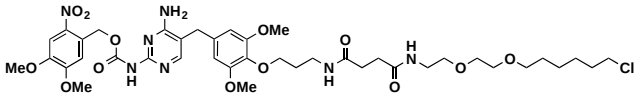
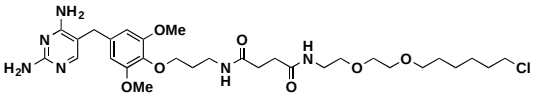
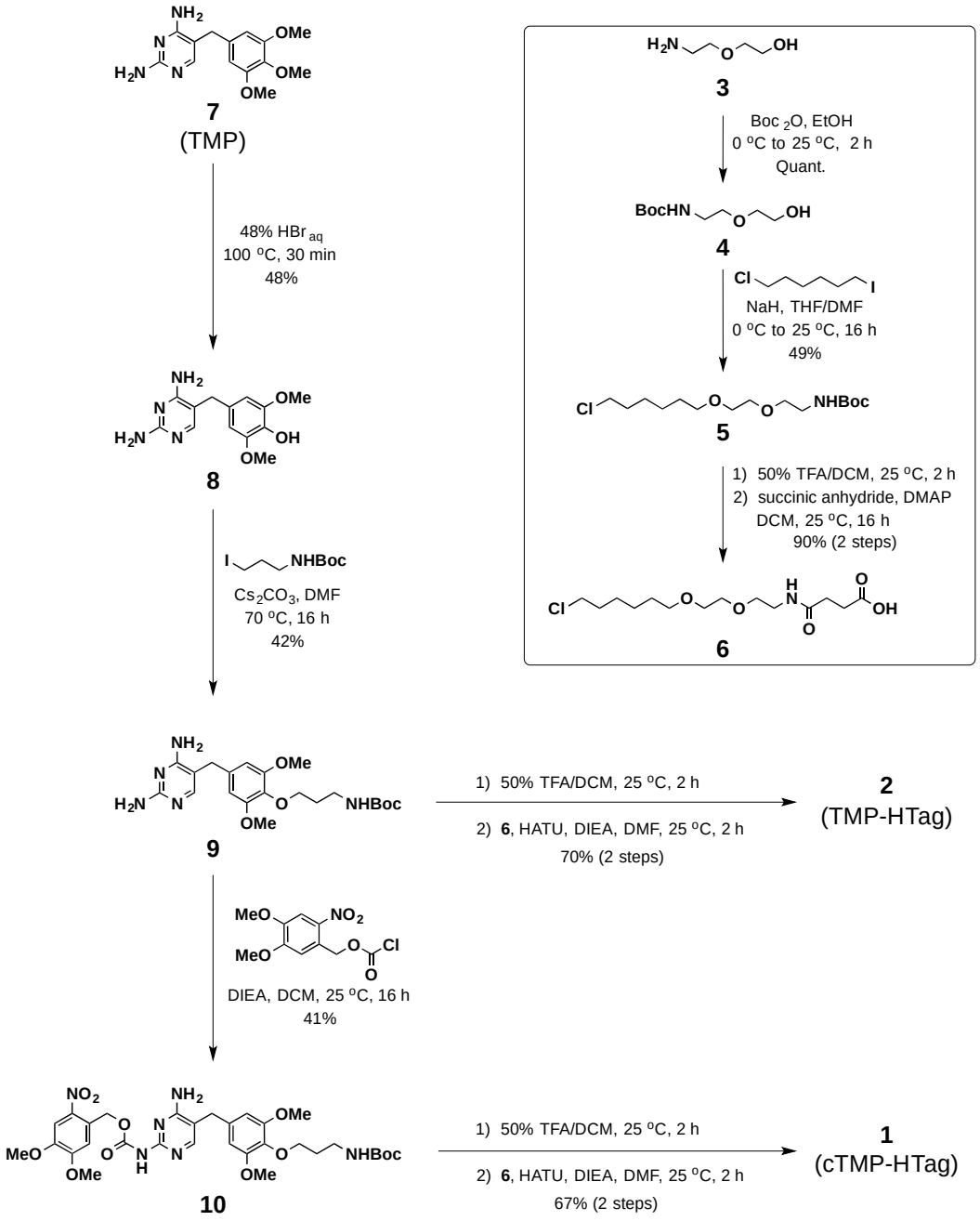


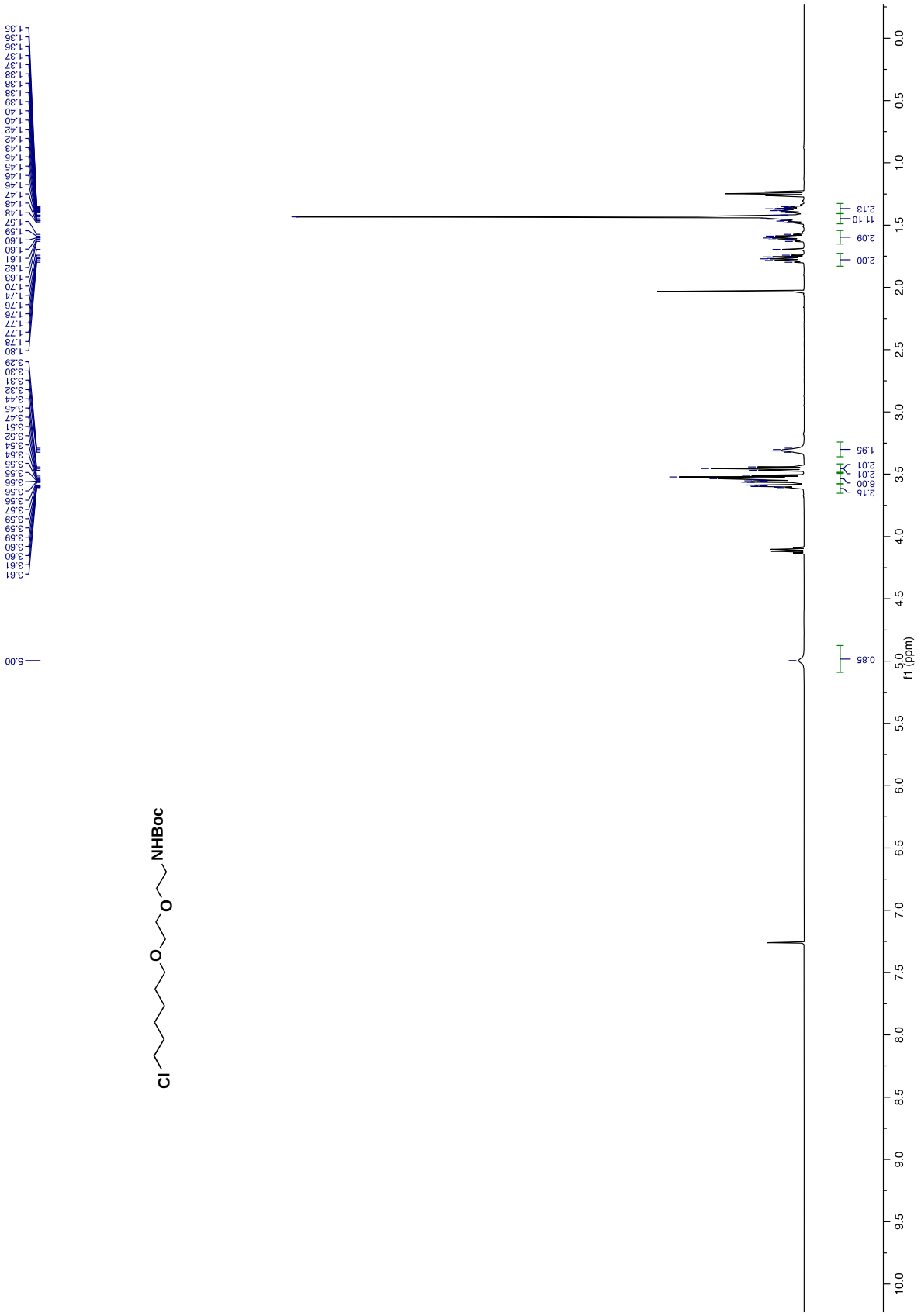
1
(cTMP-HTag)



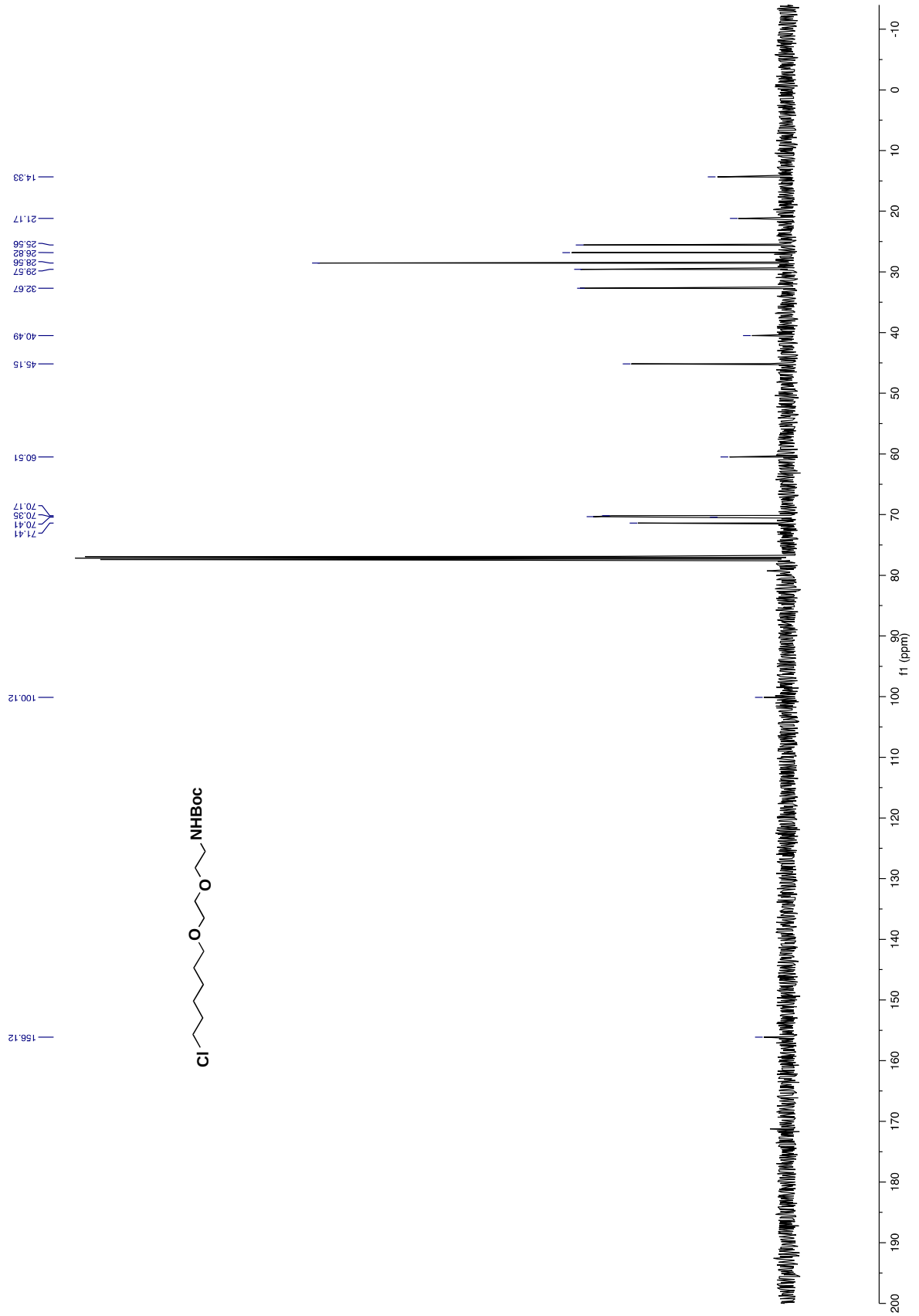
2
(TMP-HTag)



