SUPPORTING INFORMATION

H-Gemcitabine: A New Gemcitabine Prodrug for Treating Cancer

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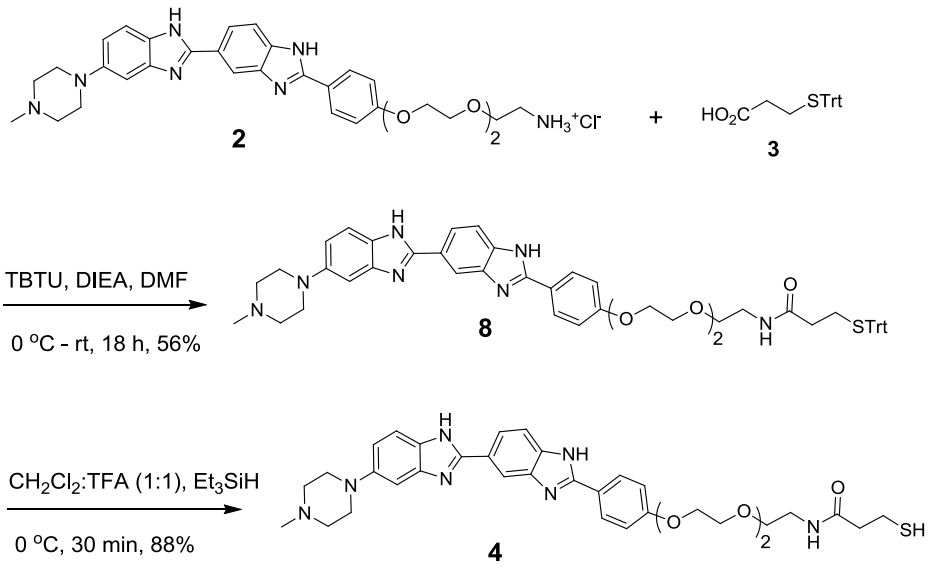
1. SYNTHETIC METHODS



Scheme 1. Synthesis of Hoechst thiol (**4**).

Synthesis of Tritylthio-Hoechst 8

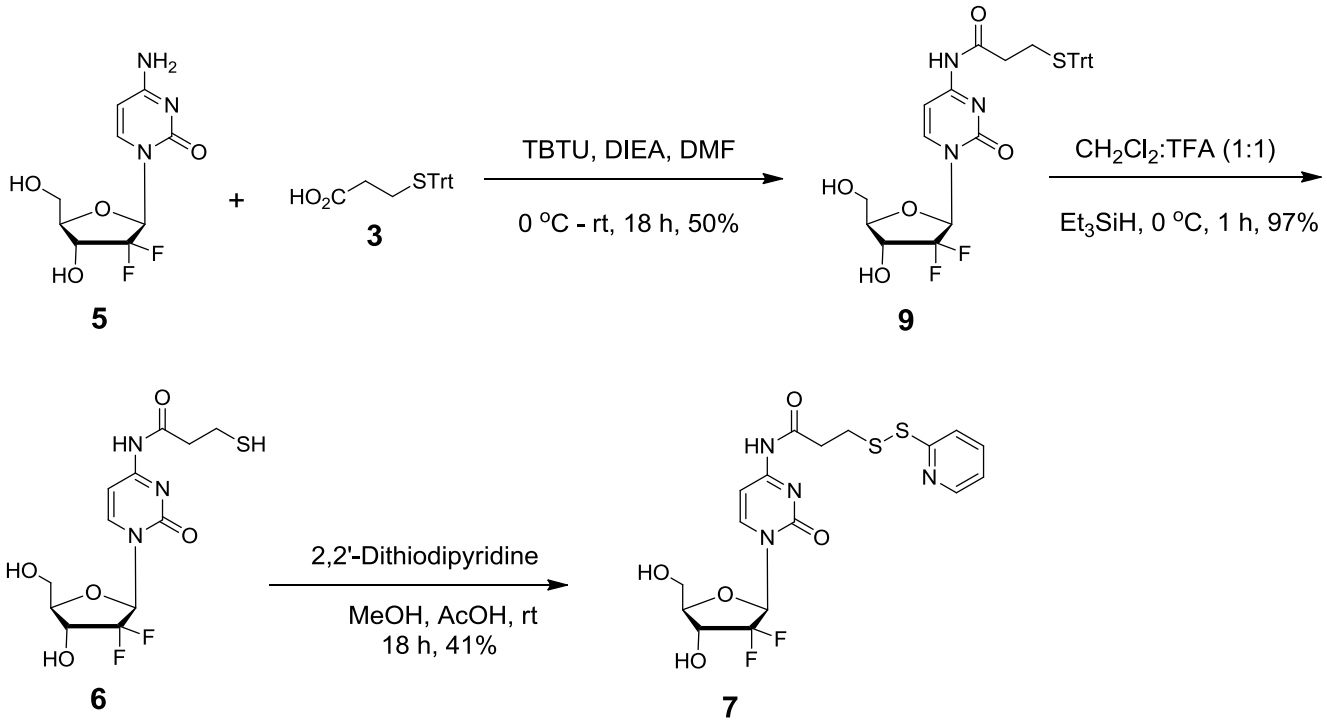
3-(Tritylthio)propionic acid **3** (50.0 mg, 0.1 mmol) was dissolved in anhydrous DMF (1 mL) and brought to 0 °C. *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TBTU) (54.9 mg, 0.1 mmol) was added to this solution followed by *N,N'*-Diisopropylethyl amine (0.6 mL, 3.5 mmol). The reaction mixture was stirred for 0.5 h, and Hoechst amine **2**[1] (0.1 g, 0.1 mmol) was then added and stirred for an additional 0.5 h at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred overnight. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography, using a gradient of 5-10% methanol in dichloromethane containing 0.1 % Et_3N. The compound **8** was obtained as a pale yellow solid (0.1 g, 56% yield). ^{1}H NMR (400 MHz, CD_3OD): δ 8.24 (s, 1H, Ar**H**), 8.01 (d, *J* = 8.8 Hz, 2H, Ar**H**), 7.93 (dd, *J* = 8.4, 1.6 Hz, 1H, Ar**H**), 7.76 (d, *J* = 7.6 Hz, 1H, Ar**H**), 7.69–7.66 (m, 2H, Ar**H**), 7.51 (d, *J* = 8.8 Hz, 1H, Ar**H**), 7.40–7.20 (m, 12H, Ar**H**), 7.17–7.15 (m, 3H, Ar**H**), 7.05 (d, *J* = 8.8 Hz, 1H, Ar**H**), 4.14 (t, *J* = 4.4 Hz, 2H, OC$\mathbf{H_2}$), 3.81 (t, *J* = 4.4 Hz, 2H, OC$\mathbf{H_2}$), 3.67–3.58 (m, 4H, 2 OC$\mathbf{H_2}$), 3.51 (t, *J* = 5.2 Hz, 2H, OC$\mathbf{H_2}$), 3.46–3.35 (m, 8H, 4 NC$\mathbf{H_2}$), 3.32 (m, 2H, C$\mathbf{H_2}$NHCO), 2.91 (s, 3H, NC$\mathbf{H_3}$), 2.35 (t, *J* = 7.2

(1) Dasari, M.; Lee, S.; Sy, J.; Kim, D.; Brown, M.; Davis, M.; Murthy, N. *Org. Lett.* **2010**, *12*, 3300.

