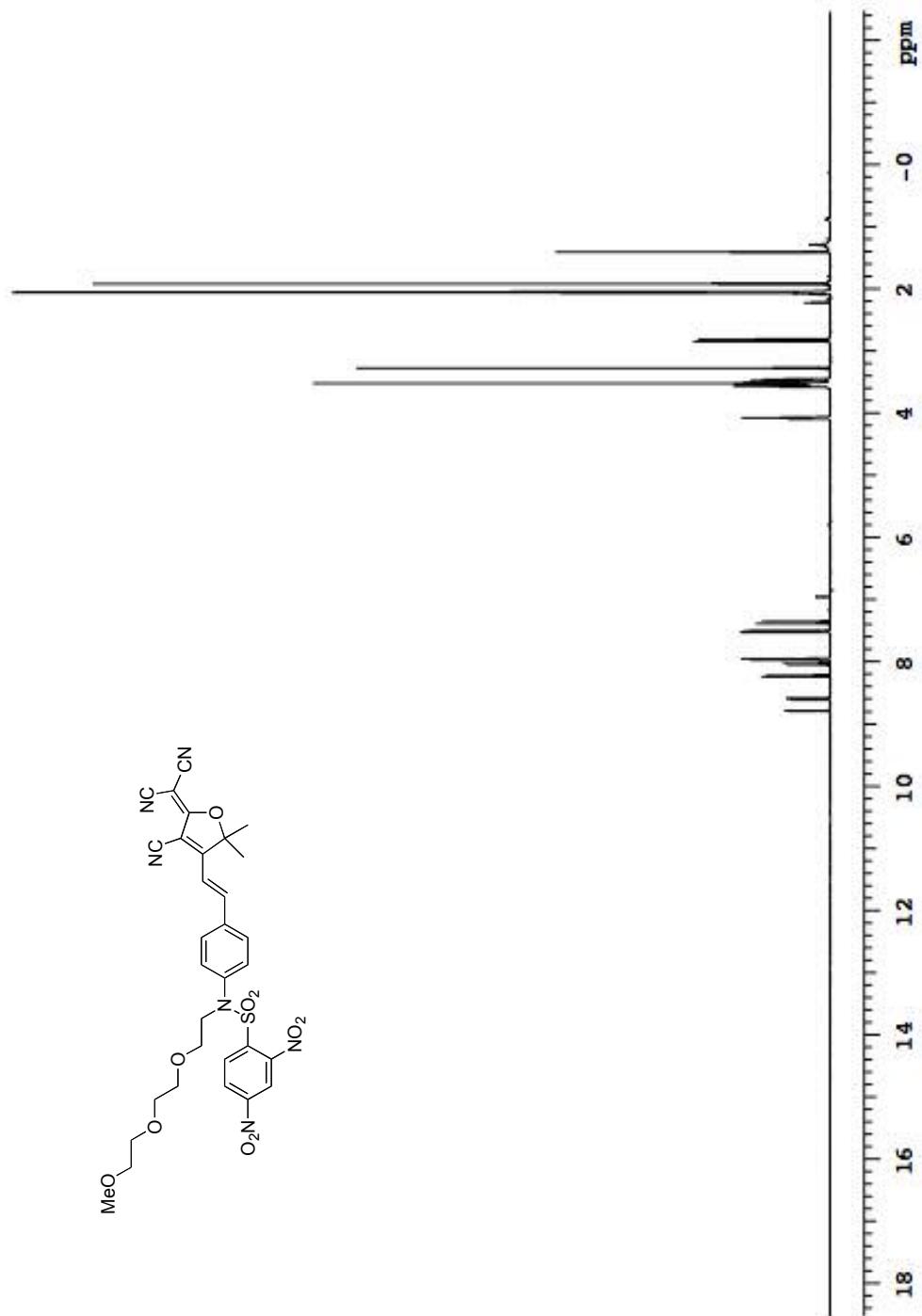
¹H-NMR Spectrum of **2** in CDCl₃ (500 MHz):



