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Chemistry & Drug Discovery

Supporting Information

Ribonucleoside 3′-Phosphates as Pro-Moieties for an Orally Administered Drug

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Supporting Information

Materials

Uridine phosphoramidite and iodine oxidizing solution were from Glen Research (Sterling, VA). Metronidazole, 3-Å molecular sieves, tetrabutylammonium fluoride (TBAF), methylbenzimidazole, Brucella broth powder, vitamin K, Hemin, and all other commercial reagents were from Sigma–Aldrich (St. Louis, MO). Methylbenzimidazole triflate was prepared from methylbenzimidazole.[1] Round, 96-well, U-bottom plates and 95-pin inoculator assemblies were from Evergreen Scientific (Los Angeles, CA).

Instrumentation and statistical calculations

^{1}H NMR spectra were acquired at the National Magnetic Resonance Facility at Madison (NMRFAM) at 298 K with a Bruker DMX-400 Avance spectrometer (Bruker AXS, Madison, WI, ^{1}H, 400 MHz; ^{13}C, 101 MHz; ^{31}P, 162 MHz). ^{13}C NMR spectra were also acquired on an Avance III 500 MHz spectrometer with a ^{13}C/^{15}N{^{1}H} 5-mm cryogenic probe from Bruker AXS (Madison, WI, ^{13}C, 126 MHz). ^{13}C and ^{31}P spectra were proton decoupled. All ^{1}H and ^{13}C NMR spectra were referenced to TMS. All ^{31}P NMR spectra were referenced to an internal insert standard of H_3PO_4. Protein absorbance values were measured with a Varian Cary 50 UV–Vis Spectrometer from Agilent Technologies (Santa Clara, CA). ^{1}H NMR spectra for the NpMet release kinetics were acquired at the NMRFAM at 310 K on an Avance III 500 MHz spectrometer with a TCI 500 H-C/N-D cryogenic probe from Bruker AXS (Madison, WI, ^{1}H, 500 MHz). Photos of 96-well plates to determine the MIC values of various compounds were taken with an iPhone 4S from Apple Computer (Cupertino, CA).

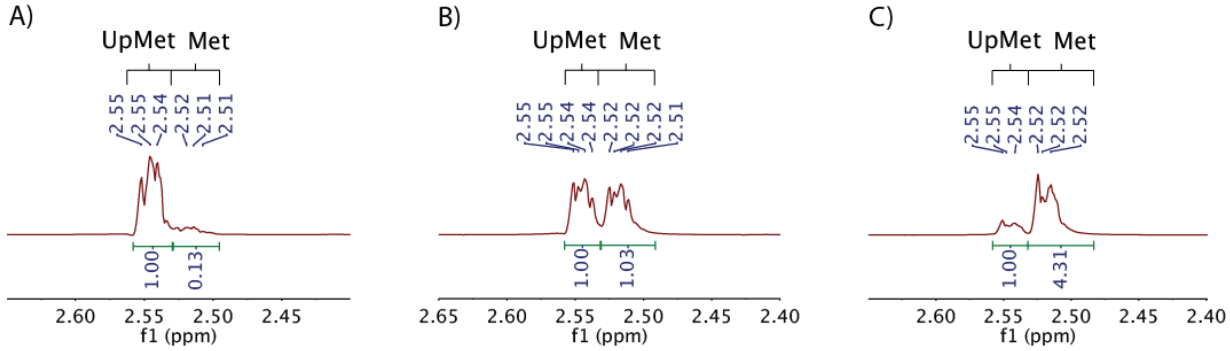


Figure S1. Representative ^{1}H NMR spectra for assessing the rate of prodrug hydrolysis. Degradation was assessed by monitoring the integration of the peak corresponding to the methyl group of UpMet (2.55) and Met (2.52). The assay solution contained RNase 1 (0.1 mg/mL) in simulated intestinal fluid. (**A**) 6 min, (**B**) 64 min, and (**C**) 193 min.