Hz, 2H, C**H**$_2$S), 2.15 (t, J = 6.8 Hz, 2H, NHCOC**H**$_2$). ^{13}C NMR (100 MHz, CD_3OD): δ 173.7, 162.1, 155.1, 153.6, 149.4, 146.1, 144.5, 130.6, 129.5, 128.9, 126.7, 125.5, 125.2, 125.0, 123.0, 122.5, 118.7, 116.0, 112.4, 71.7, 71.2, 70.6, 70.5, 68.6, 67.7, 66.8, 56.0, 51.4, 45.9, 40.4, 35.8, 28.9; HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{53}H_{56}N_7O_4S$, 886.4109; found, 886.405.

Synthesis of Hoechst thiol 4

To compound **8** (149 mg, 0.1 mmol) was added, 0.5 mL of TFA:CH_2Cl_2 (1 to 1 ratio) followed by triethylsilane (134 µL, 0.8 mmol), and stirred for 0.5 h at room temperature. The solvents were removed under reduced pressure to obtain an oil, which was washed with diethyl ether (2 x 5 mL) and dried under reduced pressure to obtain **4** as a solid (96 mg, 88% yield), which was used without further purification and used for coupling with **7** to obtain final conjugate **1**.



Scheme 2. Synthesis of gemcitabine-dithiopyridine (**7**).

Synthesis of Tritylthio-gemcitabine 9

3-(Tritylthio)propionic acid **3** (0.24 g, 0.6 mmol) was dissolved in anhydrous DMF (2 mL) and the reaction mixture was brought to 0 °C. *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoro borate (TBTU) (0.26 g, 0.8 mmol) was added to this solution followed by *N,N*-

diisopropylethyl amine (0.2 mL, 1.3 mmol). After 0.5 h, gemcitabine **5** (0.2 g, 0.7 mmol) was added and stirred for another 0.5 h at 0 ^{o}C. The reaction mixture was then allowed to warm to room temperature and stirred for 18 h. The solvents were evaporated under reduced pressure and the crude product was purified by flash chromatography, using 0 – 4% methanol as a gradient in dichloromethane, to obtain pure **9** as a white solid (0.2 g, 50% yield). ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3OD, 10:1 v/v): δ 8.35 (d, *J* = 7.6 Hz, 1H, C**H**=CH), 7.58–7.53 (m, 6H, Ar**H**), 7.42–7.39 (m, 7H, 6 Ar**H** and CH=C**H**), 7.35–7.31(m, 3H, Ar**H**), 6.39–6.36 (m, 1H, **H-1'**), 4.47–4.39 (m, 1H, **H-3'**), 4.14–4.10 (m, 2H, **H-5'**), 3.97-3.93 (m, 1H, **H-4'**), 2.64 (t, *J* = 7.6 Hz, 2H, SC**H$_2$**), 2.49 (t, *J* = 7.2 Hz, 2H, NHCOC**H$_2$**). ^{13}C NMR (100 MHz, $CDCl_3$ + CD_3OD 10:1 v/v): δ 171.9, 162.6, 155.8, 144.5, 144.3, 129.2, 127.7, 126.5, 97.2, 81.2, 66.7, 58.8, 36.1, 26.2; HRMS (FAB) m/z: $[M+H]^+$ calcd for $C_{31}H_{30}F_2N_3O_5S$, 594.1869; found, 594.1935.

Synthesis of gemcitabine-dithiopyridine 7

To compound **9** (0.3 g, 0.5 mmol) at 0 ^{o}C was added, 2.0 mL of TFA:CH_2Cl_2 (1 to 1 ratio) followed by triethylsilane (0.2 mL, 1.2 mmol), the resulting solution was stirred for 1 h. The solvents were then removed under reduced pressure to obtain an oil, which was washed with diethyl ether (2 x 5 mL) and dried under reduced pressure to obtain the thiol **6** as a solid (0.14 g, 97%), which was used for the next reaction without further purification.

The thiol **6** (0.12 g, 0.3 mmol) and 2, 2'-dithiodipyridine (0.1 g, 0.7 mmol) were dissolved in anhydrous methanol (4 mL) containing glacial acetic acid (10.3 µL, 0.1 mmol). The reaction mixture was stirred at room temperature for 18 h and the solvent was removed under reduced pressure to obtain the crude product. The crude product was purified by flash chromatography (using a 0 – 7% methanol gradient in dichloromethane), to obtain **7** as a white solid (0.09 g, 41% yield). ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3OD, 2:1 v/v): δ 8.40 (d, *J* = 4.8 Hz, 1H, Ar**H**), 8.32 (d, *J* = 8.0 Hz, 1H, C**H**=CH), 7.79-7.70 (m, 2H, Ar**H**), 7.45 (d, *J* = 7.6 Hz, 1H, CH=C**H**), 7.19-7.16 (m, 1H, Ar**H**), 6.27-6.23 (m, 1H, **H-1'**), 4.33-4.25 (m, 1H, **H-3'**), 3.98-3.94 (m, 2H, **H-5'**), 3.81-3.77 (m, 1H, **H-4'**), 3.10 (t, *J* = 6.4 Hz, 2H, C**H$_2$**S), 2.89 (t, *J* = 6.4 Hz, 2H, NHCOC**H$_2$**). ^{13}C NMR (100 MHz, $CDCl_3$ + CD_3OD, 2:1 v/v): δ 171.9, 162.9, 159.3, 156.1, 149.2, 144.7, 137.6, 121.1, 120.1, 97.4, 81.4, 81.3, 58.9, 36.3, 33.3; HRMS (FAB) m/z: $[M+H]^+$ calcd for $C_{17}H_{19}N_4O_5F_2S_2$, 461.0759; found, 461.0742.

2. Determination of the dissociation constant (K_d) of H-gemcitabine with DNA (Figure S1)

The 21bp oligonucleotide 5'-GCGACTGCAATTTCGACGTCC-3' (Integrated DNA Technologies, Coralville, IA) was dissolved in deionized water to generate a 20 nM concentration. Various quantities of H-gemcitabine were added to this DNA solution, to generate H-gemcitabine concentrations ranging from (2 nM – 100 nM). The solution was allowed to stand for 5 minutes and the fluorescence spectra of the solution was measured with a Shimadzu spectrophotometer, using an excitation wavelength of 340 nm and an emission wavelength of 440 nm. All measurements were corrected for the fluorescence intensity of H-gemcitabine by itself. The corrected fluorescence intensity data points were fitted using a non-linear regression analysis (Graph Pad software), to obtain the saturation binding curve and the dissociation constant (K_d).