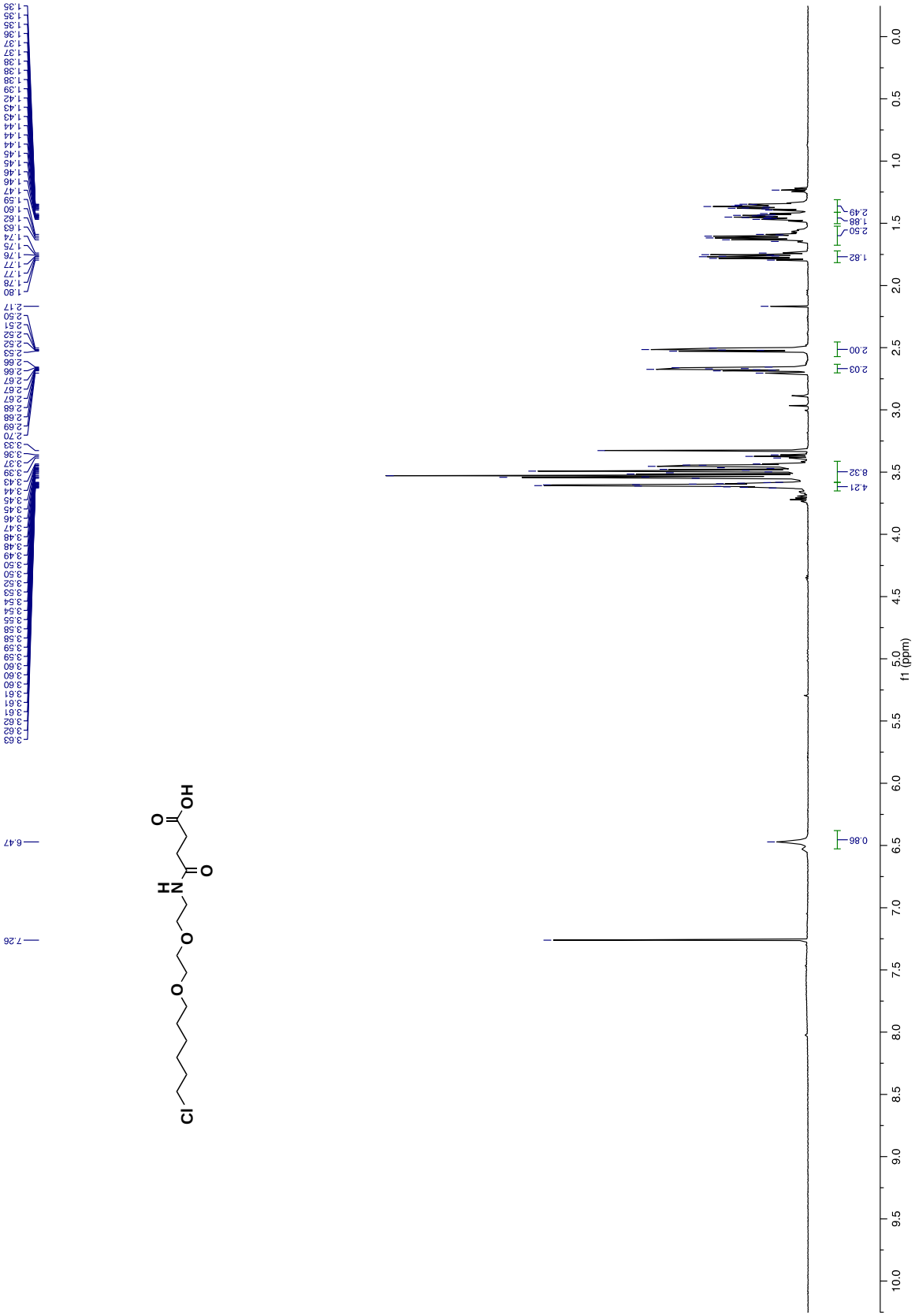
Supplementary Figure 1: Structures and synthesis scheme for compounds **1** and **2**.



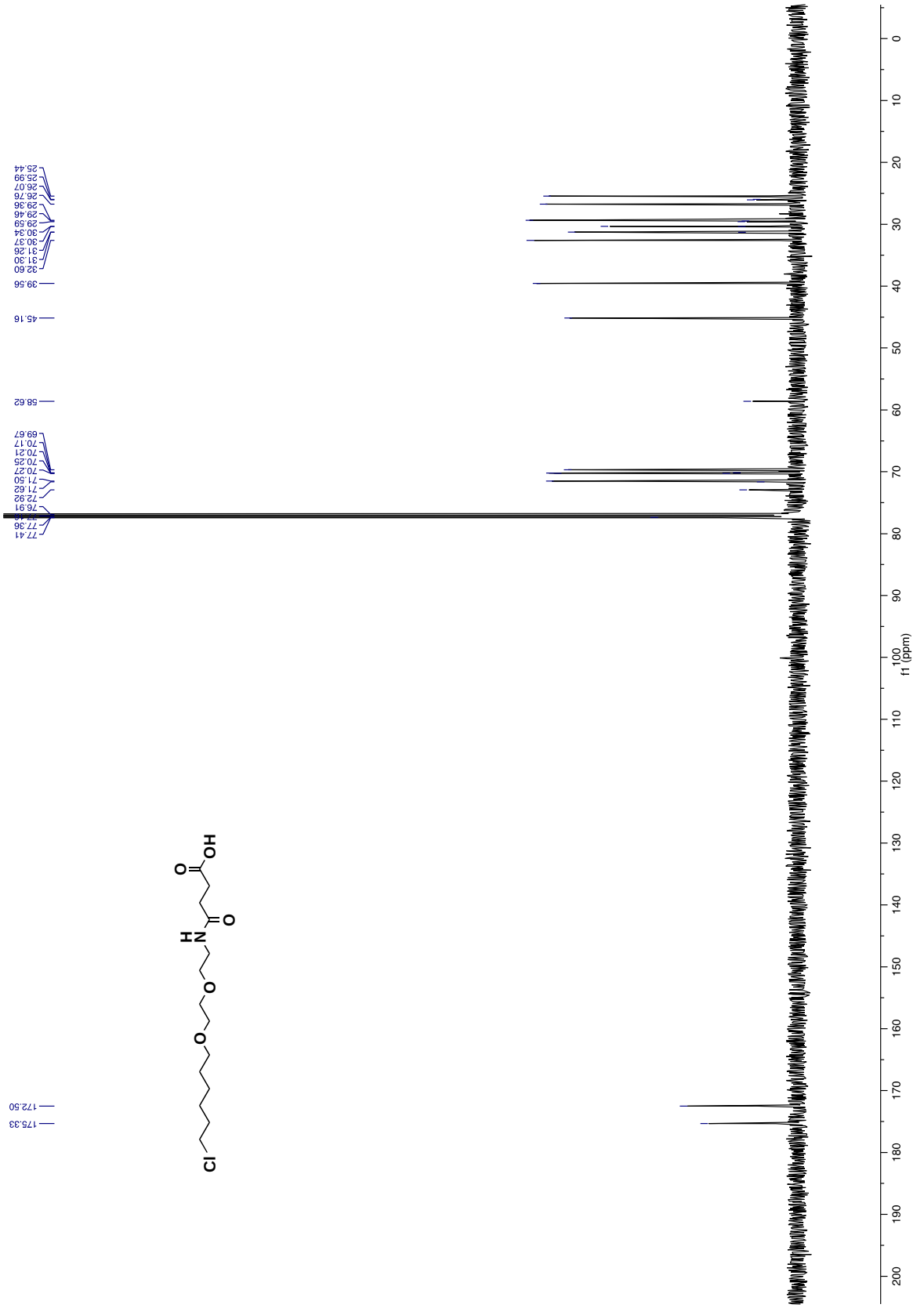
Supplementary Figure 2: ^{1}H NMR spectrum of **5** in $CDCl_3$ (500 MHz).