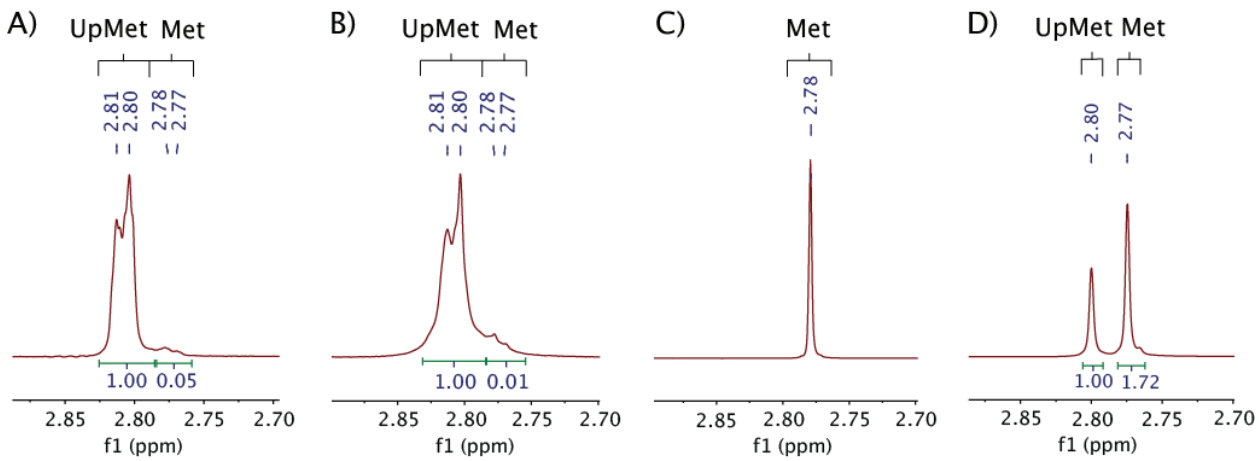


Figure S2. Representative ^{1}H NMR spectra for 3-h incubations in simulated gastric fluid (0.10 N HCl, 2% v/v D_2O, pH 1.1, United States Pharmacopeia) to assess the stability of these molecules in a stomach. Degradation was assessed by monitoring the integration of the peak corresponding to the methyl group of UpMet (2.80) and Met (2.78). (**A**) Spectrum of UpMet at 0 min. (**B**) Spectrum of UpMet at 3 h. (**C**) Control spectrum of Met. (**D**) Spectrum of UpMet after the 3-h incubation with added Met to confirm that the minor peak at 2.78 ppm was indeed Met. After 3 h, there was no increase in the amount of Met present; hence, UpMet is stable under these conditions. (Note: For spectra in panels C and D, the spectrometer was re-shimmed, which allowed for better resolution of the peaks compared to those in panels A and B.)

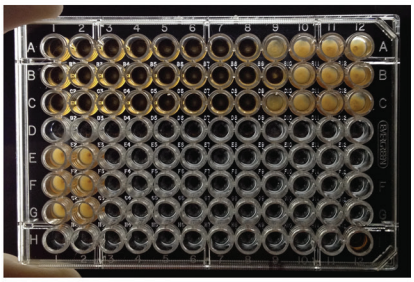
Effect of DEPC-treatment on the MIC of metronidazole for *B. fragilis*

Metronidazole is marginally soluble in aqueous solutions (~10 mg/mL).[2] Accordingly, for the initial dissolution of metronidazole, dimethyl sulfoxide (DMSO, 10% v/v) was added to the medium. For MIC test solutions used in the 96-well plates, <0.5% of the final volume of liquid was DMSO. For each plate, ~10 mg of metronidazole, taking note of the exact mass to make the appropriate dilutions, was added to a 4-mL glass vial, and then 1 mL of culture medium containing DMSO (10% v/v) was added. Serial dilutions (log 2) were made according to guidelines from the Clinical and Laboratory Standards Institute[3] in the indicated medium and dispensed in triplicate wells (90 μL per well) into a 96-well, U-bottom plate. DEPC-treatment of the medium had no affect on the MIC of metronidazole for *B. fragilis.*

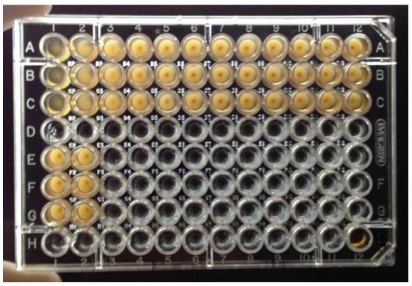
Values are in µg/mL of Met

	1	2	3	4	5	6	7	8	9	10	11	12
A	128	64	32	16	8	4	2	1	0.5	0.25	0.13	0.06
B	128	64	32	16	8	4	2	1	0.5	0.25	0.13	0.06
C	128	64	32	16	8	4	2	1	0.5	0.25	0.13	0.06
D												
E	0.03	0										
F	0.03	0										
G	0.03	0										
H												sterile