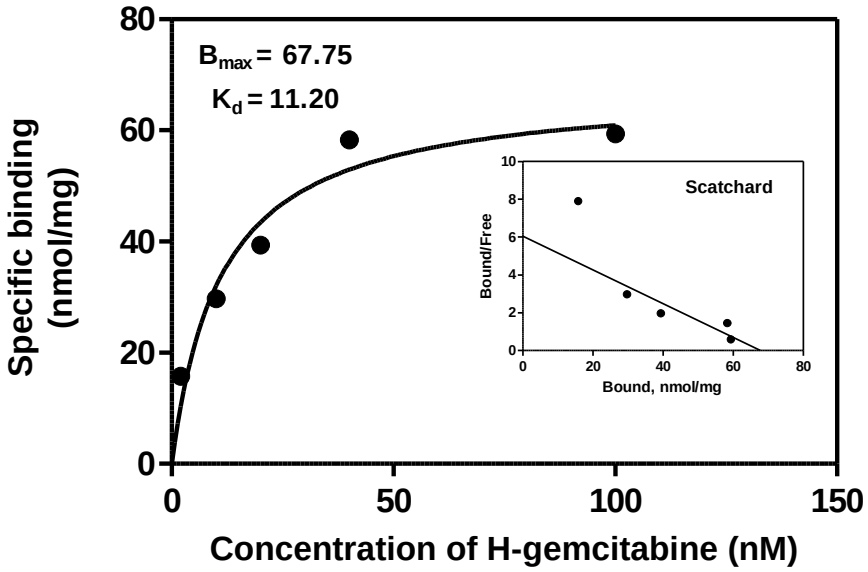


Figure S1. Binding of H-gemcitabine to oligomeric DNA.

3. Hydrolysis of H-gemcitabine (Figure 1)

H-gemcitabine was dissolved in methanol at 100 µg/mL and stored at -20 °C. 2 mL of the H-gemcitabine stock (100 µg/mL) was added to 2 mL of PBS (pH 7.4) to obtain a final concentration of 50µg/mL. The blank sample was prepared by the addition of 1 mL methanol to 1 mL of PBS (pH 7.4). Samples were prepared in triplicate and shaken at 100 rpm protected from light at 37 ^{o}C. A 100µL aliquot was removed from each triplicate at specific time points and analyzed by high-performance liquid chromatography (HPLC). Liquid chromatography was performed on a Shimadzu L6AD unit

with an analytical X-bridge C8 column and an analytical detector system. The area under the curve for each peak was integrated using LC solution software (Shimadzu). The relative percentage of gemcitabine released was calculated by dividing the area under the curve for gemcitabine by the sum of area under the curve of gemcitabine and H-gemcitabine at a given time point.

4. INVITRO EXPERIMENTS

Cell permeability of H-gemcitabine (Figure 2a)

The cell permeability of H-gemcitabine was measured *in vitro* using HT29 human colon cancer cells (ATCC, Manassas, VA, USA). HT29 cells were cultured in a humidified atmosphere at 37 ^{o}C, 5% CO_2 in RPMI-medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 0.2% penicillin/streptomycin (Gibco, California, USA). Cells were incubated with 10 μM of H-gemcitabine in medium containing 10% serum for 30 min at 37 ºC. The cell media was then replaced by PBS and the cellular fluorescence was visualized with a fluorescence microscope (Nikon E600, Nikon, Melville, NY, USA), using an excitation wavelength of 340 nm and an emission filter range of 400-500 nm. In addition, HT29 cells were fixed and permeabilized with methanol (10% v/v), and then incubated with H-gemcitabine (10 μM) for 30 min at 37 ^{o}C. The cell media was then replaced by PBS and the cellular fluorescence was visualized as described above.

Cell toxicity of H-gemcitabine (Figure 2b)

The time dependent cell killing efficacy of H-gemcitabine, gemcitabine, and gemcitabine-SH were tested *in vitro*. T75 flasks containing HT29 cells were treated with a 0.2% (w/v) trypsin-0.1% (w/v) EDTA solution (Gibco, California, USA) and were seeded onto 96 well plates at a density of 5×10^5 cells/mL. The cells were allowed to proliferate for 24 h and then incubated with, free gemcitabine, gemcitabine-SH, or H-gemcitabine at concentrations ranging from 0.1 μM-1 mM for 4 hours. The cell viability was quantified using the MTT assay.

5. IN VIVO EXPERIMENTS

Tumor development in mice

HT29 tumors were grown in Balb/c nude mice (Simonsen Lab Inc., Gilroy, CA). The mice were accommodated in autoclaved micro-isolator cages that were housed in a positive pressure containment

rack and maintained under the guidelines of an approved animal protocol from the Georgia Institute of Technology Institutional Animal Care and Use Committee (animal protocol #: A08051). Xenografts of HT29 cells were developed by subcutaneously implanting 2×10^6 cells/mL in the right flanks of nude mice. When the tumor volume reached between 50-150 mm^3, mice were randomly assigned to three groups (6-8 mice per group), H-gemcitabine treated, gemcitabine treated, and control groups.

Inhibition of tumor growth with H-gemcitabine, survival (Figure 3a and 3b) and toxicity (Figure 4)

The ability of H-gemcitabine to inhibit tumor growth in nude mice bearing HT29 tumors was measured. Mice were given HT29 tumors as described above, and were given 4 consecutive doses of H-gemcitabine or gemcitabine, each dose was given 3 days apart, on days 0, 3, 6, and 9. The H-gemcitabine (18.84 mg/mL, 95 μmol) and gemcitabine (20 mg/mL, 380 μmol) formulations were prepared using 60% PEG400+10% EtOH+30% saline. A 60-80 uL of H-gemcitabine at a dose of 94.2 mg/kg or gemcitabine at 100 mg/kg was injected intravenously into the mice, via the jugular vein using a 25G5/8 needle. The control group of mice were injected with a solution containing a mixture of 60% PEG400, 10% EtOH, and 30% saline, which was also given via the jugular vein with a 25G5/8 needle. The tumor volume was measured using vernier caliper. The tumor volume was calculated by the following equation: $V = (w)^2 \times (l)/2$, where (w) and (l) are the width and length of the tumor. The survival of the mice was determined following the IACUC protocol of the Georgia Institute of Technology, which states that the animals in these experiments have to be sacrificed when they meet any of the following conditions: (1) 15 % loss from initial body weight, (2) the size of the tumor ≥ 1.5 cm in any dimension, (3) the mouse becomes lethargic, sick or unable to feed (4) the mouse develops an ulcerated tumor. The mice were also weighed regularly to monitor the weight loss. The weights of the mice were normalized to the weight obtained on day 0 and plotted against the number of days. The toxicity was defined as the dose that caused a maximal weight loss of 15% of their initial body weight.

Chronic toxicity of H-gemcitabine (Figure S2)

The toxicity of H-gemcitabine was assessed in using non-tumor bearing female C57/B16 mice, 4-5 weeks old, weighing 15-16 g. Mice were accustomed to laboratory conditions for at least 1 week before experimental use. Mice were randomly assigned to three groups (6-8 mice per group), H-

gemcitabine treated, gemcitabine treated, and control. The mice were treated intraperitonially for 5 consecutive doses (on days 0, 1, 2, 3 and 4) with either H-gemcitabine, gemcitabine, or control (no drug). All formulations were prepared fresh prior to the experiment using 60% PEG400+10% EtOH+30% saline. A 60 – 80 µL volume of either H-gemcitabine (representing doses of 10, 25 or 50 mg/kg gemcitabine mole equivalents) or gemcitabine (at 10, 25 or 50 mg/kg) was injected intraperitoneally into the mice, using a 25G5/8 needle. The mice were weighed regularly to monitor the weight loss. Weights of the mice were normalized to the weight obtained on day 0 and plotted against the number of days, toxicity was defined as the dose that caused a maximal weight loss of 15% of their initial body weight.

