Materials and Methods.

General. All reagents were purchased from Sigma-Aldrich, Acros Organics or Fisher Scientific and used as received unless otherwise stated. Gemcitabine hydrochloride > 99 % was used as received without any further purification from LC Laboratories. Dichlorodiisopropylsilane > 97 % and L - cysteine hydrochloride monohydrate were used as received from Sigma-Aldrich. Ethylenediaminetetraacetic acid (EDTA) and sodium chloride were obtained from Fisher Scientific. Tris(2 - carboxyethyl)phosphine hydrochloride (TCEP), BupH borate buffer packs and BupH phosphate buffer packs were obtained from Thermo Scientific. Herceptin® (Trastuzumab) was purchased from the University of North Carolina at Chapel Hill Hospital Pharmacy. Solvents were used as received without any further purification or drying. All reactions were carried out under normal atmospheric conditions at room temperature unless otherwise noted. (*N* - (5 – hydroxypentyl)maleimide was synthesized from literature methods, briefly described below. The extinction coefficient of Trastuzumab (Herceptin®) was taken from literature. The reported antibody drug conjugates were prepared and purified from modified literature methods.

NMR measurements were recorded on a Bruker AVANCE III spectrometer at room temperature. ^{1}H NMR measurements were collected at 600 MHz and ^{13}C NMR measurements were collected at 150 MHz. All chemical shifts (δ) are reported in parts per million (ppm). Electrospray ionization mass spectrometry (ESI - MS) measurements were recorded on a TriVersa Nanomate Quattro II.

Synthesis.

(*N* - (5 - hydroxypentyl)maleimide)[1]

In a dry 500 mL round bottom flask, purged with N_2, 5 – amino – 1 - pentanol (2.66 g, 25.7 mmol) was dissolved in saturated sodium bicarbonate (120 mL). After cooling the solution to 0˚C in an ice bath, *N* - methoxycarbonylmaleimide (4.0 g, 25.7 mmol) was added over 30 minutes. After stirring for 30 minutes at 0˚C the ice bath was removed and the solution was allowed to warm to room temperature over 60 minutes. The aqueous solution was extracted with methylene chloride (3 x 100 mL). The organic layer was dried with excess anhydrous magnesium sulfate, filtered and the solvent removed *in vacuo*. The product was purified by column chromatography (silica, 99:1 methylene chloride: methanol, R_f = 0.04) and dried *in vacuo*. Yield: 2.27 g (12.4 mmol, 48.2 %), white solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 1.32 (m, 2H), 1.57 (m, 4H), 1.89 (s, 1H), 3.49 (t, 2H, *J* = 7.2 Hz), 3.58 (t, 2H, *J* = 6.5 Hz), 6.67 (s, 2H). ^{13}C NMR (150 MHz $CDCl_3$): δ = 22.96, 28.36, 32.13, 37.71, 62.55, 134.15, 171.02. MS (m/z) observed for $C_9H_{13}NO_3$, $[M + H^+]^+ = 184.04$ ($[M + H^+]^+_{theoretical} = 184.10$), $[M + Na^+]^+ = 206.01$ ($[M + Na^+]^+_{theoretical} = 206.08$), $[M + K^+]^+ = 222.02$ ($[M + K^+]^+_{theoretical} = 222.19$).

Gemcitabine Silyl Ether Maleimide

In a dry 100 mL round bottom flask, purged with N_2, dichlorodiisopropylsilane (0.301 mL, 1.66 mmol) was dissolved in anhydrous DMF (6 mL) and anhydrous pyridine (0.272 mL, 3.33 mmol) and cooled to 0°C. (*N* – (5 – hydroxypentyl)maleimide) (0.305 g, 1.66 mmol) was dissolved in anhydrous DMF (6 mL) and added to the reaction dropwise over 2 h. After 2 h, the reaction was allowed to warm from 0°C to room temperature and was stirred for an additional 2 h at room temperature. The reaction mixture was then added to a solution gemcitabine hydrochloride (0.250 g, 0.83 mmol) in anhydrous DMF (12 mL). After 2 h the reaction was diluted with ethyl acetate (150 mL) and washed with saturated sodium chloride (3 x 100 mL). The organic layer was dried with excess anhydrous magnesium sulfate, filtered and the solvent removed *in vacuo*. The product was purified by column chromatography (silica, 95:5 methylene chloride:methanol, R_f = 0.11) and dried *in vacuo*. Yield: 0.021 g (0.037 mmol, 18.0 %), white solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 1.00 (s, 14H), 1.32 (m, 2H), 1.55 (sep, 4H, *J* = 6.5 Hz), 3.48 (t, 2H, *J* = 7.0 Hz), 3.71 (t, 2H, *J* = 6.3 Hz), 4.01 (m, 2H), 4.10 (d, 1H, *J* = 10.9 Hz), 4.35 (q, 1H, *J* = 8.3 Hz), 5.80 (d, 1H, *J* = 7.5 Hz), 6.32 (m, 1H, *J* = 7.5 Hz), 6.68 (s, 2H), 7.66 (d, 1H, *J* = 7.5 Hz). ^{13}C NMR (150 MHz $CDCl_3$): δ = 11.90, 11.99, 17.33, 23.08, 28.33, 32.10, 37.89, 60.63, 62.81, 69.32 (dd, J_{C-F} = 19.30 Hz, J_{C-F} = 27.17 Hz), 81.34, 81.37, 84.06 (dd, J_{C-F} = 21.9 Hz, J_{C-F} = 39.5 Hz), 96.10, 122.45 (t, J_{C-F} = 258.6 Hz), 134.23, 141.18, 154.76, 164.46, 171.25. MS (m/z) observed for $C_{24}H_{37}F_2N_4O_7Si$, $[M + H^+]^+$ = 559.28 ($[M + H]^+_{theoretical}$ = 559.24).