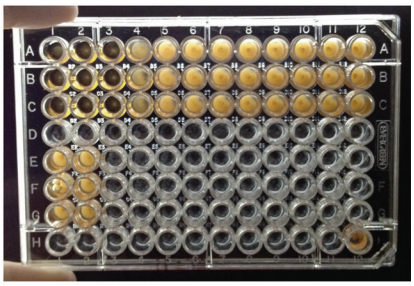
Met



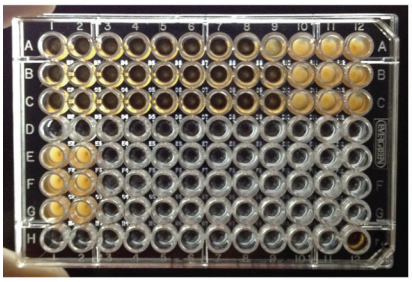
ApMet



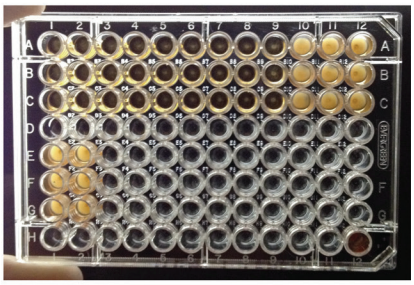
UpMet



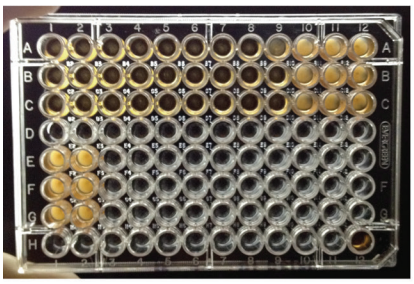
Met + 0.1 mg/mL RNase 1 over night



UpMet + 0.1 mg/mL RNase 1 over night



Met + 0.01 mg/mL RNase 1 in all wells



UpMet + 0.01 mg/mL RNase 1 in all wells

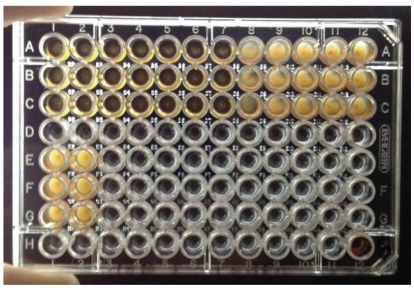
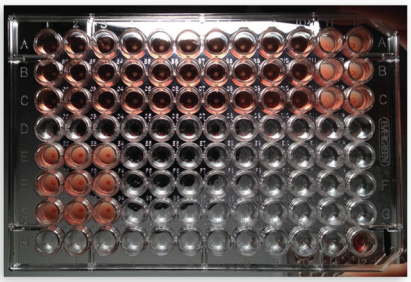


Figure S3. Photographs of the plates used to determine the MICs. Assays followed the protocols of the Clinical and Laboratory Standards Institute.[3] (Note: These photographs were taken with a different light source than those in Figure S4 and were in an oxygen environment for several hours prior to taking the photograph, causing oxygenation of the media and an ensuing divergence of color.)

KEY (Values are in µg/mL of Met)

	1	2	3	4	5	6	7	8	9	10	11	12
A	256	128	64	32	16	8	4	2	1	0.5	0.25	0.13
B	256	128	64	32	16	8	4	2	1	0.5	0.25	0.13
C	256	128	64	32	16	8	4	2	1	0.5	0.25	0.13
D												
E	0.06	0.03	0									
F	0.06	0.03	0									
G	0.06	0.03	0									
H												sterile

Normal medium



DEPC-treated medium

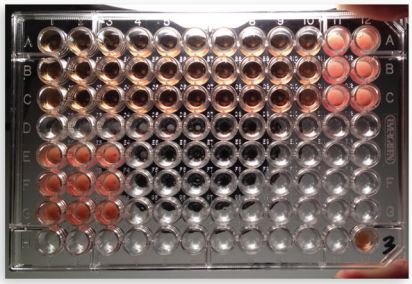


Figure S4. Effect of DEPC-treatment on the MIC of metronidazole for *B. fragilis*. The MIC for normal medium and DEPC-treated medium was 0.5 µg/mL, indicating that treatment with DEPC did not interfere with MIC determination. For quality control purposes, the Clinical and Laboratory Standards Institute[3] defines the acceptable MIC of this strain of *B. fragilis* to be 2.00–0.25 µg/mL.

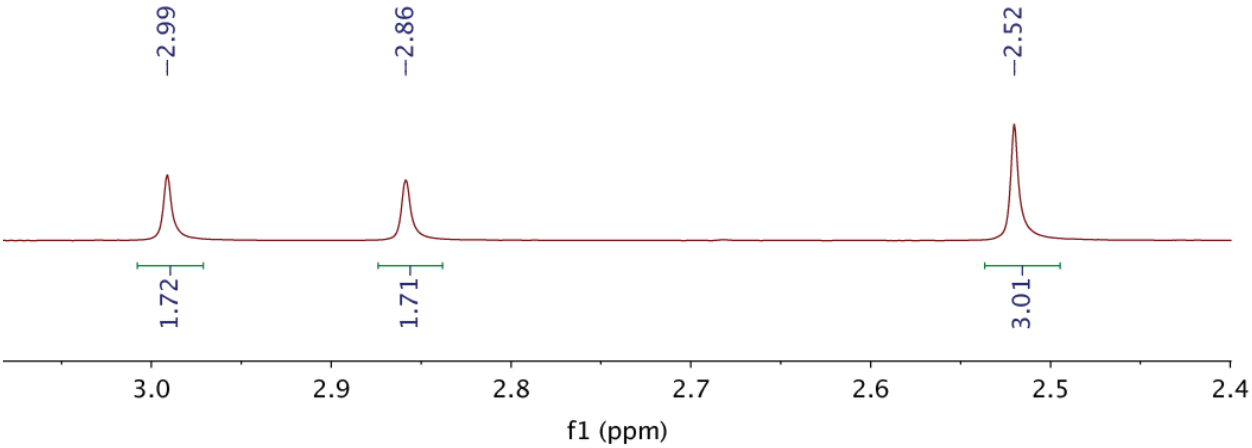
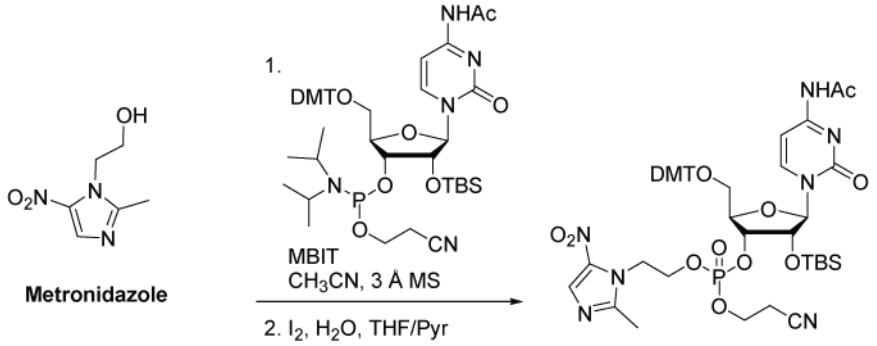