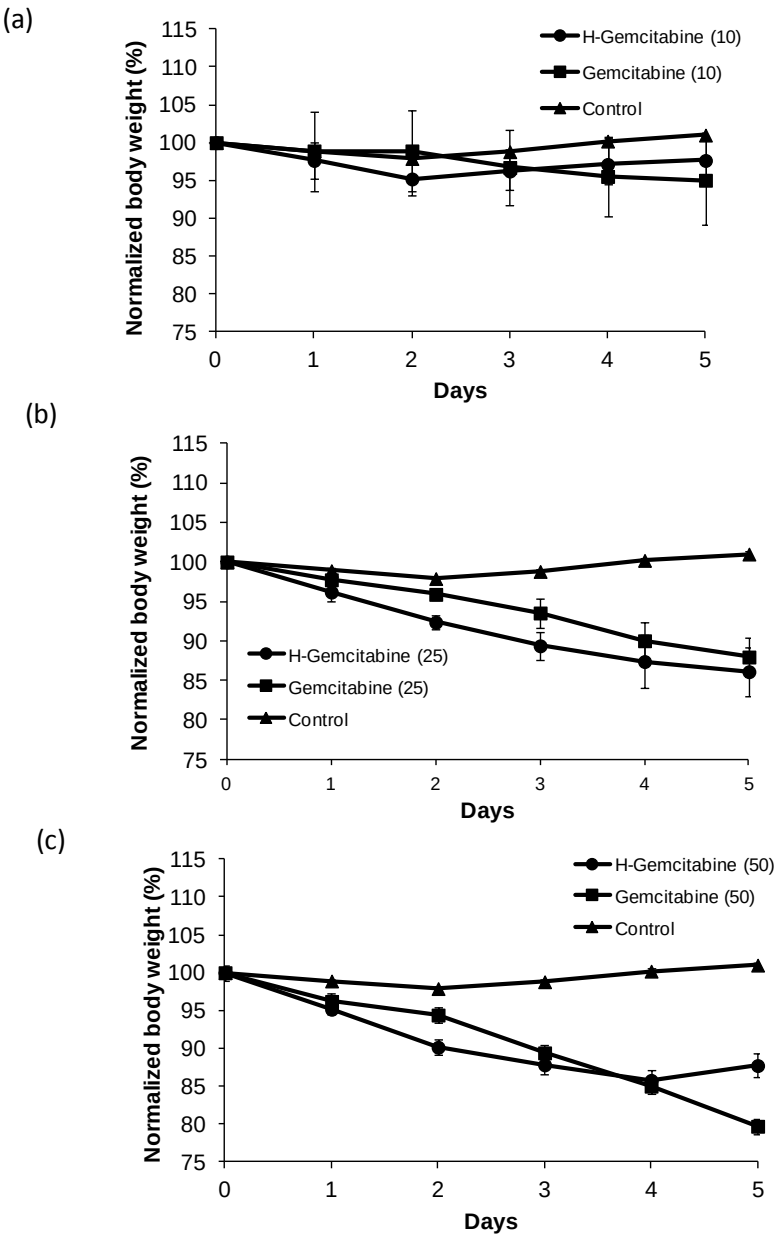


Figure S2. Chronic toxicity of H-gemciabine and gemcitabine at three different doses. a) H-gemcitabine at 10 mg/kg gemcitabine equivalents (7.5 mg/mL, 38 µmol) or gemcitabine at 10 mg/kg (2 mg/mL, 38 µmol) was given intraperitoneally for 5 consecutive days. b) H-gemcitabine at 25 mg/kg gemcitabine equivalents (19 mg/mL, 95 µmol) or gemcitabine at 25 mg/kg (5 mg/mL, 95 µmol) was given intraperitoneally for 5 consecutive days. c) H-gemcitabine at 50 mg/kg gemcitabine equivalents (37.8 mg/mL, 190 µmol) or gemcitabine at 50 mg/kg (10 mg/mL, 190 µmol) was given intraperitoneally for 5 consecutive days.

Accumulation of H-gemcitabine in tumors in vivo

The ability of H-gemcitabine to target tumors was evaluated in nude mice. Xenografts of HT29 cells were developed by subcutaneously implanting 2×10^6 cells/mL in the right flanks of nude mice. When the tumor volume reached between 50-150 mm^3, mice were randomly assigned to two groups (3 mice per group), H-gemcitabine treated and control (untreated) groups. H-gemcitabine (18.84 mg/mL, 95 µmol) formulations were prepared using 60% PEG400+10% EtOH+30% saline. A 60-80 uL of H-gemcitabine at a dose of 94.2 mg/kg was injected intravenously into the mice, via the jugular vein using a 25G5/8 needle. The control (untreated) group of mice were injected with the solution containing mixture of 60% PEG400, 10% EtOH, and 30% saline, which was also given via the jugular vein with a 25G5/8 needle. After 24 h of dose administration, the mice were euthanized with CO_2 asphyxiation and the tumor tissue was harvested. Tumor tissues weighing 100 mg were incubated with 1 mL of 0.2% (w/v) trypsin-0.1% (w/v) EDTA solution for 2 hours, and homogenized using a tissue homogenizer (Fisher Scientific). To reduce tissue background fluorescence, each sample was mixed with 1 mL deionized water and sodium borohydride (20 mg) and incubated at 0 ^oC overnight. The mixture was centrifuged to remove residual tumor tissue and the fluorescence intensity of the supernatant was measured with a Shimadzu spectrofluorometer, using an excitation wavelength of 340 nm and an emission wavelength of 450 nm. The fluorescence intensity of H-gemcitabine treated tumors was subtracted from the control tumors, to determine the H-gemcitabine accumulation. The fluorescence intensity was fitted to the linear calibration curve generated for H-gemcitabine and the percentage of Hoechst accumulated per gram of tumor tissue was quantified.

Statistical evaluation

Treatment groups were analyzed for statistical significance ($*p < 0.05$) by a one-way analysis of variance (ANOVA), using a post-test analysis when necessary.