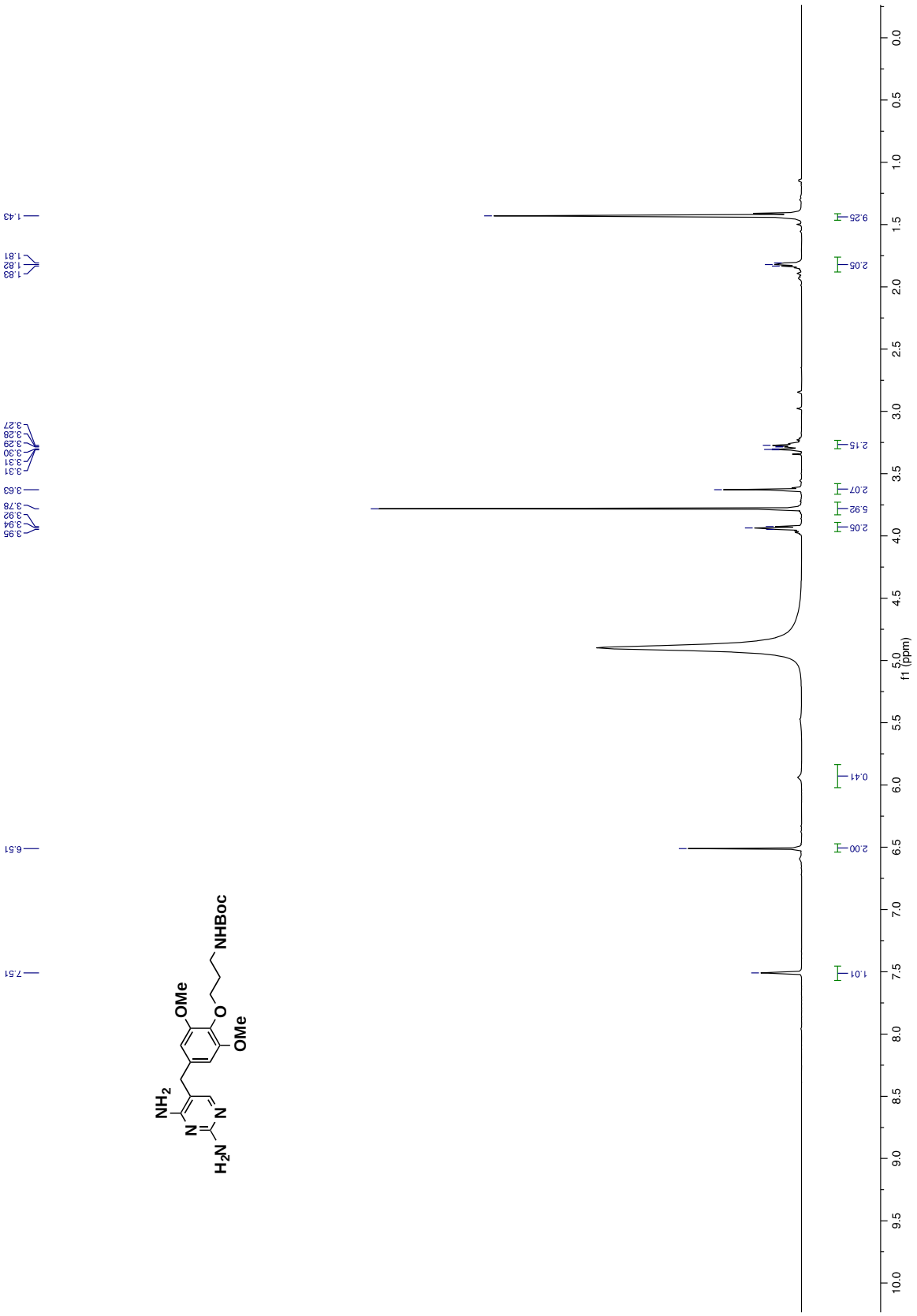
Supplementary Figure 3: ^{13}C NMR spectrum of **5** in $CDCl_3$ (126 MHz).



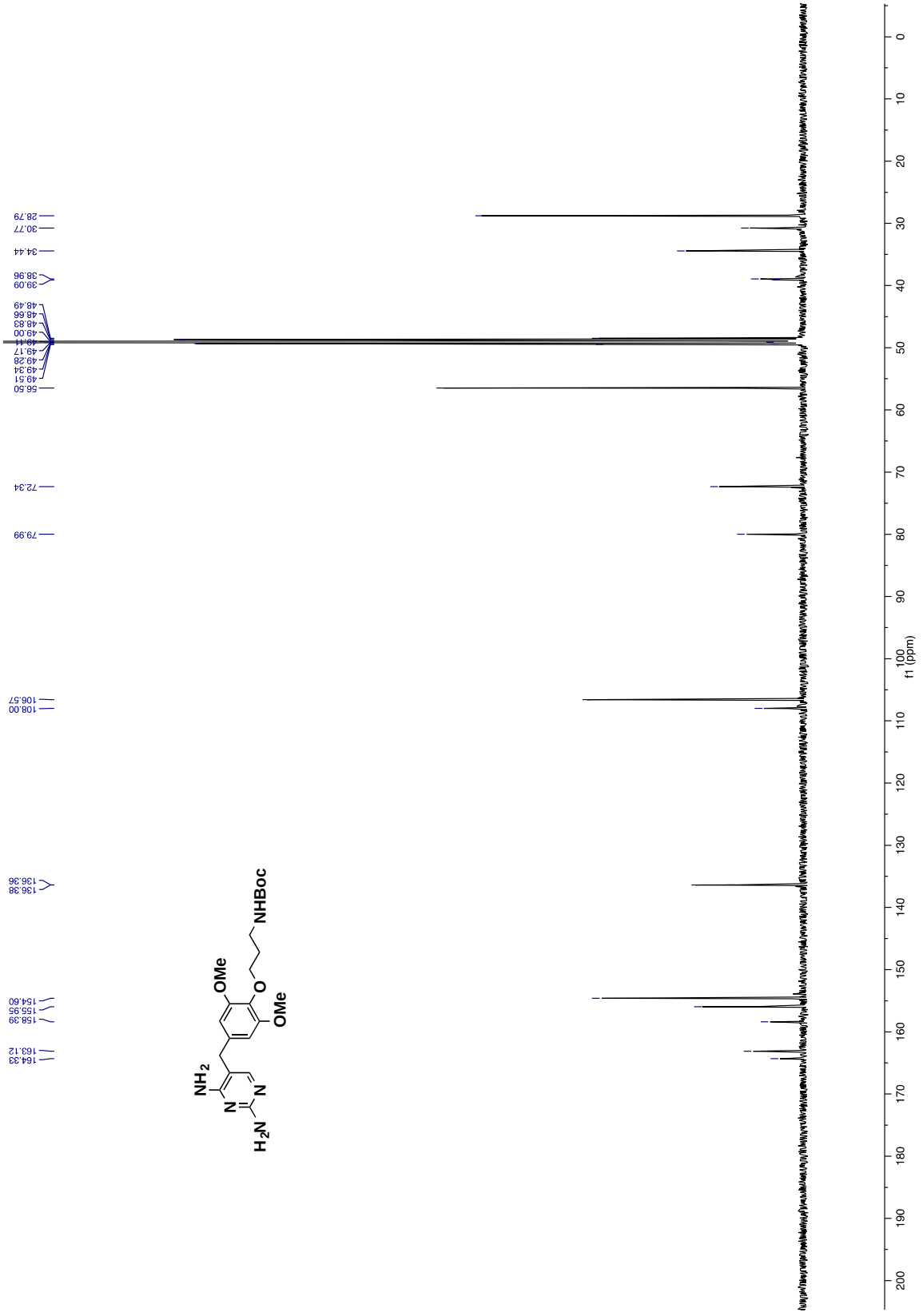
Supplementary Figure 4: ^{1}H NMR spectrum of **6** in $CDCl_3$ (500 MHz).



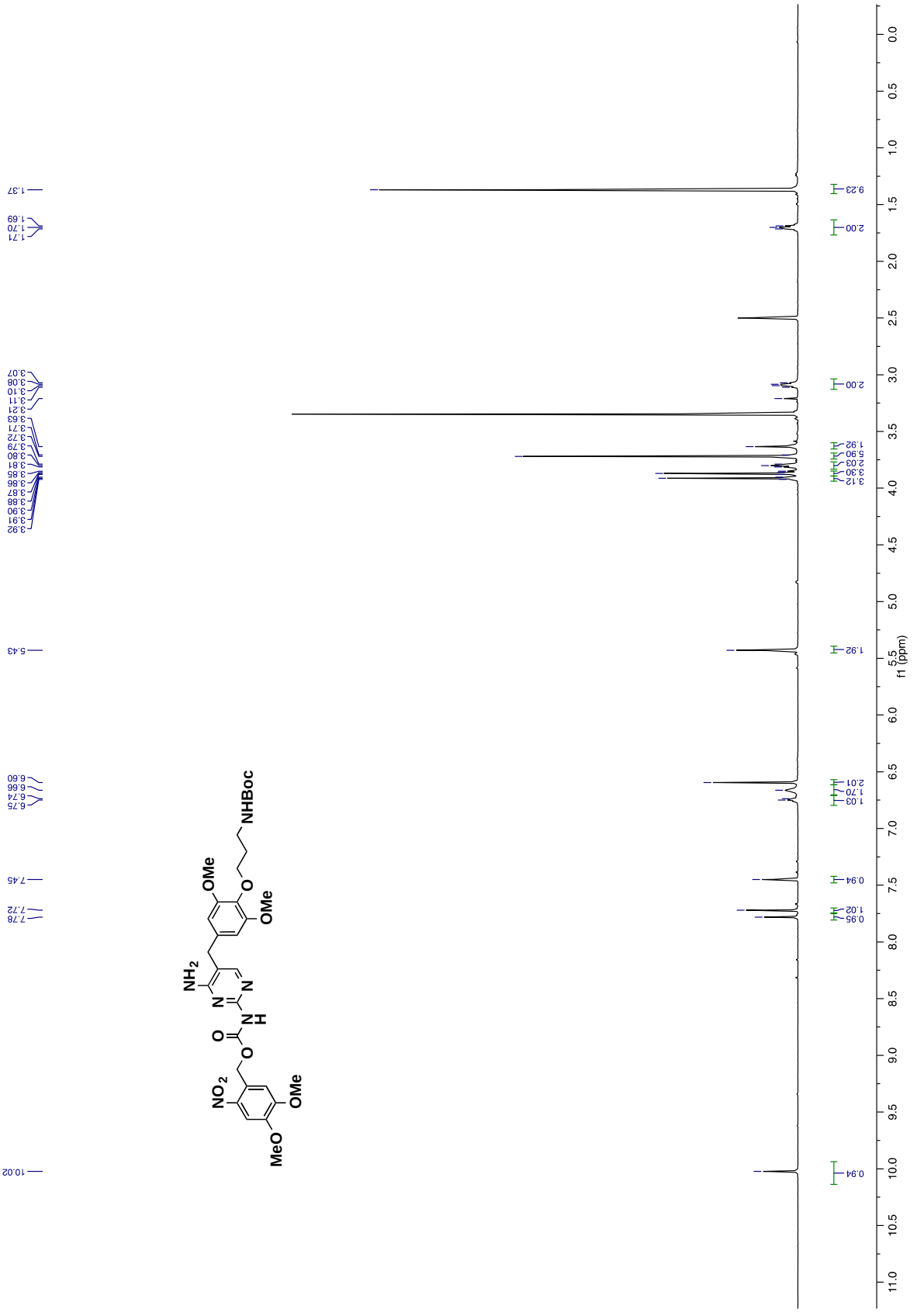
Supplementary Figure 5: ^{13}C NMR spectrum of **6** in $CDCl_3$ (126 MHz).