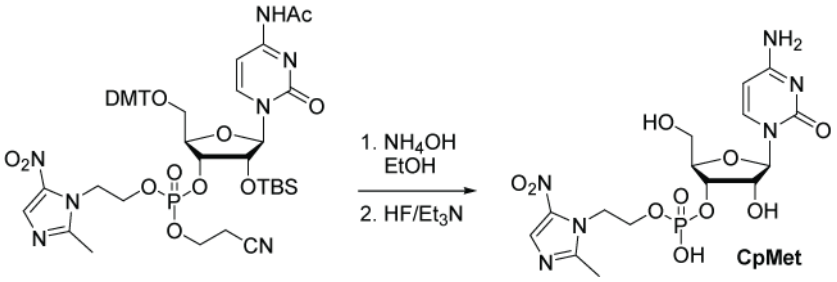
Figure S5. Representative NMR spectrum for determination of Met (or NpMet) concentration. DMF methyl peaks corresponded to the peaks at 2.99 and 2.86 ppm, and the methyl imidazole peak of metronidazole corresponded to the peak at 2.52 ppm.

Synthesis of cytidine 3′-(4-metronidazole phosphate)



Metronidazole (0.173 g, 1.010 mmol) and phosphoramidite (1.000 g, 1.110 mmol) were added to a round-bottom flask that had been charged with 3-Å molecular sieves (approximately 20 beads) under Ar(g), and that contained 11 mL of CH_3CN. After 10 min, methylbenzimidazole triflate (MBIT) (0.285 g, 1.010 mmol) was added, and the reaction mixture was stirred for 3 h. The CH_3CN was removed by vacuum, and the reaction mixture was filtered through a plug of silica (100% v/v ethyl acetate). Ethyl acetate was removed by vacuum, and 55.0 mL of I_2 (0.02 M) in THF/H_2O/pyridine was added. The reaction mixture turned from brown to clear yellow after 1 h. The solvent was removed by vacuum, and the resulting solid was purified with silica gel chromatography (ethyl acetate and then 10% methanol/90% dichloromethane) to yield pure product (1.026 g, 93% over 2 steps).

^{1}H NMR (400 MHz, CD_3OD) δ (Mixture of isomers) 8.46–8.39 (m, 1H), 7.95 (s, 0.39H), 7.90 (s, 0.61H), 7.42 (d, J = 7.6, 2H), 7.38–7.25 (m, 7H), 7.05 (d, J = 7.6, 0.39H), 7.00 (d, J = 7.5, 0.61H), 6.90 (d, J = 8.7, 4H), 5.91–5.86 (m, 1H), 4.95–4.90 (m, 1H), 4.69–4.52 (m, 3H), 4.45–4.26 (m, 3H), 4.2–4.05 (m, 2H), 3.81 (s, 6H), 3.71 (t, J = 11.2, 1H), 3.51 (t, J = 10.4, 1H), 2.79 (t, J = 5.8, 1.22H), 2.66 (s, 0.78H), 2.49 (s, 1.17H), 2.46 (s, 1.83H), 2.16 (s, 3H), 0.92–0.87 (m, 9H), 0.18–0.07 (m, 6H); **^{13}C NMR (126 MHz, CD_3OD)** (Mixture of isomers. When the diastereomer peaks resolve, the peaks are listed in parentheses) δ 173.0, 164.5, (160.6 & 160.6), (158.1 & 158.0), (153.1 & 153.0), (146.0 & 145.9), (145.7 & 145.7), 140.3, (136.5 & 136.4), (133.1 & 133.1), 131.7, (129.8 & 129.7), 129.3, 128.6, (118.5 & 118.5), 114.5, 98.5, (92.2 & 92.0), (89.0 & 88.9), (82.8 & 82.6), (76.7 & 76.4), (68.5 & 68.4), (68.2 & 68.2), (64.8 & 64.7), 62.4, 55.9, (47.2 & 47.1), 26.4, 24.7, (20.3 & 20.3), 19.2, (14.5 & 14.5), (–4.3 & –4.7); **^{31}P NMR (162 MHz, CD_3OD)** (Mixture of isomers) δ = –3.10, –3.76; **HRMS** (ESI) m/z 988.3672 [calc'd for $C_{47}H_{59}N_7O_{13}PSi$ (M+H) 988.3673].

