Tunable Stability of Imidazotetrazines Leads to a Potent Compound for Glioblastoma

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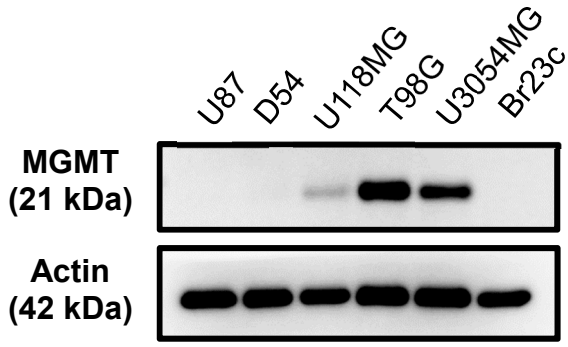
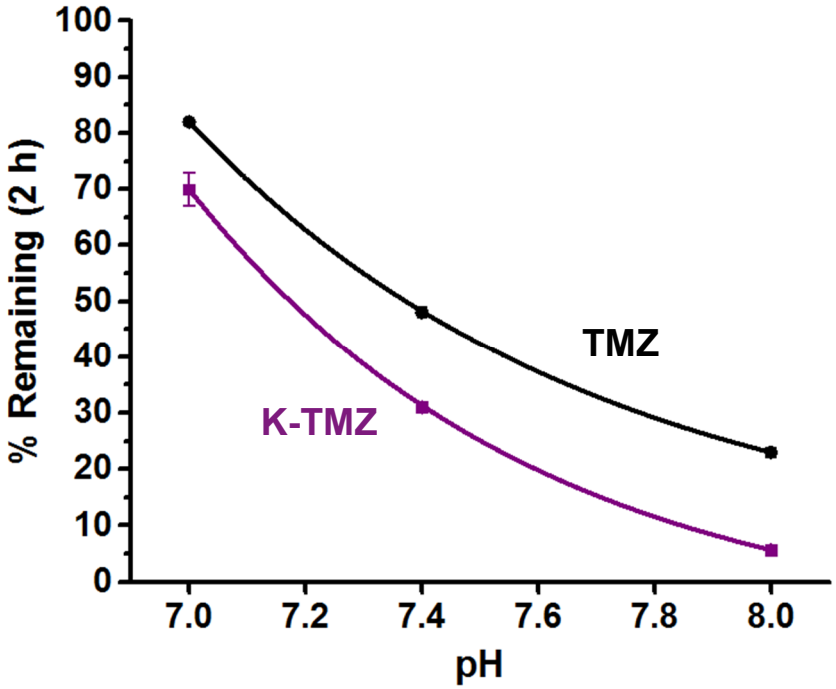
1. Table S1. Panel of C8-substituted imidazotetrazines and associated 7 day IC_{50} values (μM) in multiple GBM cell lines; the four compounds below were tested and this supporting table is a complement to Table 1. Cell viability was assessed using the Alamar Blue assay. Error is SEM, n≥3.

		MGMT			
		–	–	+	+
Compound	R	U87	D54	U118MG	T98G
TMZ	$CONH_2$	51 ± 8	12 ± 1	322 ± 7	660 ± 10
18	Br	26 ± 7	20 ± 1	80 ± 20	60 ± 10
24	C_6H_4-4-F	13 ± 2	ND	ND	18 ± 2
25	C_6H_4-4-CF_3	16 ± 1	ND	ND	>100
26	C_6H_4-4-Cl	9 ± 1	7 ± 1	5 ± 1	19 ± 3

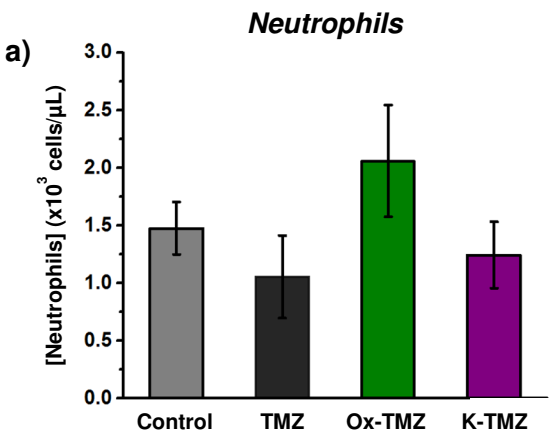
2. Figure S1. Western blot for MGMT status of all cell lines used.

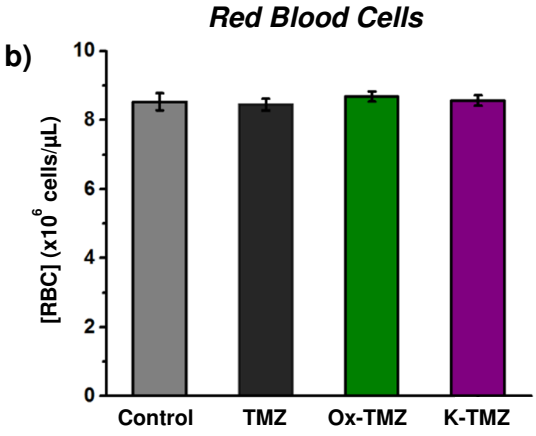


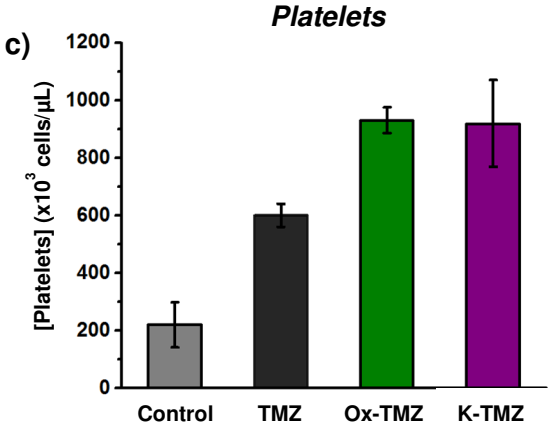
3. Figure S2. The hydrolytic stabilities of TMZ and K-TMZ assessed in saline at pH 7.0, 7.4, and 8.0 by calculating the percentage of parent compound remaining after 2 h at 37°C.



4. Figure S3. Assessment of the hematological toxicity of imidazotetrazines *in vivo*. Mice were treated with a single IV dose of 125 mg/kg imidazotetrazine and a complete blood count was obtained for each mouse after 7 days. (a) Neutrophil concentrations (b) RBC concentrations (c) Platelet concentrations (d) Cohort weights of mice prior to treatment and at the time of blood collection 7 days post-treatment. Error is SEM, number of mice per cohort = 4.







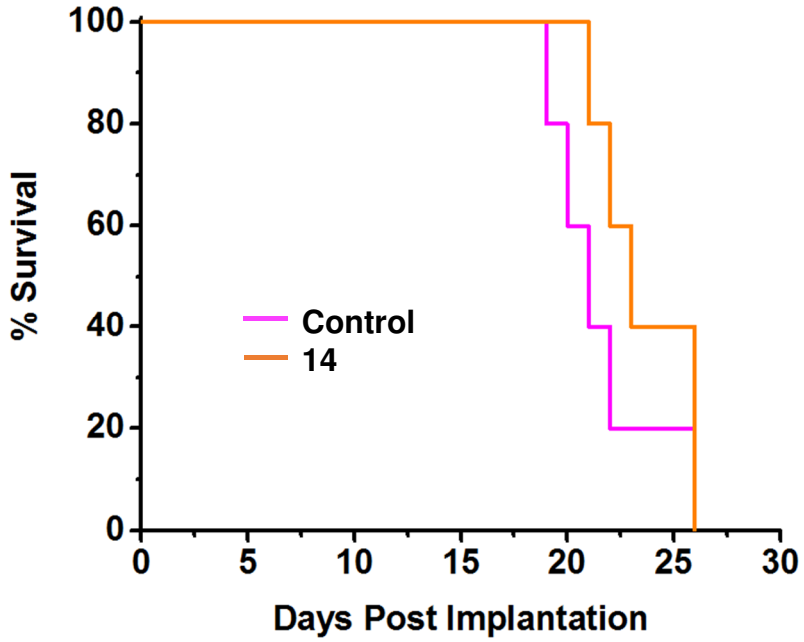
d)

Compound	Pre-treatment (g)	7 days Post (g)
Control	31 ± 1	32 ± 1
TMZ	31 ± 1	32 ± 1
K-TMZ	30 ± 1	31 ± 1
Ox-TMZ	30.7 ± 0.1	31.6 ± 0.3

5. Table S2. 7 day IC_{50} values (μM) for TMZ and lead C8-substituted imidazotetrazines in the Br23c GBM oncosphere cell line. Cell viability was assessed using the Alamar Blue assay. Error is SEM, n≥3.

		MGMT
		–
Compound	R	Br23C
TMZ	$CONH_2$	5.2 ± 0.2
4 (Me-TMZ)	CONHMe	6 ± 1
5 (DiMe-TMZ)	$CONMe_2$	6 ± 1
17 (K-TMZ)	COMe	5.2 ± 0.3

6. Figure S4. GBM oncosphere Br23c cells were intracranially implanted into female athymic nude mice. Treatment was started 5 days post implantation. Mice were administered compound 14 (12.8 mg/kg, equimolar to 15 mg/kg TMZ) orally once-per-day for 5 doses. Compound was formulated in 10% PEG in PBS. n≥5. This experiment was run alongside that presented in Figure 5b, and thus the control group is the same for Figure 5b and Figure S4.



7. Experimental Information for Biological Data.

Cell Culture and Reagents

All cell lines were grown in a 37°C, 5% CO_2, humidified environment, in media containing 1% penicillin/streptomycin. Cell culture conditions are as follows: traditional cell lines U87 and T98G were grown in EMEM with 10% FBS. Traditional cell lines D54 and U118MG were grown in DMEM with 10% FBS. HGCC patient-derived cell line U3054MG[1] was cultured under serum-free stem cell conditions (1:1 neurobasal: DMEM/F12 media supplemented with B27, N2, hEGF, and hFGF). GBM oncosphere cell line Br23c[2] was cultured with the NeuroCult NS-A proliferation kit (Stem Cell Technologies) supplemented with 0.0002% heparin, hEGF, and hFGF. Temozolomide (TMZ) was purchased from AK Scientific. TMZ analogs were synthesized as described below. Compounds were dissolved in DMSO (1% final concentration, Fisher Chemical) for cell culture studies.

Cell Viability Assays

Cells were harvested, seeded in a 96-well plate and allowed to adhere. After three hours, compound was added to each well in DMSO (1% final concentration). Cells were incubated for seven days before viability was assessed by the Alamar Blue Assay. Raptinal (20 μM) was used as a dead control.

Mouse Liver Microsome Stability Assay

A mixture of PBS (pH 7.4), NADPH regenerating system solution A (Corning Life Sciences), and NADPH regenerating system solution B (Corning Life Sciences) was incubated at 37ºC in a shaking incubator for 5 min. Next, compound was added in DMSO (final concentration 50 μM, 0.5% DMSO) before ice-cold mouse liver microsomes (Thermo Fisher, male CD-1 mice, pooled) were added (final protein concentration of 1 mg/mL). An aliquot was immediately removed, quenched with an equal volume of 100 μM internal standard and 0.5% hydrochloric acid in ice-cold acetonitrile, and centrifuged at 13,000 rcf for 3 min. The supernatant was diluted 1:5 in ddH_2O and analyzed by LC-MS. The reactions were incubated at 37ºC in a shaking incubator for 2 h. A second aliquot was removed, quenched and diluted as before and analyzed by LC-MS. The ratio of the areas of analyte: internal standard at 2 hours was compared to the ratio at t_0 to determine the percentage of compound remaining. Analysis was performed on an Agilent 6230 LC/MS TOF

system with a 1.8 µm, 2.1x50 mm Agilent ZORBAX Eclipse Plus C18 column. Internal standard = N3-propyl TMZ.

O^6-Methyldeoxyguanosine Quantitation

U87 cells were plated at $1x10^6$ c/w in a 6-well plate before they were treated with compound at the indicated concentration (1% final concentration DMSO). After 8 h incubation, the cells were harvested and pelleted. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, ID: 69504). DNA was then precipitated using the following procedure: 1/10 v/v 3M sodium acetate (pH 5.2) and 2.5x v/v ethanol was added to each sample which was then kept at –80°C for 1 h. The mixture was centrifuged at max at 4°C for 30 min and decanted to afford a pellet of DNA, which was re-suspended in ddH_2O containing 10 mM tris base (pH 7.5) and 1 mM EDTA. The concentration of DNA in each sample was quantified measuring absorbance on a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher). DNA (10 µg) from each sample was added to DNA hydrolysis buffer[3] and incubated at 37°C for 6 h. Hydrolyzed samples were then submitted for LC-MS/MS quantitation. Samples were analyzed with a 5500 QTRAP LC/MS/MS system (AB Sciex) with a 1200 series HPLC system (Agilent).

in vivo Blood-Brain Barrier Permeability

All experimental procedures were reviewed and approved by the University of Illinois Institutional Animal Care and Use Committee. CD-1 IGS mice were administered compound in 1% DMSO (Figure 2b) or 10% DMSO (Figure 2c-e) in PBS at 25 mg/kg via lateral tail vein injection. Five minutes post injection, mice were sacrificed and blood was collected by lacerating the right auricle with iris scissors. An 18 gauge angiocatheter was inserted through the left ventricle, and all residual circulatory volume was removed by perfusing 0.9% saline solution via an analog peristaltic pump. Blood samples were immediately centrifuged at 13,000 rcf for five minutes and the supernatant collected and acidified with 8.5% aqueous H_3PO_4. Brains were harvested from the cranial vault, acidified with 0.3% aqueous H_3PO_4 and flash frozen. Homogenized brain samples were centrifuged twice at 13,000 rcf for ten minutes and supernatant and tissue debris were separated. The resultant supernatant was analyzed, along with plasma, by LC-MS/MS to determine compound concentrations. In order to calculate absolute brain:serum ratios (ng $drug_{brain}$:ng $drug_{serum}$), a mouse blood volume of 58.5 mL/kg was assumed for each mouse.

in vivo Efficacy Models

Human GBM Br23c stem-like neurosphere cells were intracranially implanted in female athymic nude mice (150,000 cells/mouse). Beginning day 5 after implantation of the tumor cells, drugs were formulated in 10% PEG 400 in saline and 15 mg/kg TMZ (or equimolar dose of C8 analog) was administered via oral gavage once-per-day for 7 weeks (Figure 5a) or once-per-day for 5 total treatments (Figure 5b). TMZ and C8 analogs were dissolved fresh for each use. Mice were observed daily for any signs of deterioration, neurotoxicity, or movement disorders. They were inspected for signs of pain and distress, as in accordance with the Johns Hopkins Animal Care and Use Guidelines. If the symptoms persisted and resulted in debilitation, the animals were euthanized according to protocol.

Assessment of Hematological Toxicity

Male CD-1 IGS mice (n=4 mice/group) were administered a single dose of 125 mg/kg compound intravenously. Imidazotetrazines were formulated with SBEβCD in sterile water immediately prior to injection. Seven days post-treatment, mice were humanely sacrificed and whole blood was collected for assessment of total white blood cells, lymphocytes, neutrophils, platelets, and red blood cells.

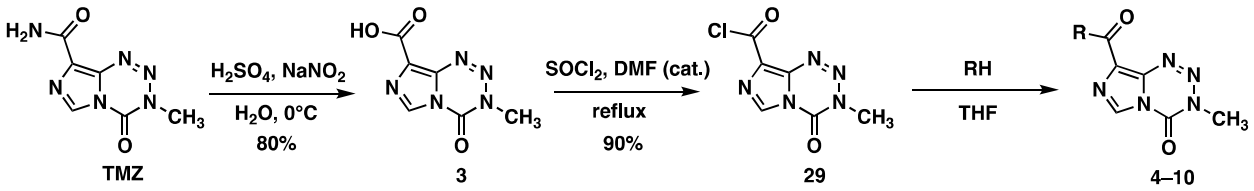
8. Materials and Methods

Chemical reagents were purchased from commercial sources and used without further purification. Flash chromatography was performed using silica gel (230-400 mesh). Anhydrous solvents were dried after being passed through columns packed with activated alumina under positive pressure of nitrogen. Unless otherwise noted, all reactions were carried out in oven-dried glassware with magnetic stirring under nitrogen atmosphere. ^{1}H and ^{13}C NMR spectra were recorded on Bruker 500 (500 MHz, ^{1}H; 125 MHz, ^{13}C) or Varian Unity Inova 500 (500 MHz, ^{1}H) MHz spectrometers. Spectra are referenced to residual chloroform (δ = 7.26 ppm, ^{1}H; 77.16 ppm, ^{13}C) or dimethyl sulfoxide (δ = 2.50 ppm, ^{1}H; 39.52 ppm, ^{13}C). Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). Coupling constants J are reported in Hertz (Hz). High resolution mass spectrometry (HRMS) was performed on a Waters Q-Tof Ultima or Waters Synapt G2-Si instrument with electrospray ionization (ESI) or electron impact ionization (EI).

9. Preparation and Characterization of C8 Analogs

Note: Experimental information for compounds **3**,[4] **9**,[5] **11**,[6] **15**,[7] **16**,[8] **29**,[9] **31**,[10] and **33**[6] has been previously reported.

General Scheme for Preparation of Amide, Ester, and Thioester Derivatives 4–10



Experimental data for compounds **3**, **9**, and **29** has been published.[4,5,9]

General procedure for preparation of 4–10: In an oven-dried 25 mL round bottom flask, acyl chloride **29** (148.6 mg, 0.70 mmol, 1 eq.) was dissolved in anhydrous THF (2.8 mL, 0.25 M). Methylamine (33% w/w in ethanol, 0.09 mL, 0.73 mL, 1.05 eq.) was then added and the reaction was stirred for 3 h at room temperature. When complete, the reaction was stopped and the solvent was evaporated. The crude solid was purified by flash silica gel chromatography (100% ethyl acetate) to yield 98.3 mg (68%) of pure **4** as a white solid.