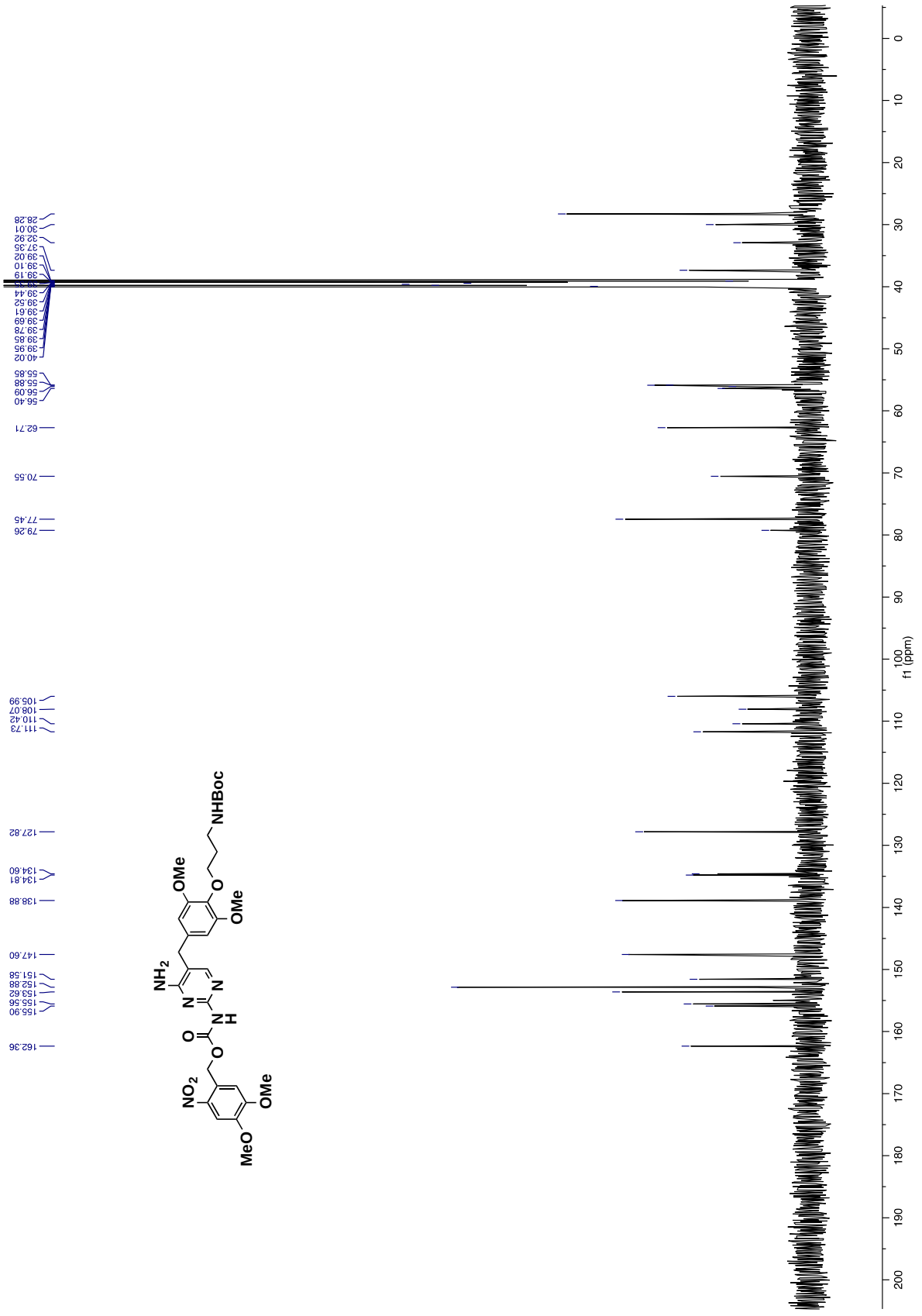
Supplementary Figure 6: ^{1}H NMR spectrum of **9** in MeOD (500 MHz).



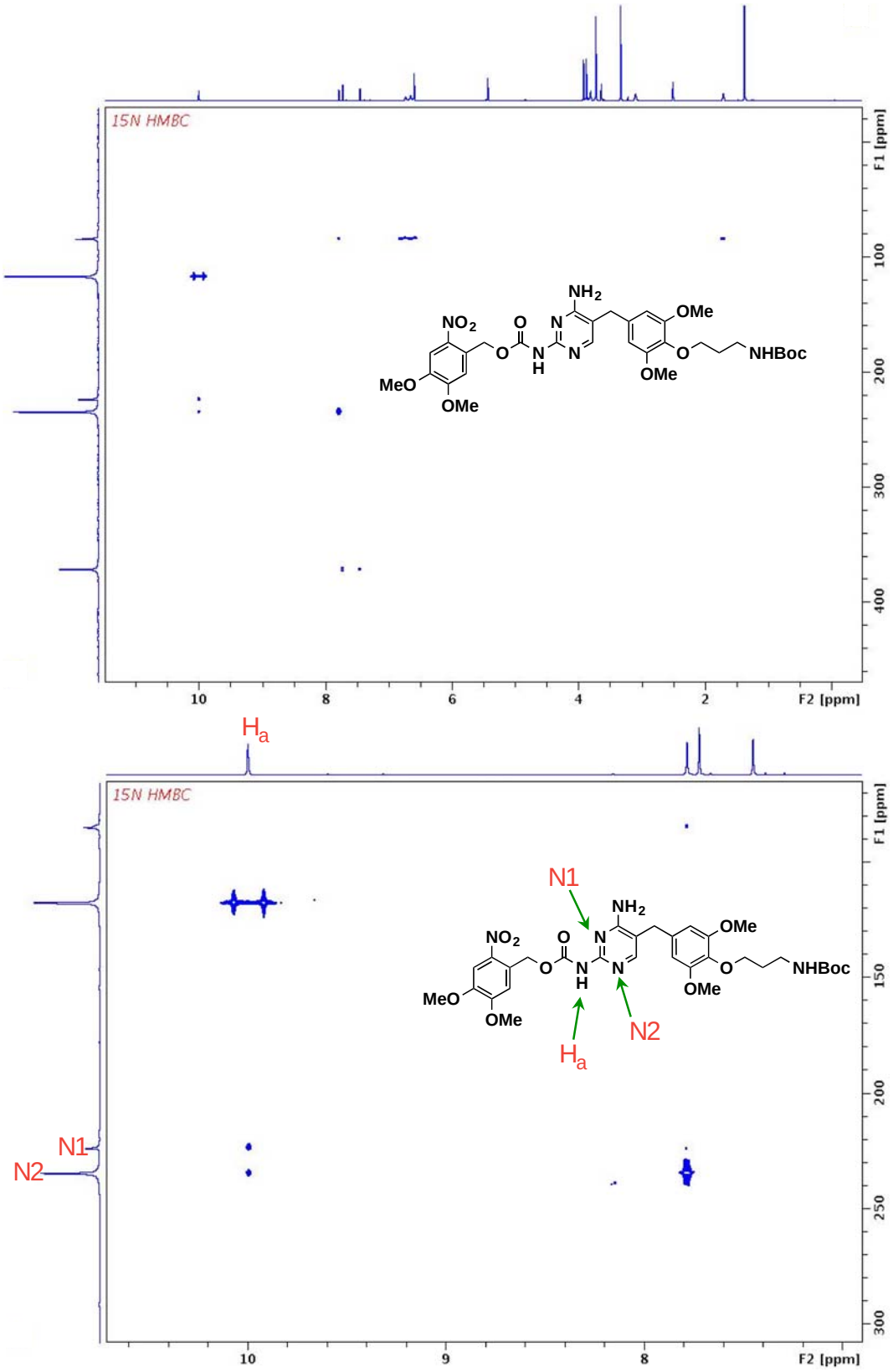
Supplementary Figure 7: ^{13}C NMR spectrum of **9** in MeOD (126 MHz).



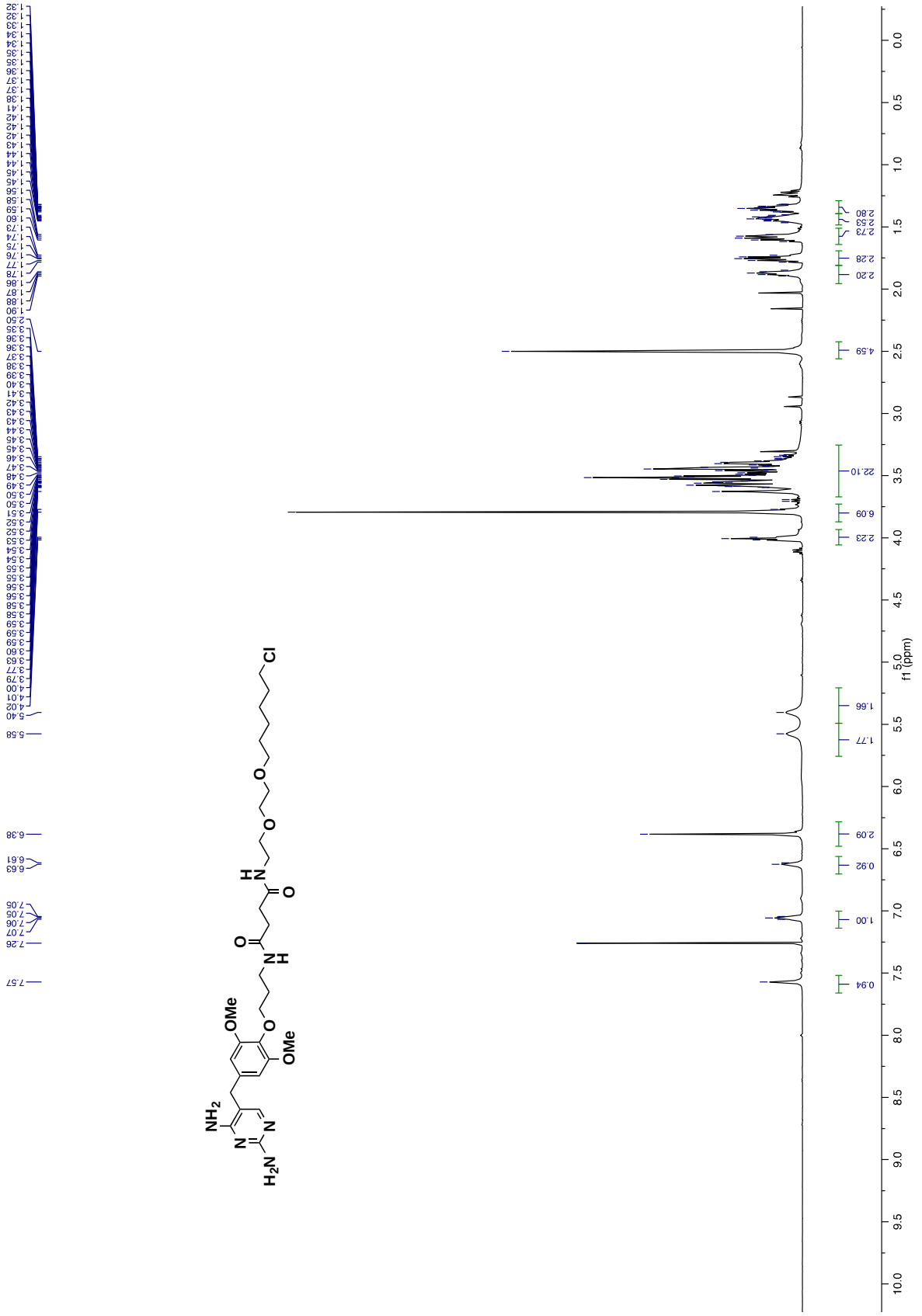
Supplementary Figure 8: ^{1}H NMR spectrum of **10** in DMSO-d_6 (500 MHz).



