

Tuning the pK_a of Fluorescein to Optimize Binding Assays

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Page	Contents
S-1	Table of Contents
S-2	Figure S-1: pH-Dependence of the fluorescence of 1 and 7 Figure S-2: Absorption and emission spectra of 1 and 7
S-3	Figure S-3: Fluorescence change of FI–RNase and DEF–RNase upon addition of excess RI Figure S-4: Data from microplate assays for determination of the K_d values of RI-ribonuclease complexes.
S-4	Scheme S-1: Scheme for the synthesis of 2',7'-diethylfluorescein 7
S-5–S-7	Procedures for Chemical Synthesis
S-8	^1H NMR Spectrum of Compound 6
S-9	^{13}C NMR Spectrum of Compound 6
S-10	^1H NMR Spectrum of Compound 7
S-11	^{13}C NMR Spectrum of Compound 7
S-12	^1H NMR Spectrum of Compound 10
S-13	^{13}C NMR Spectrum of Compound 10
S-14	^1H NMR Spectrum of Compound 11
S-15	^{13}C NMR Spectrum of Compound 11
S-16	^1H NMR Spectrum of Compound 12
S-17	^{13}C NMR Spectrum of Compound 12
S-18	^1H NMR Spectrum of Compound 13
S-19	^{13}C NMR Spectrum of Compound 13
S-20	^1H NMR Spectrum of Compound 14
S-21	^{13}C NMR Spectrum of Compound 14
S-22	HPLC Chromatograms for Compound 14

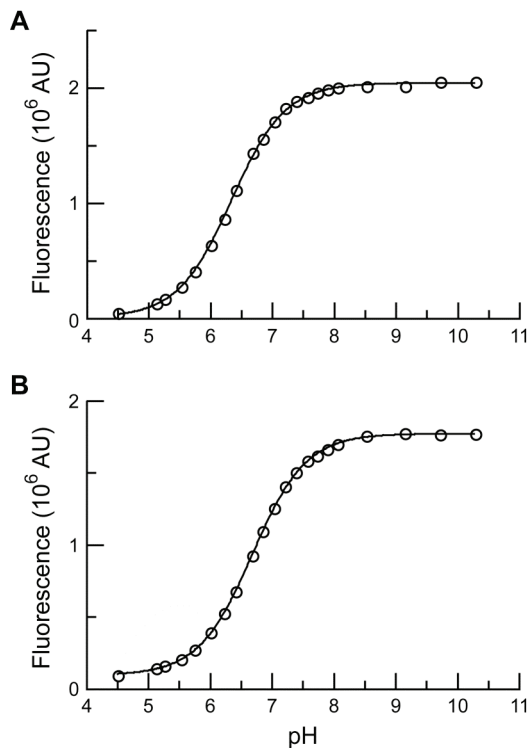


Figure S-1. pH-Dependence of the fluorescence of fluorescein (**1**, A) and 2',7'-diethylfluorescein (**7**, B) ($\lambda_{\text{ex}} = 493 \text{ nm}$, $\lambda_{\text{em}} = 515 \text{ nm}$). Values of $\text{p}K_{\text{a}}$ were found to be 6.30 ± 0.02 for **1**, and 6.61 ± 0.03 for **7**.

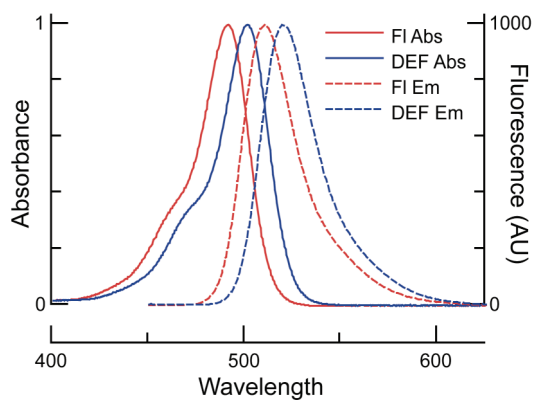


Figure S-2. Normalized absorption and emission spectra of fluorescein (**1**) and 2',7'-diethylfluorescein (**7**) in 0.1 M NaOH.

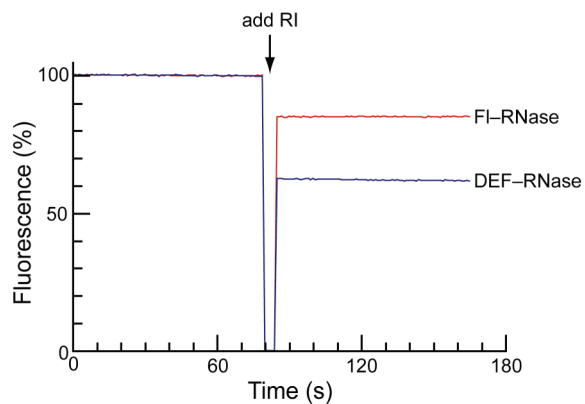


Figure S-3. Comparison of the fluorescence change of FI-RNase (red) and DEF-RNase (blue) upon addition of excess RI ($\lambda_{\text{ex}} = 493 \text{ nm}$, $\lambda_{\text{em}} = 515 \text{ nm}$) in DPBS containing BSA ($2 \mu\text{g/mL}$).

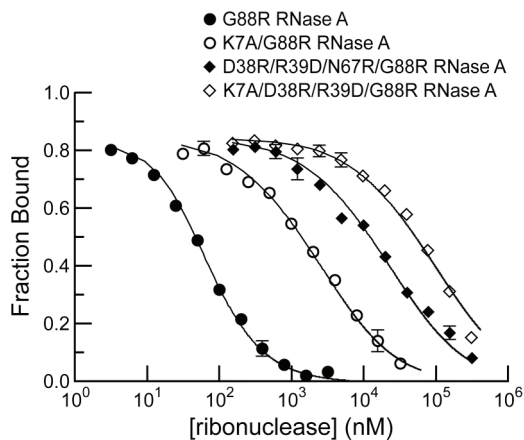
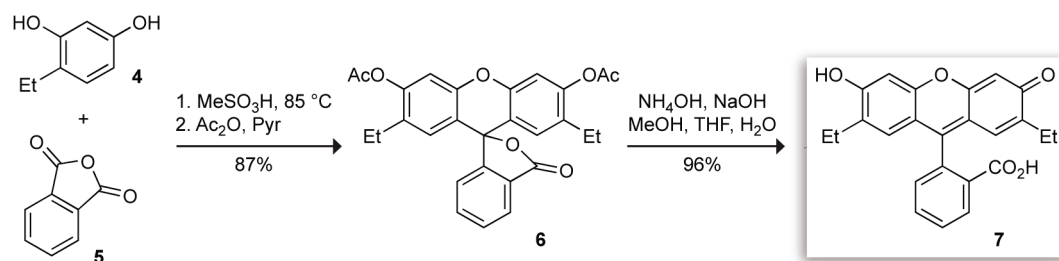


Figure S-4. Data from microplate assays for determination of the K_d values of RI-ribonuclease complexes in DPBS containing BSA (0.1 mg/mL).

Scheme S-1. Synthesis of 2',7'-Diethylfluorescein

General Experimental. 4-Nitrophthalic anhydride was from TCI America (Portland, OR). Dimethylformamide (DMF), tetrahydrofuran (THF), and dichloromethane (CH_2Cl_2) were drawn from a Baker CYCLE-TAINER solvent delivery system. All other reagents were obtained from Aldrich Chemical (Milwaukee, WI) or Fisher Scientific (Hanover Park, IL) and used without further purification. $\text{NaHS}\cdot\text{H}_2\text{O}$ was obtained as “sodium hydrosulfide hydrate” (Aldrich; Product 161527). $\text{HCl}(\text{aq})$ was prepared by dilution of concentrated $\text{HCl}(\text{aq})$ into H_2O in the indicated proportions.

Thin-layer chromatography was performed using aluminum-backed plates coated with silica gel containing F_{254} phosphor and visualized by UV illumination or staining with I_2 , ceric ammonium molybdate, or phosphomolybdic acid. Flash chromatography was performed by using open columns loaded with silica gel-60 (230–400 mesh), or on a FlashMaster Solo system (Argonaut Inc., Redwood City, CA) with Isolute Flash Si II columns (International Sorbent Technology Ltd., Hengoed, Mid Glamorgan, UK). The term “high vacuum” refers to a vacuum (≤ 1 mm Hg) achieved by a mechanical belt-drive oil pump. The term “concentrated under reduced pressure” refers to the removal of solvents and other volatile materials using a rotary evaporator at water-aspirator pressure (< 20 mm Hg) while maintaining the water-bath temperature below 40 °C. The term “concentrated under high vacuum” refers to the removal of solvents and other volatile materials using a rotary evaporator at high vacuum while maintaining the water-bath temperature below 40 °C.

NMR spectra were obtained with a Bruker DMX-400 Avance spectrometer in the National Magnetic Resonance Facility at Madison (NMRFAM), or with Bruker AC-300 or Avance-360 spectrometers in the Magnetic Resonance Facility (MRF) in the Department of Chemistry. Mass spectrometry was performed with a Micromass LCT (electrospray ionization, ESI) mass spectrometer in the Mass Spectrometry Facility in the Department of Chemistry. Analytical HPLC was performed on a system from Waters (Milford, MA) with an 250×4.6 mm C-18 Microsorb column from Varian (Palo Alto, CA) using a linear gradient of CH_3CN (20–60% v/v) in H_2O . HPLC solvents contained TFA (0.1% v/v).