***N*,3-dimethyl-4-oxo-3,4-dihydroimidazo[5,1-*d*][1,2,3,5]tetrazine-8-carboxamide (4, Me-TMZ)**

^{1}H NMR (500 MHz, d-DMSO) δ 8.84 (s, 1H), 8.45 (d, J = 4.9 Hz, 1H), 3.86 (s, 3H), 2.81 (d, J = 4.8 Hz, 3H). **^{13}C** (125 MHz, d-DMSO) δ 160.13, 139.23, 134.27, 130.54, 128.44, 36.14, 25.80. **HRMS** (ESI) calc. for $C_7H_8N_6O_2Na$, $[M+Na]^+$: 231.0606, found: 231.0608.

***N*,*N*,3-trimethyl-4-oxo-3,4-dihydroimidazo[5,1-*d*][1,2,3,5]tetrazine-8-carboxamide (5, DiMe-TMZ)**

76% yield as a white solid.

^{1}H NMR (500 MHz, d-DMSO) δ 8.81 (s, 1H), 3.85 (s, 3H), 3.06 (s, 6H). **^{13}C NMR** (125 MHz, d-DMSO) δ 161.76, 139.22, 133.57, 132.05, 128.59, 38.12, 36.05, 34.84. **HRMS** (ESI) calc. for $C_8H_{11}N_6O_2$, [M+H]: 223.0938, found: 223.0943.

***N*,*N*-diethyl-3-methyl-4-oxo-3,4-dihydroimidazo[5,1-*d*][1,2,3,5]tetrazine-8-carboxamide (6)**

91% as a white solid.

^{1}H NMR (500 MHz, d-DMSO) δ 8.81 (s, 1H), 3.84 (s, 3H), 3.49 (q, J = 7.1 Hz, 2H), 3.38 (q, J = 7.0 Hz, 2H), 1.18 (t, J = 7.1 Hz, 3H), 1.11 (t, J = 7.0 Hz, 3H). **^{13}C NMR** (125 MHz, d-DMSO) δ 161.35, 139.24, 133.54, 132.72, 128.45, 42.53, 36.01, 14.43, 12.80. **HRMS** (ESI) calc. for $C_{10}H_{15}N_6O_2$, $[M+H]^+$: 251.1256, found: 251.1250.

***N*,*N*-dibutyl-3-methyl-4-oxo-3,4-dihydroimidazo[5,1-*d*][1,2,3,5]tetrazine-8-carboxamide (7)**

85% yield as a white solid.

^{1}H NMR (500 MHz, d-DMSO) δ 8.80 (s, 1H), 3.84 (s, 3H), 3.45 (m, 2H), 3.34 (m, 2H), 1.59 (m, 2H), 1.49 (m, 2H), 1.35 (h, J = 7.4 Hz, 2H), 1.11 (h, J = 7.4 Hz, 2H), 0.94 (t, J = 7.4 Hz, 3H), 0.76 (t, J = 7.4 Hz, 3H). **^{13}C NMR** (125 MHz, d-DMSO) δ 161.74, 139.23, 133.35, 132.80, 128.42, 47.66, 44.41, 35.99, 30.54, 29.20, 19.65, 19.17, 13.79, 13.55. **HRMS** (ESI) calc. for $C_{14}H_{23}N_6O_2$, $[M+H]^+$: 307.1882, found: 307.1881.

3-methyl-8-(pyrrolidine-1-carbonyl)imidazo[5,1-*d*][1,2,3,5]tetrazin-4(3*H*)-one (8)

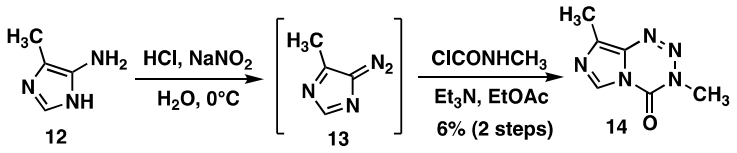
55% yield as pale yellow solid.

^{1}H NMR (500 MHz, d-DMSO) δ 8.81 (s, 1H), 3.85 (s, 3H), 3.63 (m, 2H), 3.53 (m, 2H), 1.88 (m, 4H). **^{13}C NMR** (125 MHz, d-DMSO) δ 159.73, 139.21, 134.07, 132.54, 128.33, 48.05, 46.09, 36.05, 25.80, 23.63. **HRMS** (ESI) calc. for $C_{10}H_{13}N_6O_2$, $[M+H]^+$: 249.1100, found: 249.1105.

***S*-ethyl 3-methyl-4-oxo-3,4-dihydroimidazo[5,1-*d*][1,2,3,5]tetrazine-8-carbothioate (10)**

92% as a white solid.

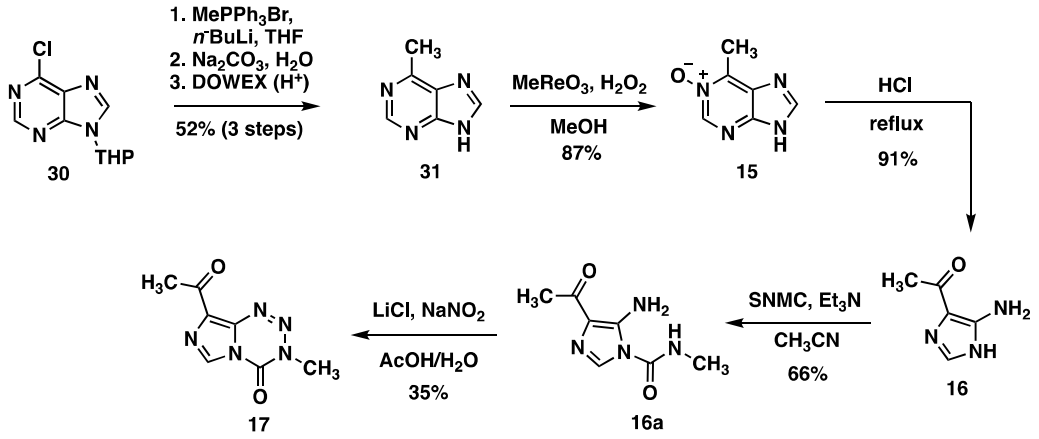
^{1}H NMR (500 MHz, d-DMSO) δ 8.86 (s, 1H), 3.89 (s, 3H), 3.02 (q, J = 7.4 Hz, 2H), 1.28 (t, J = 7.4 Hz, 3H). **^{13}C NMR** (125 MHz, d-DMSO) δ 184.57, 138.95, 133.80, 131.55, 129.19, 36.50, 22.15, 14.70. **HRMS** (ESI) calc. for $C_8H_{10}N_5O_2S$, $[M+H]^+$: 240.0555, found: 240.0551.



3,8-dimethylimidazo[5,1-*d*][1,2,3,5]tetrazin-4(3*H*)-one (14)

Procedure: To a 15 mL round bottom flask, 4-methyl-1*H*-imidazol-5-amine dihydrochloride **12** (44.2 mg, 0.3 mmol, 1 eq.) was added and dissolved in 1M HCl (0.4 mL, 0.65 M) before sodium nitrite (26.2 mg, 0.4 mmol, 1.5 eq.) in water (0.4 mL, 0.65 M) was added at 0ºC in the dark. The solution was stirred 30 minutes then concentrated and azeotroped twice with toluene to afford crude diazo **13**. To the crude diazo suspended in ethyl acetate (1.3 mL, 0.2 M), anhydrous triethylamine (0.08 mL, 0.6 mmol, 2.2 eq.) and methylcarbamic chloride (79 mg, 0.8 mmol, 3.2 eq.) were added in the dark. The reaction was stirred overnight before being purified via flash silica gel chromatography (4:1 hexanes: ethyl acetate) to afford 2.4 mg (6%) **14** as a pale yellow solid. *Note*: To minimize decomposition of the crude diazo species, concentration was done (without heating) in the dark as quickly as possible.

^{1}H NMR (500 MHz, d-$CHCl_3$) δ 8.35 (s, 1H), 3.94 (s, 3H), 2.66 (s, 3H). **^{13}C NMR** (125 MHz, d-$CHCl_3$) δ 139.71, 139.64, 132.51, 127.94, 35.76, 12.53. **HRMS** (EI) calc. for $C_6H_7N_5O$, $[M]^+$: 165.0651, found: 165.0654.



8-acetyl-3-methylimidazo[5,1-*d*][1,2,3,5]tetrazin-4(3*H*)-one (17, K-TMZ)

Experimental data for intermediates **31**, **15**, and **16** has been published.[7,8,10]

Procedure: To an oven-dried 25 mL round bottom flask, **16** (186 mg, 0.89 mmol, 1 eq.) and *N*-succinimidyl *N*-methylcarbamate (321 mg, 1.86 mmol, 2.1 eq.) were added and suspended in anhydrous acetonitrile (1.5 mL, 0.6 M). Next, under nitrogen, dry triethylamine (0.34 mL, 2.4 mmol, 2.7 eq.) was added slowly and the solution was stirred overnight at room temperature. Upon completion, the mixture was concentrated and purified by silica gel flash chromatography (100% dichloromethane to 4:1 dichloromethane: methanol) to afford 106 mg (66%) of intermediate **16a** as a gold solid.

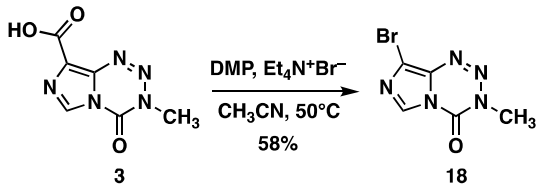
In a 15 mL round bottom flask, LiCl (802 mg, 19 mmol, 36 eq.) was dissolved in distilled water (1.3 mL, 0.4 M) and AcOH (0.10 mL, 5.3 M) and stirred for thirty minutes until the exotherm dissipated. Intermediate **16a** (96.3 mg, 0.53 mmol, 1 eq.) was added in one portion and stirred for thirty minutes. The suspension was then cooled to 0°C in an ice bath before a solution of $NaNO_2$ (57 mg, 0.8 mmol, 1.5 eq.) in a minimal amount of distilled water was added dropwise. The resultant mixture was stirred at 0°C for 30 minutes, then warmed to room temperature and stirred an additional 5 hours. Upon completion, the reaction mixture was diluted with CH_2Cl_2 and the organic layer was separated. The aqueous layer was extracted with dichloromethane (x6) and the combined organic layers were dried over sodium sulfate and concentrated to yield crude solid which was purified by flash silica chromatography (1:1 ethyl acetate: hexanes) to afford 36 mg (35%) of **17** as a white solid.

*Intermediate **16a***

^{1}H NMR (500 MHz, d-DMSO) δ 8.17 (br s, 1H), 7.97 (s, 1H), 7.56 (s, 2H), 3.36 (d, J = 4.5 Hz, 3H), 2.73 (s, 3H).

K-TMZ (17)

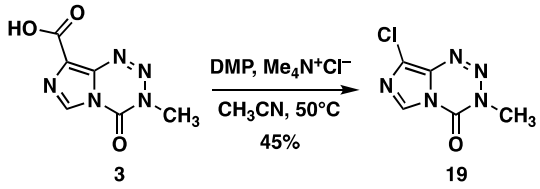
^{1}H NMR (500 MHz, d-DMSO) δ 8.86 (s, 1H), 3.90 (s, 3H), 2.68 (s, 3H). **^{13}C NMR** (125 MHz, d-DMSO) δ 191.47, 139.01, 135.56, 133.35, 129.11, 36.43, 28.31. **HRMS** (ESI) calc. for $C_7H_8N_5O_2$, $[M+H]^+$: 194.0678, found: 194.0683.



8-bromo-3-methylimidazo[5,1-d][1,2,3,5]tetrazin-4(3*H*)-one (18)

Procedure: To a stirred suspension of Dess-Martin periodinane (477 mg, 1.12 mmol, 2.2 eq) in anhydrous CH_3CN (2.6 mL, 0.2 M), tetraethylammonium bromide (240 mg, 1.12 mmol, 2.2 eq) was added. Reaction was stirred 5 min at room temperature before **3** (100 mg, 0.51 mmol, 1 eq) was added. The resultant reaction mixture was heated at 50°C for 2 h. Upon completion, the solvent was concentrated under reduced pressure to give the crude product that was purified by flash silica gel chromatography (9:1 hexanes: ethyl acetate) to afford 73 mg (58%) of **18** as a white solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.37 (s, 1*H*), 3.98 (s, 3*H*). **^{13}C NMR** (125 MHz, $CDCl_3$) δ 138.74, 133.32, 128.56, 117.16, 36.43. **HRMS** (ESI) calc. for $C_5H_5N_5OBr$, $[M+H]^+$: 229.9677, found: 229.9684.

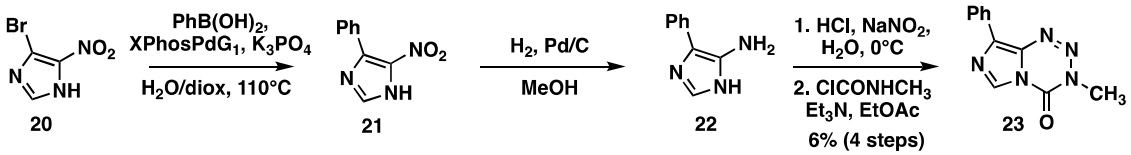


8-chloro-3-methylimidazo[5,1-d][1,2,3,5]tetrazin-4(3*H*)-one (19)

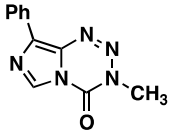
Procedure: To a stirred suspension of Dess-Martin periodinane (477 mg, 1,12 mmol, 2.2 eq.) in anhydrous CH_3CN (2.6 mL, 0.2 M), tetramethylammonium chloride (123 mg, 1.12 mmol, 2.2 eq.) was added. The reaction was stirred 5 min at room temperature before **3** (100 mg, 0.51 mmol, 1 eq.) was added. The resultant reaction mixture was heated at 50°C for 2 hours. Upon completion, the solvent was concentrated under reduced pressure to give the crude product that was purified by flash silica chromatography (9:1 hexanes: ethyl acetate) to afford 43 mg (45%) of **19** as a white solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.33 (s, 1*H*), 3.98 (s, 3*H*). **^{13}C NMR** (125 MHz, $CDCl_3$) δ 138.74, 130.84, 129.81, 127.25, 36.37. **HRMS** (ESI) calc. for $C_5H_5N_5OCl$, $[M+H]^+$: 186.0183, found: 186.0186.

General Scheme for Preparation of Aryl Derivatives 23–26



General Procedure for Preparation of 23-26: (a) *Suzuki Coupling:* A mixture of 4-bromo-5-nitro-1H-imidazole **20** (400 mg, 2.08 mmol,1 eq.), phenyl boronic acid (507 mg, 4.17 mmol, 2 eq.), XPhosPdG$_1$ (164 mg, 0.2 mmol, 0.1 eq.) and K_3PO_4 (1.32 g, 6.24 mmol, 3 eq.) under nitrogen was suspended in degassed 1:1 H_2O: dioxane (16 mL, 0.13 M). The resulting mixture was stirred at 110°C for 16 h. The reaction was cooled to room temperature and H_2O was added. The aqueous layer was extracted x3 with ethyl acetate and the combined organic layers were dried over Na_2SO_4 and concentrated. The residue obtained was purified by flash silica gel chromatography (100% ethyl acetate) to afford crude product **21** that was used for next step without further purification.
(b) *Nitro Reduction:* Crude **21** was dissolved in dry MeOH (10 mL, 0.2 M) containing 10% Pd/C before H_2 (1 atm) was introduced. The reaction was stirred for 16 h at room temperature before the catalyst was filtered over Celite. The filtrate was concentrated under reduced pressure and purified by flash silica gel chromatography (95:5 DCM: MeOH) providing compound **22** that was used for next step without further purification.
(c) *Cyclization:* To a suspension of intermediate **22** in 1 M HCl (2.9 mL, 0.7 M) at 0°C was added a pre-formed solution of $NaNO_2$ (186 mg, 2.7 mmol, 1.3 eq.) in H_2O (2.9 mL, 0.9 M) dropwise. The resultant mixture was stirred at 0°C in the dark for 30 min. Upon completion, the solvent was evaporated and the crude diazo compound was dissolved in ethyl acetate (9.6 mL, 0.2 M) before triethylamine (544 µL, 4.6 mmol, 2 eq.) and methylcarbamic chloride (1010 mg, 10.8 mmol, 5.2 eq.) were added. The reaction mixture was stirred at room temperature for 16 h protected from light. Upon reaction completion, the solvent was concentrated under reduced pressure and the residue was purified by flash silica gel chromatography (9:1 hexanes: ethyl acetate) to afford 28 mg (6%) of pure **23** as a white solid.

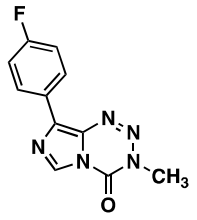


23

3-methyl-8-phenylimidazo[5,1-d][1,2,3,5]tetrazin-4(3*H*)-one (23)

Product was obtained using general procedure. White solid, 6% yield (4 steps).