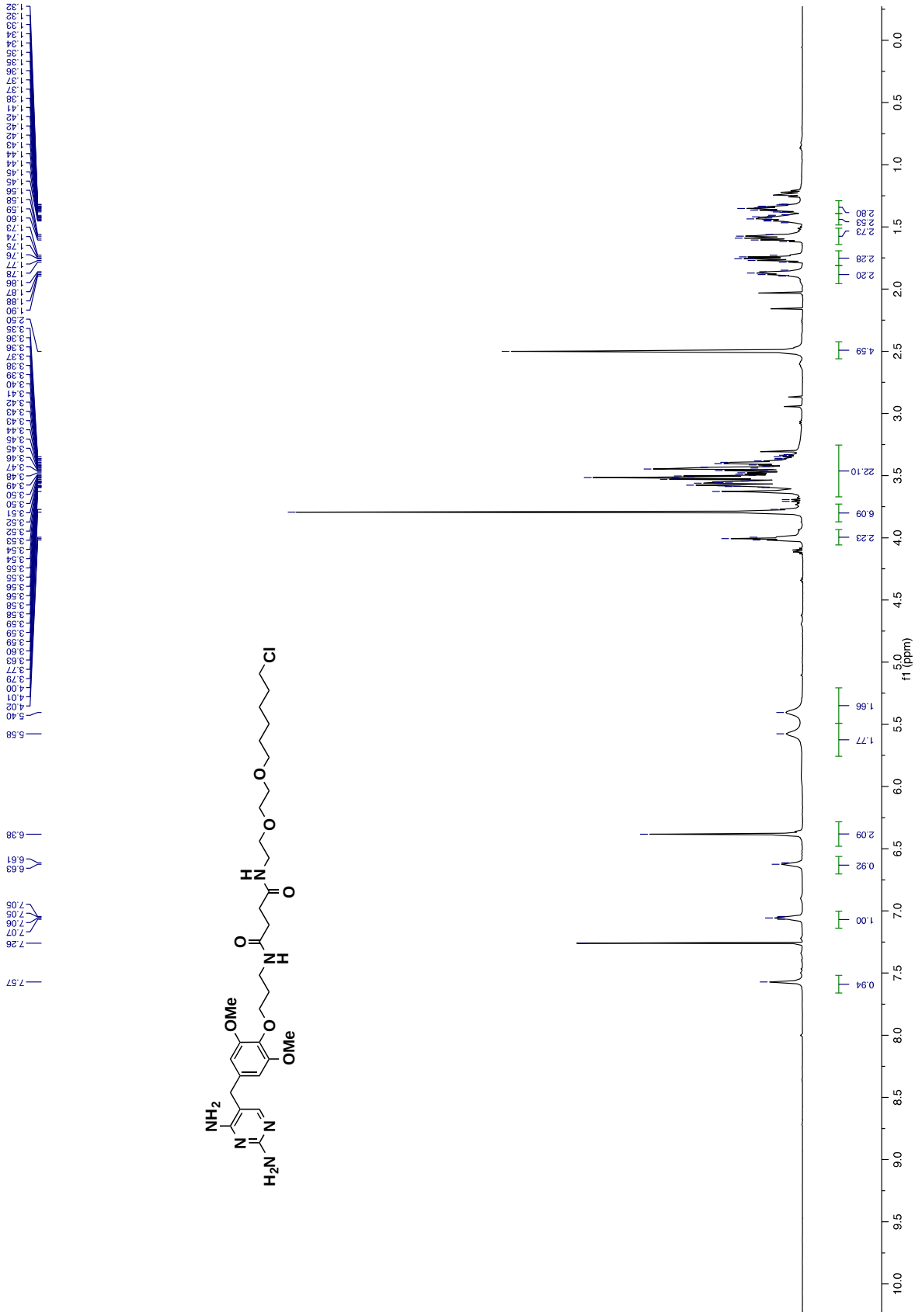
Supplementary Figure 9: ^{13}C NMR spectrum of **10** in DMSO-d_6 (126 MHz).



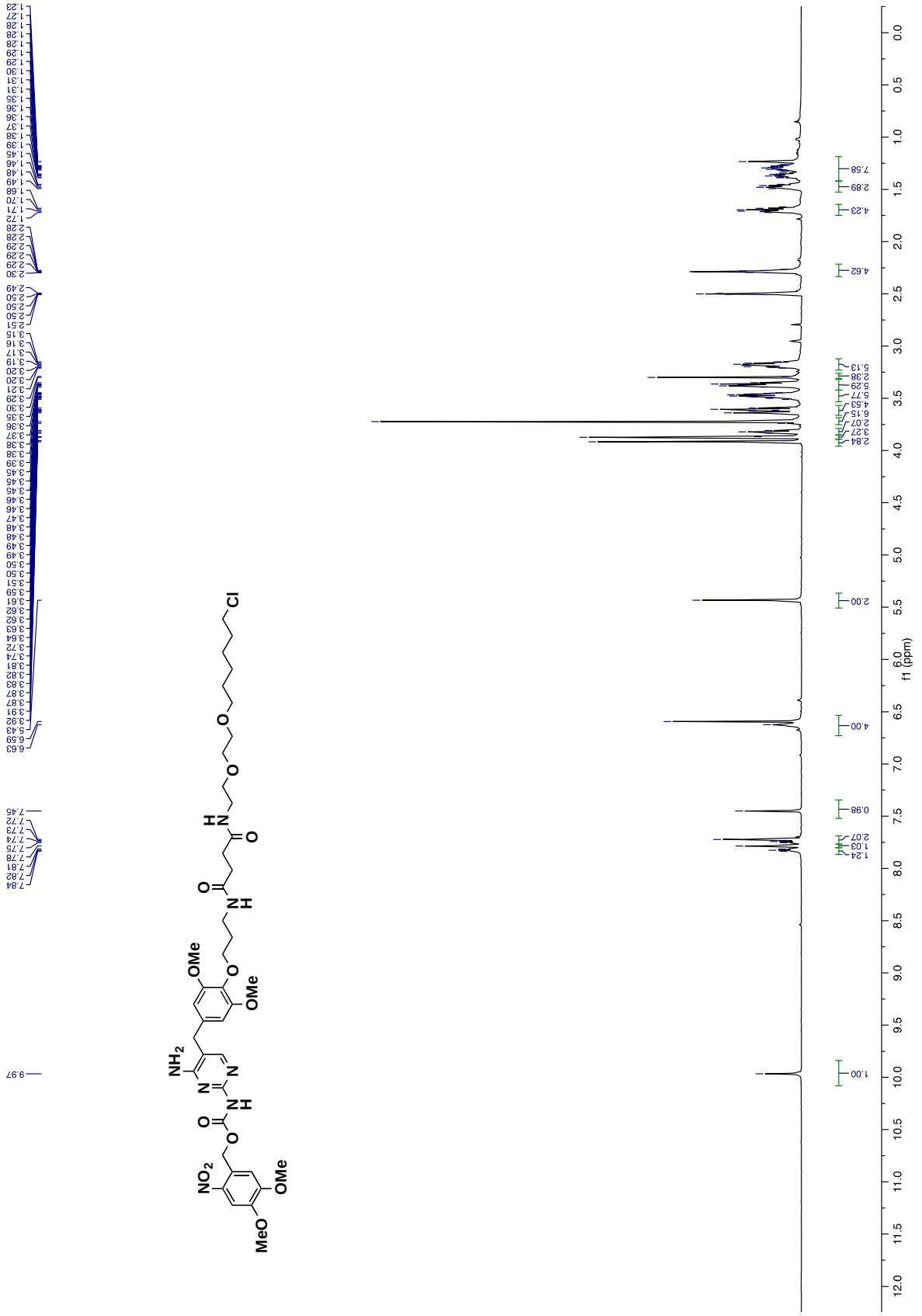
Supplementary Figure 10: ^{1}H-^{15}N HMBC NMR spectrum of **10** in DMSO-d_6 (600 MHz). Upper panel shows complete spectrum, lower panel shows magnification of indicated spectral region with key peak assignments highlighted.