2',7'-Diethylfluorescein Diacetate (6). 4-Ethylresorcinol (**4**, 5.00 g, 36.2 mmol) and phthalic anhydride (**5**, 2.68 g, 18.09 mmol) were dissolved in methanesulfonic acid (40 mL). The reaction mixture was stirred at 85 °C for 48 h. The dark brown solution was poured into ice water (250 mL), and the yellow-brown precipitate was collected by filtration and air dried. This material was dissolved in acetic anhydride (80 mL) and pyridine (80 mL), and stirred at 85 °C for 4 h. The reaction mixture was concentrated under high vacuum, and the brown residue was dissolved into CH_2Cl_2 . This solution was washed with $\text{HCl}(\text{aq})$ (5% v/v), water, and saturated brine. The organic layer was then dried over anhydrous $\text{MgSO}_4(\text{s})$ and concentrated under reduced pressure. The brown residue was suspended in cold EtOH (300 mL), and the resulting pale brown solid was collected by filtration. This material was purified further by crystallization from $\text{CHCl}_3/\text{MeCN}$ (1:1) and the crystals were washed with cold EtOH (excluded from the mother liquor). The mother liquor was concentrated under reduced pressure and a second crop of crystals was obtained by crystallization from acetic anhydride. The combined crystals were dried under high vacuum. Compound **6** was isolated as a pale yellow crystalline solid (7.41 g, 87%). ^1H NMR (400 MHz, CDCl_3) δ (ppm): 8.05 (d, $J = 7.1$ Hz, 1H), 7.69 (ddd, $J = 7.6, 7.1, 1.6$ Hz, 1H), 7.64 (ddd, $J = 7.6, 7.5, 1.2$ Hz, 1H), 7.19 (d, $J = 7.4$ Hz, 1H), 7.01 (s, 2H), 6.64 (s, 2H), 2.39 (m, 4H), 2.33 (s, 6H), 1.01 (t, $J = 7.8$ Hz, 6H). ^{13}C NMR (100 MHz, CDCl_3) δ (ppm): 169.46, 168.88 (2C), 153.23, 150.11 (2C), 149.64 (2C), 135.20 (2C), 131.96, 129.87, 128.07 (2C), 125.93, 125.13 (2C), 124.09, 116.57, 110.87 (2C), 82.09, 22.94 (2C), 20.84 (2C), 14.02 (2C). HRMS (ESI): m/z 495.1417 (MNa^+ [$\text{C}_{28}\text{H}_{24}\text{O}_7\text{Na}$] = 495.1420).

2',7'-Diethylfluorescein (7). 2',7'-Diethylfluorescein diacetate (**6**, 2.00 g, 4.23 mmol) was dissolved in THF (20 mL) and MeOH (20 mL). To this solution was added 29% w/w $\text{NH}_4\text{OH}(\text{aq})$ (5.12 mL, 42.2 mmol), and the mixture was stirred at ambient temperature for 2 h. NaOH (1.69 g, 42.2 mmol) was added as a solution in H_2O (20 mL) and MeOH (5 mL), and the reaction mixture stirred for an additional 1 h at ambient temperature. The mixture was diluted with 200 mL of H_2O , and concentrated under reduced pressure to remove the organic solvents. The dark brown solution was filtered and then acidified with $\text{HCl}(\text{aq})$ (10% v/v) to pH ~ 3 . The resulting yellow-orange precipitate was collected by filtration, washed with H_2O , and dried under high vacuum. Compound **7** was isolated as an orange solid (1.57 g, 96%). An analytical sample was prepared by column chromatography (silica gel, EtOAc). ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ (ppm): 10.09 (bs, 2H), 8.00 (d, $J = 7.8$ Hz, 1H), 7.78 (ddd, $J = 7.5, 7.4, 1.2$ Hz, 1H), 7.71 (ddd, $J = 7.5, 7.4, 1.0$ Hz, 1H), 7.24 (d, $J = 7.4$ Hz, 1H), 6.71 (s, 2H), 6.34 (s, 2H), 2.35 (m, 4H), 0.92 (t, $J = 7.6$ Hz, 6H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ (ppm): 168.83, 157.11 (2C), 152.62, 149.95 (2C), 135.53,

130.01, 127.23 (2C), 127.12 (2C), 126.21, 124.55, 124.00, 109.02 (2C), 101.73 (2C), 83.55, 22.35 (2C), 14.10 (2C). HRMS (ESI): m/z 387.1219 (M–H [C₂₄H₁₉O₅] = 387.1232).

5(6)-Nitro-2',7'-Diethylfluorescein (9). 4-Ethylresorcinol (**4**, 5.00 g, 36.2 mmol) and 4-nitrophthalic anhydride (**8**, 3.49 g, 18.1 mmol) were dissolved in methanesulfonic acid (40 mL). The reaction mixture was stirred at 85 °C for 48 h. The dark brown solution was poured into ice water (250 mL), and the yellow–brown precipitate was collected by filtration. This material was washed with H₂O and air dried. Crude compound **9** was isolated as a yellow-brown solid and used in the next step without further purification.

5-Nitro-2',7'-diethylfluorescein Diacetate (10). Crude 5(6)-nitro-2',7'-diethylfluorescein (**9**) from the previous step was dissolved in acetic anhydride (30 mL) and stirred at reflux for 2 h. This solution was placed at 4 °C for 48 h and the resulting tan crystals (2.61 g, 28%) were collected by filtration and washed with cold acetic anhydride. The mother liquor was concentrated under high vacuum and the residue was dissolved in acetic anhydride (15 mL). This solution was incubated at 4 °C for 48 h after which additional tan crystals formed (1.53 g, 16%). These crystals were isolated by filtration and washed with cold acetic anhydride. The combined crystals were purified further by crystallization twice from acetic anhydride and dried under high vacuum. Compound **10** was isolated as an off-white solid (1.91 g, 20%, two steps). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 8.73 (d, J = 1.7 Hz, 1H), 8.59 (dd, J = 8.4, 2.3, 1H), 7.72 (d, J = 8.4 Hz, 1H), 7.27 (s, 2H), 6.86 (s, 2H), 2.37 (m, 4H), 2.33 (s, 6H), 0.94 (t, J = 7.6 Hz, 6H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 168.92, 166.53, 156.73, 150.48, 149.26, 148.95, 132.53, 130.62, 128.53, 127.35, 125.95, 120.83, 115.09, 111.21, 81.88, 22.17, 20.63, 14.42. HRMS (ESI): m/z 518.1441 (MH⁺ [C₂₈H₂₄NO₉] = 518.1451).

5-Nitro-2',7'-diethylfluorescein (11). 5-Nitro-2',7'-diethylfluorescein diacetate (**10**, 1.00 g, 1.93 mmol) was dissolved in THF (35 mL). This solution was diluted with MeOH (35 mL) and H₂O (20 mL). To the resulting solution was added NaOH (1.55 g, 38.6 mmol), and the mixture was stirred at 0 °C for 1 h and then at ambient temperature for 1 h. This was concentrated under reduced pressure to remove the organic solvents, and the residue was diluted with 50 mL of H₂O and filtered. The resulting dark brown solution was acidified with HCl(aq) (10% v/v) to pH ~3, and the resulting yellow-orange precipitate was collected by filtration, washed with H₂O and dried under high vacuum. Compound **11** was isolated as an orange solid (0.797 g, 95%). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 10.16 (bs, 2H), 8.66 (d, J = 1.7 Hz, 1H), 8.56 (dd, J = 8.4, 2.3 Hz, 1H), 7.53 (d, J = 8.4 Hz, 1H), 6.71 (s, 2H), 6.48 (s, 2H), 2.36 (m, 4H), 0.94 (t, J = 7.5 Hz, 6H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 166.78, 157.53 (3C), 150.01 (2C), 149.00, 130.20, 127.85, 127.64 (4C), 125.90, 120.41, 107.81 (2C), 101.88 (2C), 84.34, 22.41 (2C), 14.18 (2C). HRMS (ESI): m/z 432.1075 (M–H [C₂₄H₁₈NO₇] = 432.1084).