^{1}H NMR (500 MHz, *d*-DMSO) δ 8.84 (s, 1*H*), 8.31-8.29 (m, 2*H*), 7.57-7.53 (m, 2*H*), 7.44 (tt, *J* = 7.4, 1.3 Hz, 1*H*), 3.85 (s, 3*H*). **^{13}C NMR** (125 MHz, $CDCl_3$) δ 140.02, 137.02, 132.30, 131.88, 129.86, 129.48, 129.43, 127.07, 36.29. **HRMS** (ESI) calc. for $C_{11}H_{10}N_5O$, $[M+H]^+$: 228.0885, found: 228.0878.

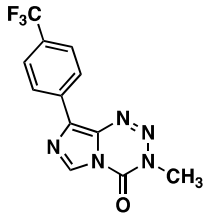


24

8-(4-fluorophenyl)-3-methylimidazo[5,1-d][1,2,3,5]tetrazin-4(3*H*)-one (24)

Product was obtain using general procedure. Yellow solid, 3% yield (4 steps).

^{1}H NMR (500 MHz, *$CDCl_3$)* δ 8.46 (s, 1*H*), 8.44-8.40 (m, 2*H*), 7.24-7.19 (m, 2*H*), 4.01 (s, 3*H*). **^{13}C NMR** (125 MHz, *$CDCl_3$)* δ 164.57, 162.58, 139.20 (d, *J* = 86.4 Hz, 1C), 131.00, 129.43 (d, *J* = 8.3 Hz, 1C), 128.56, 127.26 (d, *J* = 3.3 Hz, 1C), 116.00 (d, *J* = 21.6 Hz, 1C), 35.97. **HRMS** (ESI) calc. for $C_{11}H_9FN_5O$, $[M+H]^+$: 246.0791, found: 246.0788.

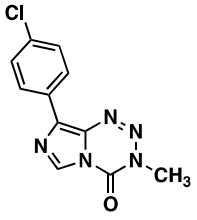


25

3-methyl-8-(4-(trifluoromethyl)phenyl)imidazo[5,1-d][1,2,3,5]tetrazin-4(3*H*)-one (25)

Product was obtained using the general procedure. Yellow solid, 5% yield (4 steps).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.52 (d, *J* = 8.2 Hz, 2*H*), 8.48 (s, 1*H*), 7.75 (d, *J* = 8.2 Hz, 2*H*), 4.02 (s, 3*H*). **^{13}C NMR** (125 MHz, $CDCl_3$) δ 139.34, 137.89, 134.30, 131.91, 131.03 (q, *J* = 32.3 Hz, 1C), 128.77, 127.61, 125.80 (q, *J* = 3.8 Hz, 1C), 122.96, 36.15. **HRMS** (ESI) calc. for $C_{12}H_9N_5OF_3$, $[M+H]^+$: 296.0759, found: 296.0754.

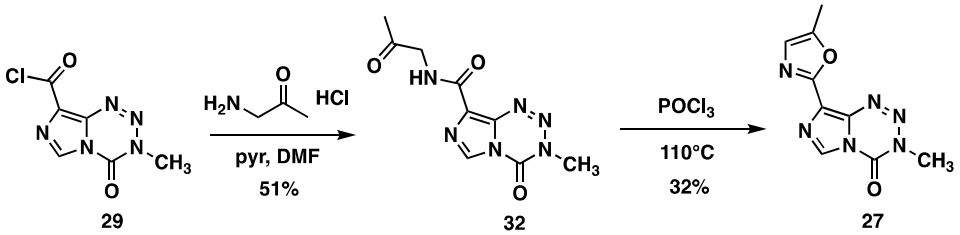


26

8-(4-chlorophenyl)-3-methylimidazo[5,1-d][1,2,3,5]tetrazin-4(3*H*)-one (26)

Product was obtained using the general procedure. Yellow solid, 1.2% yield (4 steps).

^{1}H NMR (500 MHz, *d*-DMSO) δ 8.86 (s, 1*H*), 8.30 (dt, *J* = 9.25, 2.5 Hz, 2*H*), 7.62 (dt, *J* = 9.25, 2.5 Hz, 2*H*), 3.86 (s, 3*H*). **^{13}C NMR** (125 MHz, *d*-DMSO) δ 139.92, 135.67, 133.97, 132.45, 130.75, 130.00, 129.63, 128.62, 36.37. **HRMS** (ESI) calc. for $C_{11}H_9N_5OCl$, $[M+H]^+$: 262.0496, found: 262.0489.



The route to 4-substituted oxazol-2-yls at the C8 position of imidazotetrazines is known,[11] however, the synthesis of compound **27** via intermediate **32** had never been reported.

3-methyl-4-oxo-N-(2-oxopropyl)-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide (32)

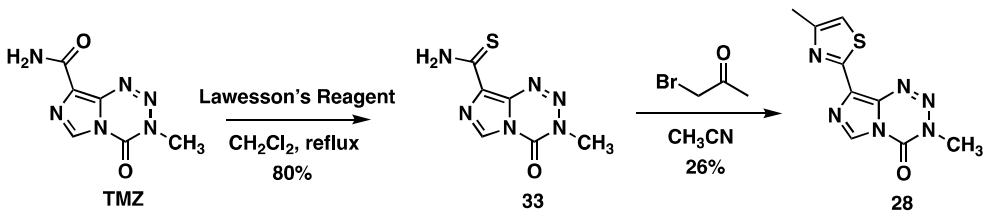
Procedure: To **29** (447 mg, 2.09 mmol, 1 eq.) and 2-aminoacetophenone hydrochloride (229 mg, 2.09 mmol, 1 eq.) was added DMF (4.4 mL, 0.47 M) and pyridine (0.9 mL). The reaction mixture was stirred for 16 h at room temperature. Water was added and the aqueous layer was extracted x5 with ethyl acetate. The combined organic layers were dried over Na_2SO_4 and concentrated. The residue obtained was purified by flash silica gel chromatography (100% ethyl acetate) to afford 266 mg (51%) of **32** as an orange solid.

^{1}H NMR (500 MHz, *d*-DMSO) δ 8.87 (s, 1*H*), 8.59 (t, *J* = 5.7 Hz, 1*H*), 4.17 (d, *J* = 5.7 Hz, 2*H*), 3.88 (s, 3*H*), 2.15 (s, 3*H*). **^{13}C NMR** (125 MHz, *d*-DMSO) δ 204.55, 160.15, 139.65, 135.10, 130.18, 129.09, 49.57, 36.67, 27.52. **LC-MS** (ESI) calc. for $C_9H_{11}N_6O_3$ $[M+H]^+$: 251.0893, found: 251.09.

3-methyl-8-(5-methyloxazol-2-yl)imidazo[5,1-d][1,2,3,5]tetrazin-4(3*H*)-one (27, Ox-TMZ)

Procedure: Intermediate **32** (266 mg, 1.06 mmol, 1 eq.) was added to phosphoryl chloride (6.5 mL, 0.16 M) and the stirred mixture was heated at 110°C for 3 h. Upon completion, ice water was added and the aqueous layer was extracted x4 with ethyl acetate. The combined organic layers were dried over Na_2SO_4 and concentrated. The residue obtained was purified by flash silica gel chromatography (100% ethyl acetate) to afford 80 mg (32%) of the product **27** as a yellow solid.

^{1}H NMR (500 MHz, *d*-DMSO) δ 8.89 (s, 1*H*), 7.13 (br d, *J* = 1.2 Hz, 1*H*), 3.87 (s, 3*H*), 2.44 (d, *J* = 1.2 Hz, 3*H*). **^{13}C NMR** (125 MHz, *d*-DMSO) δ 154.15, 150.47, 139.64, 133.64, 130.43, 126.15, 125.36, 36.56, 11.17. **HRMS** (ESI) calc. for $C_9H_9N_6O_2$, $[M+H]^+$: 233.0782, found: 233.0787.



The route to 4-substituted thiazol-2-yls at the C8 position of imidazotetrazines is known,[11] however, the synthesis of compound **28** had never been reported. Experimental data for intermediate **33** has been published.[6]

3-methyl-4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carbothioamide (28)

Procedure: To a solution of **33** (550 mg, 2.6 mmol, 1 eq.) in acetonitrile (40 mL, 0.07 M) was added α-Bromo acetone (220 µL, 2.6mmol, 1 eq.) and the solution was stirred at room temperature for 18 h. Upon completion, the reaction was stopped, and the precipitate was filtered and purified by flash silica gel chromatography (4:6 hexanes: ethyl acetate) to afford 167 mg (26%) of the desired product **28** as a yellow solid.

^{1}H NMR (500 MHz, *d*-DMSO) δ 8.87 (s, 1*H*), 7.46 (d, *J* = 0.9 Hz, 1*H*), 3.86 (s, 3*H*), 2.48 (d, *J* = 0.9 Hz, 3*H*). **^{13}C NMR** (125 MHz, *d*-DMSO) δ 158.63, 154.58, 139.75, 131.84, 131.80, 130.24, 116.56, 36.52, 17.45. **HRMS** (ESI) calc. for $C_9H_9N_6OS$, $[M+H]^+$: 249.0559, found: 249.0559.

10. References

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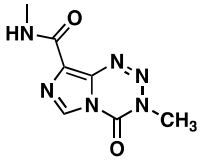
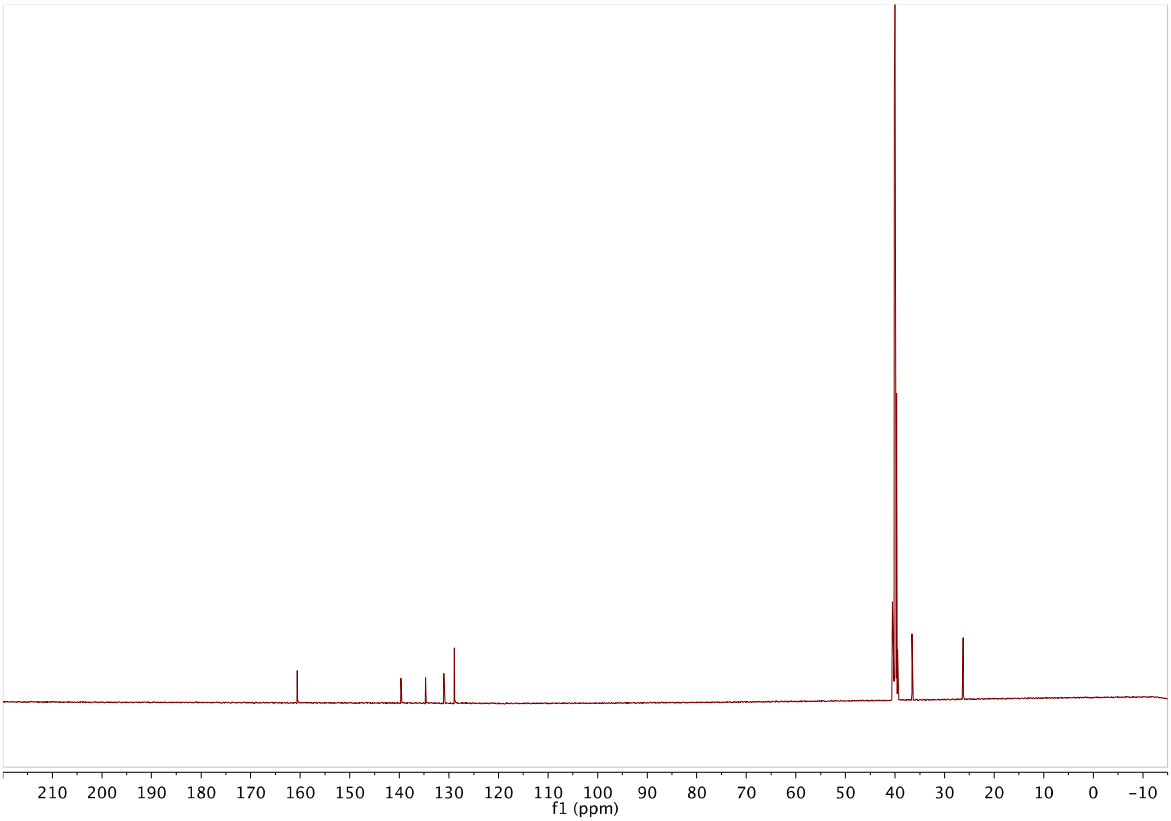
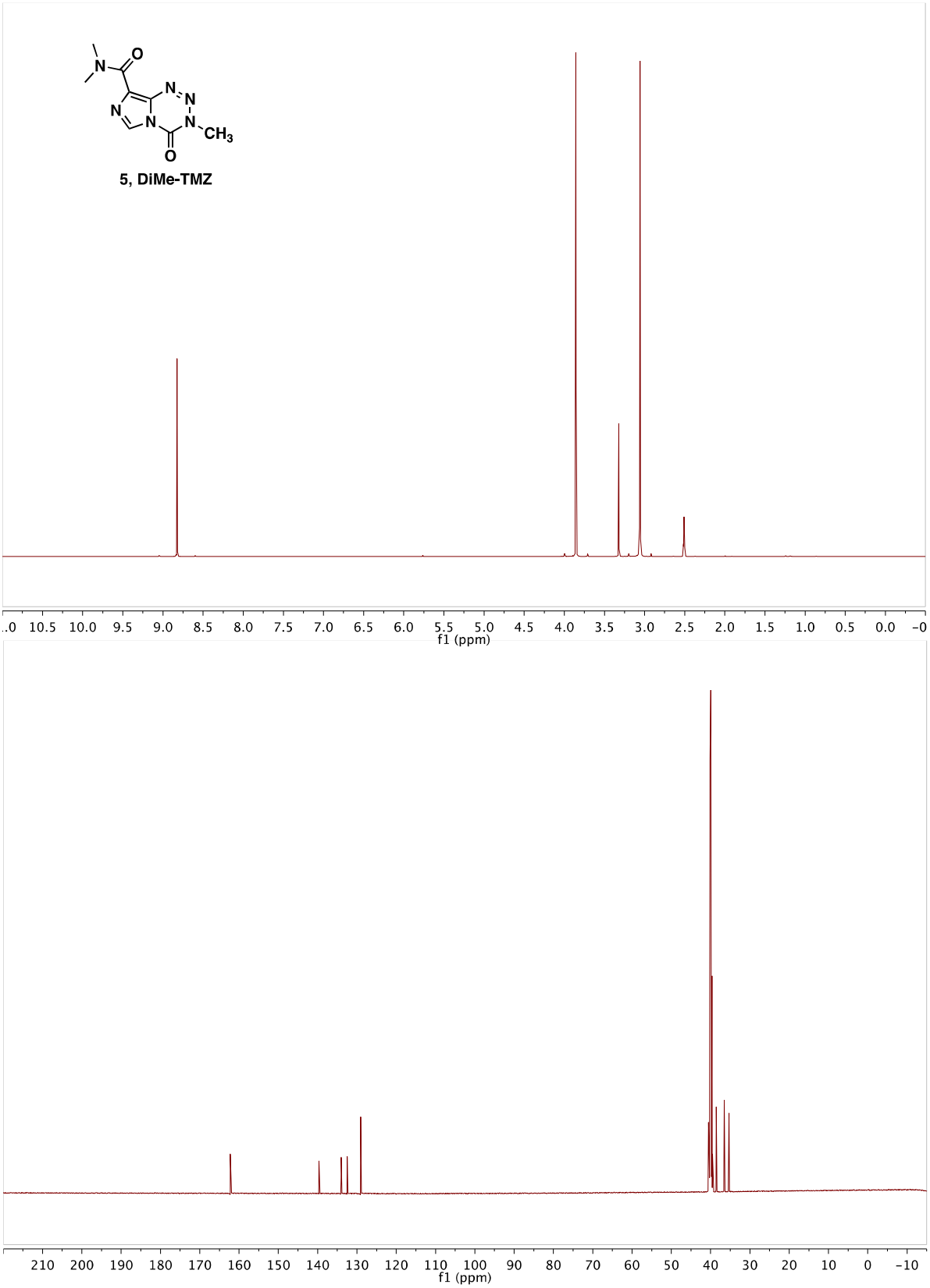
11. ^{1}H and ^{13}C NMR Spectra of C8 Analogs