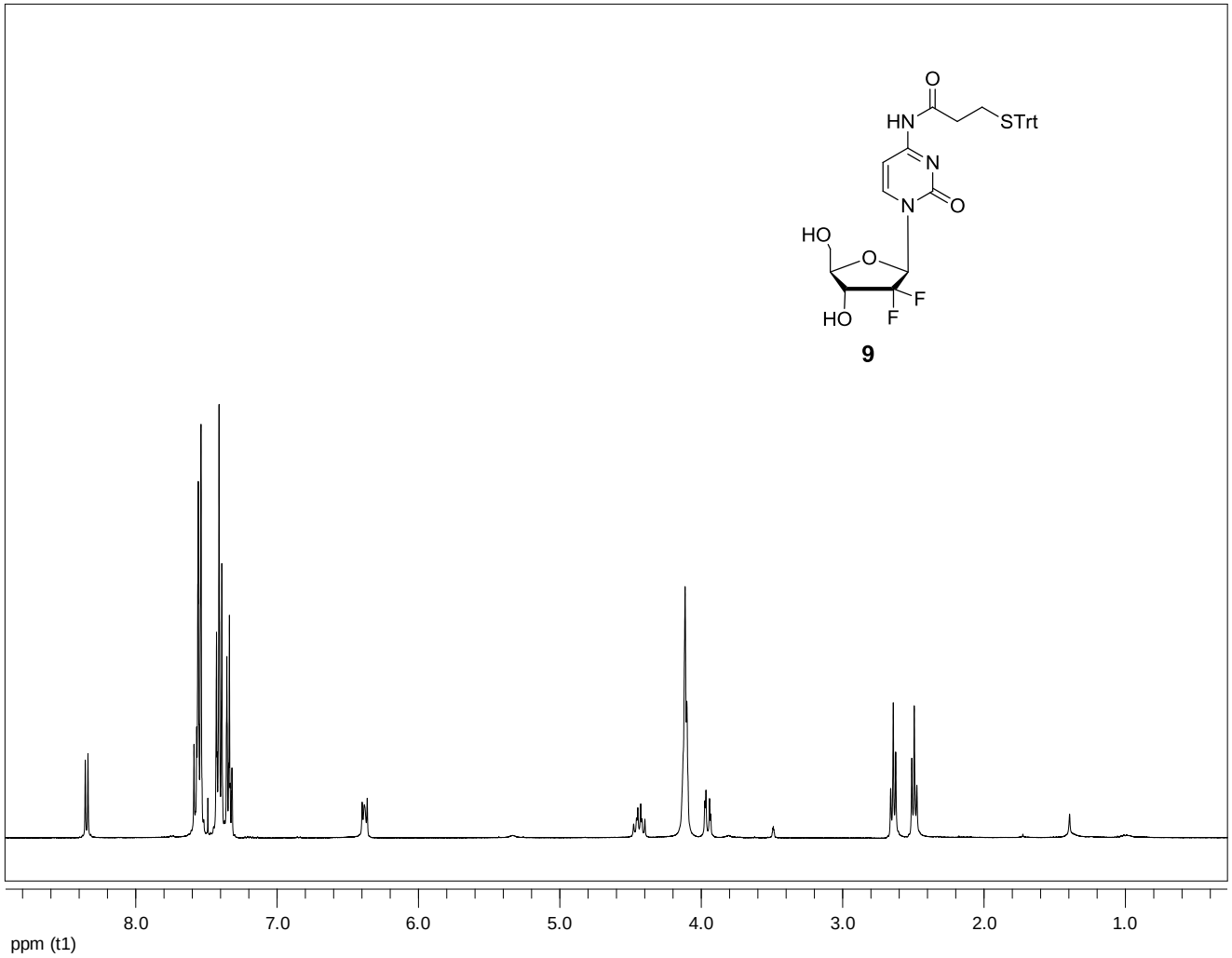


Figure S3. ^{1}H-NMR spectrum of **9**.

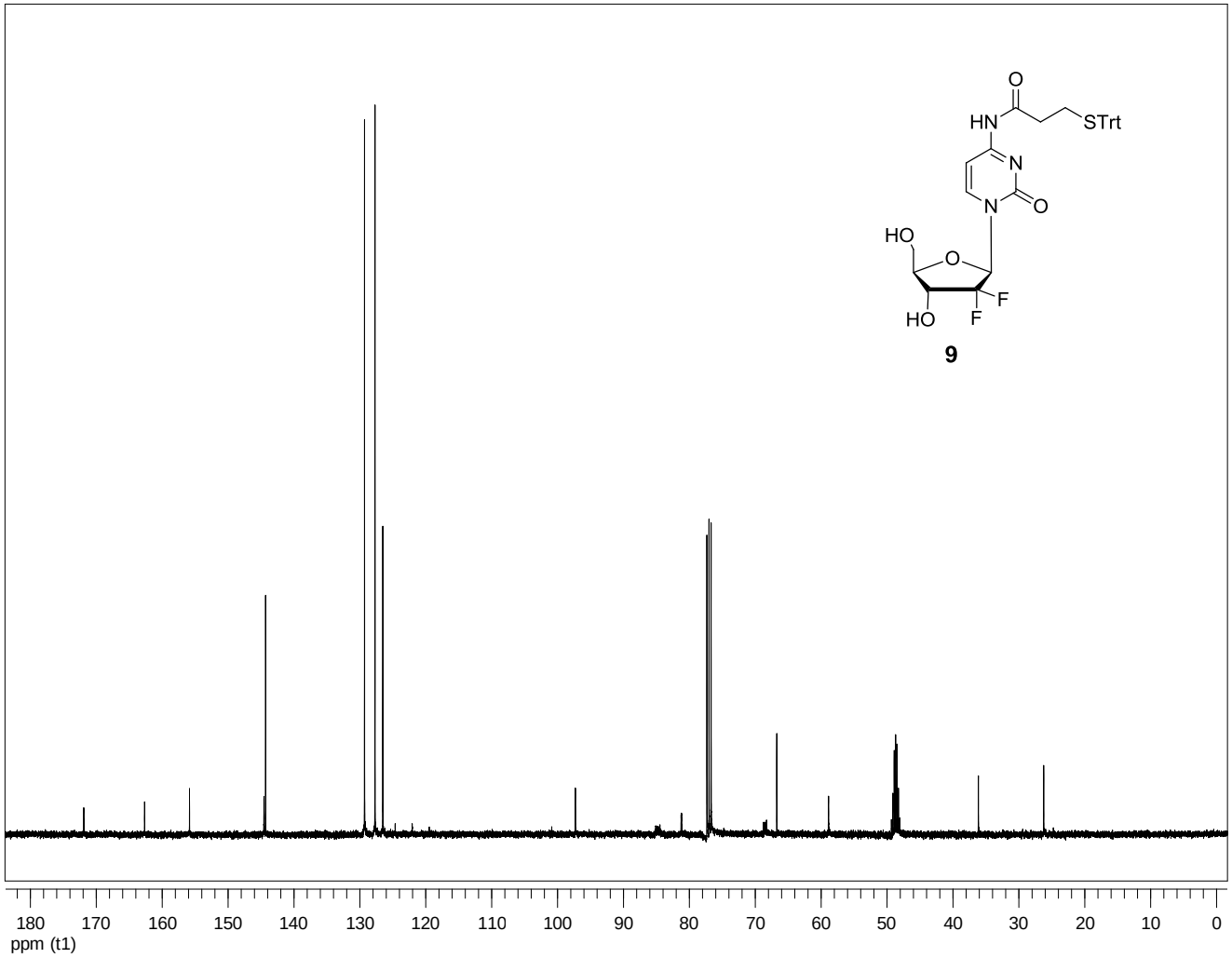


Figure S4. ^{13}C-NMR spectrum of **9**.