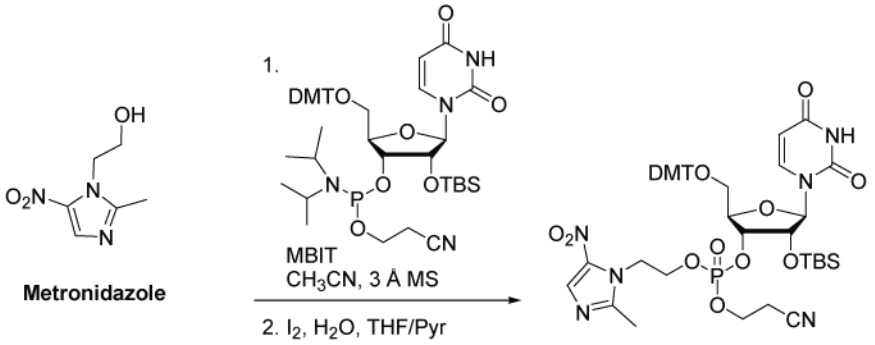


The oxidized metronidazole adduct (1.026 g, 1.035 mmol) was added to NH_4OH (72.5 μL, 2.071 mmol) in ethanol (20.7 mL). The reaction mixture was stirred for 3 h. Solvent was

removed by vacuum, $Et_3N{\cdot}HF$ (3.37 mL, 20.7 mmol) was added in CH_3CN (20.7 mL), and the reaction mixture was stirred at 65 °C for an additional 8 h. The reaction mixture was then cooled to room temperature and quenched with aqueous sodium bicarbonate solution, and the solvent was removed by vacuum. The resulting solid was purified by flash chromatography (10% methanol/90% dichloromethane to 100% methanol) to yield product (0.083 g, 17% over 2 steps).

^{1}H NMR (400 MHz, CD_3OD) δ 8.04 (d, J = 7.5, 1H), 7.92 (s, 1H), 5.90–5.85 (m, 2H), 4.61 (t, J = 4.7, 2H), 4.45–4.38 (m, 1H), 4.27–4.18 (m, 3H), 4.14–4.10 (m, 1H), 3.82 (dd, J = 10.4, 2.5, 1H), 3.74 (dd, J = 10.4, 2.5, 1H), 2.56 (s, 3H); **^{13}C NMR (126 MHz, CD_3OD)** δ 166.3, 157.0, 152.0, 141.5, 138.6, 131.2, 94.5, 90.2, 83.3, 74.2, 73.0, 64.1, 60.2, 29.4, 13.1; **^{31}P NMR (162 MHz, CD_3OD)** δ = –1.10; **HRMS** (ESI) m/z 475.0968 [calc'd for $C_{15}H_{20}N_6O_{10}P$ (M–H) 475.0984].

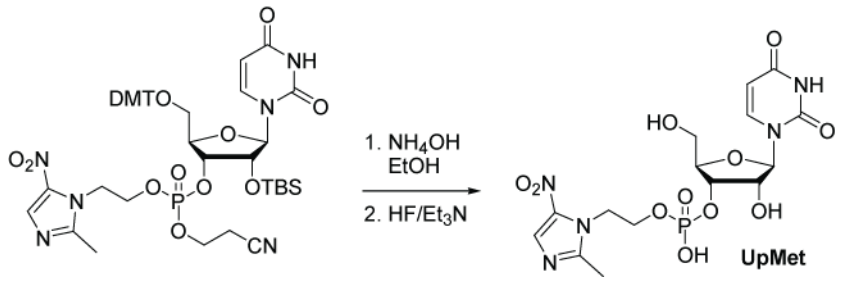
Synthesis of uridine 3′-(4-metronidazole phosphate)



Metronidazole (0.100 g, 0.585 mmol) and phosphoramidite (0.554 g, 0.643 mmol) were added to a round-bottom flask that had been charged with 3-Å molecular sieves (approximately 20 beads) under Ar(g), and that contained 5 mL of CH_3CN. After 10 min, methylbenzimidazole triflate (MBIT) (0.165 g, 0.585 mmol) was added, and the reaction mixture was stirred for 3 h. The CH_3CN was removed by vacuum, and the reaction mixture was filtered through a plug of silica (100% ethyl acetate). Ethyl acetate was removed by vacuum, and 29.0 mL of I_2 in THF/H_2O/pyridine (0.02M) was added. The reaction mixture turned from brown to clear yellow after 1 h. The solvent was removed by vacuum and the resulting solid was purified with silica gel chromatography (ethyl acetate and then 10% methanol/90% dichloromethane) to yield pure product (0.542 g, 98% over 2 steps).