Preparation of ADCs

Herceptin® was reconstituted in bacteriostatic water to give a Trastuzumab concentration of 22 mg/ml and dialyzed against a 25 mM sodium borate, pH 8.0, 25 mM NaCl, 1 mM EDTA buffer overnight in a 20 000 MWCO cassette The protein concentration was quantified using the extinction coefficient of Trastuzumab, 225000 $M^{-1}cm^{-1}$, the measured absorbance values at 280 nm and the molecular weight of Trastuzumab, 1445531.5 g/mol. To achieve 4 or 8 free thiols/mAb, Trastuzumab was treated with either 2.2 or 4.4 molar equivalents of TCEP, respectfully, in a 25 mM sodium borate, pH 8.0, 25 mM NaCl, 1 mM EDTA buffer for 2 h at 37°C. Immediately after 2 h, the mixture was cooled to 0°C. The partially reduced Trastuzumab was then reacted with either 2.2, 4.4 or 8.8 molar equivalents of gemcitabine silyl ether maleimide at 0°C for 30 mins to achieve drug/mAb ratios of 2-8, respectfully. L-cysteine hydrochloride monohydrate was added to the reaction, final concentration of 1mM, to quench excess gemcitabile silyl ether maleimide. The gemcitabine silyl ether ADCs were purified by dialyzing against phosphate buffered saline in a 20 000 MWCO cassette overnight.

Drug to antibody ratio (DAR) determination

After purification, the ADC was removed and a 60µL aliquot was diluted with 120µL methanol with 1% trifluoroacetic acid (TFA) to cleave gemcitabine and precipitate the mAb. The ADC was shaken for 15 minutes and then centrifuged at 14,000 rpm and 4°C

for 15 minutes. The supernatant was then removed and 10µL was injected onto HPLC for analysis.

High Performance Liquid Chromatography (HPLC) analysis of gemcitabine[2]

Gemcitabine was measured following previously published methods. Gemcitabine was separated using an Agilent Technologies Series 1200 HPLC with a C18 reverse phase column (Zorbax Eclipse XDB-C18, 4.6x150mm, 5 micron). Mobile phase A was 100% water with 0.1% TFA and mobile phase B was 100% acetonitrile with 0.1% TFA. A linear gradient was run from 100% A to 97.5% A 2.5% B over 15 minutes at a flow rate of 1 mL/min and temperature of 37°C. Gemcitabine was detected at 9 minutes using a wavelength of 267nm.

***In vitro* release of gemcitabine**

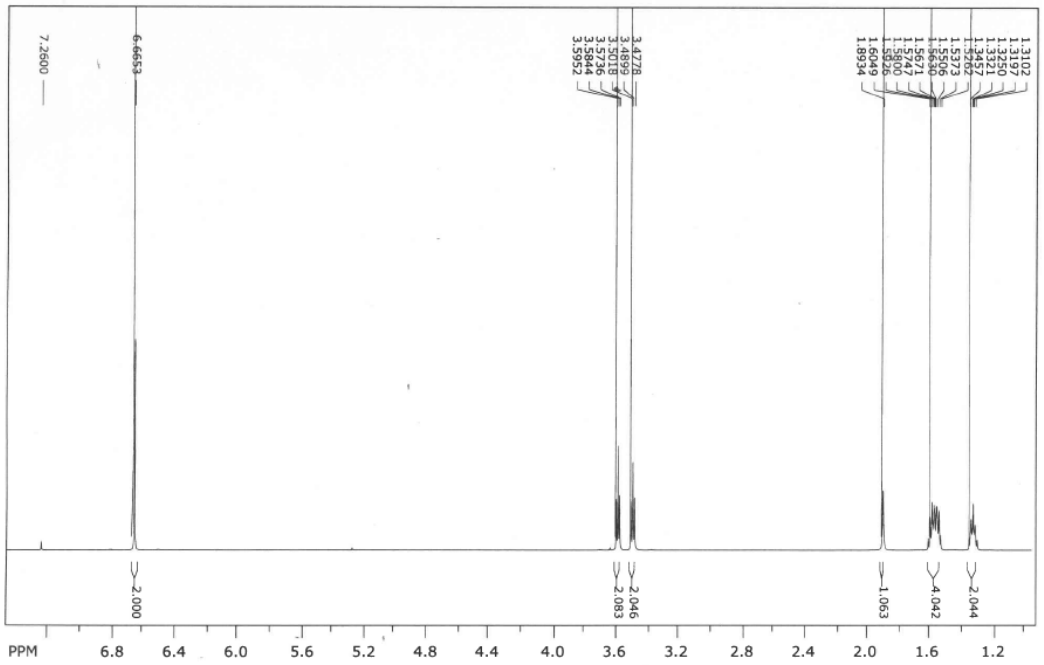
The rate of gemcitabine release from the ADC was measured in pH 7.4, pH 5.0 buffer and 50% CD-1 mouse plasma. The 7.4 buffer contained potassium phosphate monobasic and sodium hydroxide at a concentration of 50 mM. The pH 5.0 buffer contained potassium acid phthalate and sodium hydroxide at a concentration of 50 mM. The plasma was collected from CD-1 mice into K3-EDTA tubes and mixed 1:1 with 50 mM PBS. For the pH 5 study, the ADC was dialyzed against pH 5 buffer to adjust the pH prior to study start. The ADC was then shaken at 37°C and at set time points, an aliquot of the ADC solution was removed and precipitated with methanol to measure the free gemcitabine in solution. These experiments were repeated 3 times and less than 1% free drug was measured before each experiment.