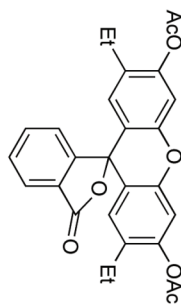
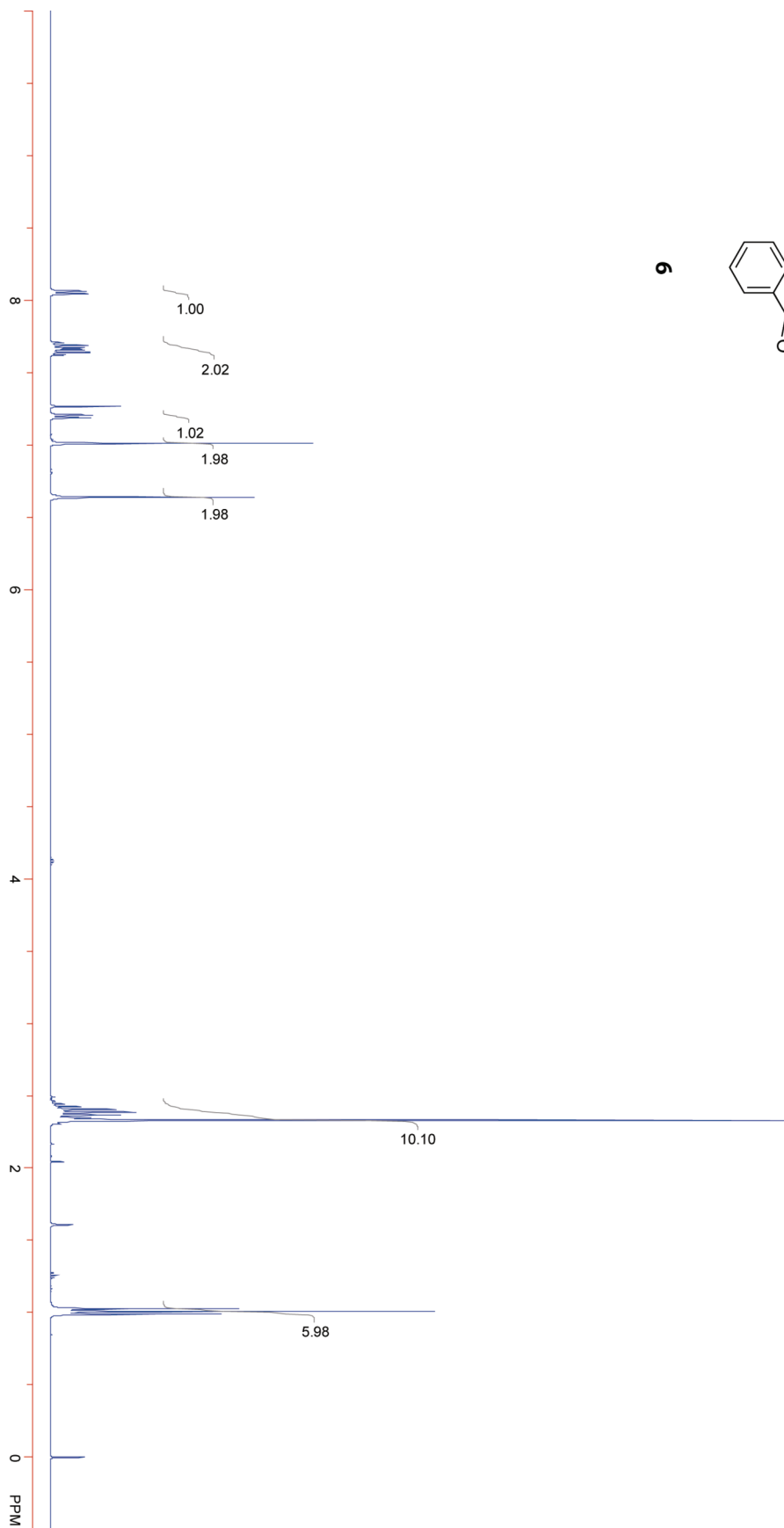
5-Amino-2',7'-Diethylfluorescein (12). Na₂S·9(H₂O) (5.54 g, 23.1 mmol) was dissolved in H₂O (75 mL). 5-Nitro-2',7'-diethylfluorescein (**11**, 2.50 g, 5.77 mmol) was added, and the resulting solution was stirred at ambient temperature for 10 min. Then, to this solution was added NaHS·H₂O (2.59 g, 46.1 mmol), and the mixture was stirred at reflux for 24 h. The solution was carefully acidified with glacial acetic acid, and the resulting dark red precipitate was collected by filtration. This solid was dissolved in 175 mL of HCl(aq) (6% v/v), heated to reflux, and filtered while hot to remove elemental sulfur. The solution was incubated at 4 °C for 48 h, and the resulting red solid was collected by filtration. This solid was dissolved in 120 mL of HCl(aq) (6% v/v) and, as before, filtered while hot and incubated at 4 °C for 48 h. The solid was collected by filtration and taken up into 210 mL of NaOH(aq) (0.5% w/v) to give a dark red solution. Glacial acetic acid (4.2 mL) was added dropwise, and the resulting red precipitate was collected via filtration and dried under high vacuum. Compound **12** was isolated as a red-orange solid (1.92 g, 82%). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 7.24 (d, J = 2.1 Hz, 1H), 7.05 (dd, J = 8.3, 2.2 Hz, 1H), 6.92 (d, J = 8.2 Hz, 1H), 6.62 (s, 2H), 6.61 (s, 2H), 2.46 (m, 4H), 1.03 (t, J = 7.5 Hz, 6H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 169.52, 156.84 (2C), 150.32 (2C), 150.04 (2C), 139.83, 127.51, 127.26 (2C), 126.85, 124.16, 121.73, 110.16 (2C), 106.16, 101.62 (2C), 83.23, 22.43 (2C), 14.18 (2C). HRMS (ESI): m/z 403.1509 (MH⁺ [C₂₄H₂₂NO₅] = 403.1498).

2',7'-Diethylfluorescein-5-chloroacetamide (13). 5-Amino-2',7'-diethylfluorescein (**12**, 157 mg, 0.389 mmol) was dissolved in THF (5.0 mL). To this solution was added chloroacetyl chloride (382 μ L, 3.89 mmol), and the mixture was stirred at reflux for 1 h. The reaction mixture was cooled to 0 °C, and MeOH (5.0 mL) was added, followed by NaOH (311 mg, 7.78 mmol) dissolved in H₂O (5.0 mL). The resulting dark orange solution was stirred at 0 °C for 2 h. The reaction was concentrated under reduced pressure to remove the organic solvents, and the resulting dark red solution was acidified with HCl(aq) (10% v/v) to pH ~3. The yellow-orange precipitate was collected by filtration, washed with H₂O, and air-

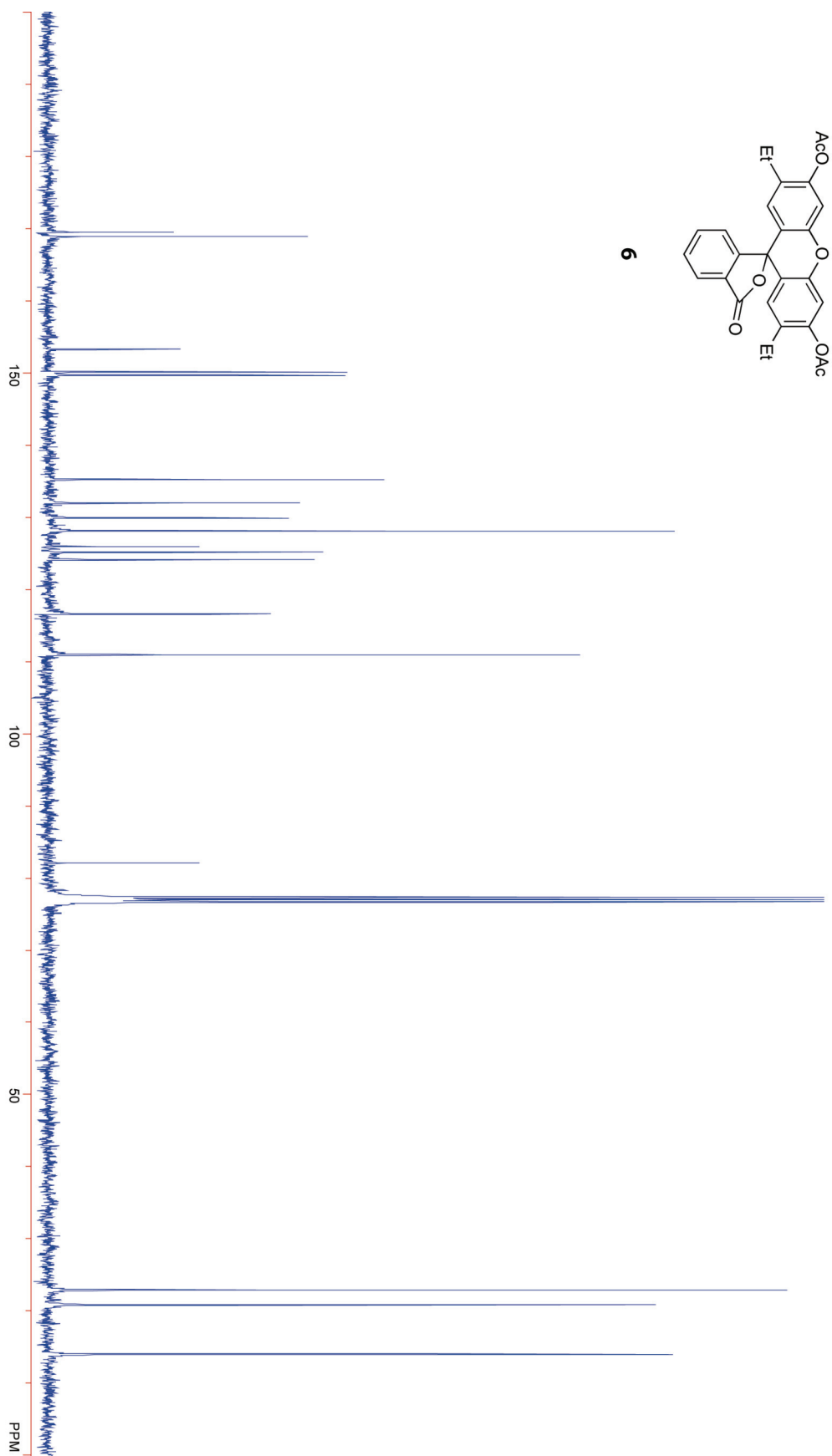
dried. Purification by column chromatography (silica gel, linear gradient of CHCl₃/MeOH/AcOH, 100:5:1–100:8:1) afforded compound **13** as an orange solid (182 mg, 97%). ¹H NMR (400 MHz, DMSO-d₆) δ (ppm): 10.82 (s, 1H), 10.27 (bs, 2H), 8.33 (s, 1H), 7.84 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.22 (d, *J* = 8.3 Hz, 1H), 6.67 (s, 2H), 6.41 (s, 2H), 4.34 (s, 2H), 2.36 (m, 4H), 0.93 (t, *J* = 7.6, 6H). ¹³C NMR (100 MHz, DMSO-d₆) δ (ppm): 168.60, 165.37, 157.26 (2C), 150.05 (2C), 147.36, 139.97, 127.32 (5C), 126.39, 124.71, 109.09, 101.75 (2C), 83.85 (2C), 43.54, 22.39 (2C), 14.19 (2C).