4, Me-TMZ




5, DiMe-TMZ
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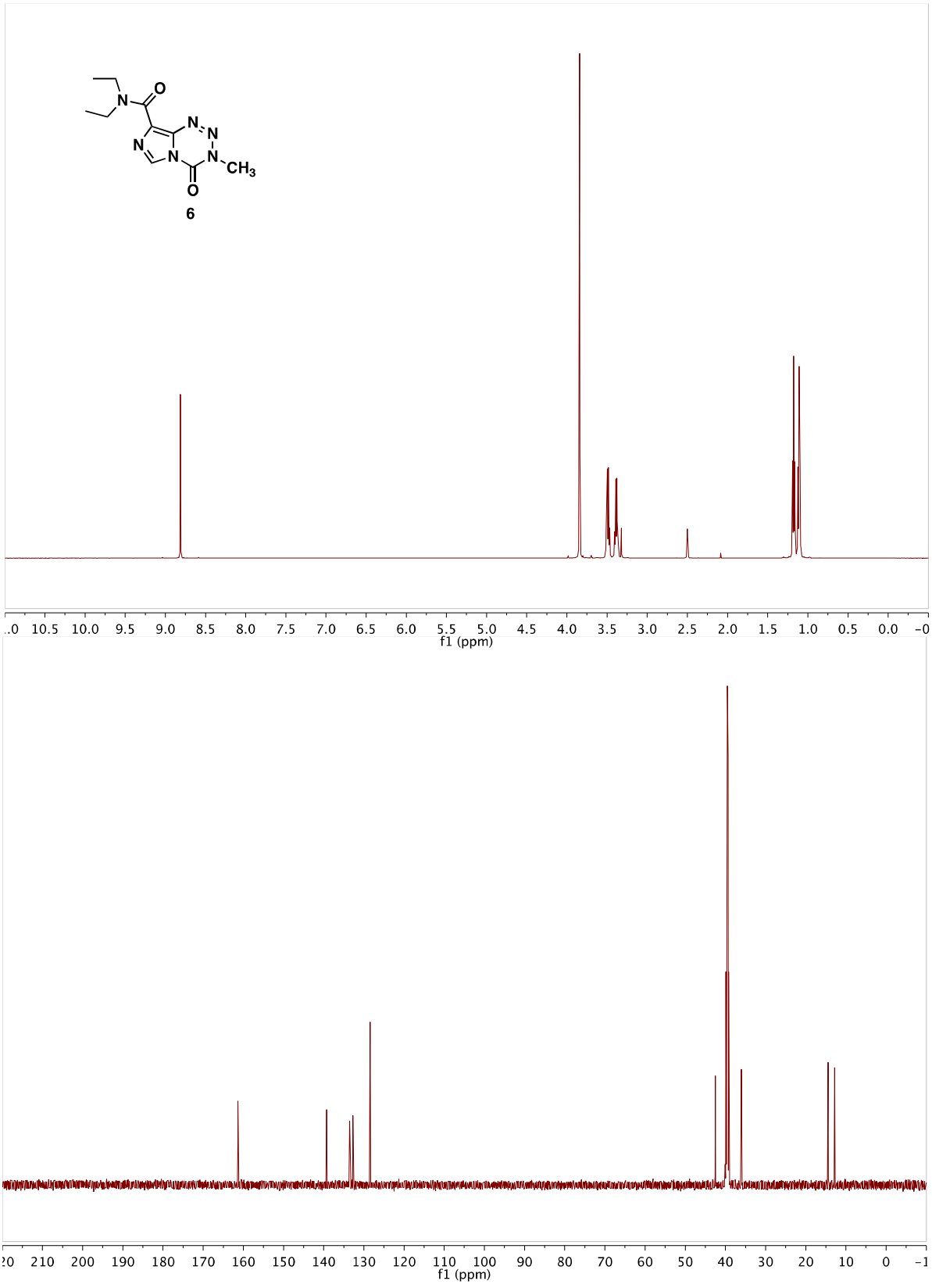
f1 (ppm)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CH3
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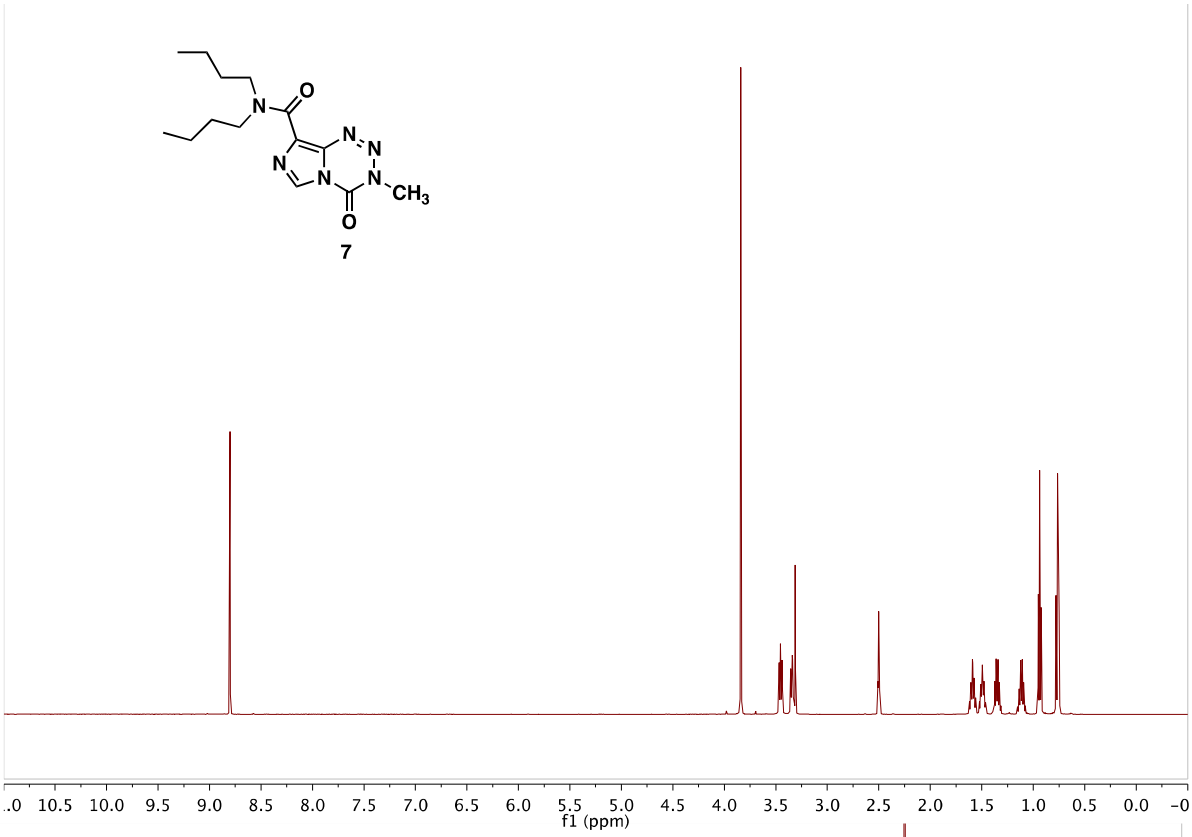
f1 (ppm)
f1 (ppm)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N
N
CH3
O
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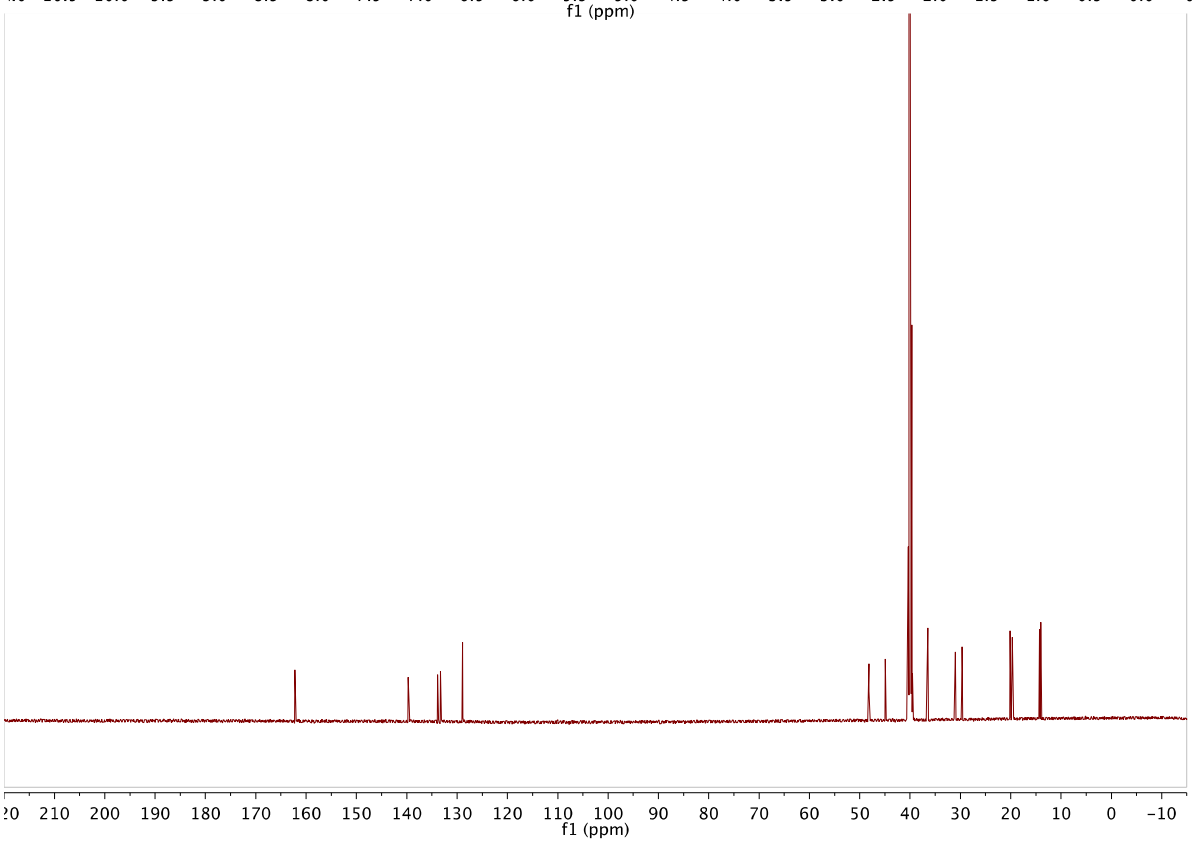
f1 (ppm)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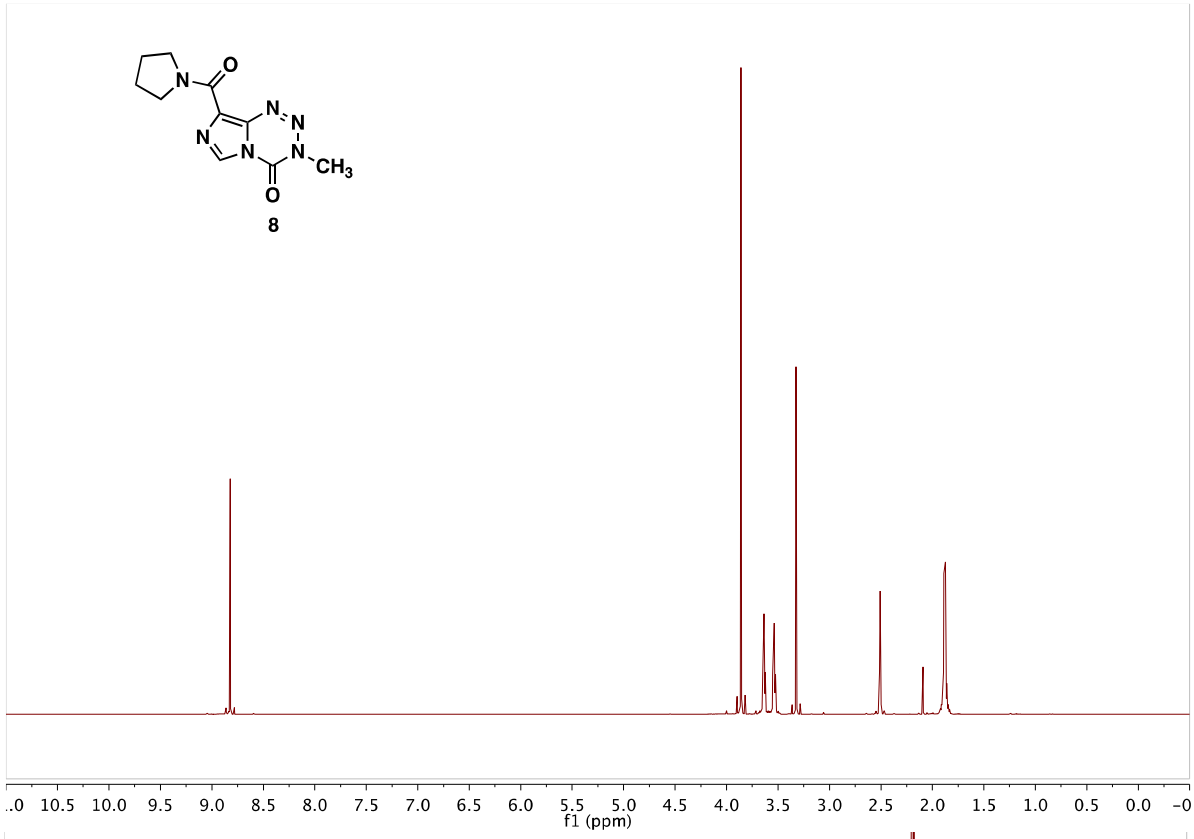
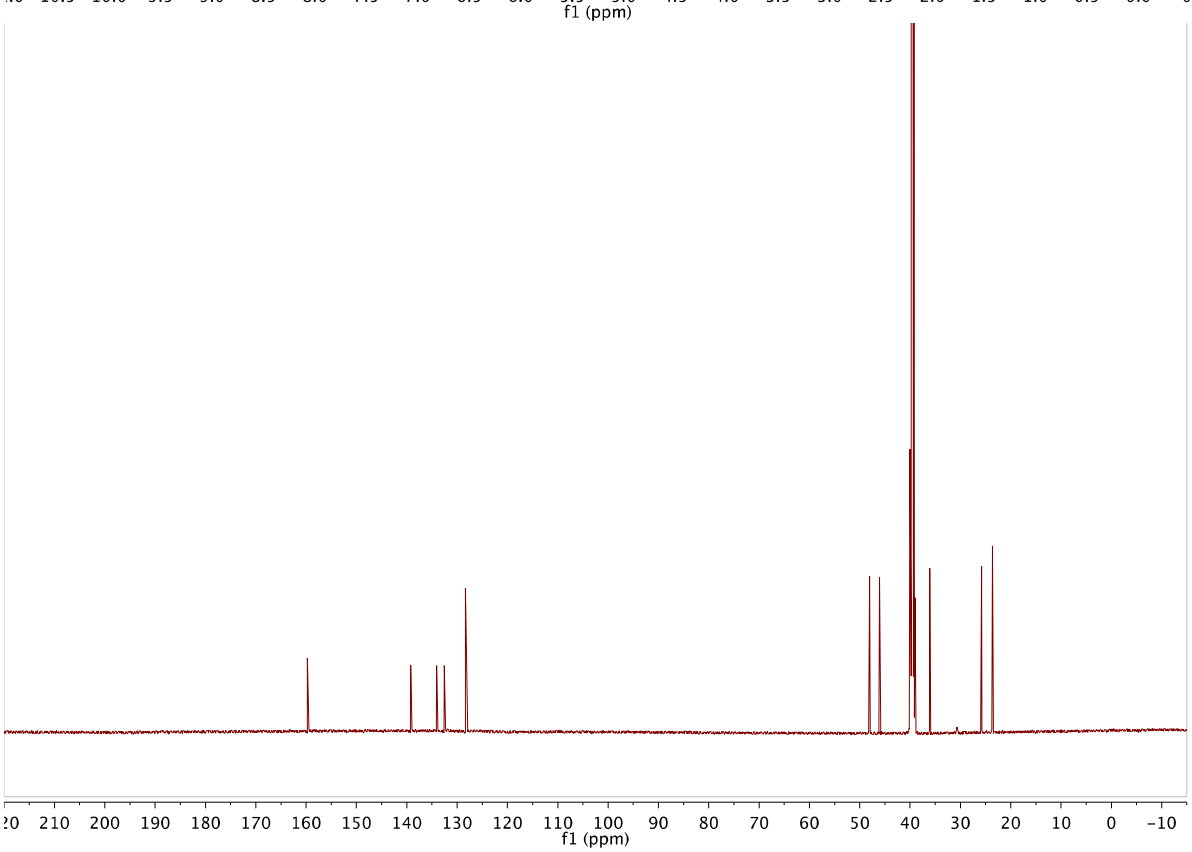
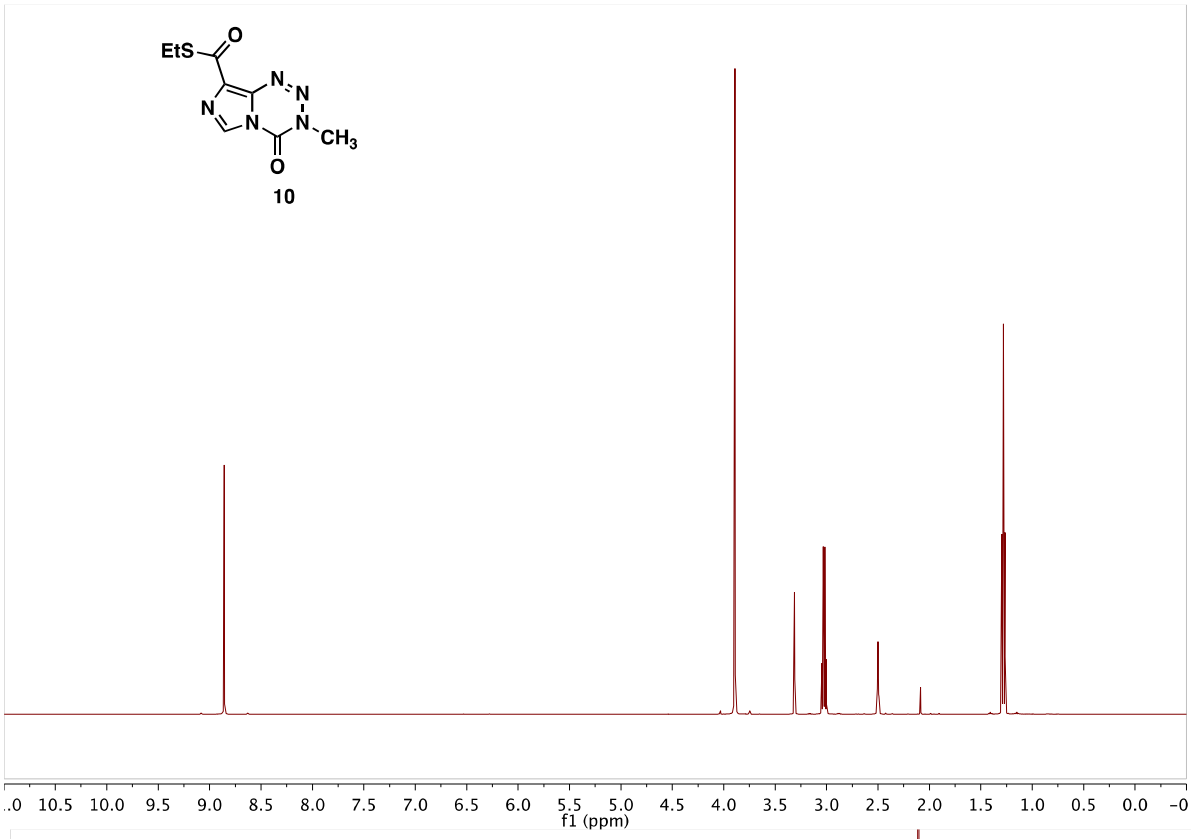