Aggregation Measurement[3]

Aggregation of the ADC was measured following previously published methods. The ADC and trastuzumab were separated by size-exclusion chromatography (SEC) using a TSKgel G3000SW_{XL} 7.8 x 300 mm column from Tosoh Biosciences. 5µL of ~0.5 mg/mL samples of the ADC and trastuzumab were injected and detected at 280 nm at 15-16 minutes. An isocratic mobile phase of 0.2 M potassium phosphate buffer (pH 6.8), 0.2 M potassium chloride and 15% isopropyl alcohol was run at a flow rate of 0.5 mL/min and 25°C.

References

1. K.A. Keller, J. Guo, S. Punna, M.G. Finn, Tetrahedron Lett, 2005, 46, 1181-84, doi: 10.1016/j.tetlet.2004.12.067
2. M.C. Parrott, M. Finniss, J.C. Luft, A. Pandya, A. Gullapalli, M.E. Napier, J.M. Desimone, J Am Chem Soc, 2012, 134, 7978-82, doi: 10.1021/ja301710z.
3. A.A. Wakankar, M.B. Feeney, J. Rivera, Y. Chen, M. Kim, V.K. Sharma, Y.J. Wang, Bioconjug Chem, 2010, 21, 1588-95. doi: 10.1021/bc900434c



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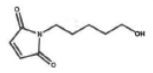

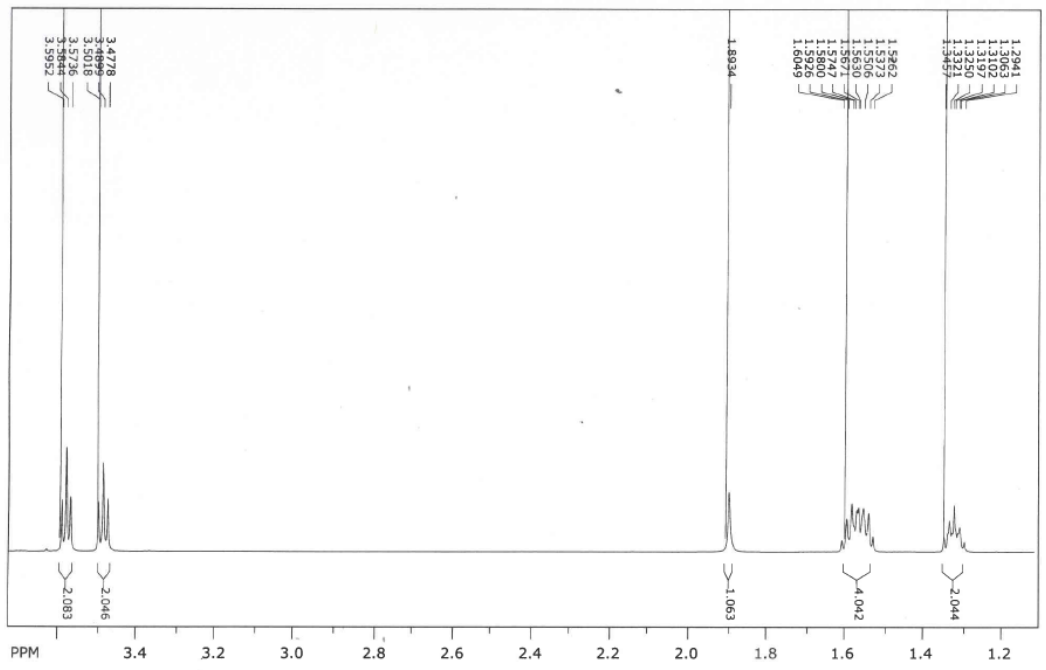

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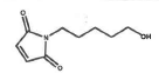