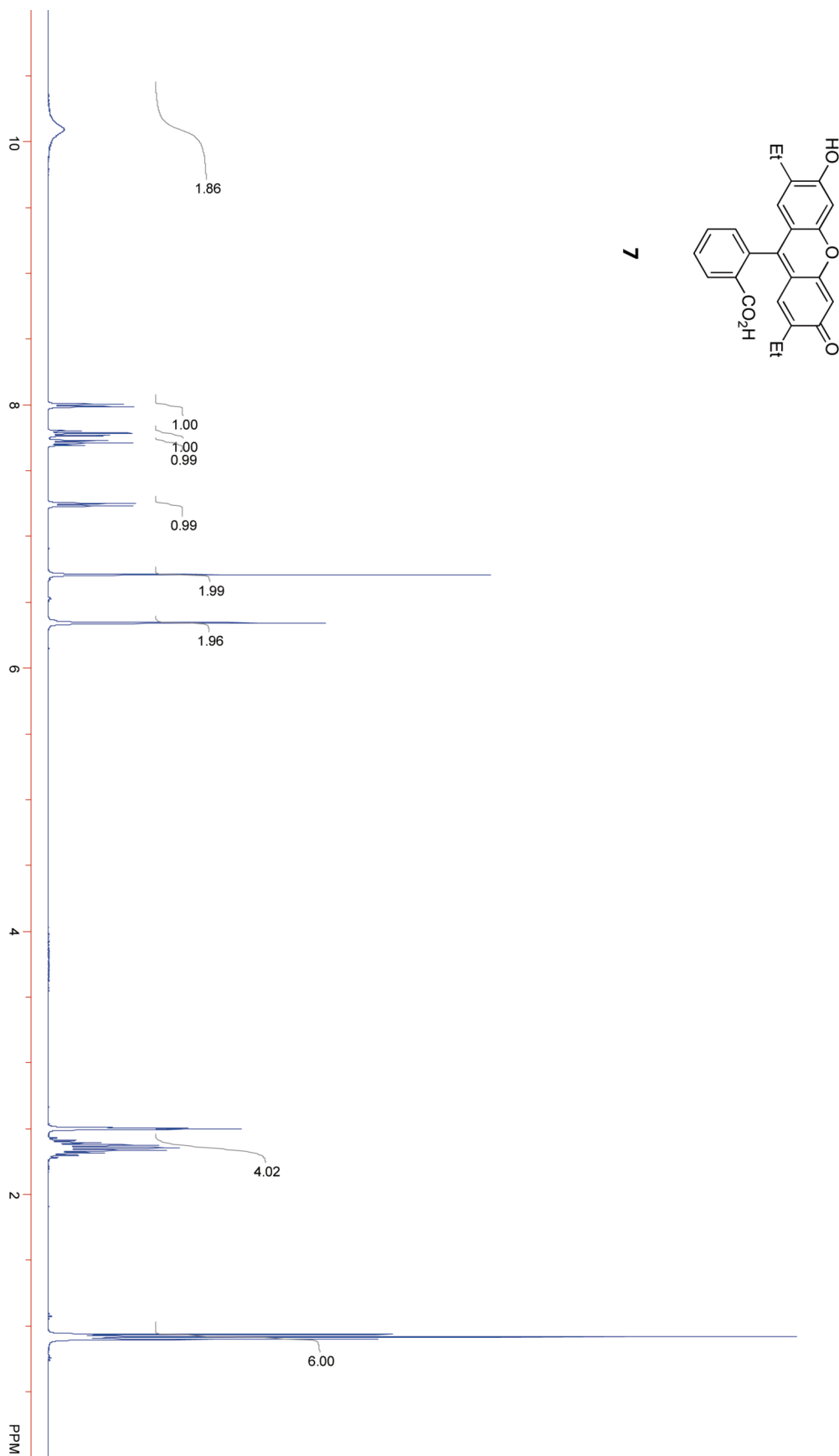
HRMS (ESI): *m/z* 480.1208 (MH⁺ [C₂₆H₂₃ClNO₆] = 480.1214).

2',7'-Diethylfluorescein-5-iodoacetamide (14, DEFIA). 2',7'-Diethylfluorescein-5-chloroacetamide (**13**, 72 mg, 0.150 mmol) was dissolved in acetone (10 mL). To this solution was added NaI (225 mg, 1.50 mmol), and the mixture was stirred at reflux for 24 h. The reaction was concentrated under reduced pressure and the residue was purified by column chromatography (silica gel, CHCl₃/MeOH/AcOH, 100:5:1). The purification afforded compound **14** as an orange solid (62 mg, 73%). An analytical sample was prepared by crystallization from CHCl₃/MeOH (9:1). ¹H NMR (300 MHz, DMSO-d₆) δ (ppm): 10.81 (s, 1H), 10.08 (bs, 2H), 8.31 (d, *J* = 1.6 Hz, 1H), 7.78 (dd, *J* = 8.4, 1.8 Hz, 1H), 7.21 (d, *J* = 8.3 Hz, 1H), 6.68 (s, 2H), 6.39 (s, 2H), 3.88 (s, 2H), 2.36 (m, 4H), 0.93 (t, *J* = 7.5, 6H). ¹³C NMR (100 MHz, DMSO-d₆) δ (ppm): 169.01, 167.85, 157.54 (2C), 150.38 (2C), 147.69, 140.73, 127.78 (3C), 127.60 (2C), 126.58, 125.03, 113.78, 109.43 (2C), 102.15 (2C), 84.04, 22.81 (2C), 14.62 (2C), 1.45. HPLC: 97% purity at λ_{abs} = 449, 254 nm. HRMS (ESI): *m/z* 572.0592 (MH⁺ [C₂₆H₂₃I NO₆] = 572.0570).

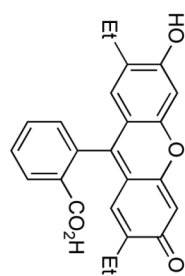
400 MHz ^1H NMR spectrum of compound **6** in CDCl_3 **6**