8

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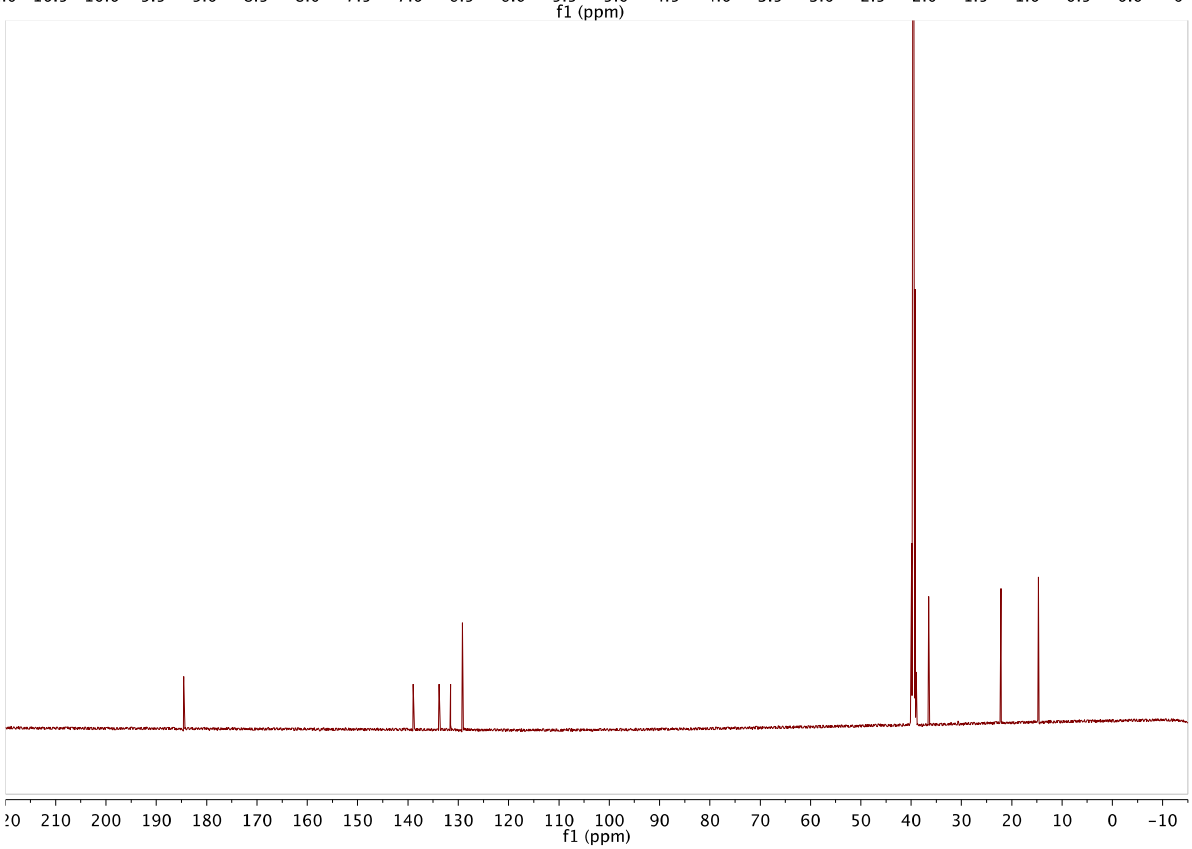
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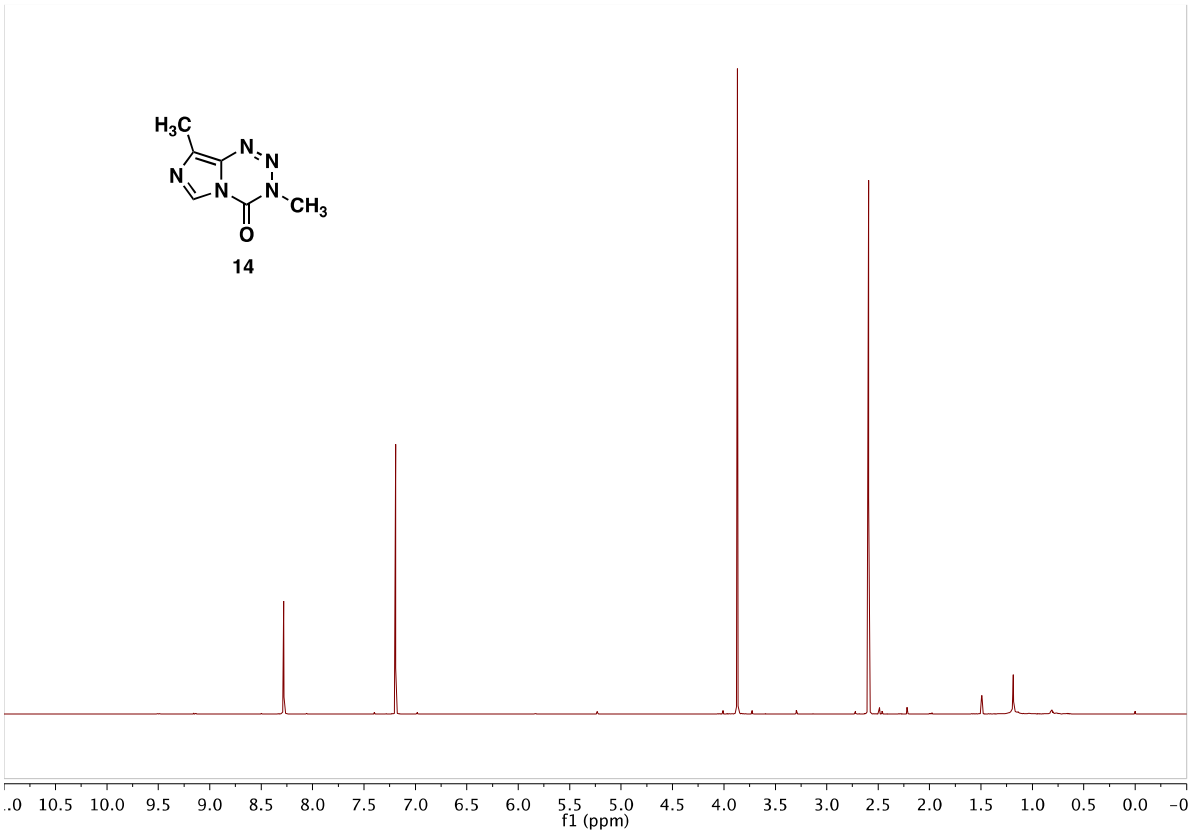
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N
CH3
O
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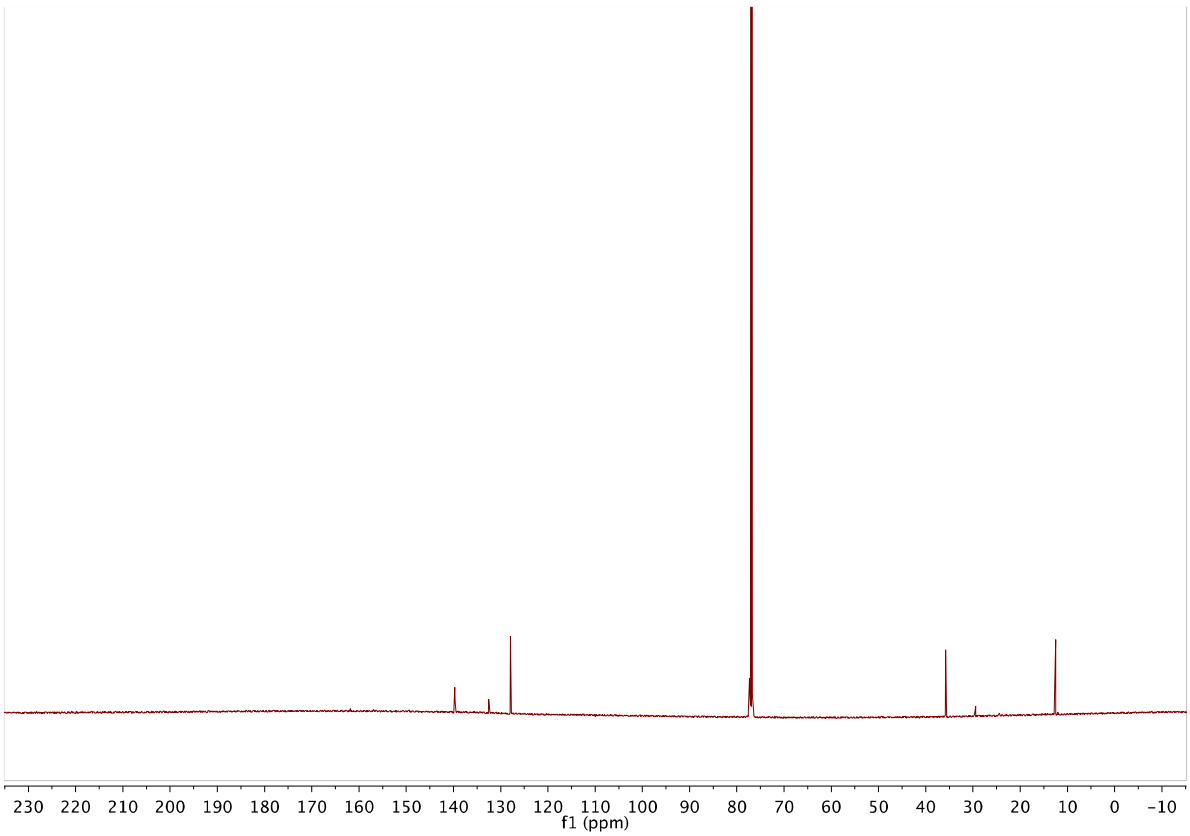
f1 (ppm)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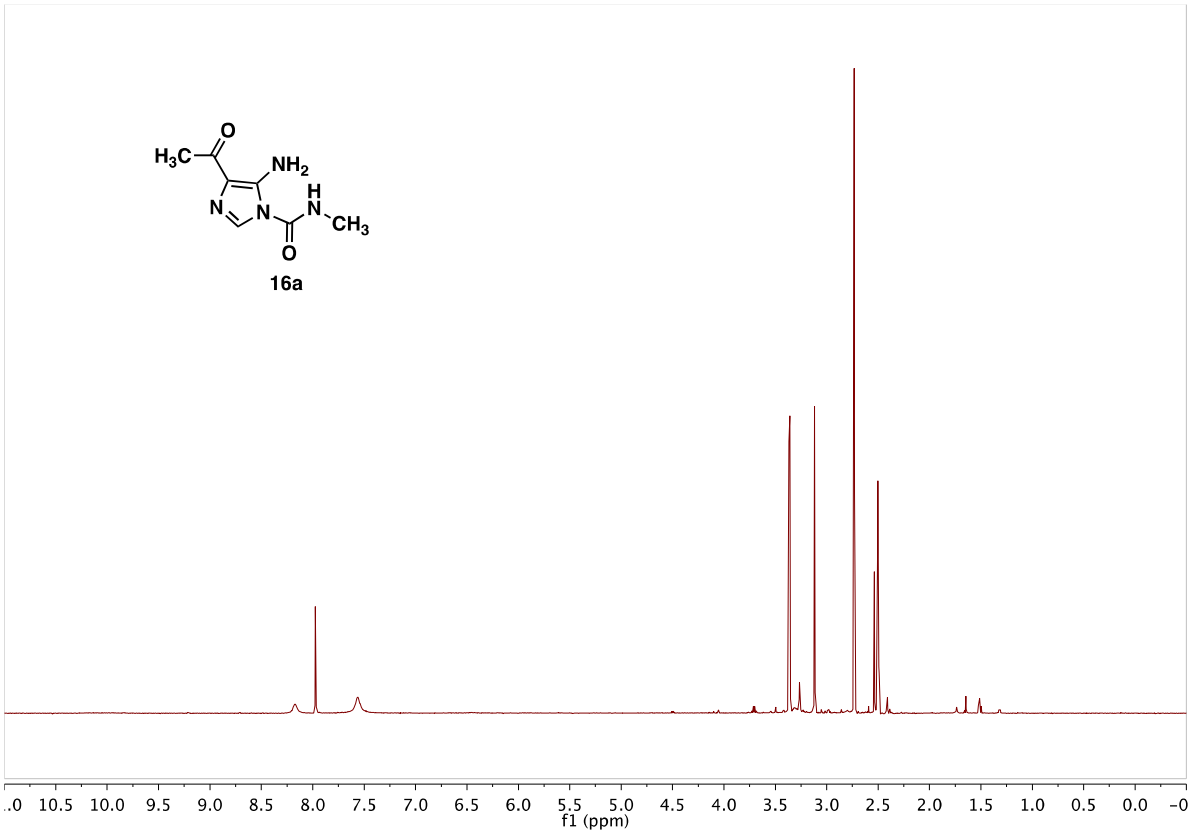
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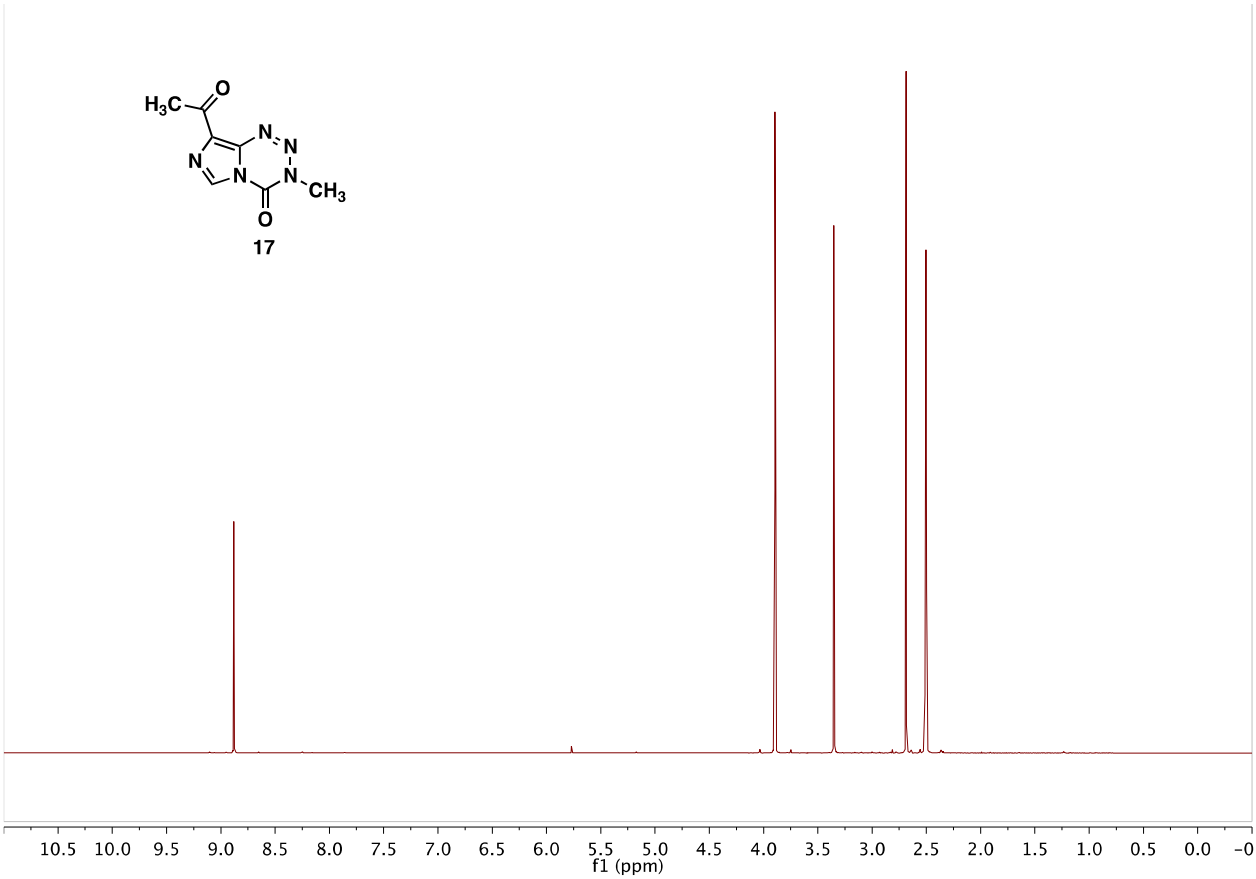
f1 (ppm)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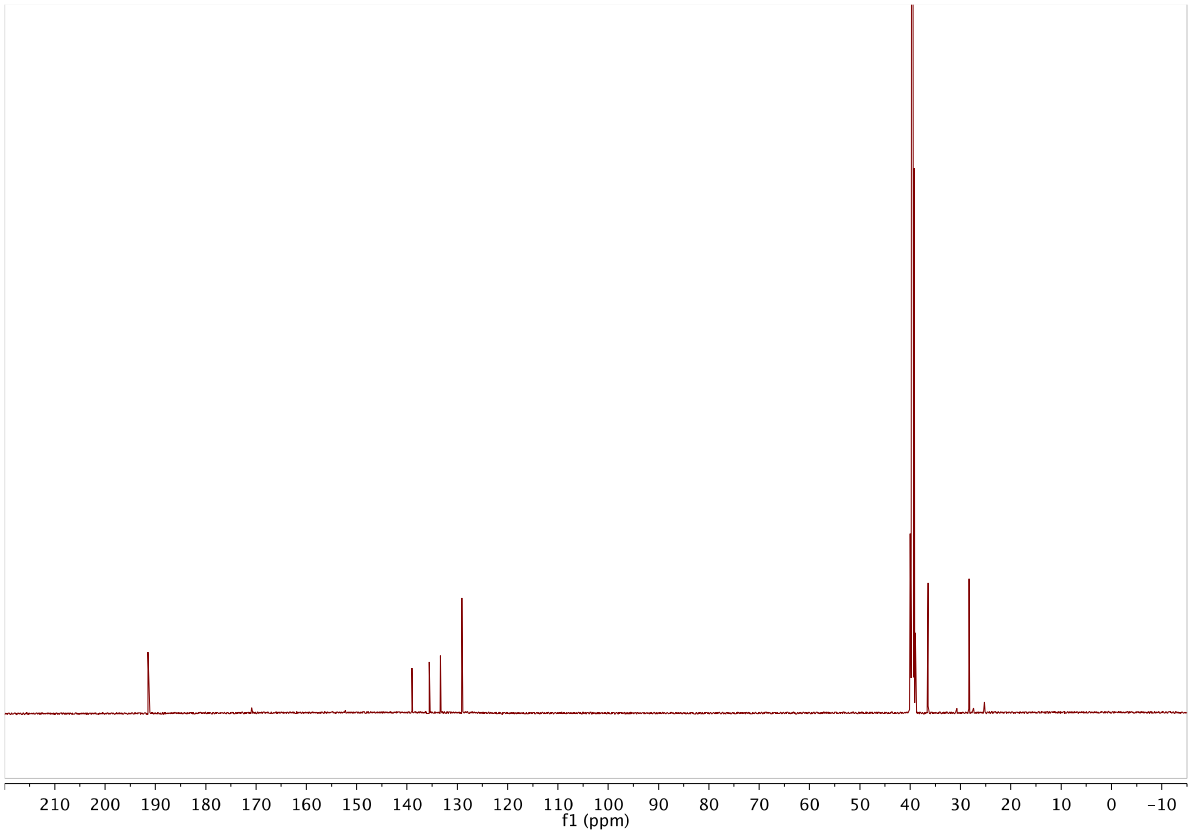
f1 (ppm)