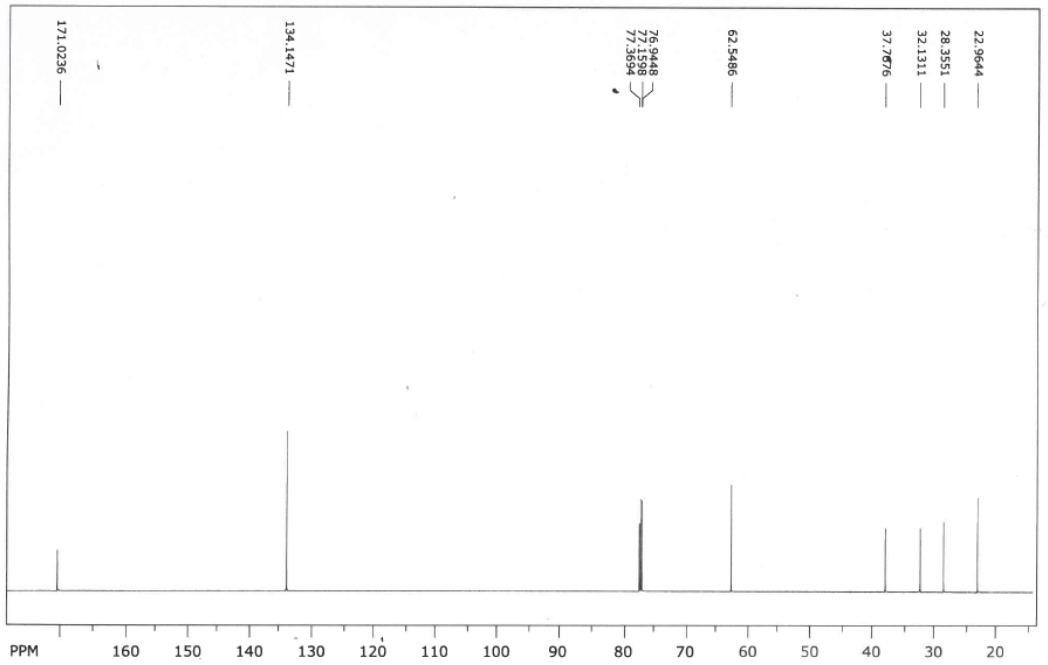

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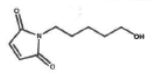