^{1}H NMR (400 MHz, CD_3OD) (Mixture of isomers) δ = 7.95 (s, 0.38H), 7.91–7.84 (m, 1.62H), 7.39 (d, J = 7.6, 2H), 7.34–7.06 (m, 7H), 6.88 (d, J = 8.8, 4H), 5.91 (t, J = 4.9, 1H), 5.33–5.27 (m, 1H), 4.68–4.56 (m, 2H), 4.53 (t, J = 4.8, 1H), 4.50–4.28 (m, 3H), 4.26–4.12 (m, 2H), 4.11–3.99 (m, 1H), 3.76 (s, 6H), 3.61–3.52 (m, 1H), 3.49–3.40 (m, 1H), 2.80 (t, J = 5.8, 1.25H), 2.68 (dd, J = 5.8, 10.2, 0.75H), 2.50 (s, 1.1H), 2.47 (s, 1.9H), 0.88 (s, 5.6H), 0.86 (s, 3.4H), 0.12 (s, 1.9H), 0.10–0.07 (m, 4.1H); **^{13}C NMR (101 MHz, CD_3OD)** (Mixture of isomers. When the diastereomer peaks resolve, the peaks are listed in parentheses) δ = 165.7, 160.4, (153.0 & 152.9), 152.0, 145.7, 141.7, 140.1, (136.2 & 136.2), (133.1 & 133.0), 131.5, 129.4, 129.1, 128.3, 118.3, 114.4, 103.1, (89.3 & 89.2), 88.8, (83.4 & 83.2), 78.4, 75.7, (68.2 & 68.0), (64.6 & 64.4), 63.4, 55.8, (47.1 & 47.0), 26.2, 20.1, 19.0, 14.4, (–4.6 & –4.7); **^{31}P NMR (162 MHz, CD_3OD)**

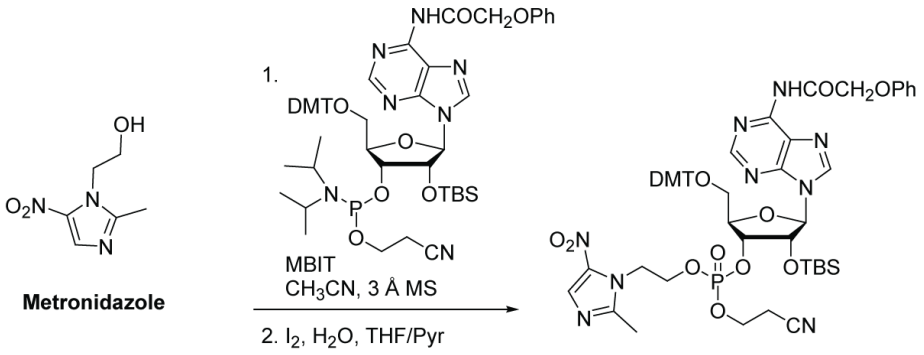
(Mixture of isomers) δ = –3.32, –3.73; **HRMS** (ESI) *m/z* 969.3218 [calc'd for $C_{45}H_{55}N_6O_{13}PSiNa$ (M+Na) 969.3227].



The oxidized metronidazole adduct (0.180 g, 0.190 mmol) was added to NH_4OH (0.13 mL, 3.800 mmol) in ethanol (3.8 mL). The resulting reaction mixture was stirred at room temperature for 3h. The solvent was removed by vacuum. $Et_3N{\cdot}HF$ (0.310 mL, 1.900 mmol) was added in CH_3CN (3.8 mL), and the reaction mixture was stirred at 65 °C for an additional 8 h. The reaction mixture was then cooled to room temperature and quenched with aqueous bicarbonate solution, and the solvent was removed by vacuum. The resulting solid was purified by flash chromatography (10% methanol/90% dichloromethane to 100% methanol) to yield product (0.075 g, 82% over 2 steps).

^{1}H NMR (400 MHz, CD_3OD) δ = 8.02 (d, *J* = 8.1, 1H), 7.93 (s, 1H), 5.90 (d, *J* = 5.4, 1H), 5.69 (d, *J* = 8.1, 1H), 4.62 (t, *J* = 4.7, 2H), 4.45–4.37 (m, 1H), 4.24 (dt, *J* = 4.3, 8.4, 2H), 4.20 (t, *J* = 5.2, 1H), 4.12 (dd, *J* = 2.5, 3.6, 1H), 3.81–3.67 (m, 2H), 2.57 (s, 3H); **^{13}C NMR (126 MHz, CD_3OD)** δ 152.0, 141.0, 138.6, 131.3, 129.1, 127.8, 127.2, 112.4, 101.5, 88.6, 84.1, 74.0, 64.1, 60.7, 13.1; **^{31}P NMR (162 MHz, CD_3OD)** δ = –1.14; **HRMS** (ESI) *m/z* 476.0845 [calc'd for $C_{15}H_{19}N_5O_{11}P$ (M–H) 476.0824].