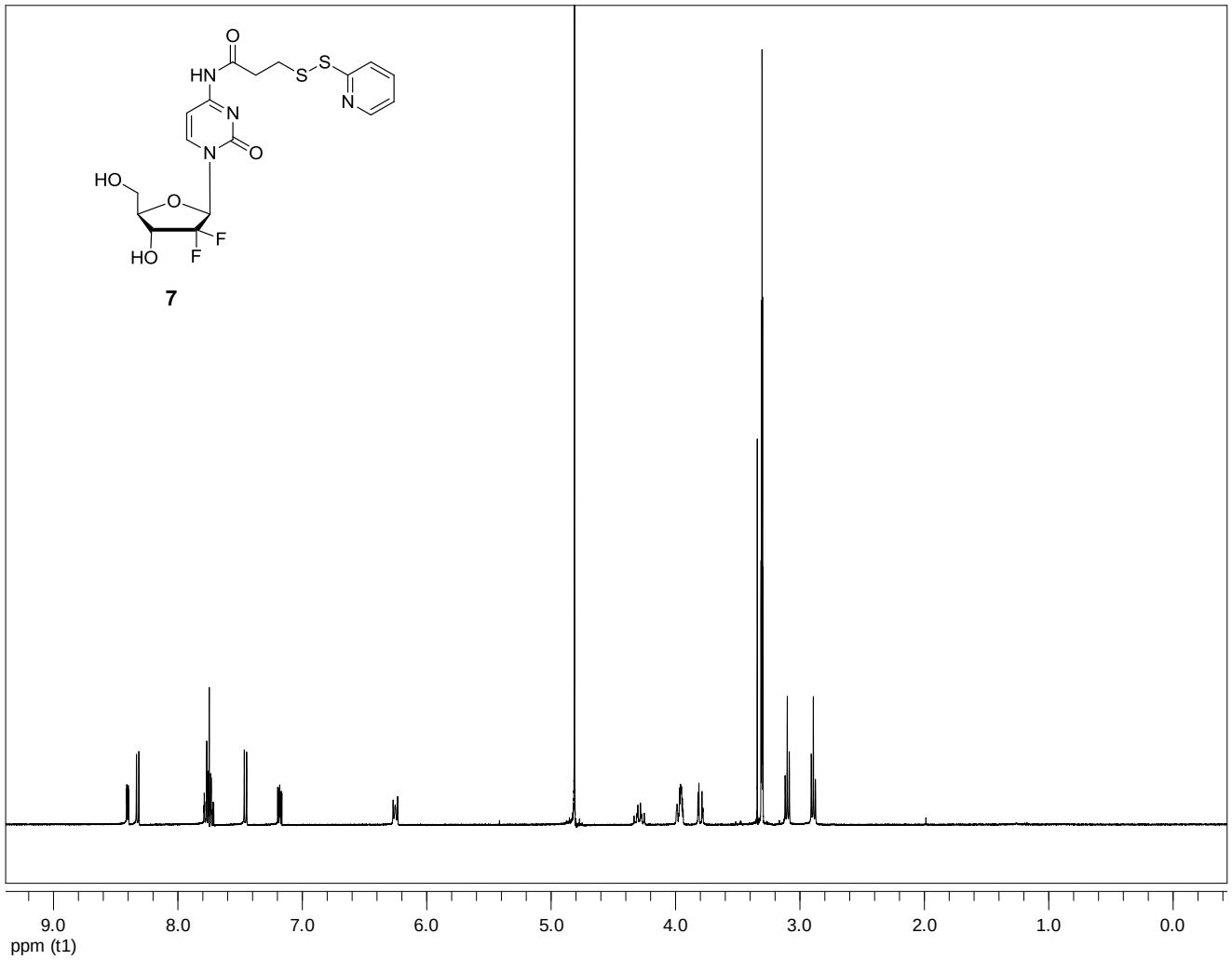


Figure S5. ^{1}H-NMR spectrum of **7.**

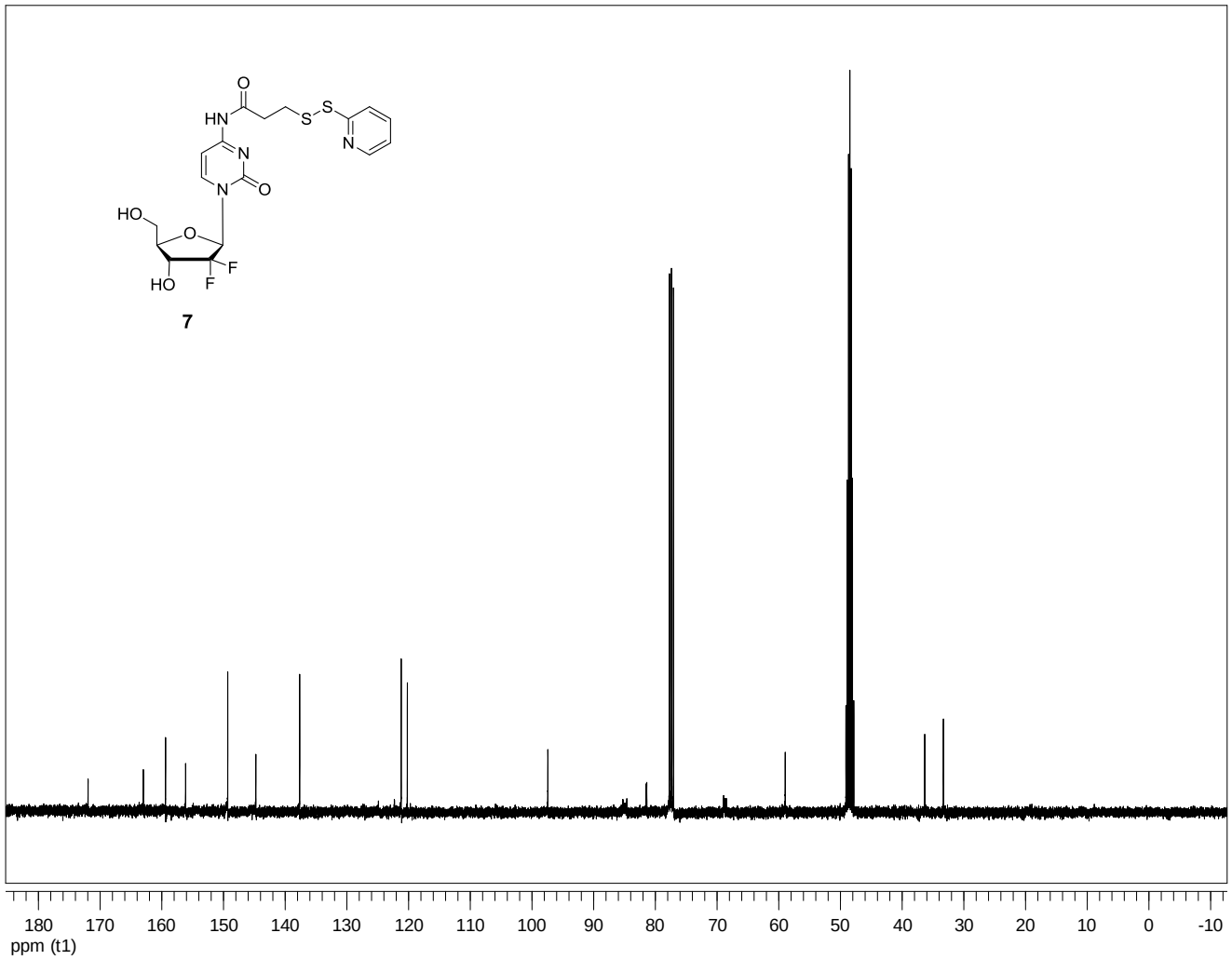


Figure S6. ^{13}C-NMR spectrum of **7**.