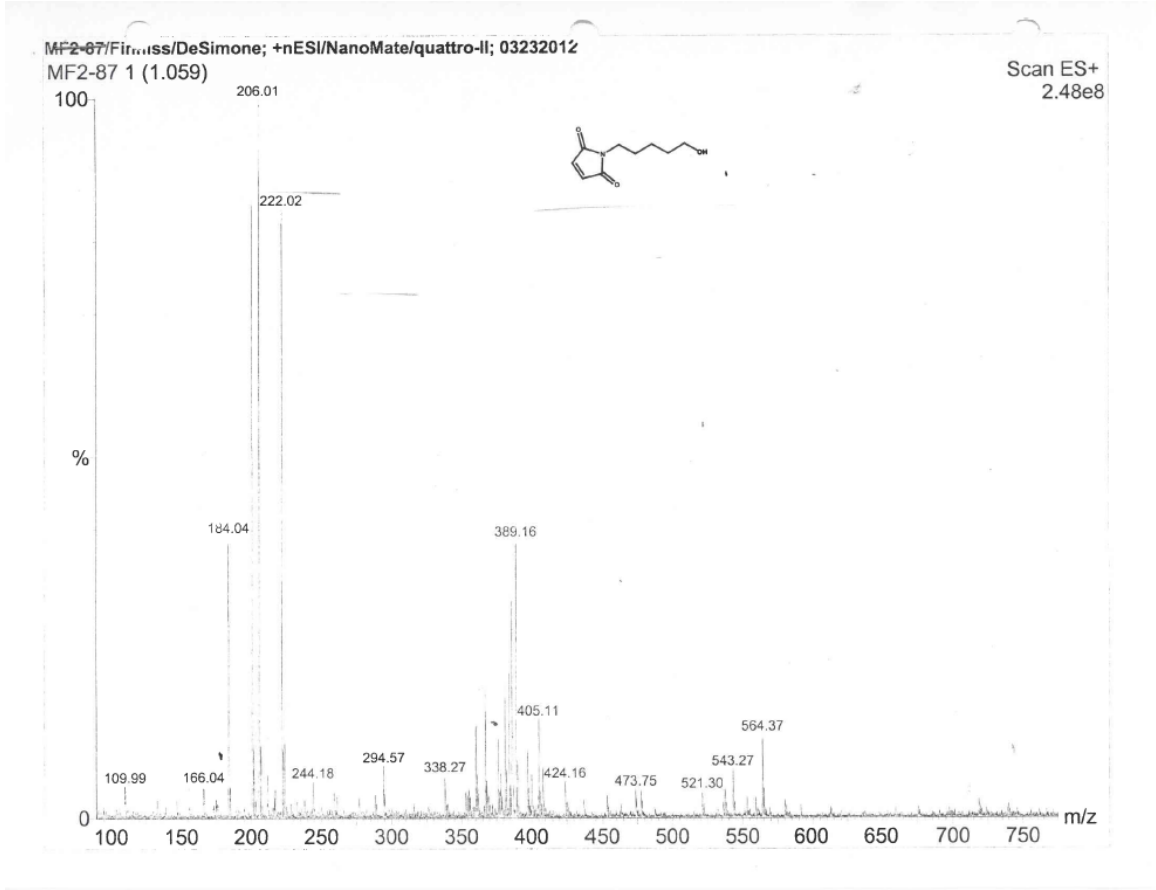
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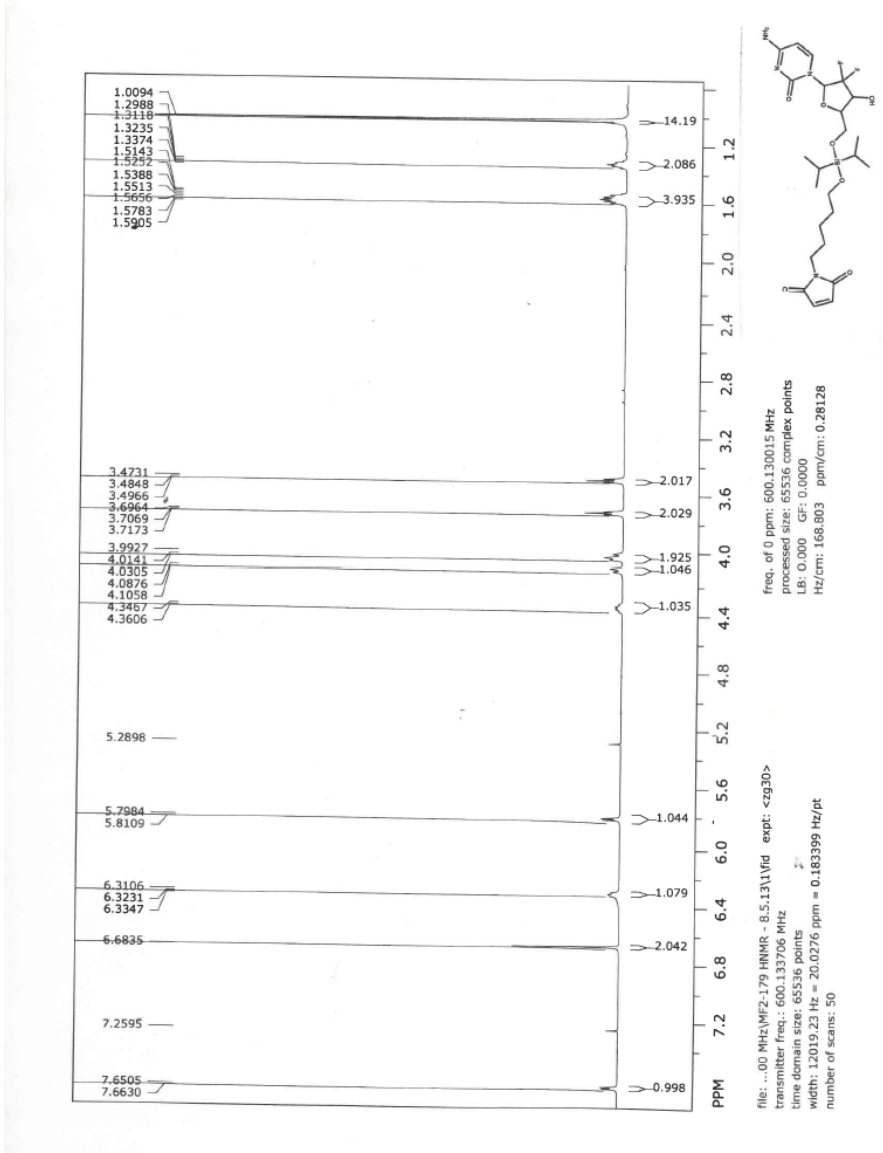
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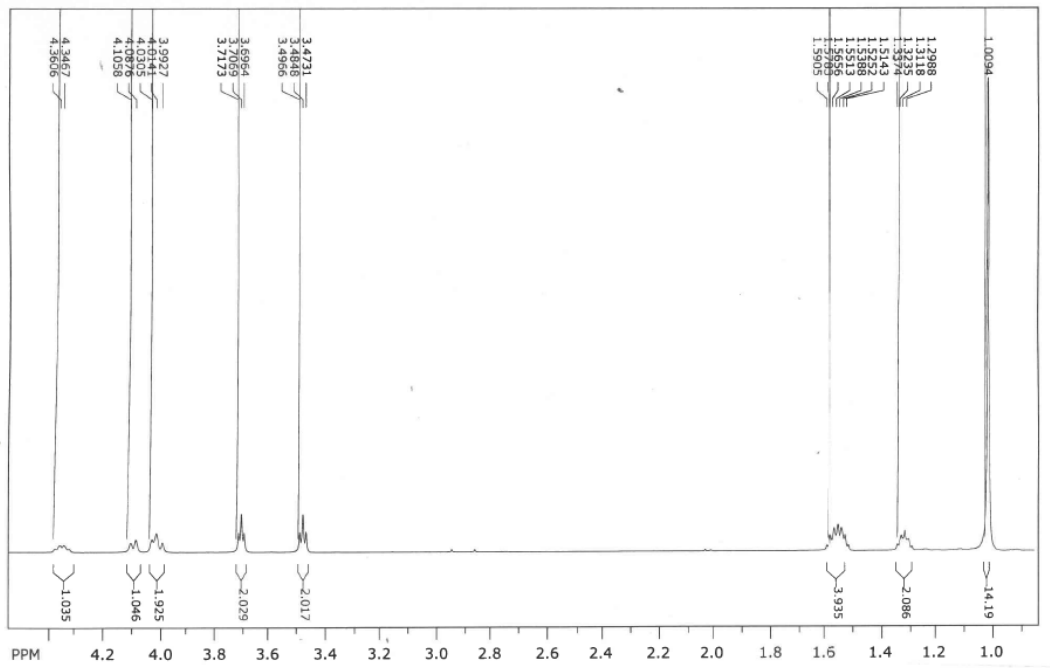

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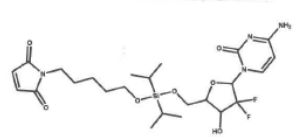