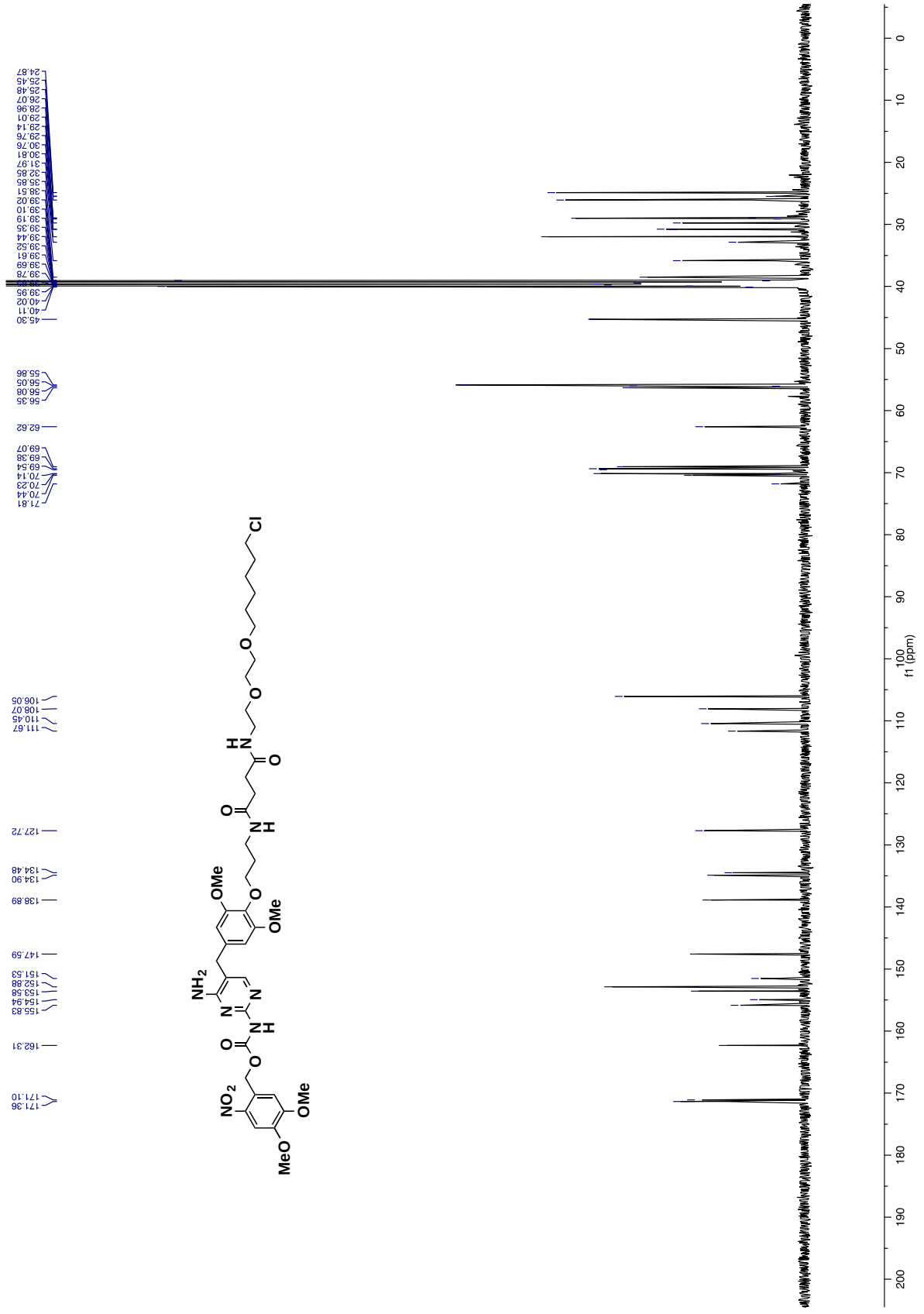
Supplementary Figure 11: ^{1}H NMR spectrum of **2** in $CDCl_3$ (500 MHz).



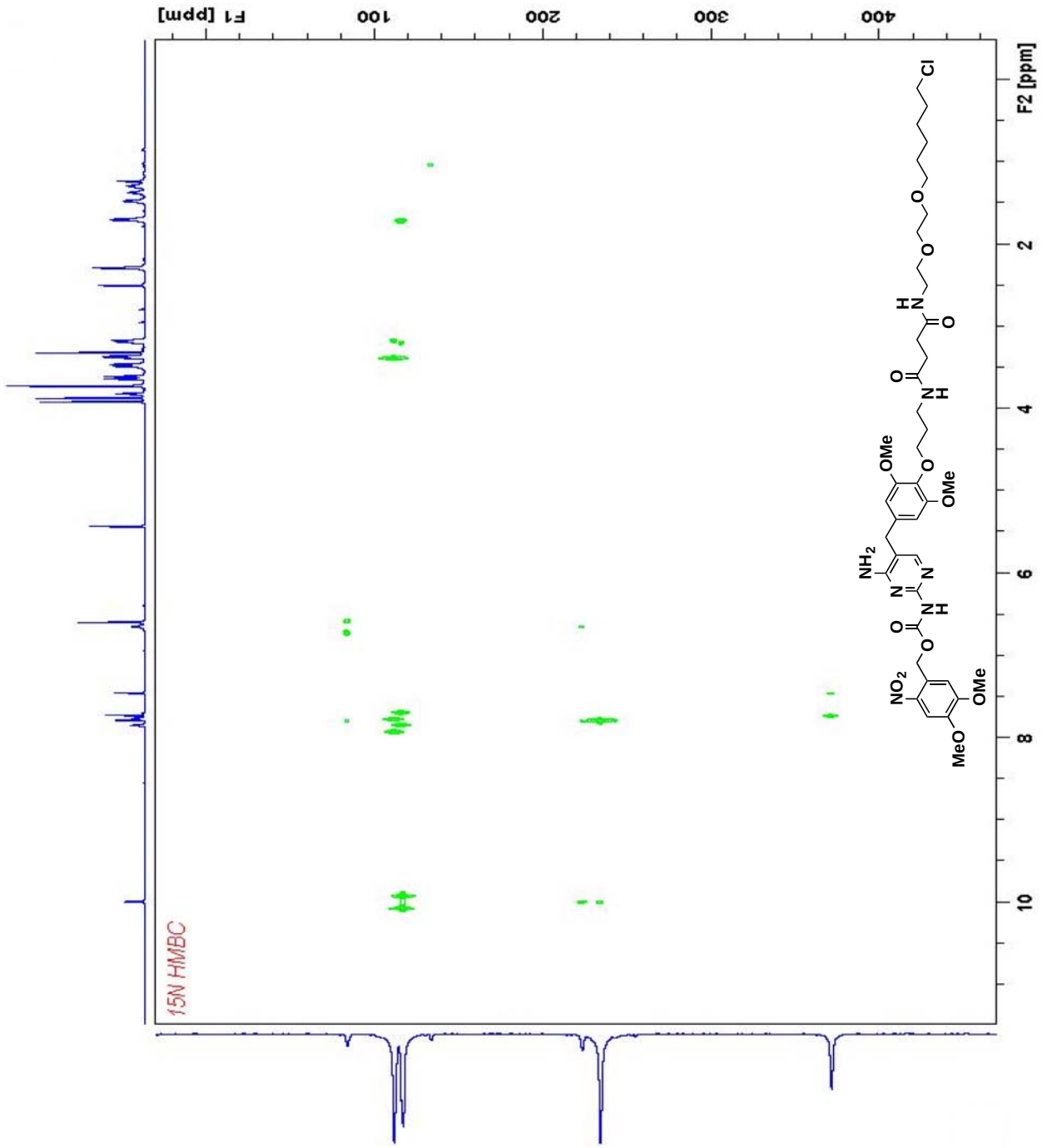
Supplementary Figure 12: ^{13}C NMR spectrum of **2** in $CDCl_3$ (126 MHz).



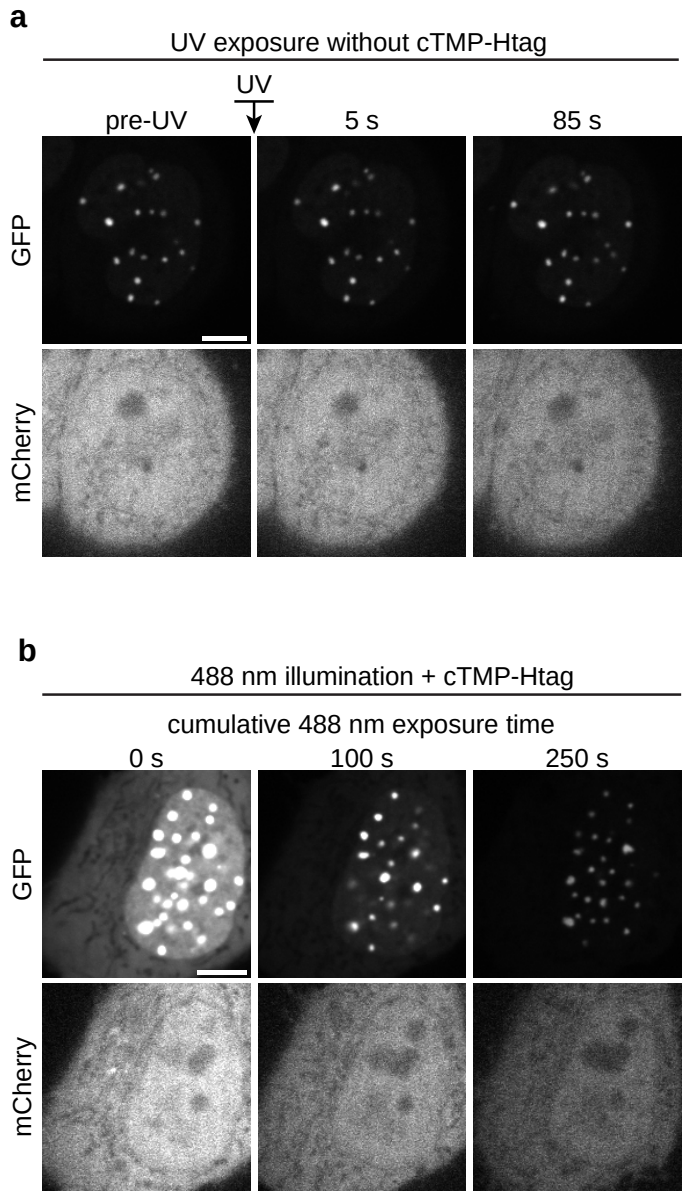
Supplementary Figure 13: ^{1}H NMR spectrum of **1** in DMSO-d_6 (500 MHz).