17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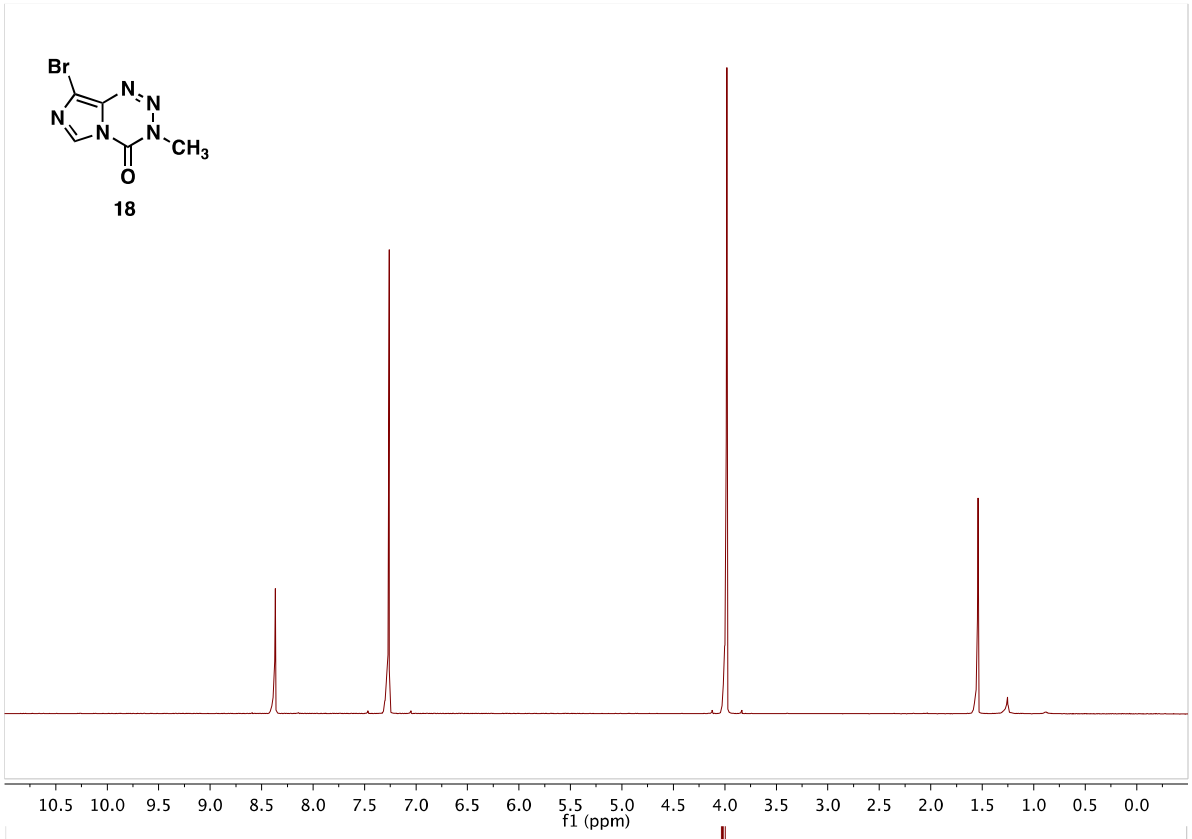
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N
CH3
O
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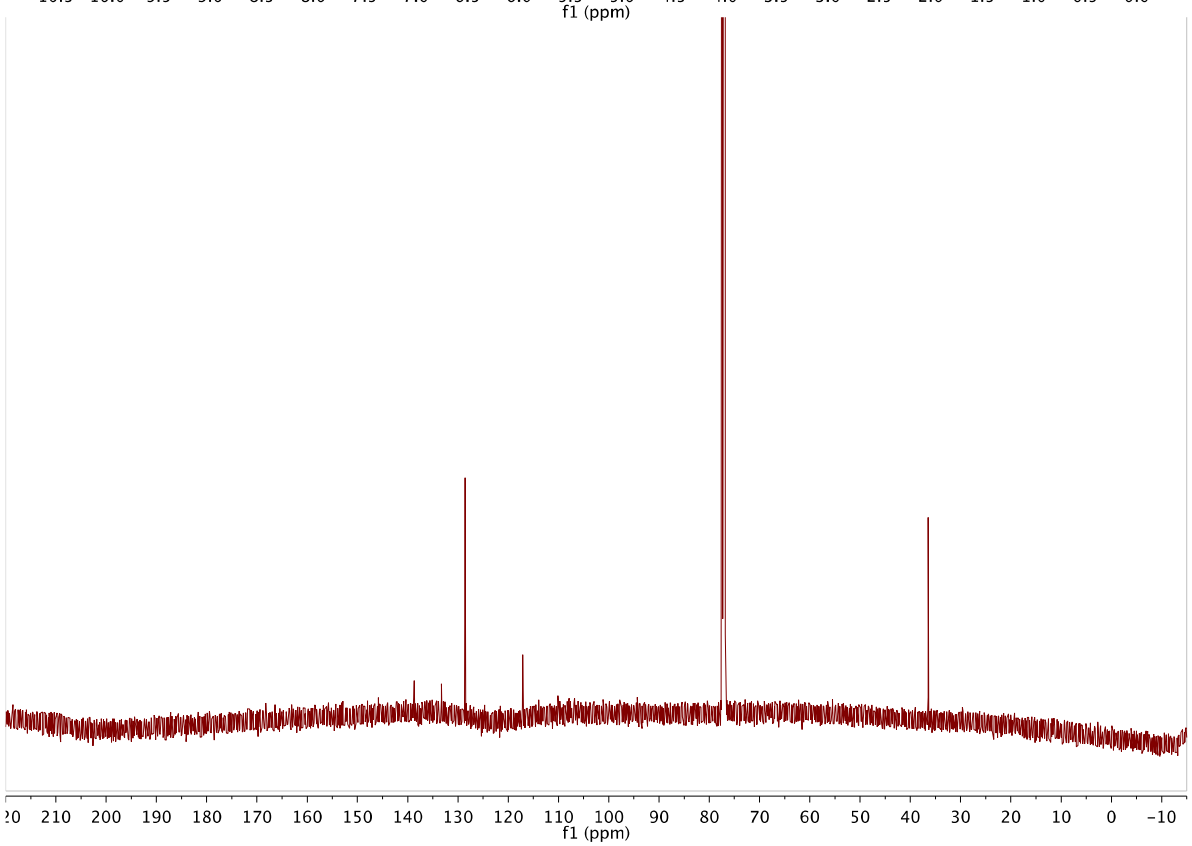
f1 (ppm)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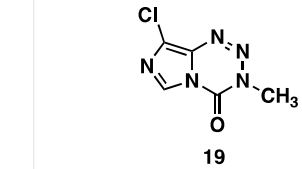
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N
N
N
CH3
O

19

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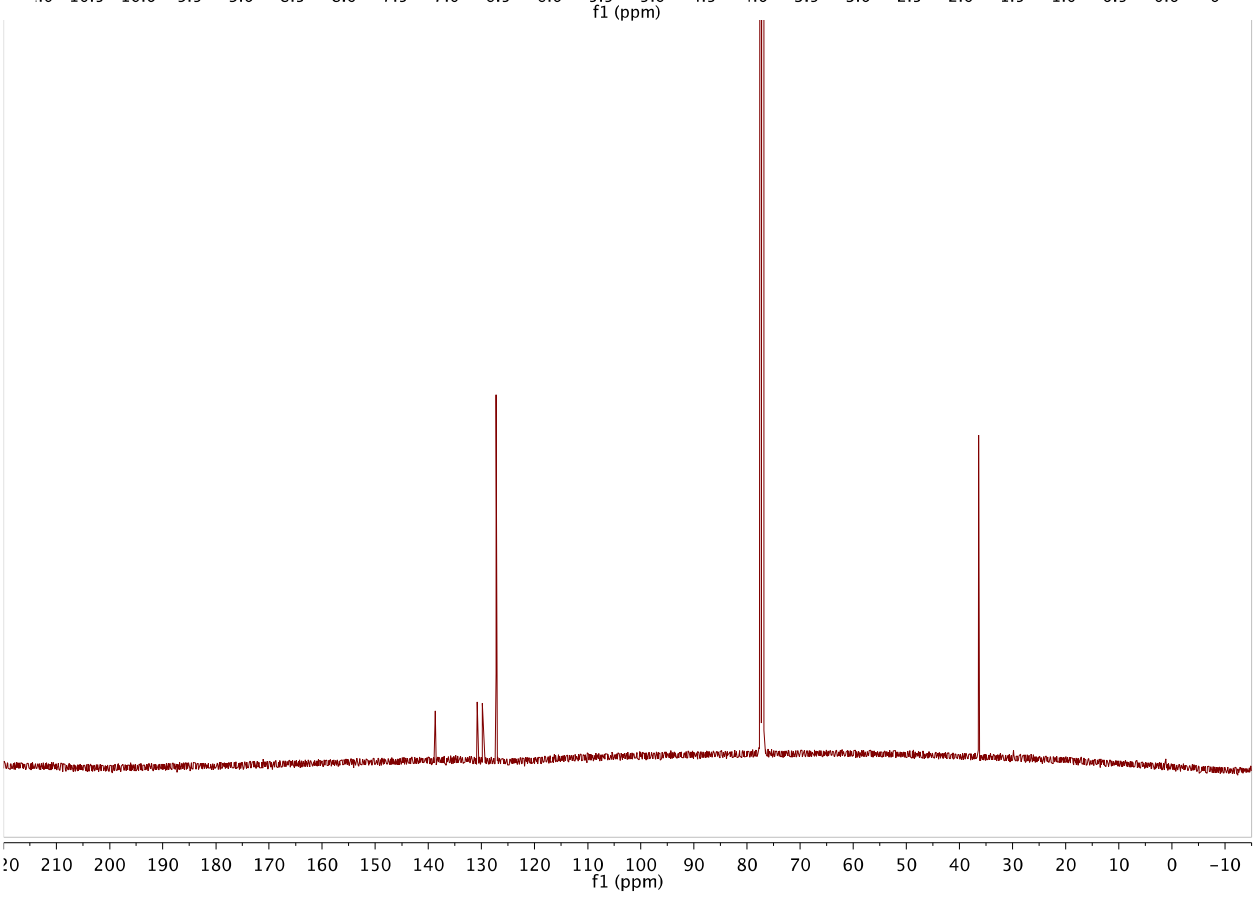
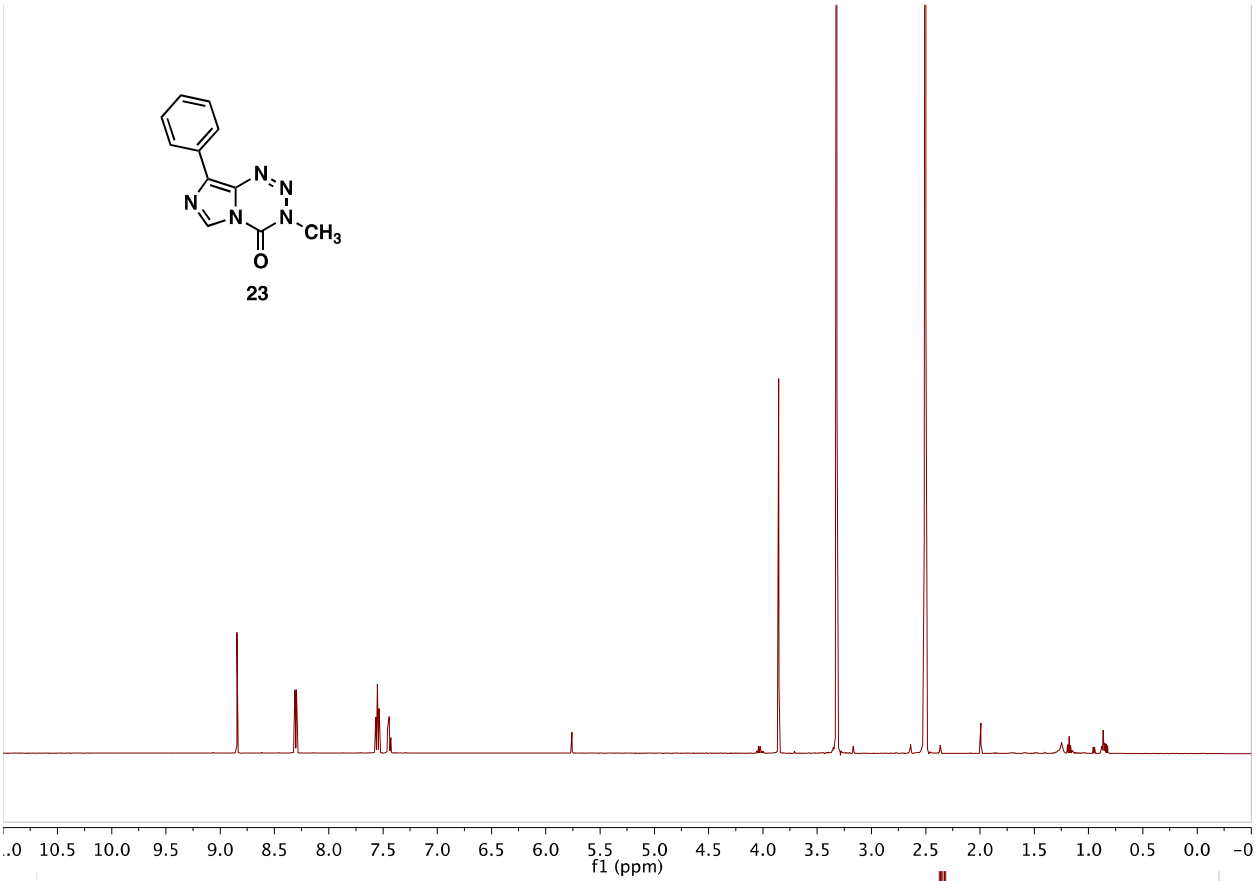
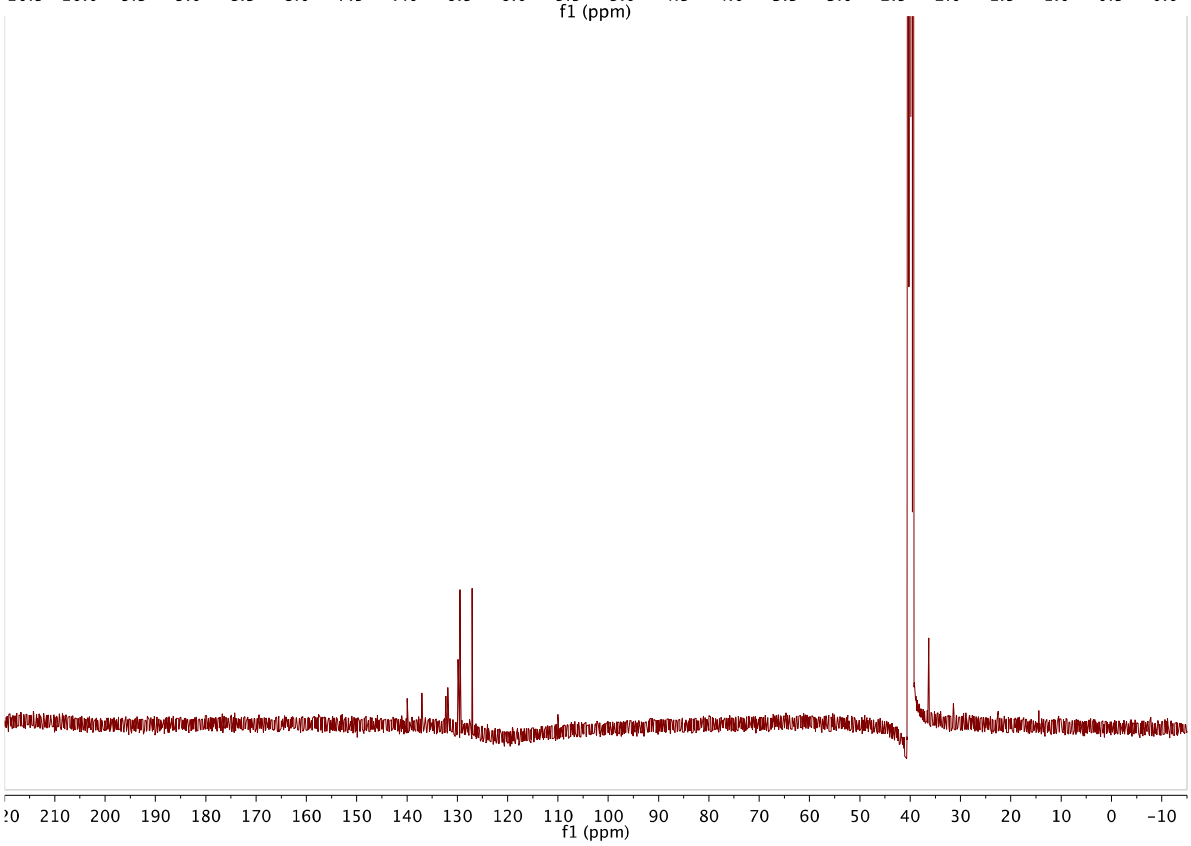
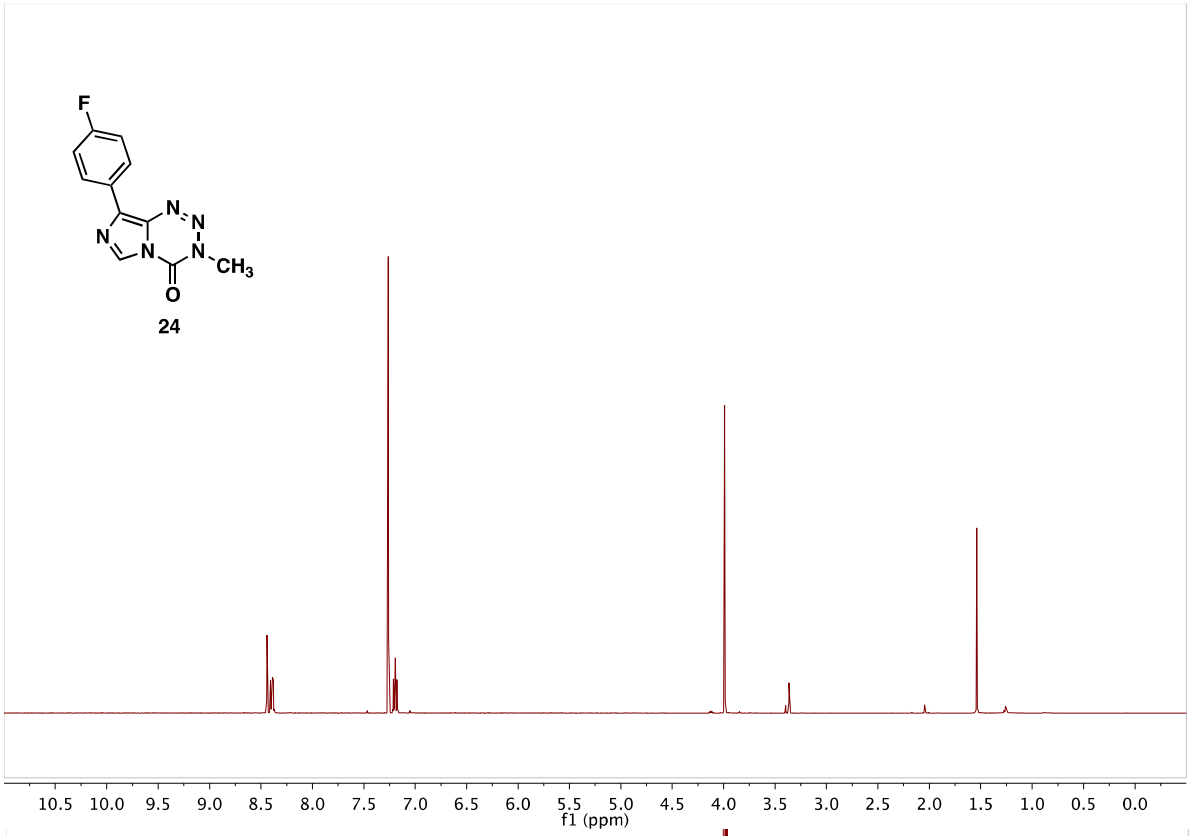
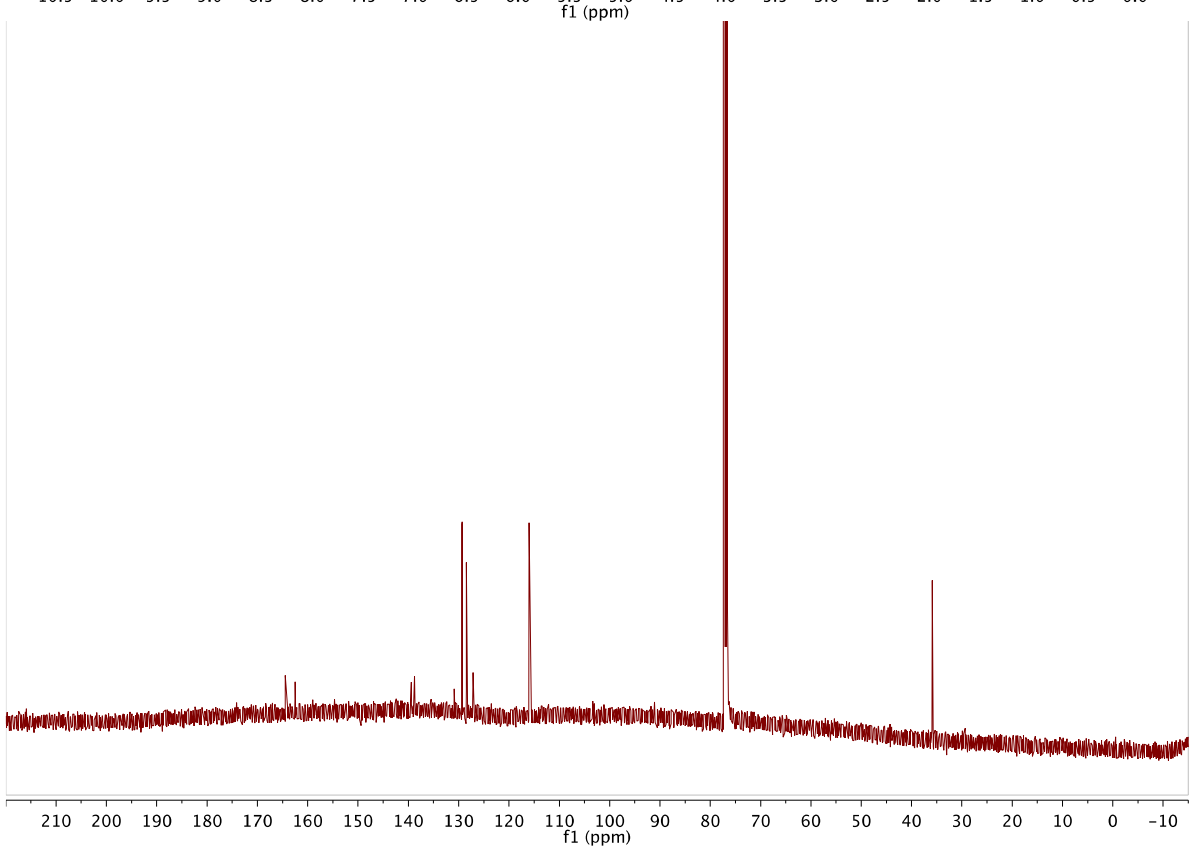