100 MHz ^{13}C NMR spectrum of compound **6** in CDCl_3

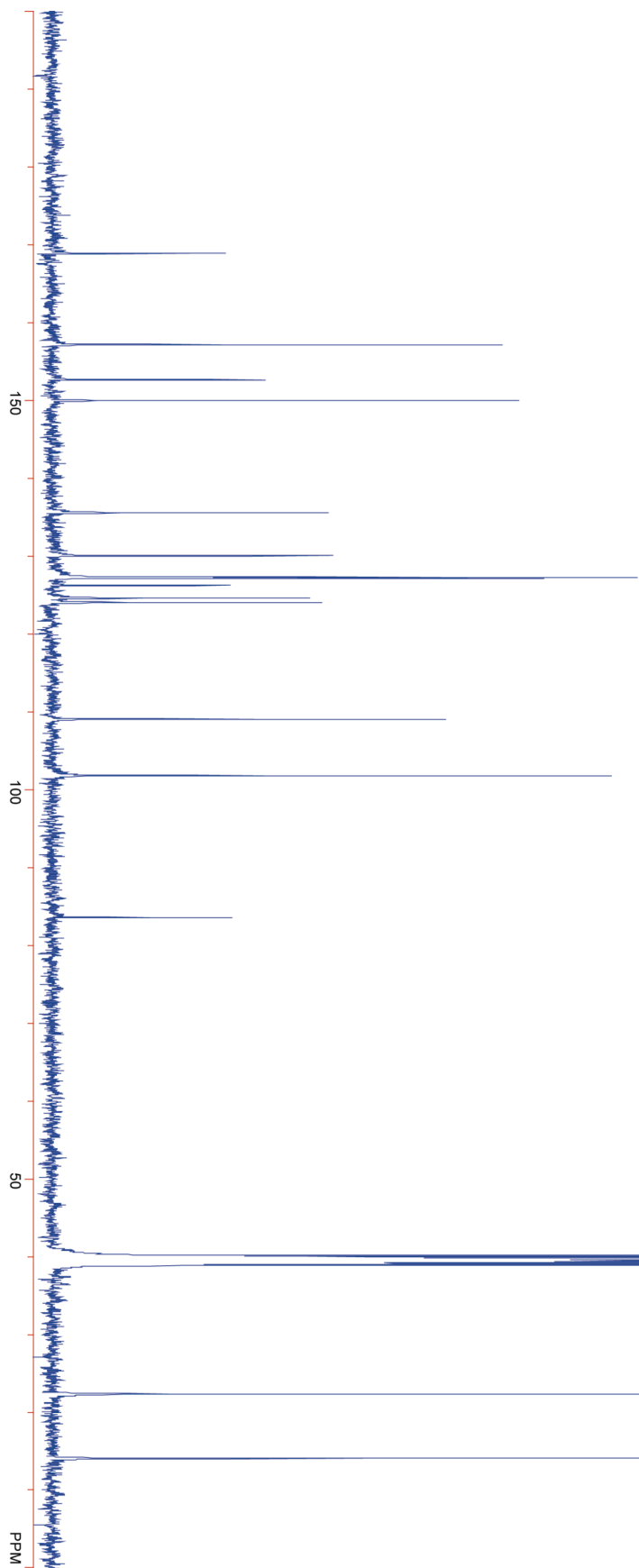


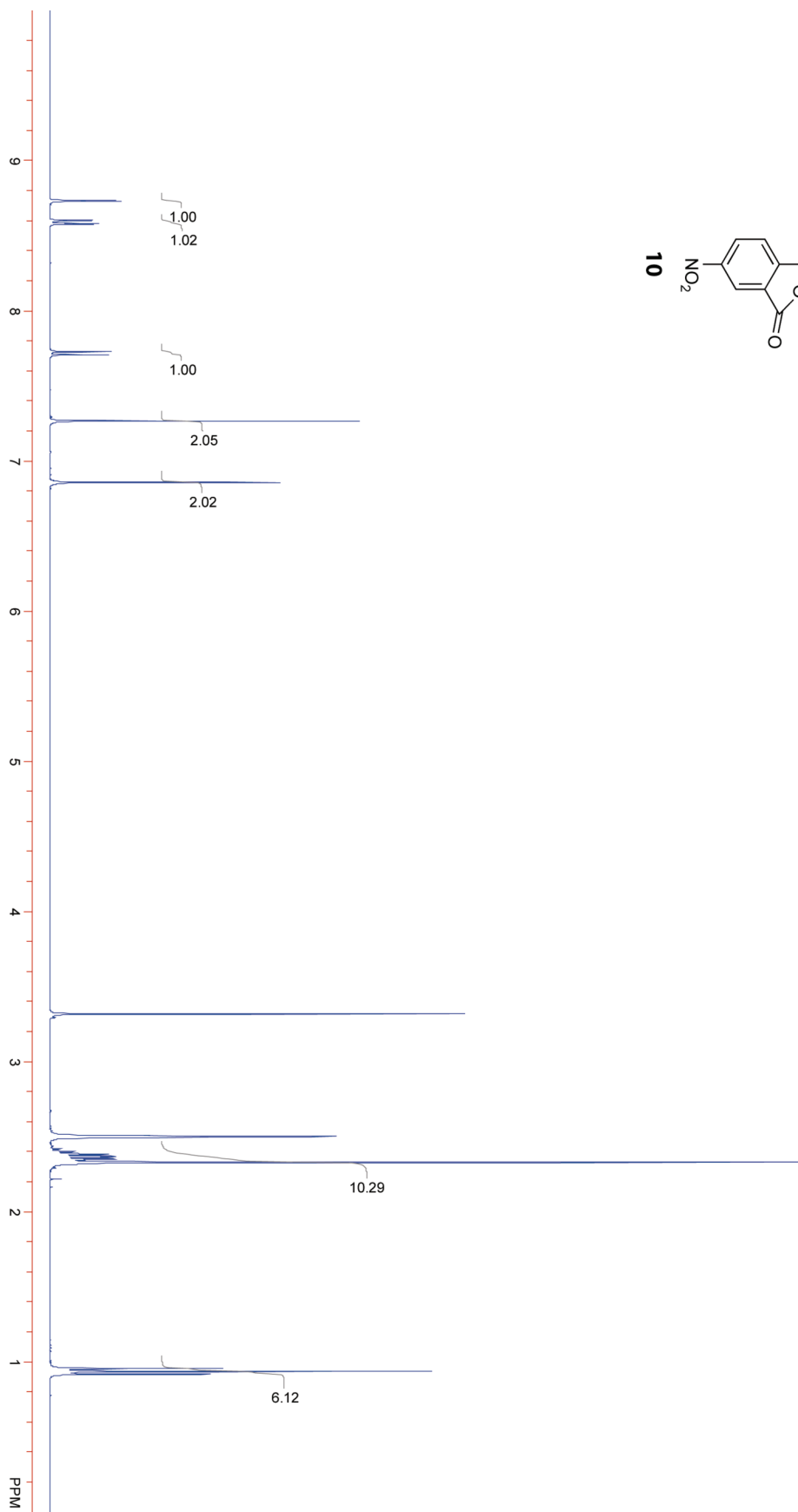
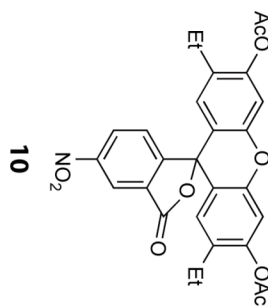
400 MHz ^1H NMR spectrum of compound **7** in DMSO-d_6 

100 MHz ^{13}C NMR spectrum of compound **7** in DMSO-d_6

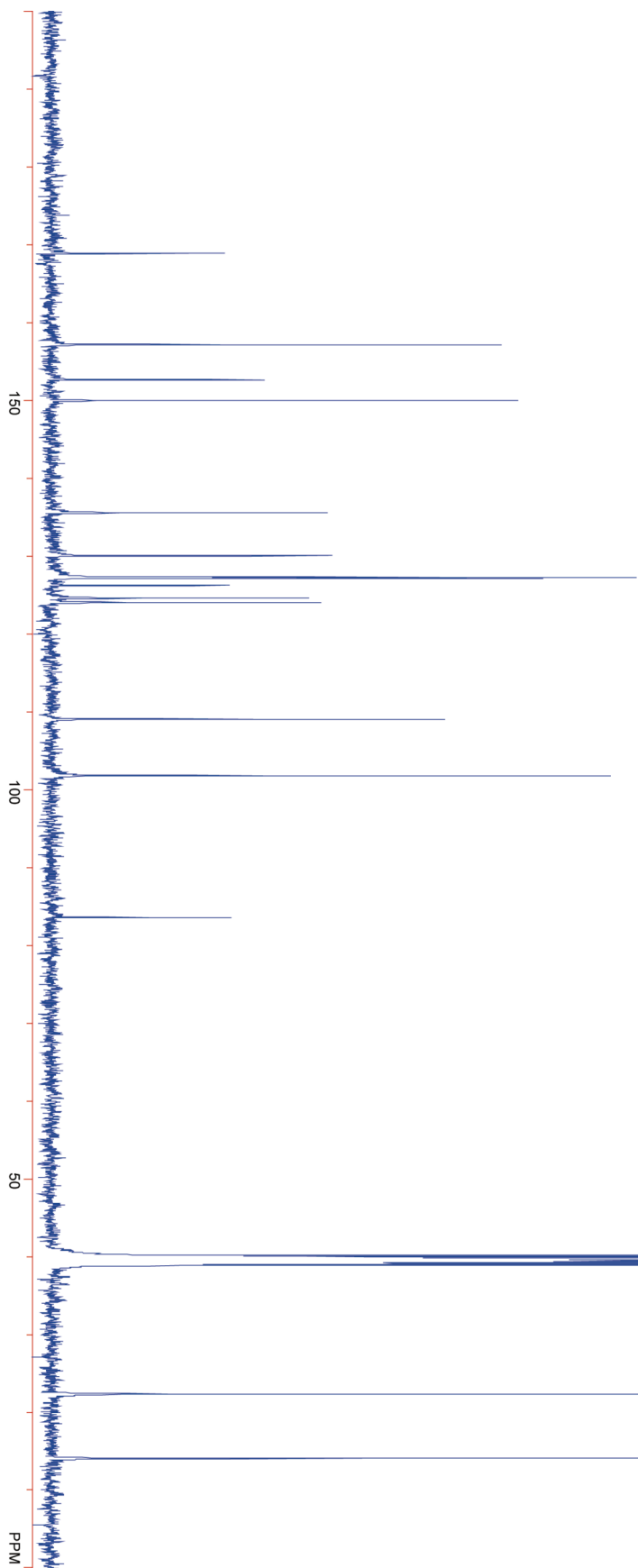
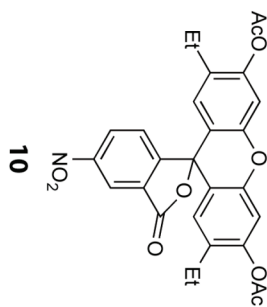


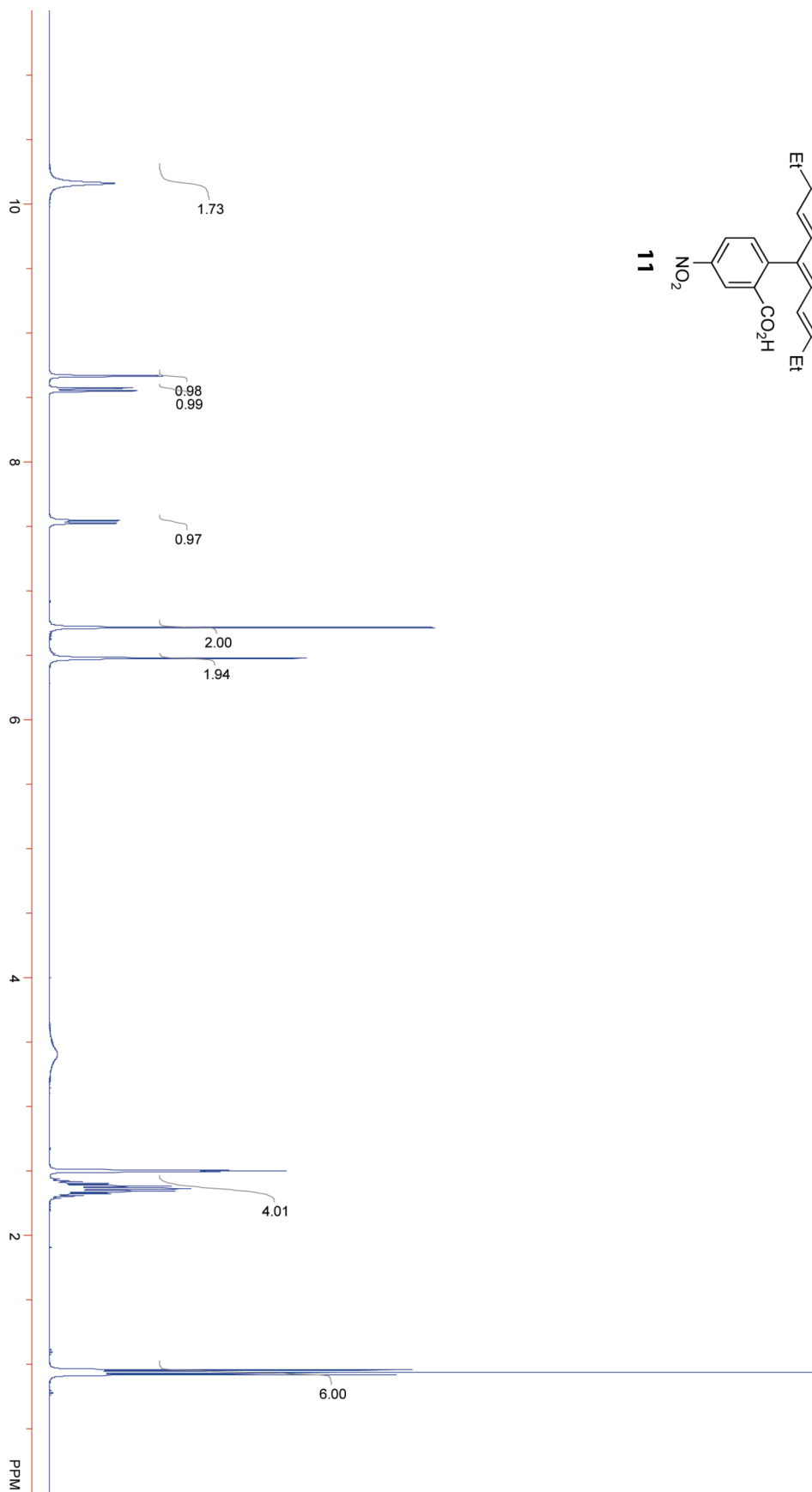
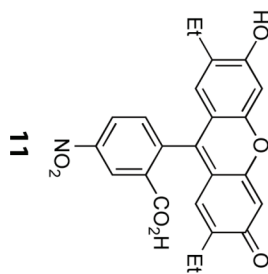
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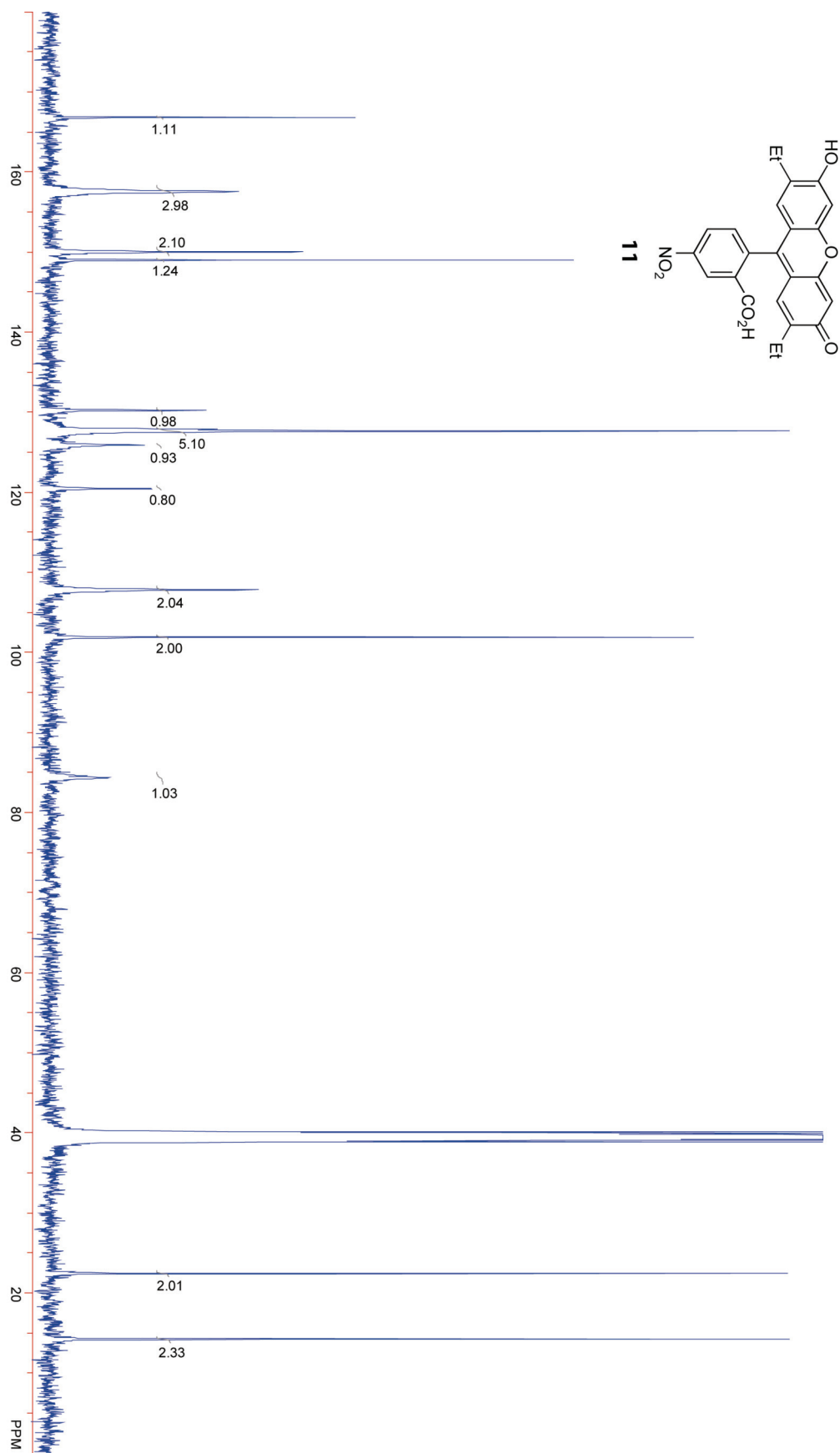
400 MHz ^1H NMR spectrum of compound **10** in DMSO-d_6 