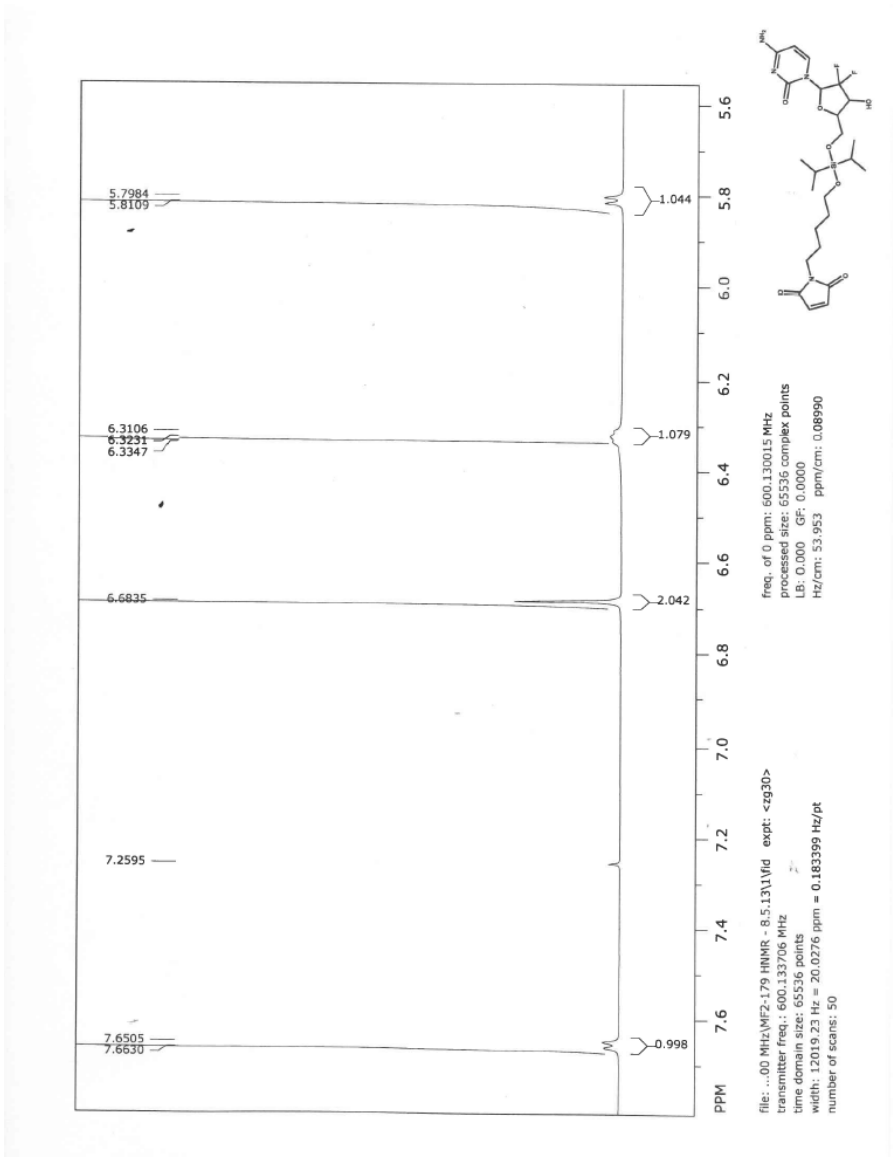
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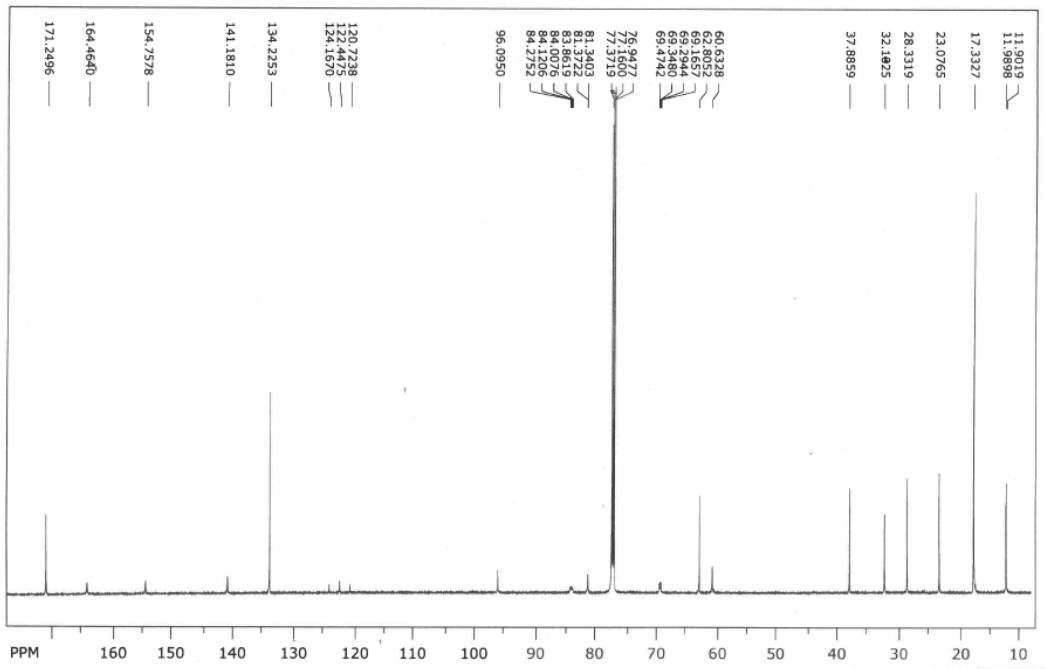