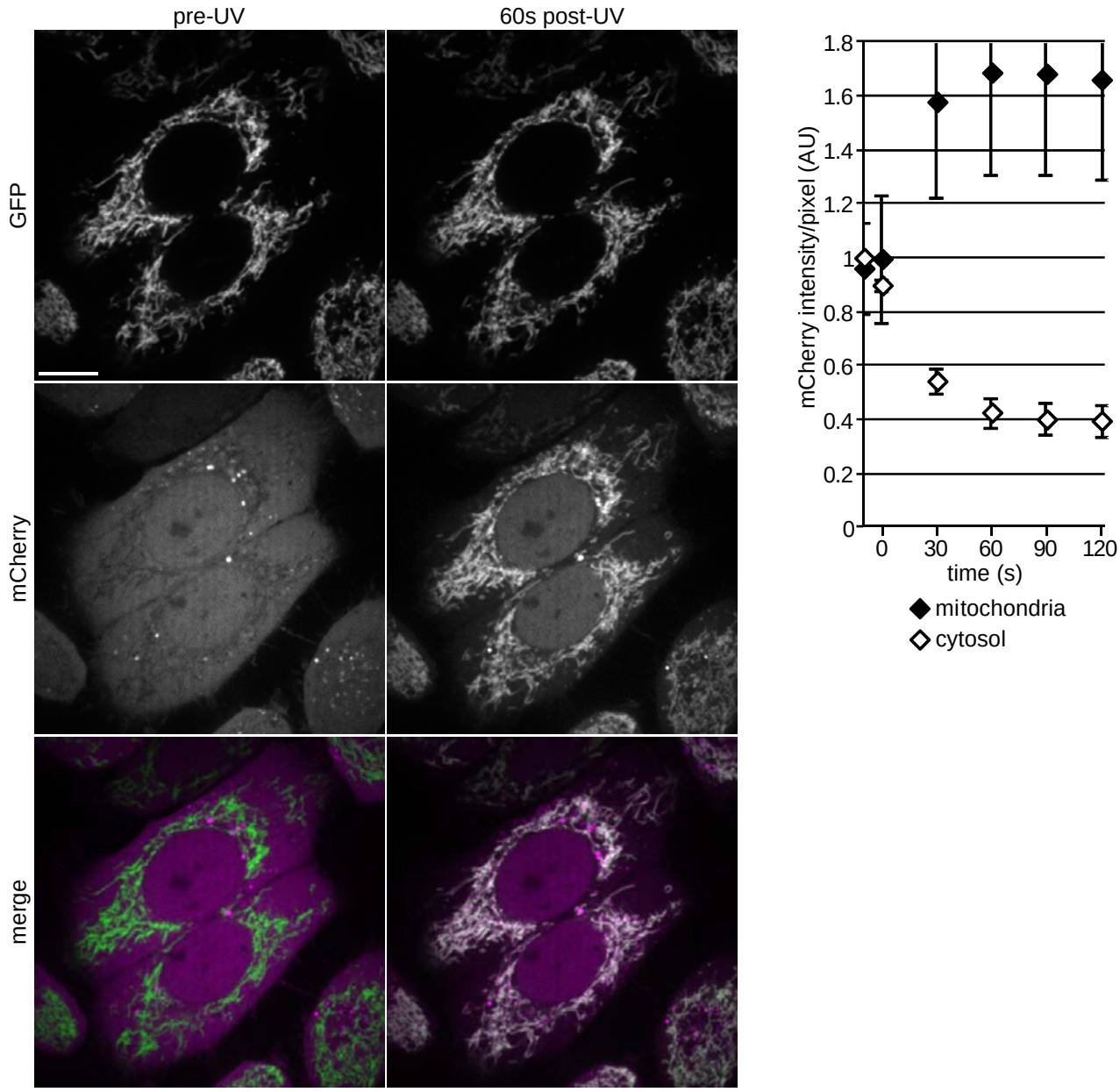
Supplementary Figure 14: ^{13}C NMR spectrum of **1** in DMSO-d_6 (126 MHz).



Supplementary Figure 15: ^{1}H-^{15}N HMBC NMR spectrum of **1** in DMSO-d_6 (600 MHz).



Supplementary Figure 16: No-dimerizer and 488 nm excitation negative controls. (**a**,**b**) Cells were expressing CENPB-GFP-Haloenzyme and mCherry eDHFR. (**a**) Cells without any small molecule treatment were exposed to a 2 second pulse of 387(±5.5) nm light, as in **Fig. 2a**. No change in mCherry-eDHFR localization was observed, indicating that UV light does not cause mCherry-eDHFR recruitment to centromeres in the absence of photocaged cTMP-Htag. (**b**) Cells treated with 20 μM cTMP-Htag for 1 hour were imaged hundreds of times in the GFP channel, using maximum 488 nm laser intensity. GFP and mCherry images after 100 s and 250 s of cumulative 488 nm laser exposure are shown. No change in mCherry-eDHFR localization is detectable, demonstrating that cTMP-Htag is functionally insensitive to 488 nm light.



Supplementary Figure 17: Cytoplasmic mCherry-eDHFR depletion. Cells expressing mitochondria-targeted ActA-GFP-Haloenzyme and mCherry-eDHFR were incubated with 10 μM cTMP-Htag for 1 hour, then washed prior to imaging. Cells were imaged before and after 500 ms exposure to 387 (+/- 6) nm UV light. Scale bar 10 μm. Average mCherry intensity/pixel in cytoplasm and mitochondria is quantified. Note that mCherry-eDHFR is depleted from the cytosol, but not the nucleus, on this experimental timescale, due to the slow kinetics of nuclear export. Mitochondria regions were defined in the GFP channel by thresholding using the ImageJ Default algorithm. Cytoplasm regions (ie, areas outside the nucleus without mitochonrdia) were defined manually. Values were normalized to pre-UV illumination value of cytoplasm region for each cell, then averaged at each timepoint over 12 cells. Error bars represent standard deviation, n = 12 cells.

Supplementary Methods

General chemistry details

All commercially available reagents and solvents were used as received. 2-(2-aminoethoxy)ethanol**,** NaH (60% in mineral oil), and 48% aq.HBr were purchased from Acros Organics. 6-chloro-1-iodohexane, 4,5-dimethoxy-2-nitrobenzyl chloroformate (NVOC), and the rest of the chemicals were purchased from Sigma Aldrich. *tert*-Butyl-3-iodopropylcarbamate was purchased from Ace Synthesis LLC. Trimethoprim was purchased from Astatech, Inc. HATU was purchased from GenScript. Flash column chromatography was performed using Silicycle silica gel (55−65 Å pore diameter). Thin-layer chromatography was performed on Sorbent Technologies silica plates (250 μm thickness). Infrared (IR) spectra were obtained on Jasco FT-IR Spectrum BX system and reported as wavenumber of the absorption maxima between 4000 cm^{-1} and 800 cm^{-1} of only major peaks. Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and Carbon nuclear magnetic resonance spectroscopy (^{13}C NMR) spectra were recorded on a Bruker AVII 500 and Biodrx 600 NMR. High-resolution mass spectra were obtained at the University of Pennsylvania's Mass Spectrometry Service Center on a Micromass AutoSpec electrospray/chemical ionization spectrometer. Ultraviolet-visible absorption spectrophotometry was performed on a JASCO V-650 spectrophotometer with a PAC-743R multichannel Peltier using quartz cells with a 1 cm cell path length.

*Synthesis of tert-butyl (2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamate, HTag-NBoc (**5**)*

5 was synthesized following the precedent of Singh *et. al.*[1]. To a solution of 2-(2-aminoethoxy)ethanol **3** in anhydrous EtOH was added Boc_2O at 0 °C. After stirring at room temperature 2 h, the reaction mixture was evaporated. The product was then extracted with CH_2Cl_2 and the combined organic layers were dried over Na_2SO_4 and evaporated under vacuum to obtain product **4** as colorless oil (quantitative yield), which was used for the next step without further purification. The spectral data were in agreement with the reported data[1].

To a solution of **4** in a 2:1 mixture of THF and DMF at 0 °C was added NaH (60% in mineral oil). After stirring at 0 °C for 30 min, 6-chloro-1-iodohexane was added to the above solution. The reaction mixture was stirred overnight and quenched with saturated NH_4Cl. The mixture was extracted with EtOAc, washed with H_2O and brine. The combined organic layers were dried over Na_2SO_4 and concentrated. The crude product was purified by silica gel column chromatography using EtOAc/Hexanes (20:80 to 30:70, v/v) to yield pure product **5** (49%) as colorless oil. The spectral data were in agreement with the reported data[1].

*Synthesis of 4-((2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)amino)-4-oxobutanoic acid, HTag-COOH (**6**)*

Compound **6** was synthesized by modification of a procedure described by Passemard *et al*.[2] To a solution of **5** (1.0 g, 3.21 mmol) in 10 mL anhydrous CH_2Cl_2 at 0 °C was slowly added TFA (10 mL, 130 mmol). Thereafter the reaction mixture was

warmed to rt and stirred for 2h. After completion of the reaction as evident by TLC analysis, the solvent was removed under high vacuum to obtain the crude product as a TFA salt, which was used without further purification.

To a solution of the above deprotected product (0.72 g, 3.21 mmol) in CH_2Cl_2 (5 mL) was slowly added triethylamine (19.26 mmol, 1.95 g) and subsequently succinic anhydride (9.63 mmol, 0.96 g) followed by stirring overnight at 25°C. Next, the reaction mixture was washed with 1 M aq. HCl (3×5 mL) followed by brine. The organic layer was dried over $MgSO_4$ and concentrated under vacuum to afford **6** as a brown oil (90% in two steps). $\mathbf{R_f}$ = 0.45 (5% MeOH:CH_2Cl_2). **IR** (NaCl, thin film): ν 3320, 2936, 2865, 1733, 1653, 1558, 1436, 1172, 1116 cm^{-1}. **^{1}H NMR** (500 MHz, $CDCl_3$) δ ppm: 6.47 (s, 1H), 3.65 - 3.58 (m, 4H), 3.58 - 3.41 (m, 8H), 2.70-2.63 (m, 2H), 2.52 (dd, *J* = 7.5, 5.8 Hz, 2H), 1.77 (dq, *J* = 8.8, 6.8 Hz, 2H), 1.68 - 1.52 (m, 2H), 1.50 - 1.41 (m, 2H), 1.41 - 1.31 (m, 2H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ ppm: 175.3, 172.5, 77.4, 71.5, 70.2, 70.2, 69.7, 45.2, 39.6, 32.6, 31.3, 30.3, 26.8, 25.4. **HRMS** (ESI, m/z): Calcd. for $C_{14}H_{26}ClNO_5$ $[M+Na]^+$: 346.1397. Found: 346.1400.