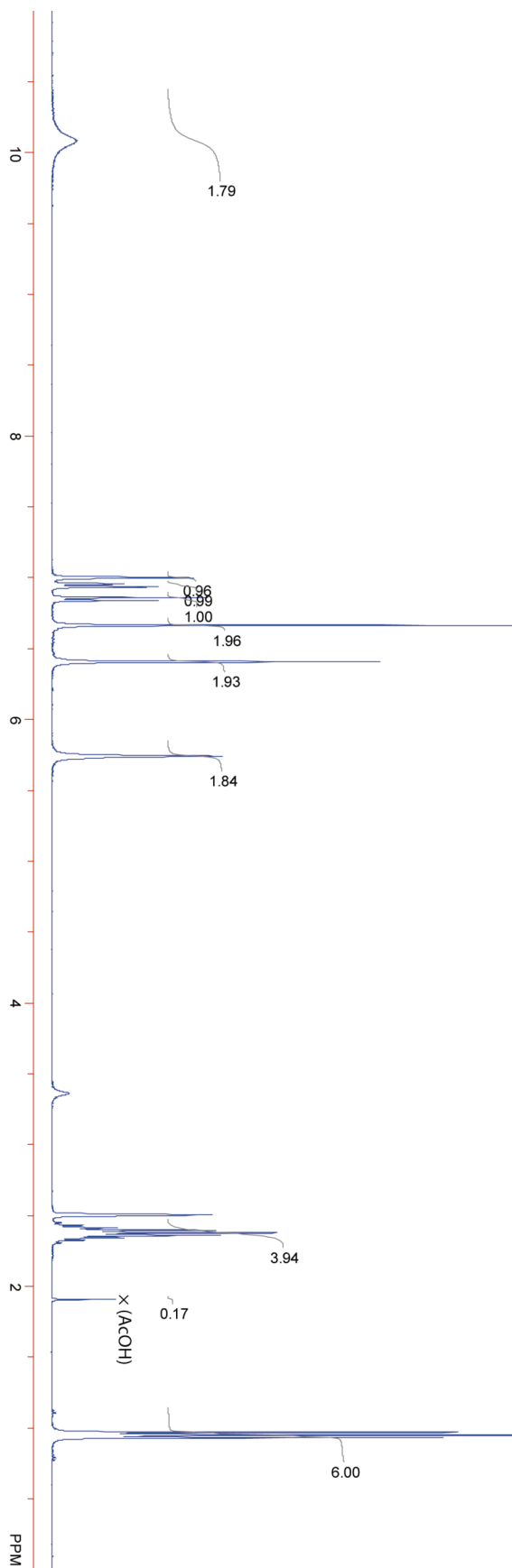
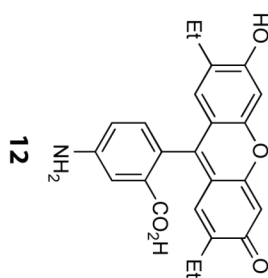
100 MHz ^{13}C NMR spectrum of compound **10** in DMSO-d_6



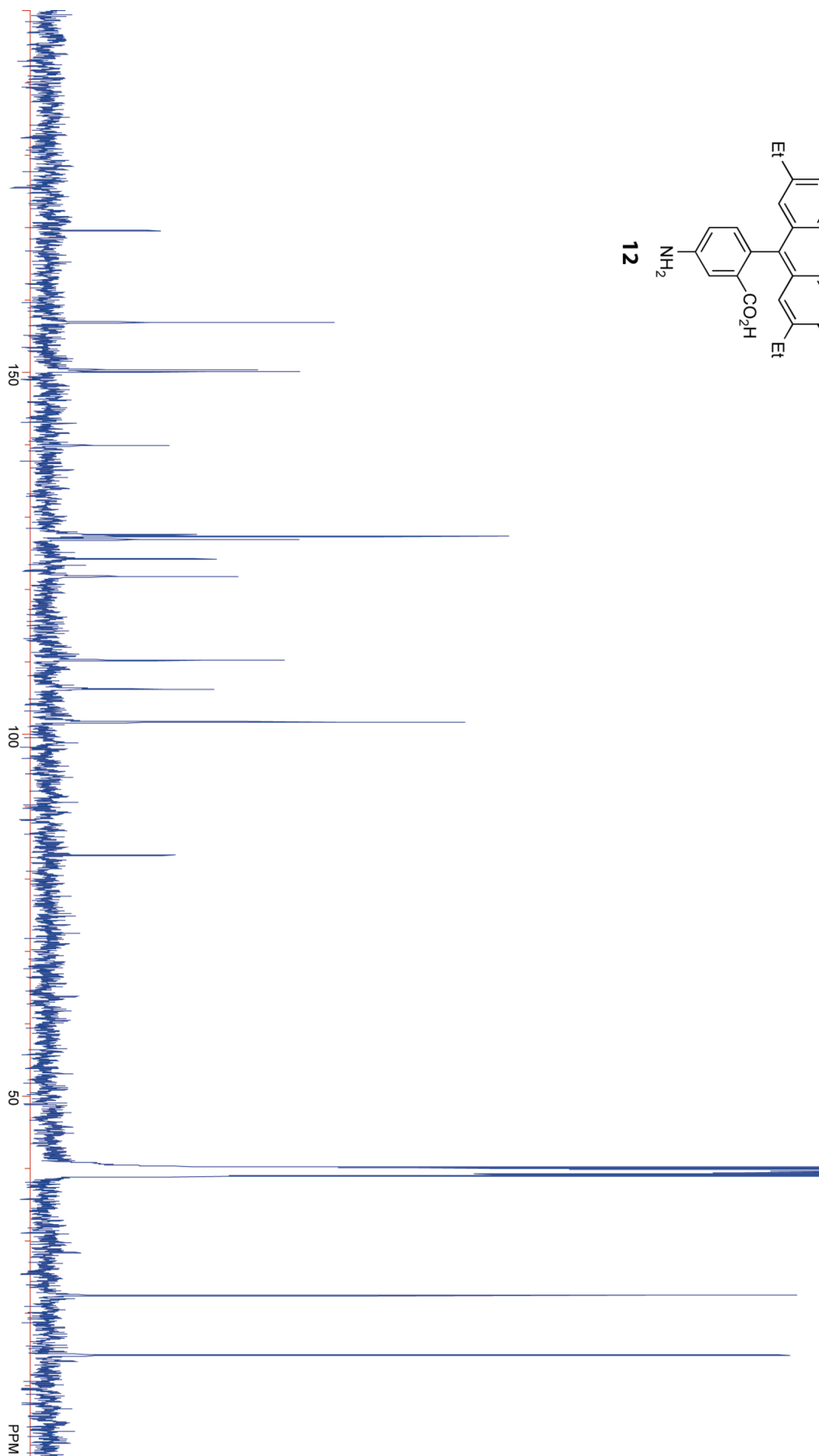
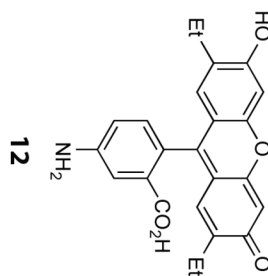
400 MHz ^1H NMR spectrum of compound **11** in DMSO-d_6 