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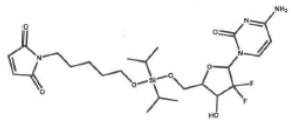

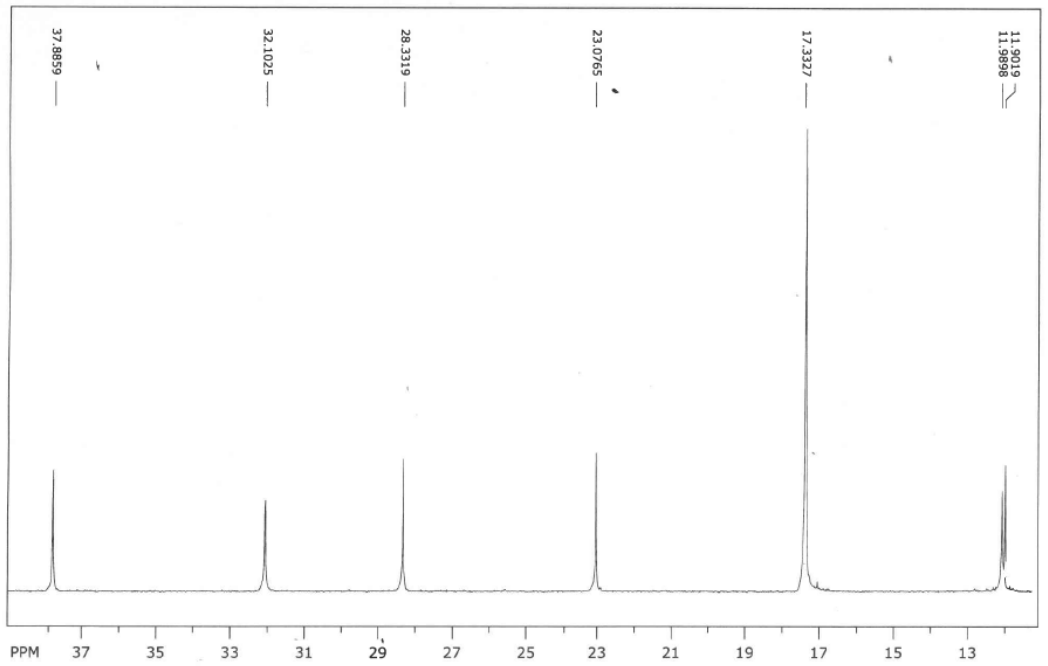

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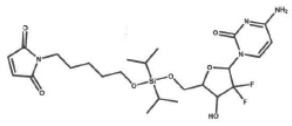

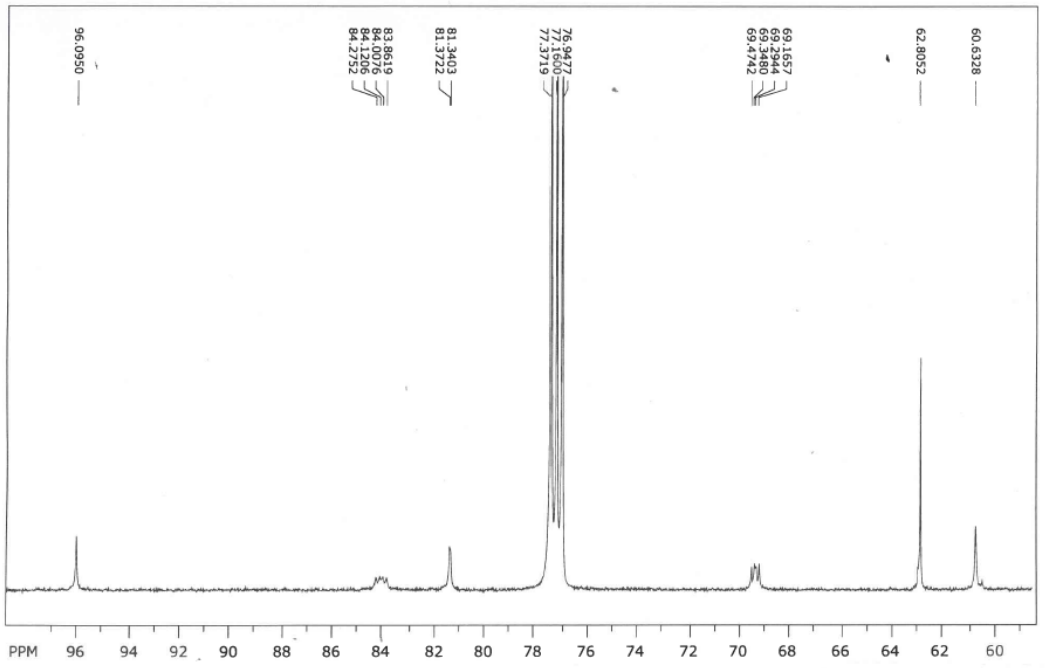

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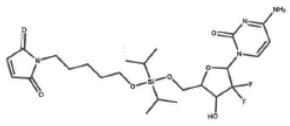