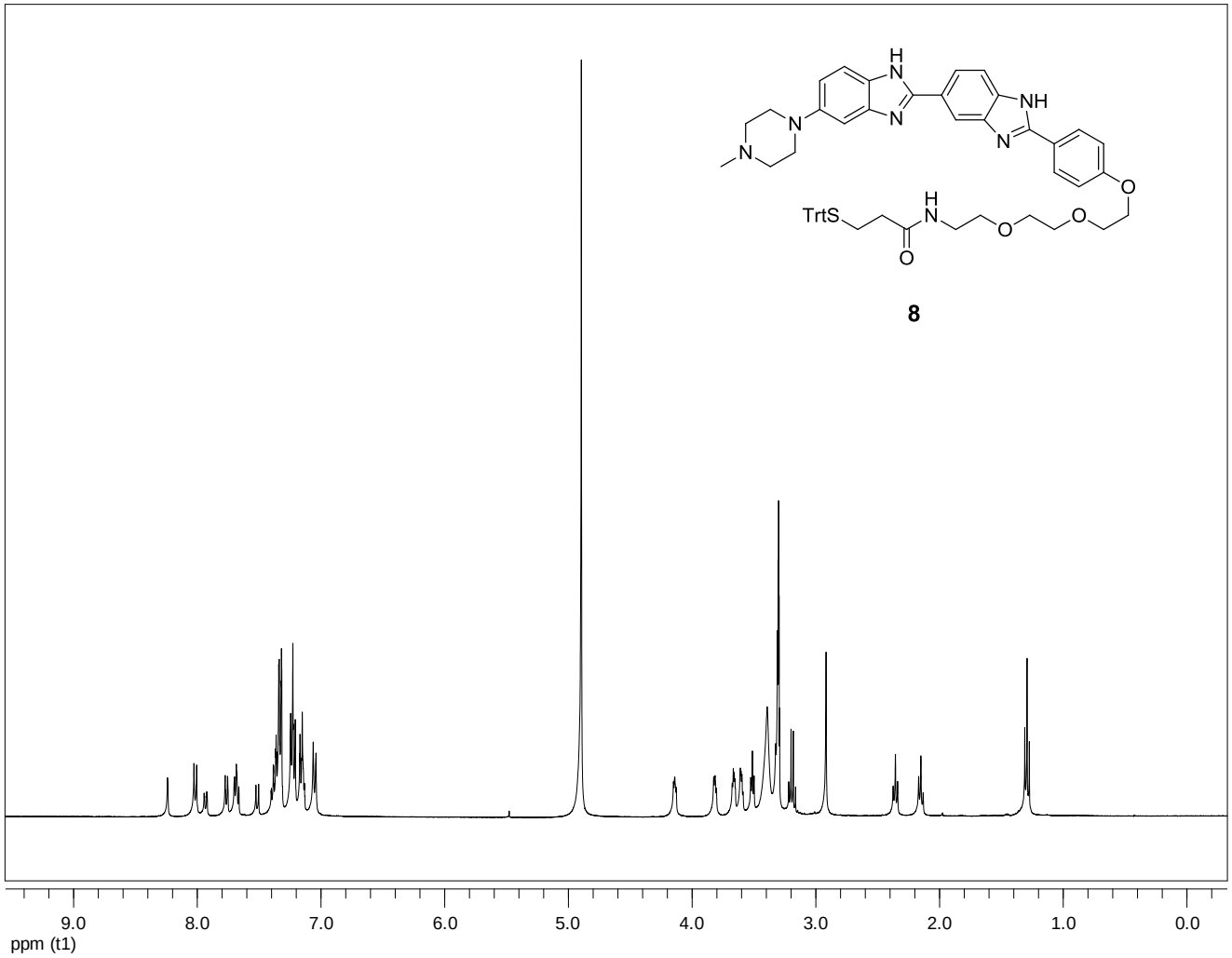


Figure S7. ^{1}H-NMR spectrum of **8**.

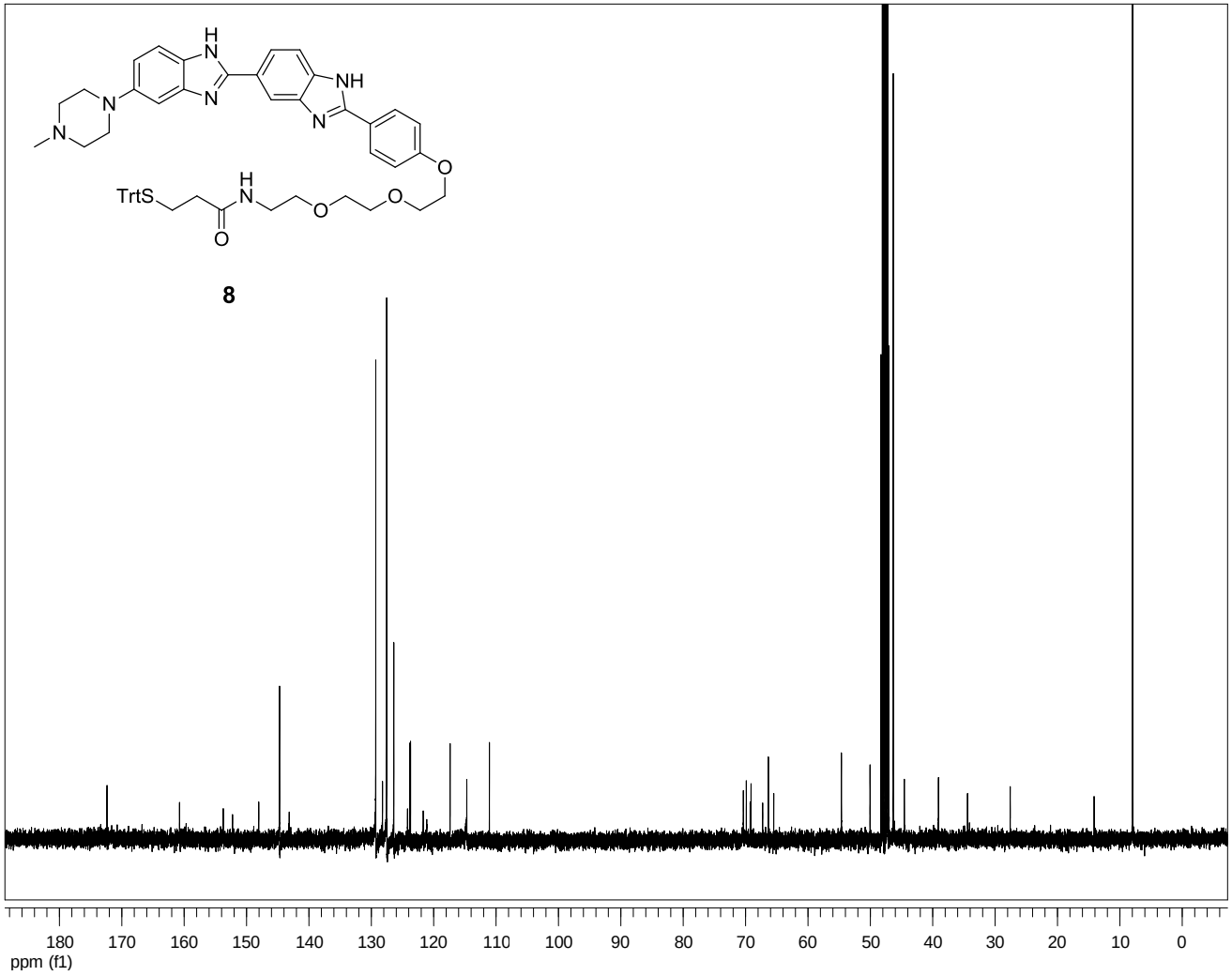


Figure S8. ^{13}C-NMR spectrum of **8**.