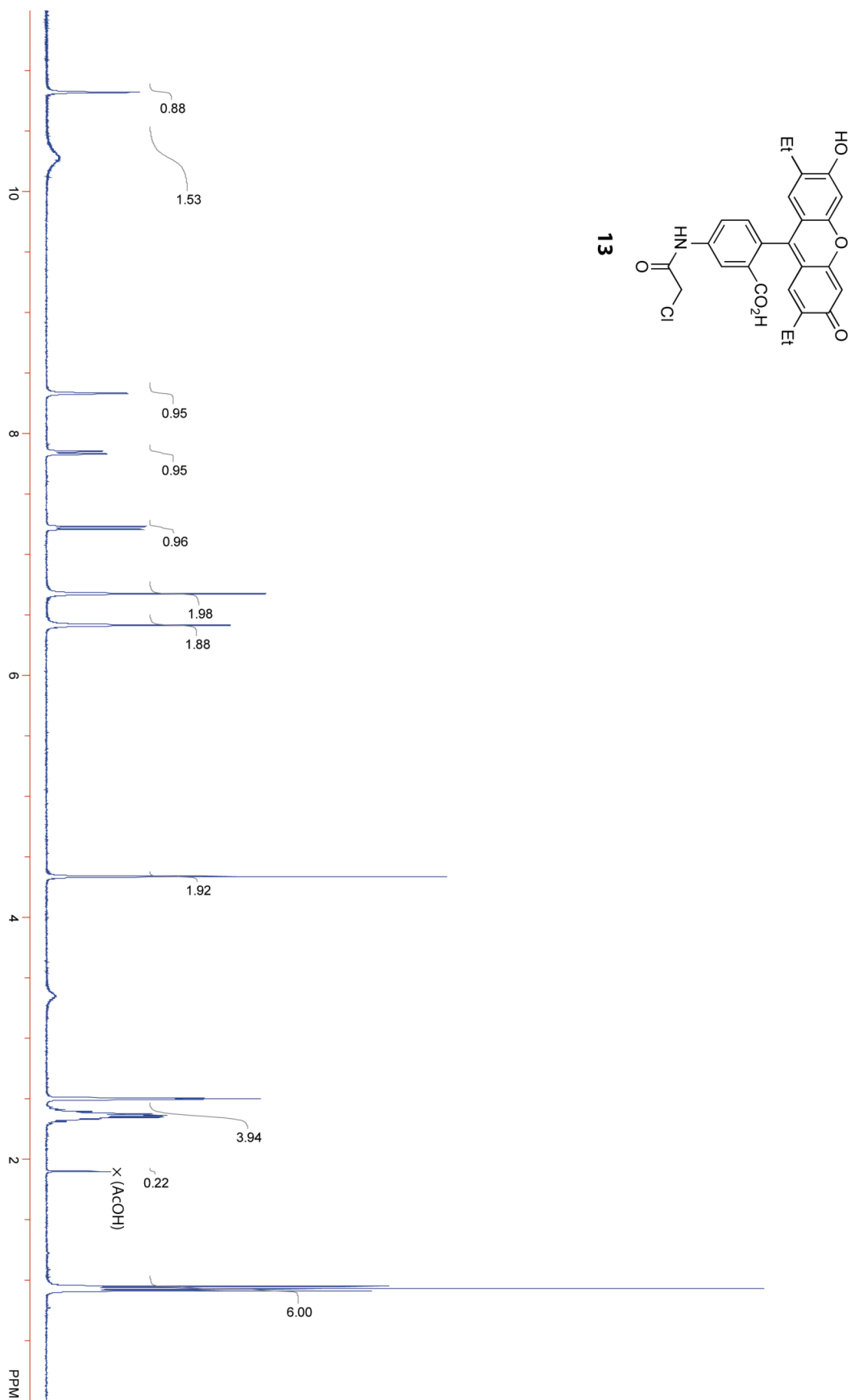
100 MHz quantitative ^{13}C NMR spectrum of compound **11** in DMSO-d_6



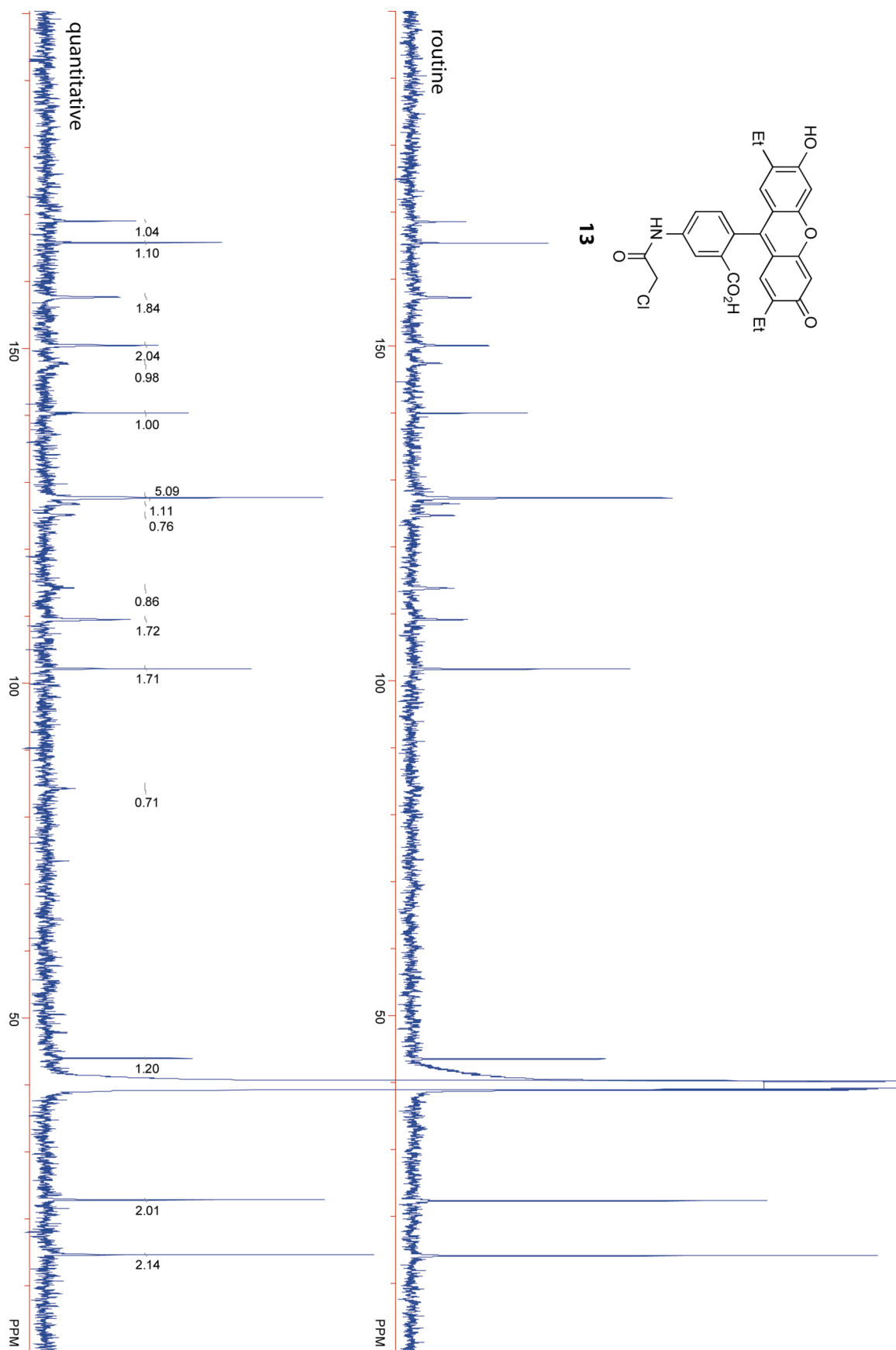
400 MHz ^1H NMR spectrum of compound **12** in DMSO-d_6 