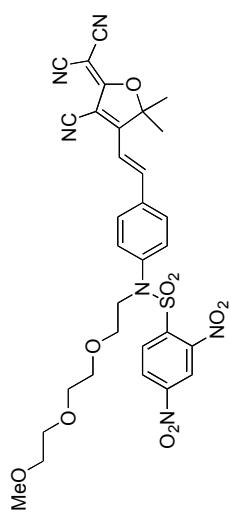
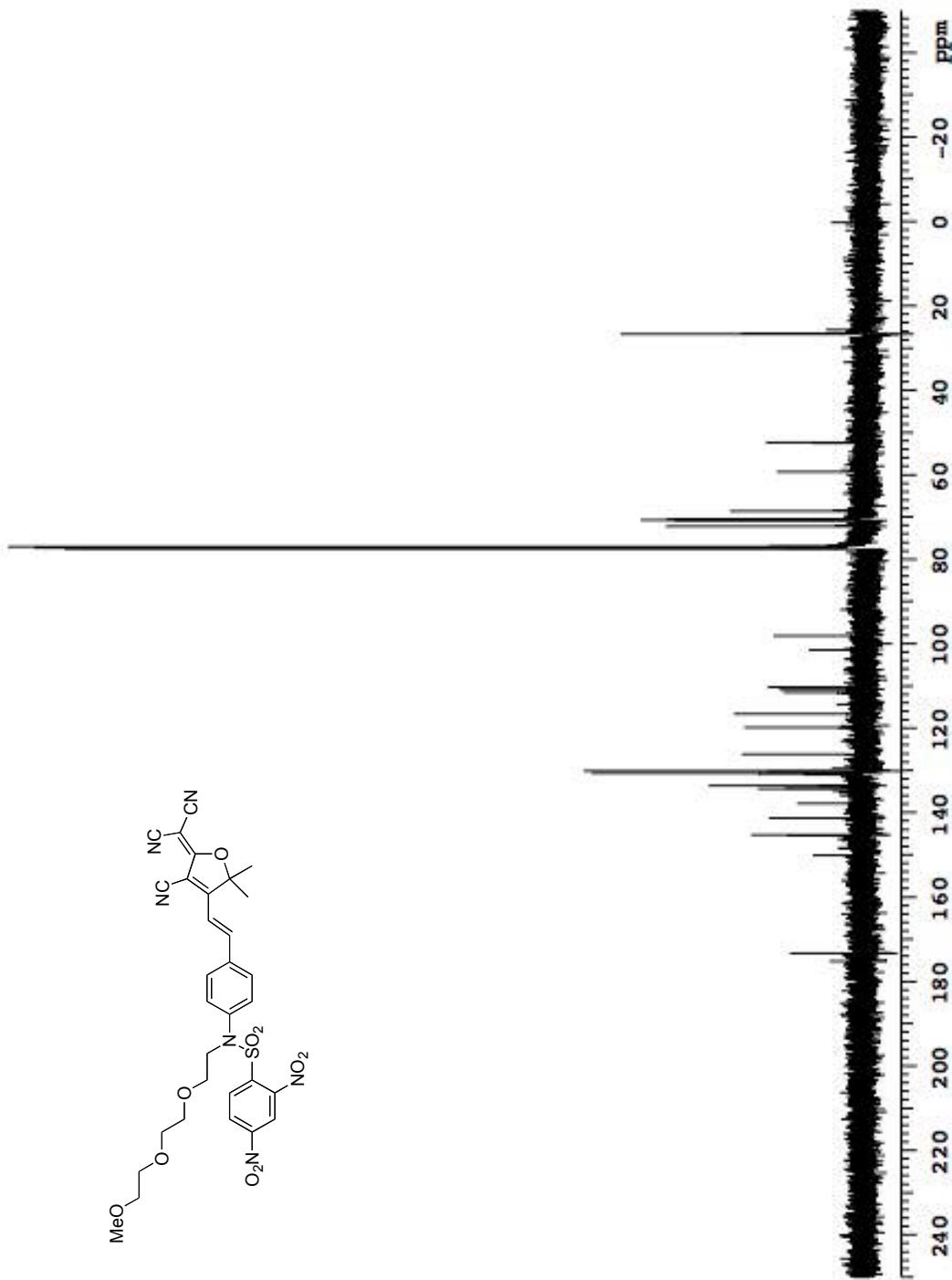
^{13}C -NMR Spectrum of **2** in CDCl_3 (125 MHz):