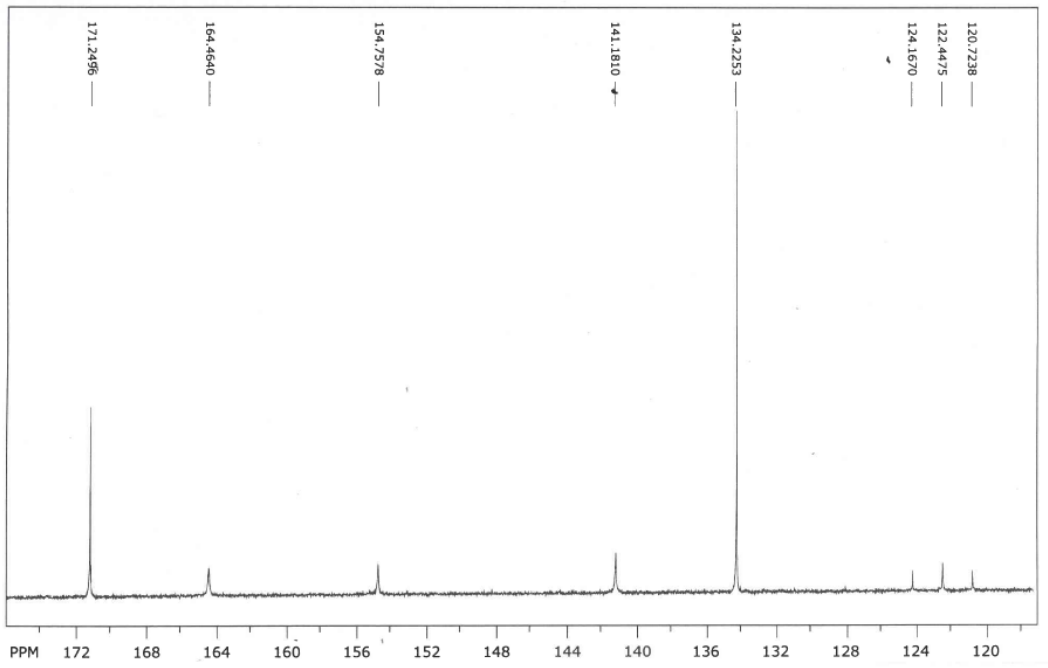

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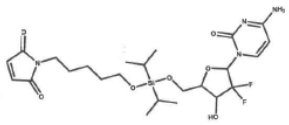

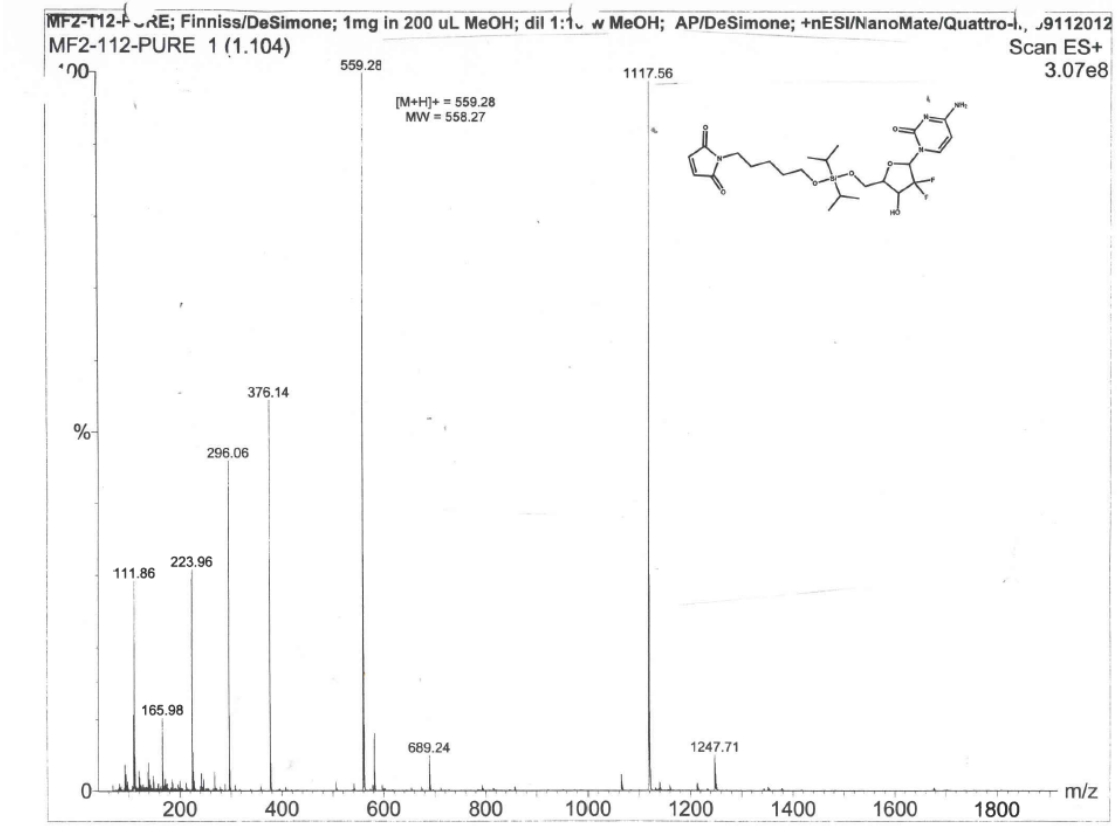
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