20 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 −10
f1 (ppm)

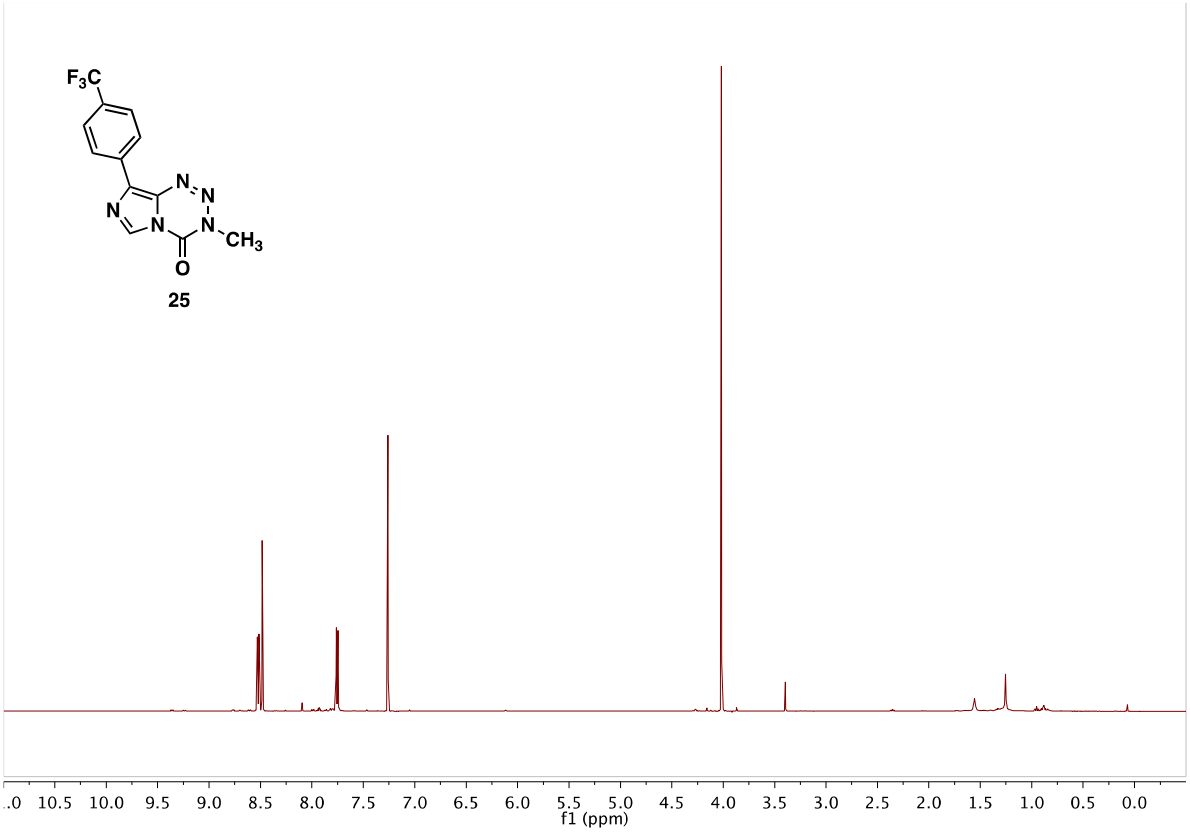

N
N
N
N
N
CH3
O
23
.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0
f1 (ppm)