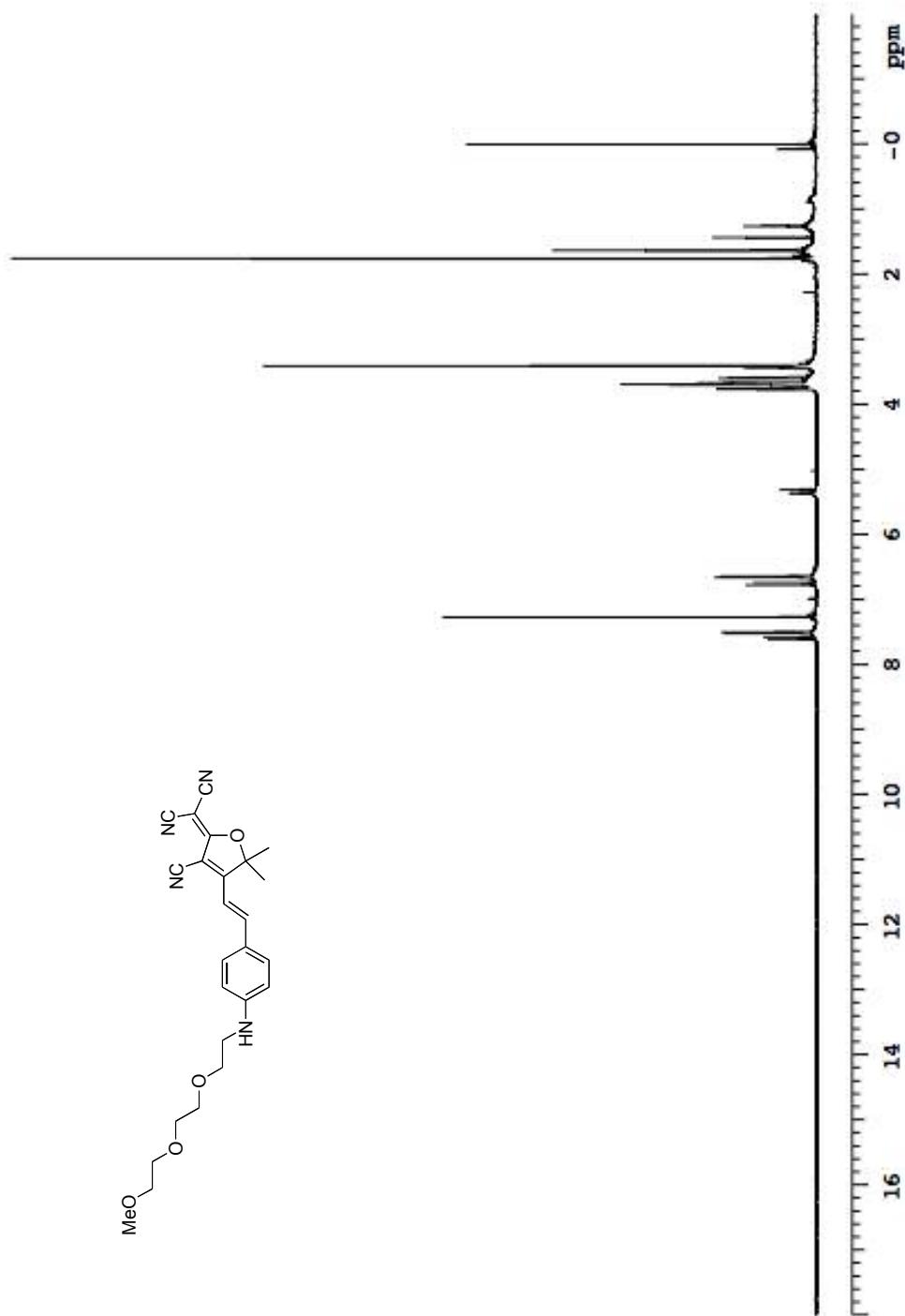
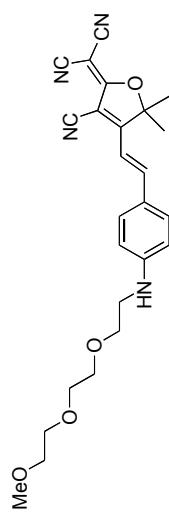
¹H-NMR Spectrum of **3** in (CD₃)₂CO (500 MHz):