*Synthesis of tert-butyl (3-(4-((2,4-diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenoxy)propyl)carbamate, TMP-NBoc (**9**)*

Compound **9** was synthesized following Jing *et al.*[3]. Compound **8**, tert-butyl N-(3-iodopropyl)carbamate, and cesium carbonate were dissolved in anhydrous DMF. The reaction mixture was heated at 70 °C for 7 h, followed by the removal of solvent in vacuo. The crude mixture was purified by column chromatography with silica gel (1:9 MeOH:CH_2Cl_2) to yield product **9** as a brown solid (42%) The spectral data were in agreement with the reported data[3]. $\mathbf{R_f}$ = 0.70 (20% MeOH:CH_2Cl_2). **UV-Vis** (λ_{max} in CH_2Cl_2) : 239, 282 nm. **IR** (NaCl, thin film) : ν 3338, 1620, 1506, 1456, 1242, 1124 cm^{-1}. **^{1}H NMR** (500 MHz, MeOD) δ ppm: 7.51 (s, 1H), 6.51 (s, 2H), 3.94 (t, *J* = 5.9 Hz, 2H), 3.78 (s, 6H), 3.63 (s, 2H), 3.28 (d, *J* = 6.7 Hz, 2H), 1.88 - 1.76 (m, 2H), 1.43 (s, 9H). **^{13}C NMR** (126 MHz, MeOD) δ ppm: 164.3, 163.1, 158.4, 155.9, 154.6, 136.4, 108.0, 106.6, 80.0, 72.3, 56.5, 39.1, 39.0, 34.4, 30.8, 28.8. **HRMS** (ESI, m/z): Calcd. for $C_{21}H_{31}N_5O_5$ $[M+H]^+$: 434.2403. Found: 434.2413.

*Synthesis of tert-butyl (3-(4-((4-amino-2-((((4,5-dimethoxy-2-nitrobenzyl)oxy)carbonyl)amino)- pyrimidin-5-yl)methyl)-2,6-dimethoxyphenoxy)propyl)carbamate, cTMP-NBoc (**10**)*

Reaction conditions were adapted from Wysocki *et al.*[4]. To a stirring solution of compound **9** (0.63 g, 1.45 mmol) and 1 equiv. of DIEA (0.188 g, 1.45 mmol) in CH_2Cl_2 was added 4,5-Dimethoxy-2-nitrobenzyl chloroformate (NVOC) (0.40 g, 1.45 mmol) in the dark. The mixture was stirred at room temperature overnight. The mixture was washed with H_2O and brine. The combined organic layers were dried over Na_2SO_4 and concentrated under vacuum. The crude product was purified by silica gel column chromatography using MeOH:CH_2Cl_2 (1% to 5%, v/v) to yield pure product **10** (41%) as a yellowish semi-solid material. $\mathbf{R_f}$ = 0.50 (5% MeOH:CH_2Cl_2). **UV-Vis** (λ_{max} in CH_2Cl_2) : 239, 282, 346 nm. **IR** (NaCl, thin film) : ν

3375, 2937, 1699, 1589, 1522, 1460, 1331, 1277, 1221, 1172, 1126, 1072, 914, 731 cm^{-1}. **^{1}H NMR (**500 MHz, DMSO-*d*6) δ ppm: 10.02 (s, 1H), 7.78 (s, 1H), 7.72 (s, 1H), 7.45 (s, 1H), 6.74 (d, *J* = 5.7 Hz, 1H), 6.66 (s, 2H), 6.60 (s, 2H), 5.43 (s, 2H), 3.91 (s, 3H), 3.87 (s, 3H), 3.80 (t, *J* = 6.3 Hz, 2H), 3.72 (s, 6H), 3.63 (s, 2H), 3.09 (q, *J* = 6.6 Hz, 2H), 1.70 (t, *J* = 6.7 Hz, 2H), 1.37 (s, 9H). **^{13}C NMR** (126 MHz, DMSO-*d*6) δ ppm: 162.4, 155.9, 155.6, 153.6, 152.9, 151.6, 138.9, 134.8, 134.6, 127.8, 111.7, 110.4, 108.1, 106.0, 77. 5, 70.5, 62.7, 56.4, 55.9, 55.9, 39.1, 37.4, 32.9, 30.0, 28.3. **HRMS** (ESI, m/z): Calcd. for $C_{31}H_{40}N_6O_{11}$ $[M+H]^+$: 673.2833. Found: 673.2835.

*Synthesis of N^1-(2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)-N^4-(3-(4-((2,4-diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenoxy)propyl)succinamide, TMP-HTag (**2**)*

Compound **9** (60 mg, 0.138 mmol) was deprotected by directly dissolving in 4 mL 1:1 TFA/CH_2Cl_2 and stirring at room temperature for 2 h. Next, excess TFA was removed under high vacuum to yield the TFA salt as a colorless solid. $\mathbf{R_f}$ = 0.02 in 10% MeOH:CH_2Cl_2. The crude product was used in the next step without further purification.

The crude mixture above was dissolved in 1 mL of DMF followed by addition of DIEA (17.9 mg, 25 µL, 0.138 mmol). After stirring for 10 minutes, a 1 mL solution of **6** (45 mg, 0.138 mmol) in DMF, DIEA (17.9 mg, 25 µL, 0.138 mmol), and HATU (52.6 mg, 0.138 mmol) was added to the crude mixture. The mixture was then allowed to stir for another 2 h before being concentrated under high vacuum. The crude mixture was purified by silica gel column chromatography using 3% MeOH:CH_2Cl_2 to yield pure product **2** (69%) as a brown oil. $\mathbf{R_f}$ = 0.55 (5% MeOH:CH_2Cl_2). **UV-Vis** (λ_{max} in CH_2Cl_2) : 210, 288 nm. **IR** (NaCl, thin film) : ν 3337, 2936, 1657, 1558, 1505, 1457, 1232, 1125 cm^{-1}. **^{1}H NMR** (500 MHz, $CDCl_3$) δ ppm : 7.57 (s, 1H), 7.06 (t, *J* = 5.8 Hz, 1H), 6.62 (d, *J* = 6.3 Hz, 1H), 6.38 (s, 2H), 5.58 (s, 2H), 5.40 (s, 2H), 4.01 (t, *J* = 5.5 Hz, 2H), 3.79 (s, 6H), 3.67 - 3.25 (m, 22H), 2.50 (s, 4H), 1.87 (p, *J* = 5.7 Hz, 2H), 1.81 - 1.69 (m, 2H), 1.59 (p, *J* = 6.9 Hz, 2H), 1.49 - 1.39 (m, 2H), 1.39 - 1.29 (m, 2H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ ppm: 172.4, 172.0, 163.2, 153.6, 135.6, 133.6, 107.1, 105.3, 77.4, 72.4, 71.4, 70.4, 70.1, 69.9, 56.3, 45.2, 39.4, 38.0, 34.6, 32.6, 31.9, 31.8, 29.5, 29.3, 26.8, 25.5. **HRMS** (ESI, m/z): Calcd. for $C_{30}H_{47}ClN_6O_7$ $[M+H]^+$: 639.3273. Found: 639.3267.

*Synthesis of 4,5-dimethoxy-2-nitrobenzyl (4-amino-5-(4-((21-chloro-5,8-dioxo-12,15-dioxa-4,9-diazahenicosyl)oxy)-3,5-dimethoxybenzyl)pyrimidin-2-yl)carbamate, cTMP-HTag (**1**)*

Compound **10** was deprotected and reacted with compound **6** using the same procedure for **2** as mentioned above. The crude mixture of product **1** was purified by silica gel column chromatography using 1% MeOH:CH_2Cl_2 to yield pure product **2** (70%) as a brown oil. $\mathbf{R_f}$ = 0.45 (5% MeOH:CH_2Cl_2). **UV-Vis** (λ_{max} in CH_2Cl_2) : 238, 280, 340 nm. **IR** (NaCl, thin film) : ν 3417, 3359, 3149, 2939, 1726, 1647, 1624, 1585, 1520, 1466, 1325, 1271, 1250, 1223, 1124, 1070, 984, 912, 839, 729 cm^{-1}. **^{1}H NMR** (500 MHz, DMSO-d_6) δ ppm: 9.97 (s, 1H), 7.83 (t, *J* = 5.7 Hz, 1H), 7.78 (s, 1H), 7.73 (d, *J* = 10.2 Hz, 2H), 7.45 (s,

1H), 6.61 (d, *J* = 15.9 Hz, 4H), 5.43 (s, 2H), 3.92 (s, 2H), 3.87 (s, 2H), 3.82 (t, *J* = 6.3 Hz, 2H), 3.72 (s, 6H), 3.61 (dd, *J* = 14.8, 8.2 Hz, 4H), 3.53 - 3.42 (m, 6H), 3.37 (dt, *J* = 10.2, 6.2 Hz, 4H), 3.30 (s, 2H), 3.23 - 3.12 (m, 4H), 2.29 (h, *J* = 1.8 Hz, 4H), 1.70 (p, *J* = 6.7 Hz, 4H), 1.47 (q, *J* = 6.9 Hz, 2H), 1.42 - 1.19 (m, 8H).

^{13}C NMR (126 MHz, DMSO-d_6) δ ppm: 171.4, 171.1, 162.3, 155.8, 154.9, 153.6, 152.9, 151.5, 147.6, 138.9, 134.9, 134.5, 127.7, 111.7, 110.5, 108.1, 106.1, 70.4, 70.1, 69.5, 69.4, 69.1, 62.6, 56.4, 56.1, 55.9, 45.3, 38.5, 35.9, 32.9, 32.0, 30.8, 30.8, 29.8, 29.0, 29.0, 26.1, 24.9. **HRMS** (ESI, m/z): Calcd. for $C_{40}H_{56}ClN_7O_{13}$ $[M+H]^+$: 878.3703. Found: 878.3704.

Supplementary References

1. Singh, V., Wang, S. & Kool, E. T. Genetically encoded multispectral labeling of proteins with polyfluorophores on a DNA backbone. *J. Am. Chem. Soc.* **135,** 6184–91 (2013).

2. Passemard, S. *et al.* Convenient synthesis of heterobifunctional poly(ethylene glycol) suitable for the functionalization of iron oxide nanoparticles for biomedical applications. *Bioorg. Med. Chem. Lett.* **23,** 5006–10 (2013).

3. Jing, C. & Cornish, V. W. A fluorogenic TMP-tag for high signal-to-background intracellular live cell imaging. *ACS Chem. Biol.* **8,** 1704–12 (2013).

4. Wysocki, L. M. *et al.* Facile and general synthesis of photoactivatable xanthene dyes. *Angew. Chemie* **50,** 11206–9 (2011).