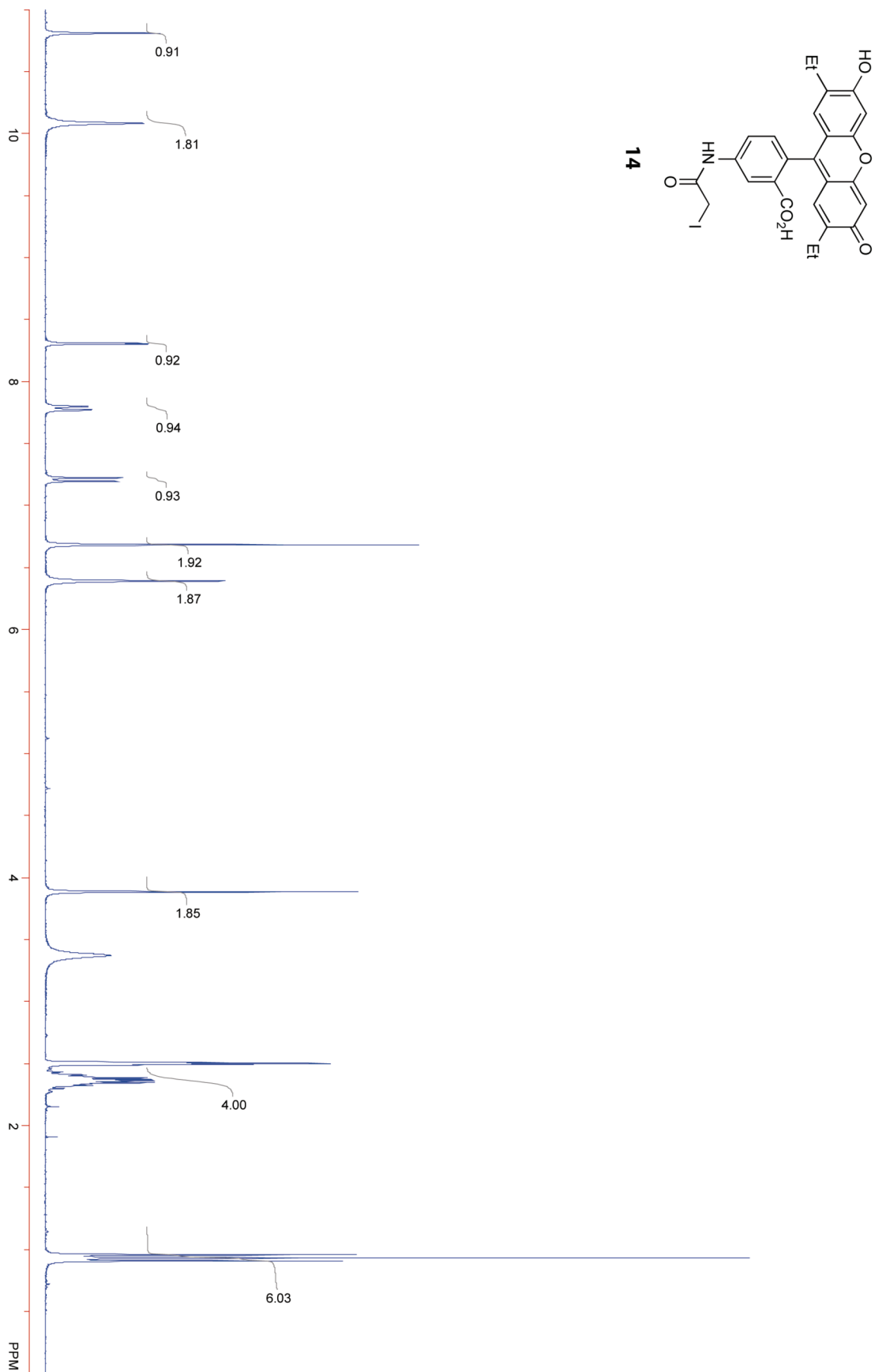
100 MHz ^{13}C NMR spectrum of compound **12** in DMSO-d_6



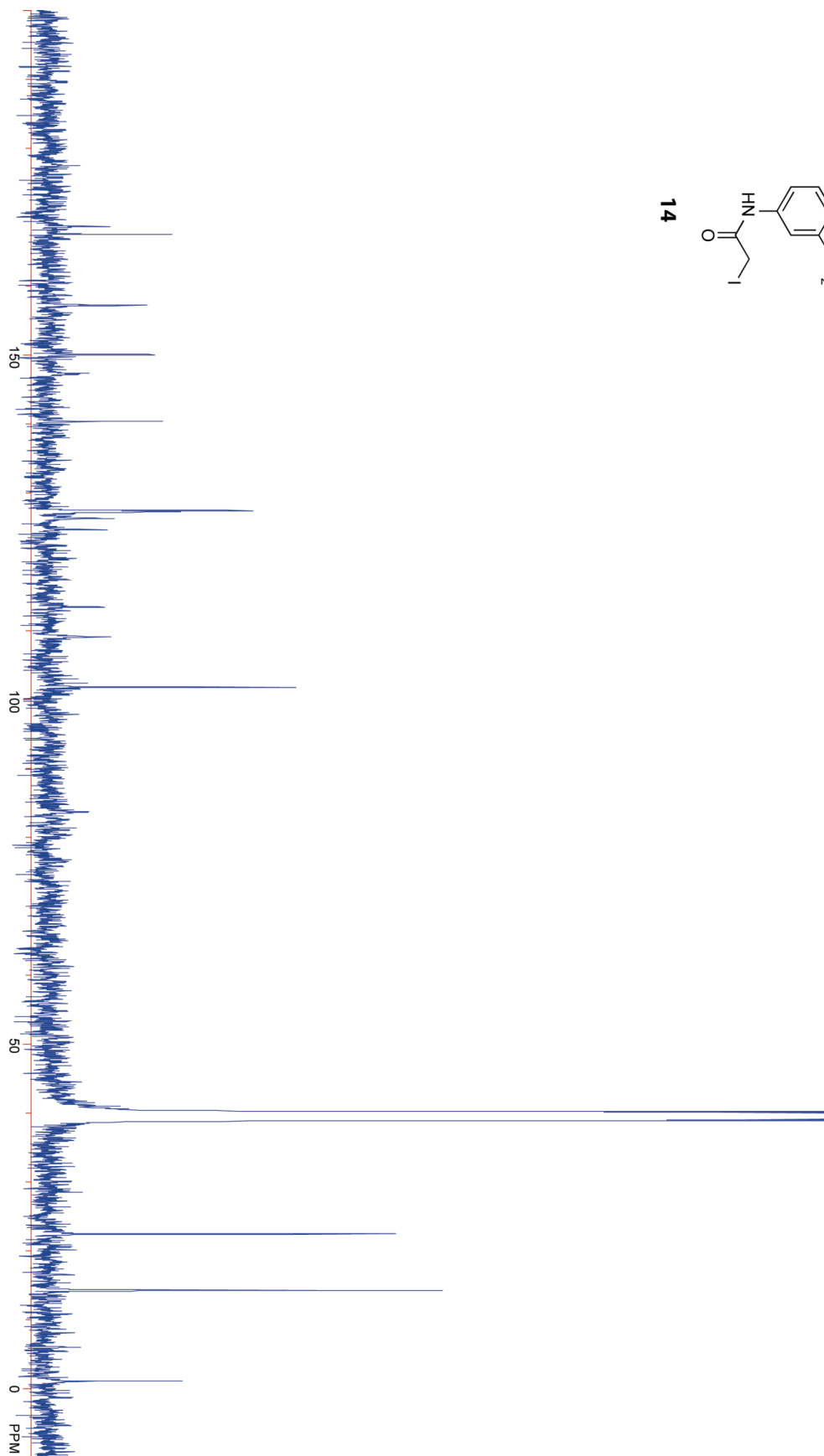
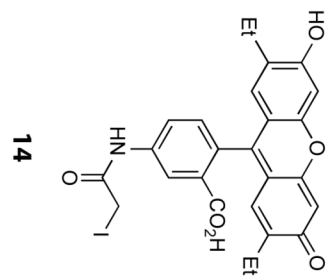
400 MHz ^1H NMR spectrum of compound **13** in DMSO-d_6 

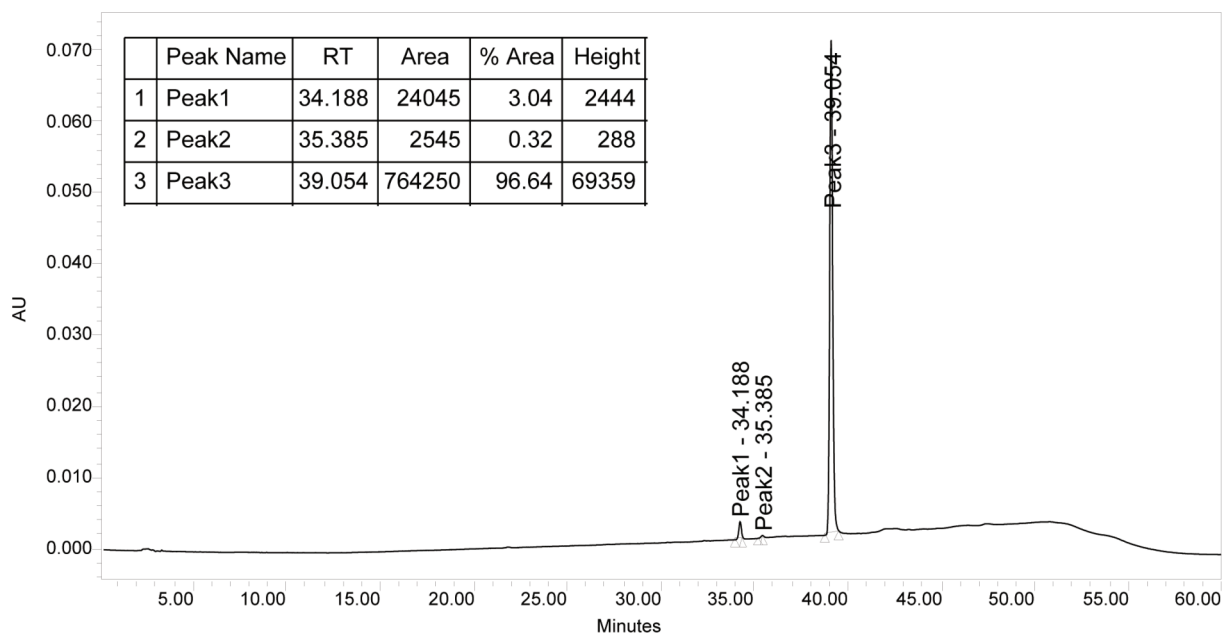
^{13}C NMR spectra of compound **13** in DMSO- d_6 (routine at 90 MHz; quantitative at 100 MHz)



300 MHz ^1H NMR spectrum of compound **14** in DMSO-d_6 

100 MHz ^{13}C NMR spectrum of compound **14** in DMSO-d_6



HPLC chromatogram for compound **14** ($\lambda = 254$ nm)HPLC chromatogram for compound **14** ($\lambda = 449$ nm)