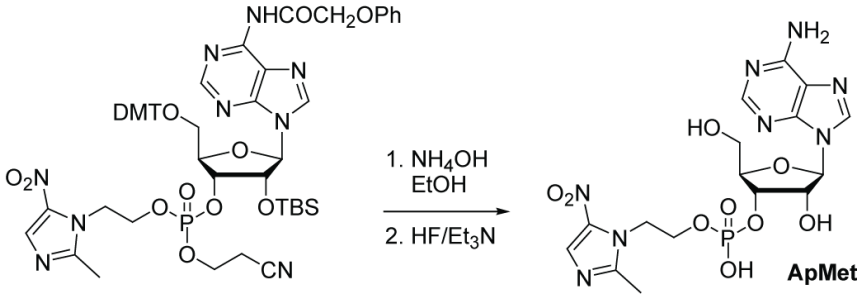
Synthesis of adenosine 3′-(4-metronidazole phosphate)



Metronidazole (0.076 g, 0.447 mmol) and phosphoramidite (0.500 g, 0.491 mmol) were added to a round-bottom flask that had been charged with 3-Å molecular sieves (approximately 20 beads) under Ar(g), and that contained 5 mL of CH_3CN. After 10 min, methylbenzimidazole triflate (MBIT) (0.126 g, 0.447 mmol) was added, and the reaction mixture was stirred for 3 h. The CH_3CN was removed by vacuum, and the reaction mixture was filtered through a plug of silica (100% v/v ethyl acetate). Ethyl acetate was removed by vacuum, and 22.4 mL of I_2 (0.02 M) in THF/H_2O/pyridine was added. The reaction mixture turned from brown to clear yellow after 1 h.

The solvent was removed by vacuum and the resulting solid was purified with silica gel chromatography (ethyl acetate) to yield pure product (0.407 g, 75% over 2 steps).

^{1}H NMR (400 MHz, CD_3OD) (Mixture of isomers) δ 8.57–8.51 (m, 2H), 7.99 (s, 0.39H), 7.88 (s, 0.61H), 7.46 (d, J = 6.3, 2H), 7.36–7.20 (m, 9H), 7.07 (d, J = 8.3, 2H), 7.00 (t, J = 7.4, 1H), 6.85 (d, J = 6.7, 4H), 6.07 (d, J = 6.2, 0.61H), 6.02 (d, J = 6.2, 0.39H), 5.21 (t, J = 4.9, 1H), 4.99 (s, 2H), 4.73–4.66 (m, 1H), 4.63 (t, J = 5.2, 0.78H), 4.58 (t, J = 5.2, 1.22H), 4.33–4.15 (m, 3H), 4.03–3.80 (m, 2H), 3.77 (s, 6H), 3.62–3.55 (m, 1H), 3.42 (td, J = 10.0, 3.8, 1H), 2.70 (t, J = 5.9, 1.22H), 2.60 (t, J = 5.8, 0.78H), 2.54 (s, 1.17H), 2.48 (s, 1.83H), 0.75 (s, 5.49H), 0.72 (s, 3.51H), –0.06 to –0.10 (m, 3H), –0.25 (s, 1.83H), –0.30 (s, 1.17H); **^{13}C NMR (101 MHz, CD_3OD)** (Mixture of isomers. When the diastereomer peaks resolve, the peaks are listed in parentheses) δ 170.1, 160.4, 159.3, (153.4 & 153.2), (150.4 & 150.2), 146.3, (145.5 & 145.3), 140.3, 138.6, (137.0 & 136.9), 133.2, (131.6 & 131.5), 130.8, 129.5, 129.1, 128.2, 125.7, 124.7, 123.1, 118.5, 116.1, 114.4, 89.8, 88.3, 84.6, 79.7, 74.3, 69.2, 68.4, (64.8 & 64.6), 61.7, 55.9, 47.3, 21.0, 20.3, 18.9, 14.6, (–4.4 & –5.0); **^{31}P NMR (162 MHz, CD_3OD)** (Mixture of isomers) δ = –3.42, –3.58; **HRMS** (ESI) *m*/*z* 1126.3859 [calc'd for $C_{54}H_{62}N_9O_{13}PSiNa$ (M+Na) 1126.3867].



The oxidized metronidazole adduct (0.468 g, 0.424 mmol) was added to NH_4OH (29.72 μL, 0.848 mmol) in ethanol (8.5 mL). The resulting reaction mixture was stirred at room temperature for 3 h. The solvent was removed by vacuum and $Et_3N{\cdot}HF$ (1.38 mL, 8.480 mmol) was added in CH_3CN (7.36 mL) and the reaction mixture was stirred at 65 °C for an additional 8 h. The reaction mixture was then cooled to room temperature and quenched with aqueous bicarbonate solution, and the solvent was removed by vacuum. The resulting solid was purified by flash chromatography (10% methanol/90% dichloromethane to 100% methanol) to yield product (0.180 g, 85% over 2 steps).