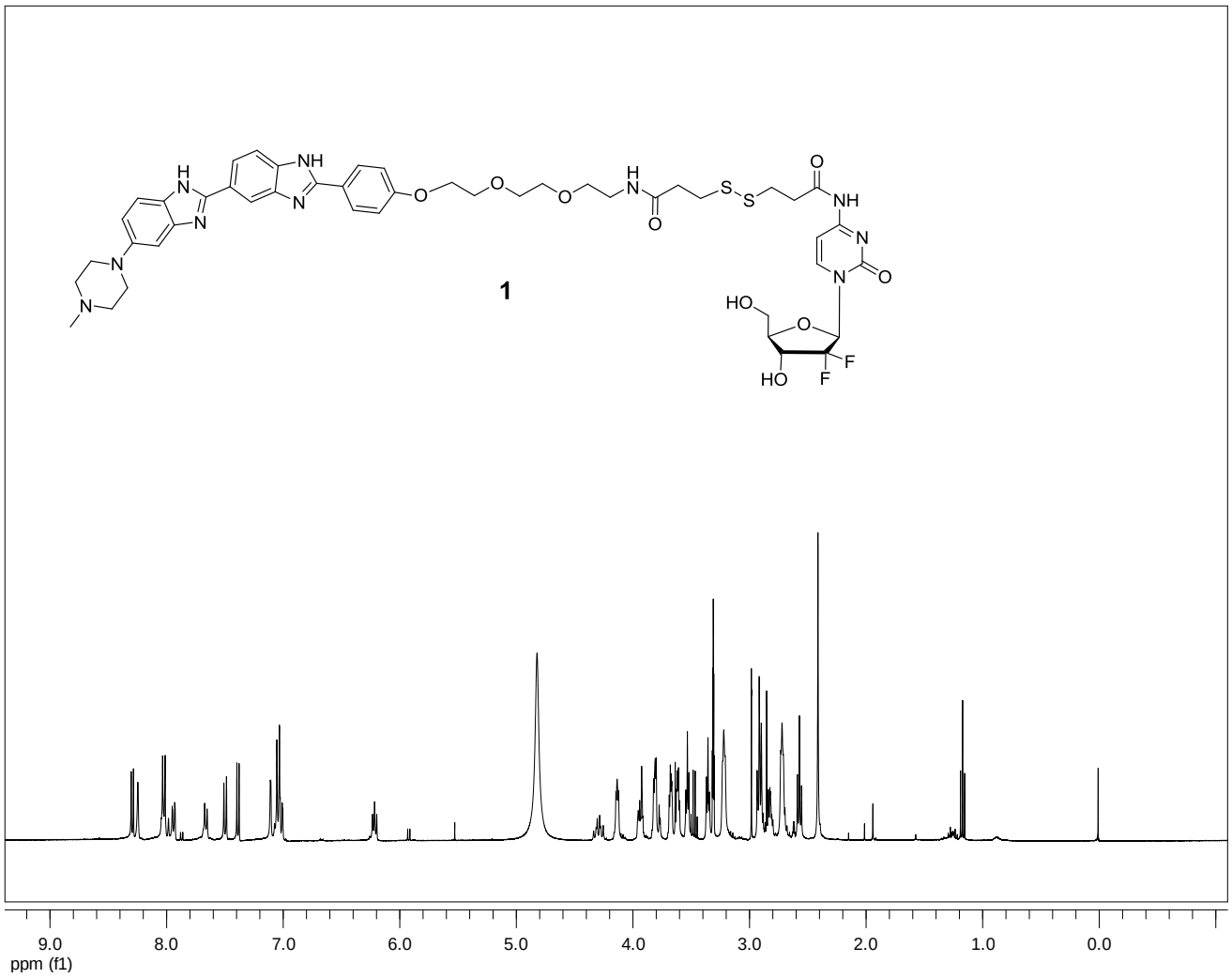


Figure S9. ^{1}H-NMR spectrum of **1**.

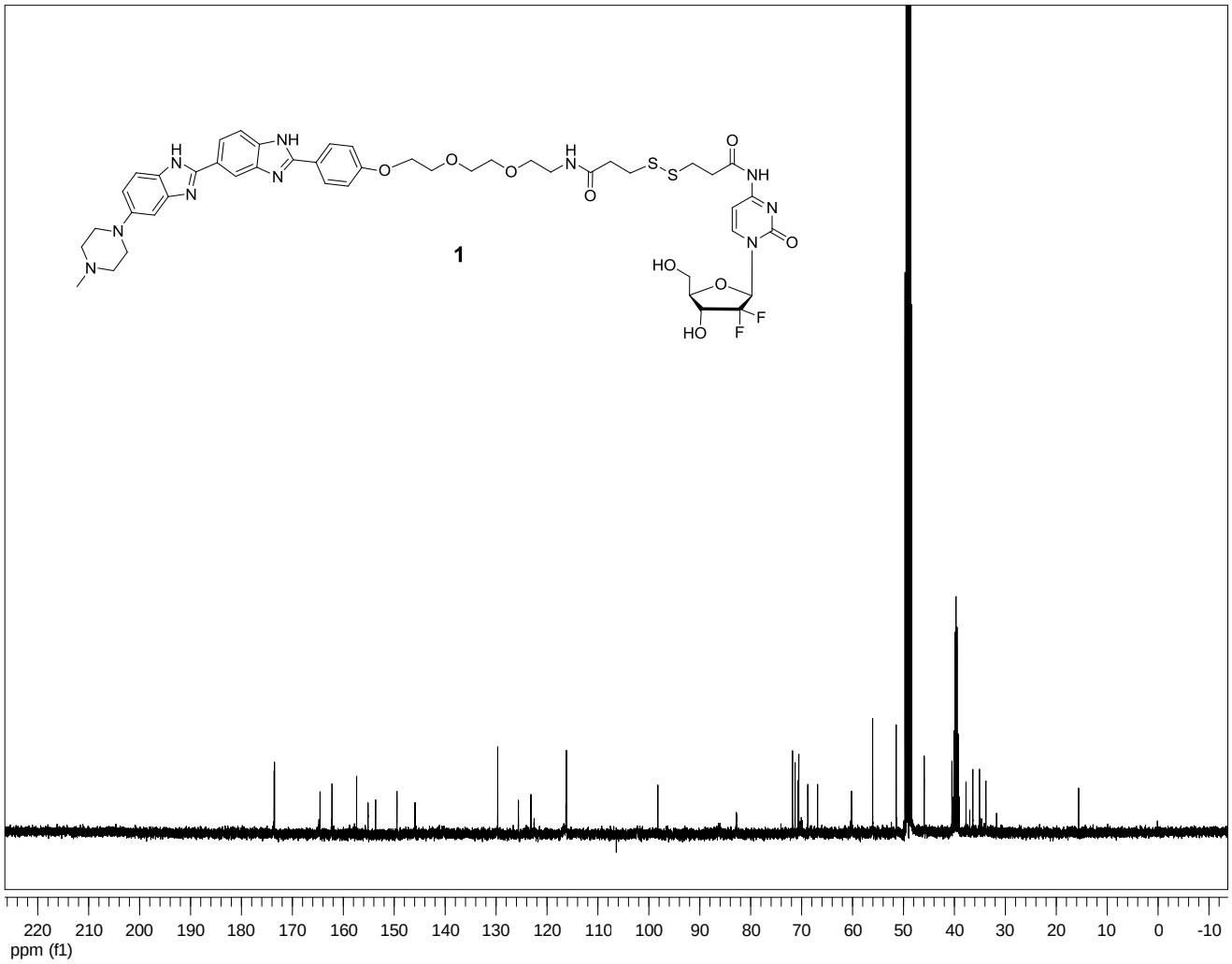


Figure S10. ^{13}C-NMR spectrum of **1**.