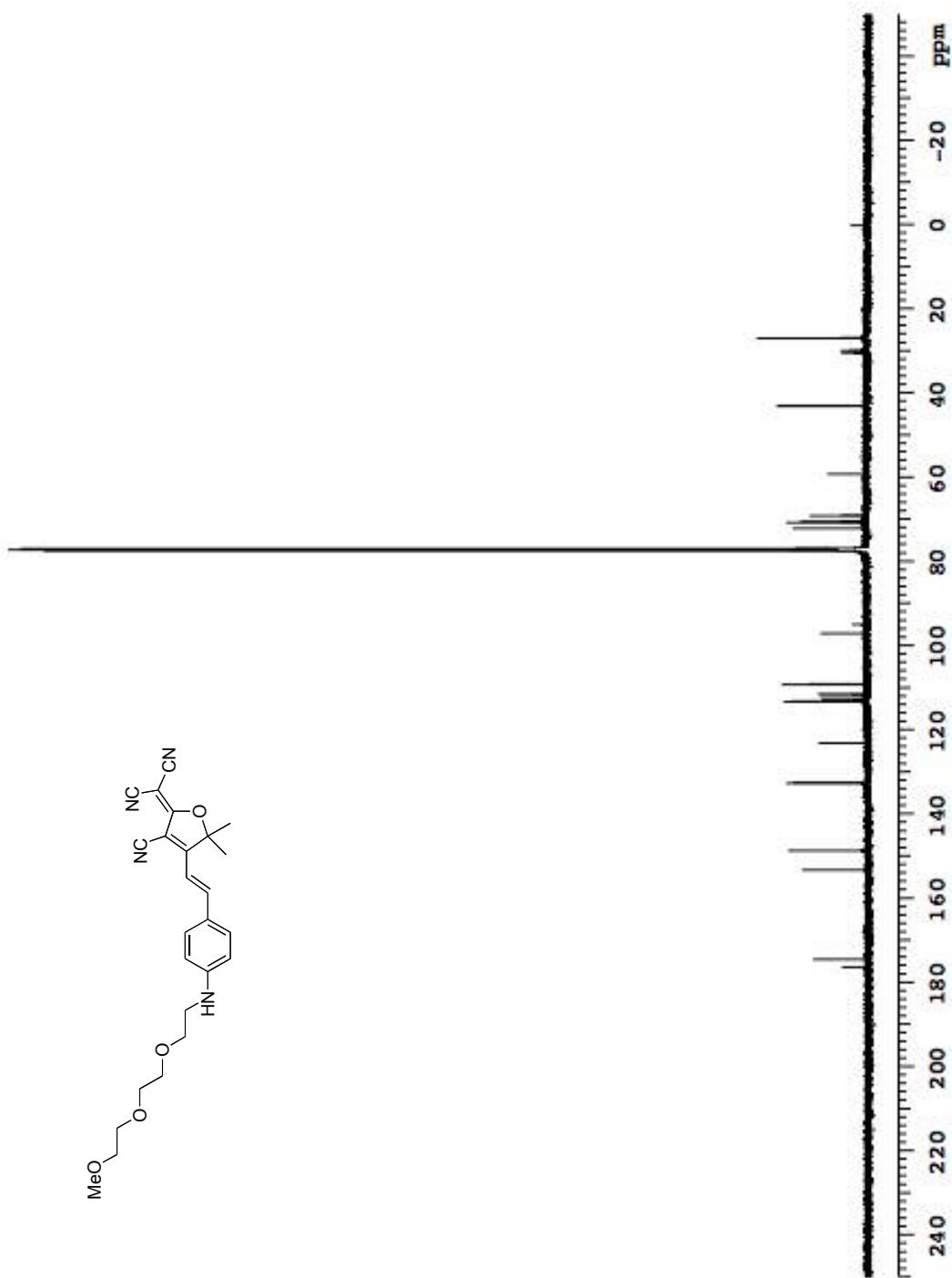
¹³C-NMR Spectrum of **3** in CDCl₃ (125 MHz):



¹H-NMR Spectrum of **4** in CDCl₃ (500 MHz):



^{13}C -NMR Spectrum of **4** in CDCl_3 (125 MHz):



ⁱ (a) Walton, R.; Lahti, Paul M. *Synth. Commun.* **1998**, *28*, 1087-1092. (b) Briggs, J. R.; Klosin, J.; Whiteker, G. T. *Org. Lett.* **2005**, *7*, 4795-4798.

ⁱⁱ Gopalan, P.; Katz, H. E.; McGee, D. J.; Erben, C.; Zielinski, T.; Bousquet, D.; Muller, D.; Grazul, J.; Olsson, Y. *J. Am. Chem. Soc.* **2004**, *126*, 1741-1747.

ⁱⁱⁱ Unclear transition, could be a LC transition. The probe **3** further melts / decomposes between 160-165°C.

^{iv} Sugio, S.; Kashima, A.; Mochizuki, S.; Noda, M.; Kobayashi, K. *Protein Eng.* **1999**, *12*, 439-446.