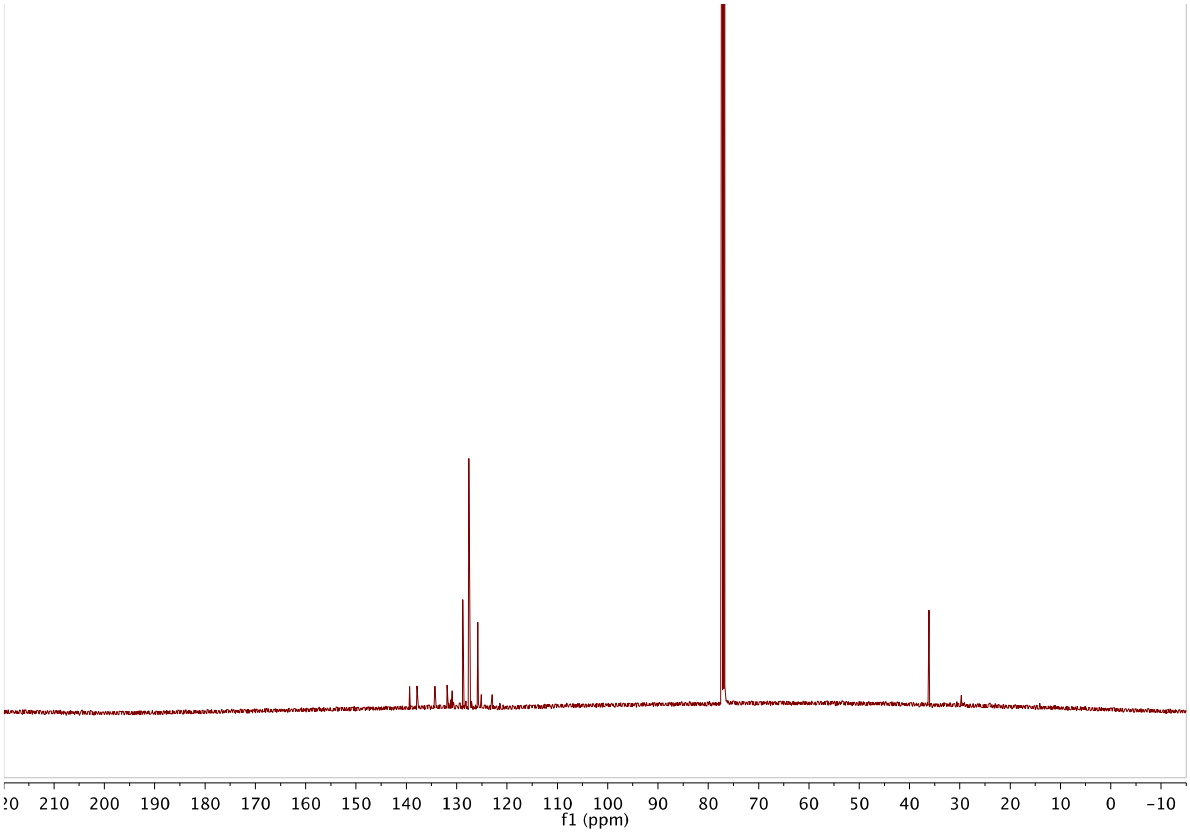

20 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

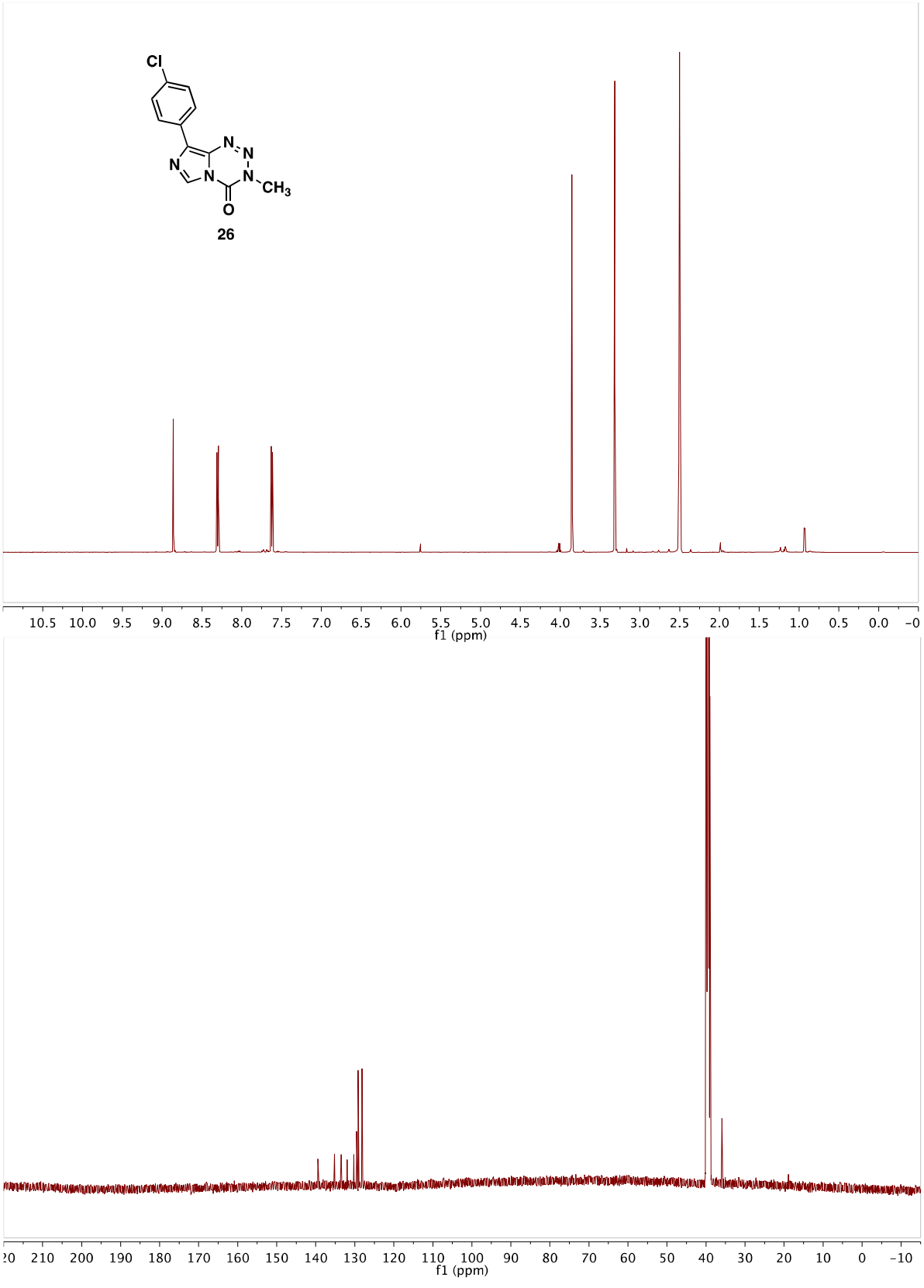

F
N
N
N
N
N
CH3
O
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

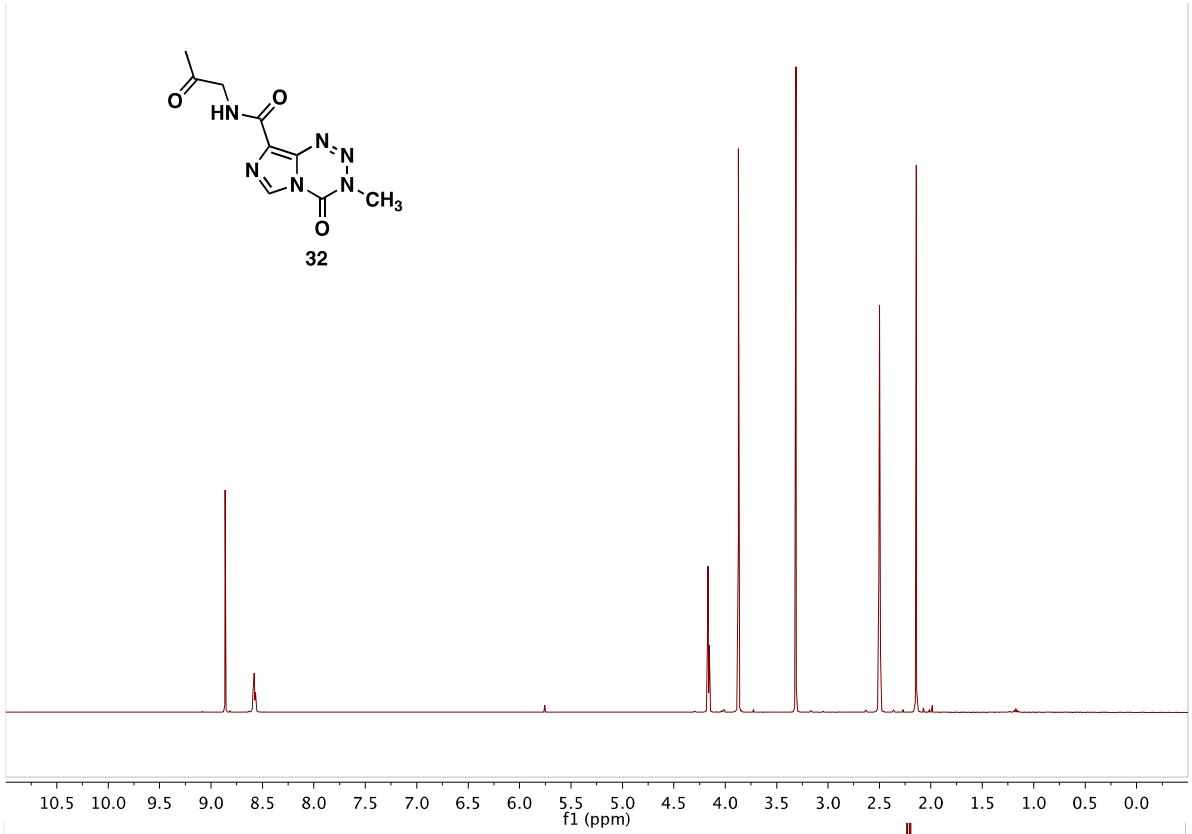
24


210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)


F3C
N
N
N
N
N
CH3
O
25
.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

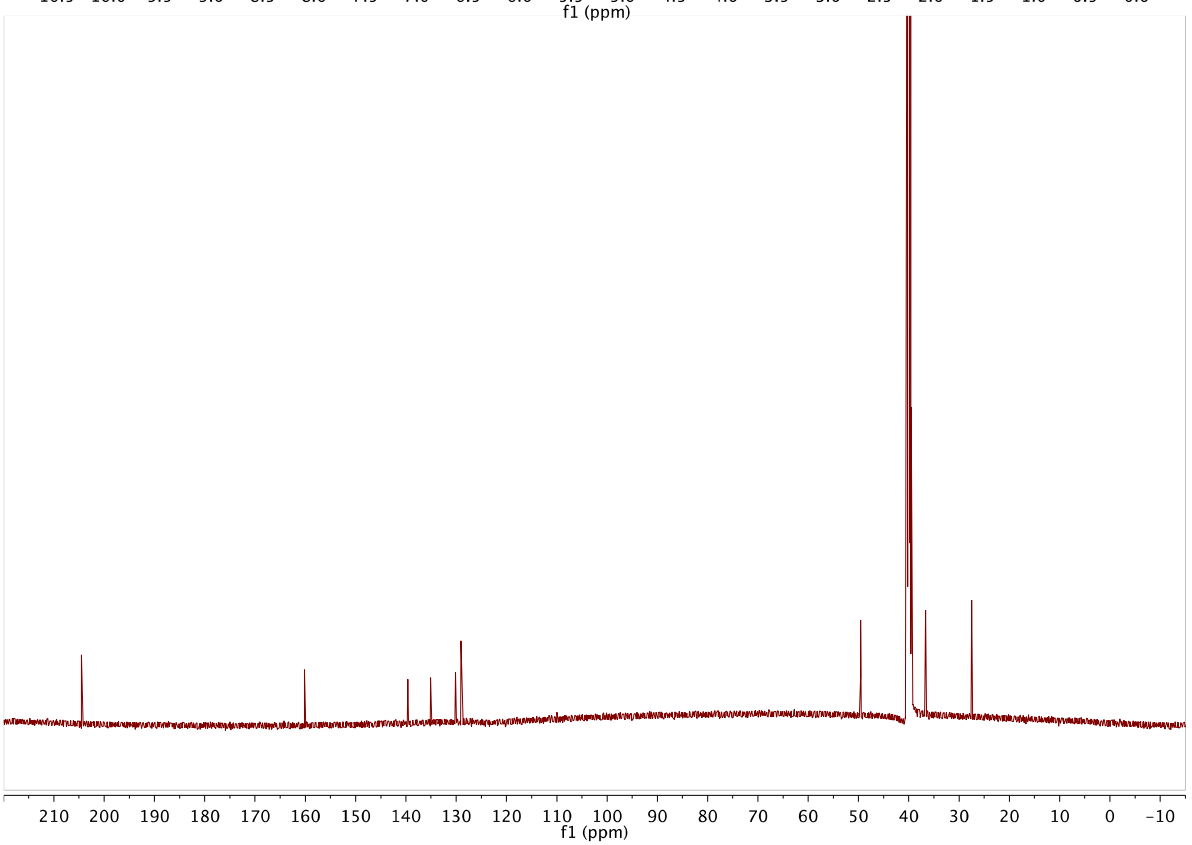

20 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

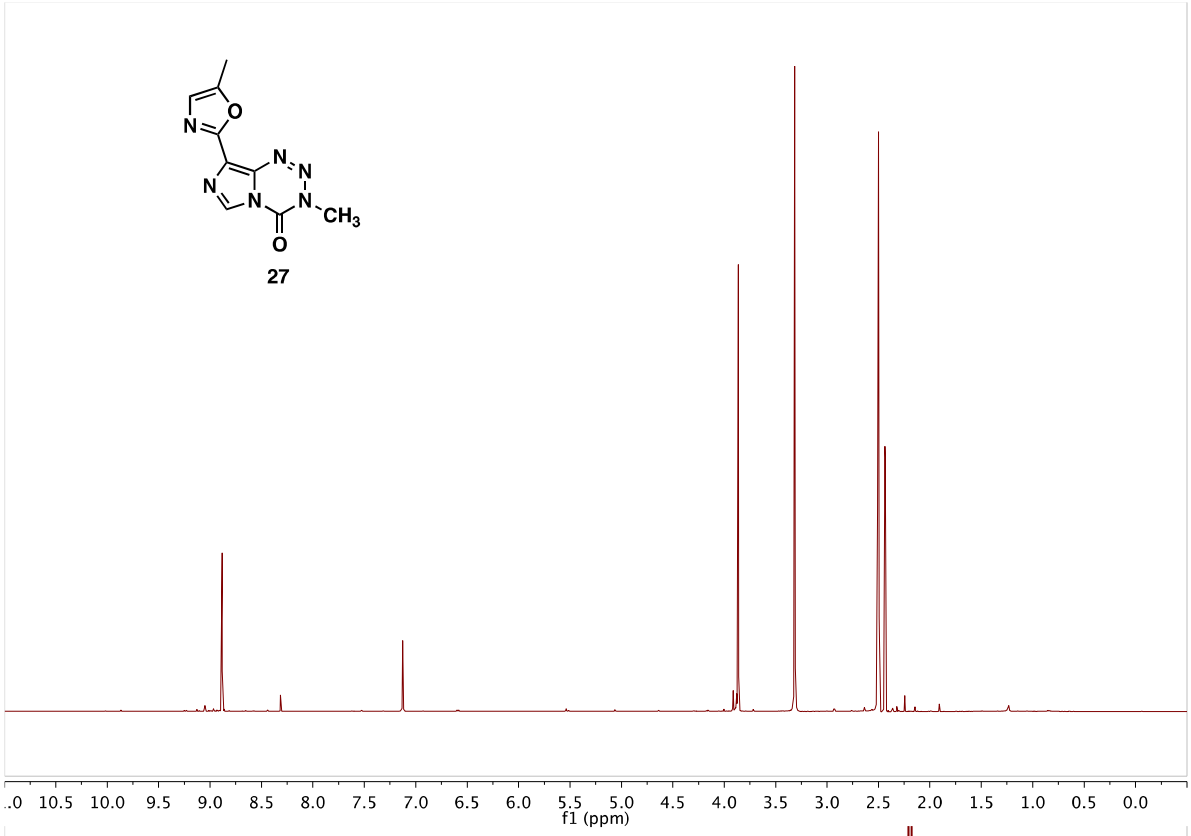

Cl
N
N
N
N
N
CH3
O
26
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0
f1 (ppm)
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

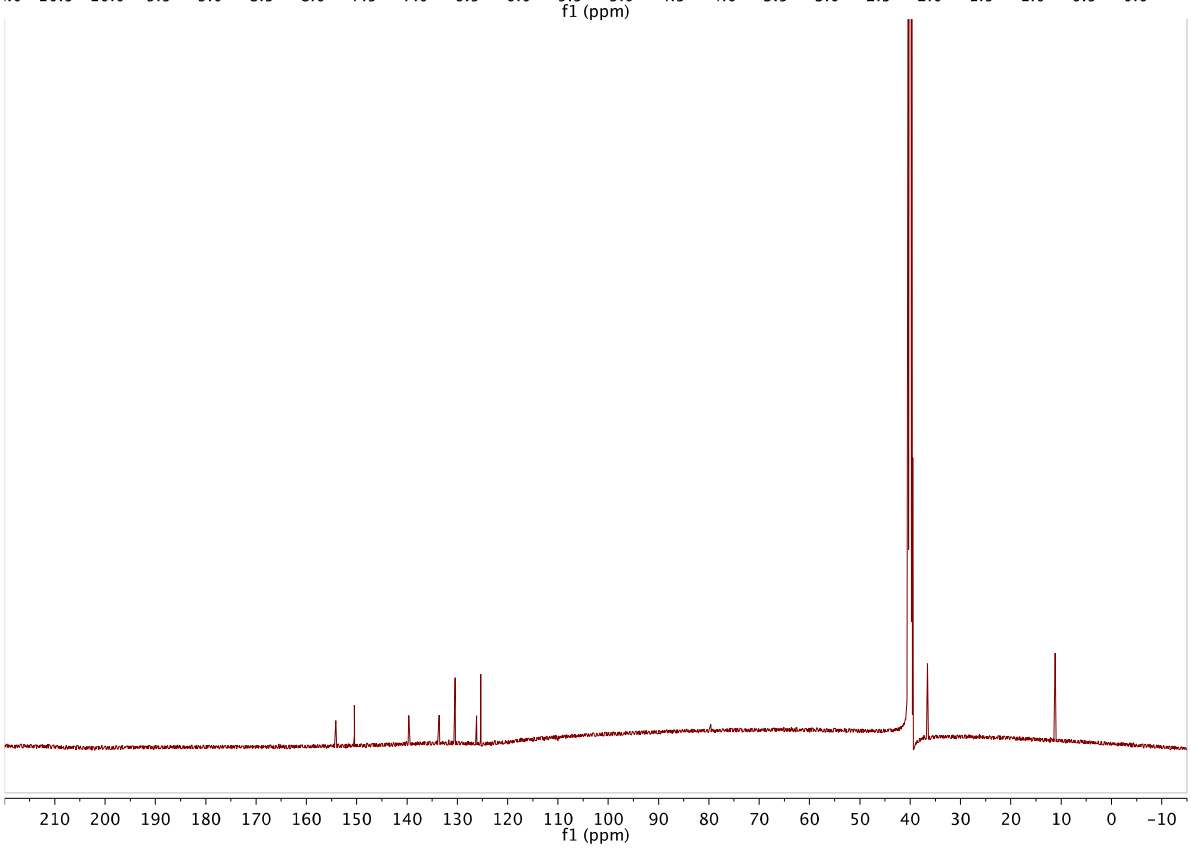

HN
O
O
N
N
N
N
N
CH3
O

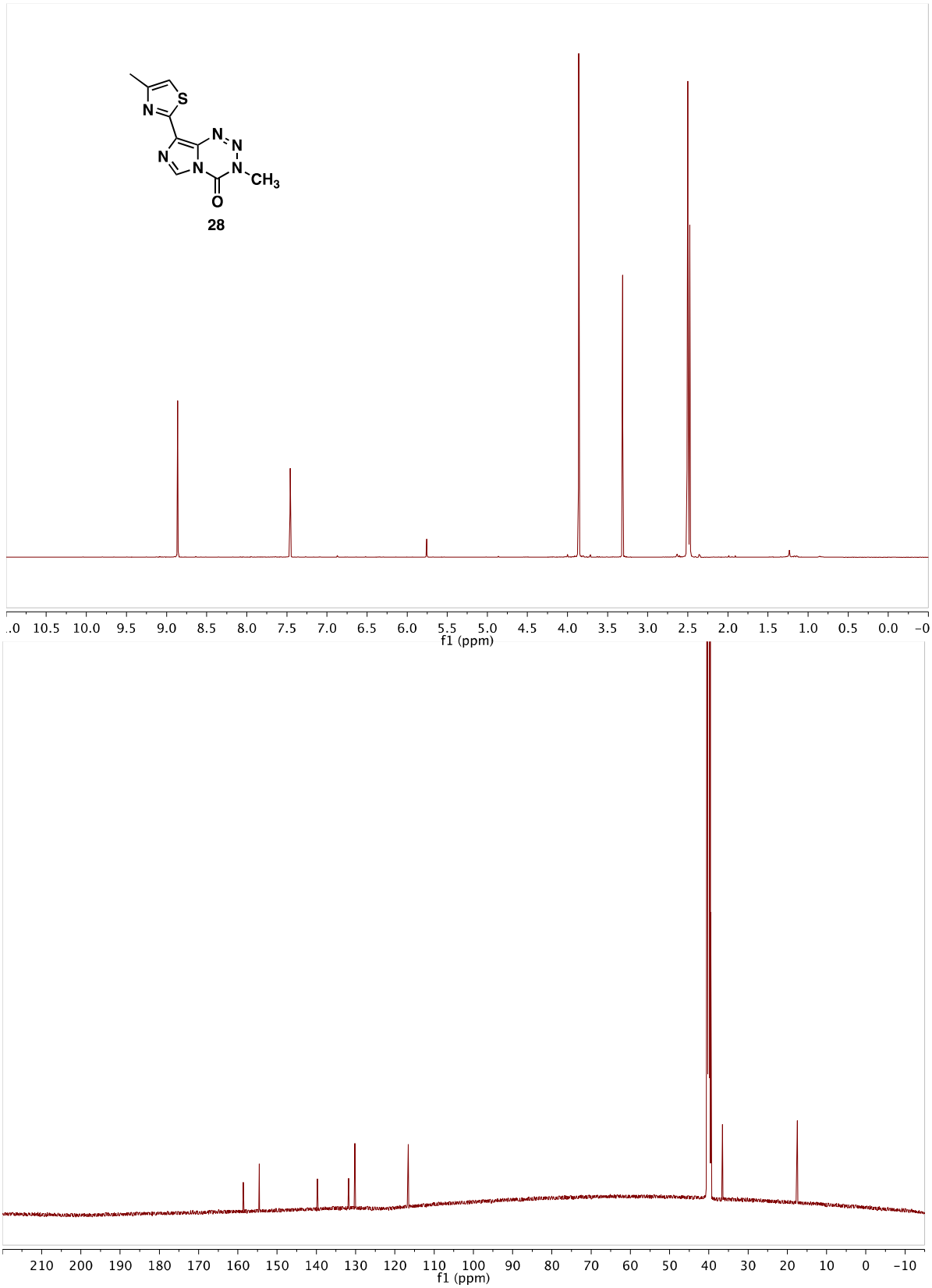
32

10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)


210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)


N
O
N
N
N
N
N
CH3
O
27
.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)


210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)


28
f1 (ppm)
f1 (ppm)