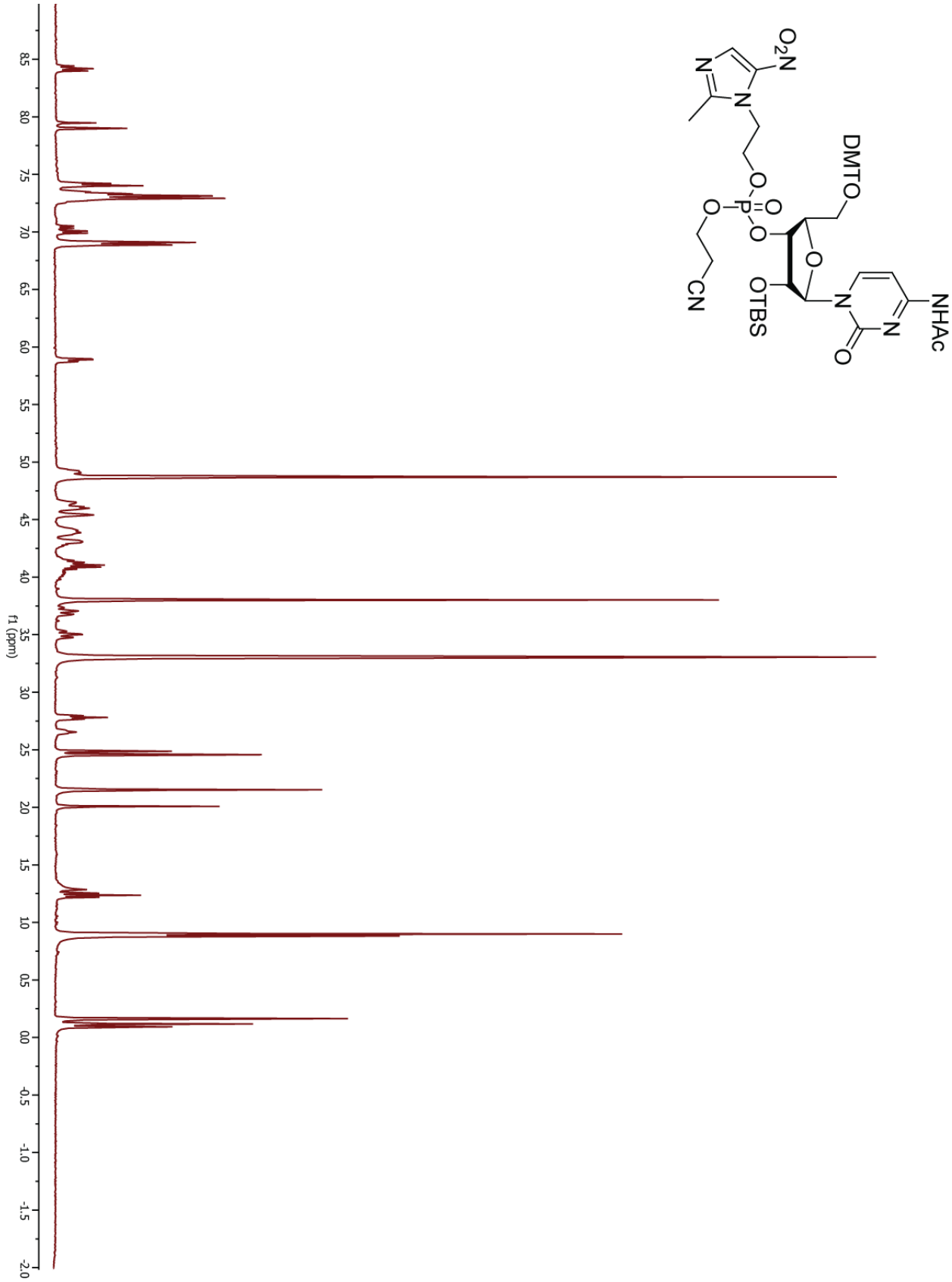
^{1}H NMR (400 MHz, CD_3OD) δ 8.31 (s, 1H), 8.18 (s, 1H), 7.93 (s, 1H), 5.93 (d, J = 6.9, 1H), 4.79–4.75 (m, 1H), 4.66–4.61 (m, 3H), 4.32–4.24 (m, 3H), 3.83 (d, J = 12.7, 1H), 3.75 (d, J = 12.7, 1H), 2.60 (s, 3H); **^{13}C NMR (126 MHz, CD_3OD)** δ 156.2, 152.1, 152.0, 148.6, 140.7, 138.6, 131.3, 119.7, 89.6, 85.9, 75.4, 73.5, 64.1, 61.8, 21.6, 13.1; **^{31}P NMR (162 MHz, CD_3OD)** δ = –1.08; **HRMS** (ESI) *m*/*z* 501.1259 [calc'd for $C_{16}H_{22}N_8O_9P$ (M+H) 501.1242].

References

[1] Y. Hayakawa, R. Kawai, A. Hirata, J. I. Sugimoto, M. Kataoka, A. Sakakura, M. Hirose, R. Noyori, *J. Am. Chem. Soc.* **2001**, *123*, 8165-8176.

[2] C. F. Rediguieri, V. Porta, G. N. DS, T. M. Nunes, H. E. Junginger, S. Kopp, K. K. Midha, V. P. Shah, S. Stavchansky, J. B. Dressman, D. M. Barends, *J. Pharm. Sci.* **2011**, *100*, 1618-1627.

[3] *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard—Seventh Edition*. CLSI document M11-A7. Wayne, PA: Clinical and Laboratory Standards Institute; 2007.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P
O
O
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N
N
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6.5
6.0
5.5
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4.0
3.5
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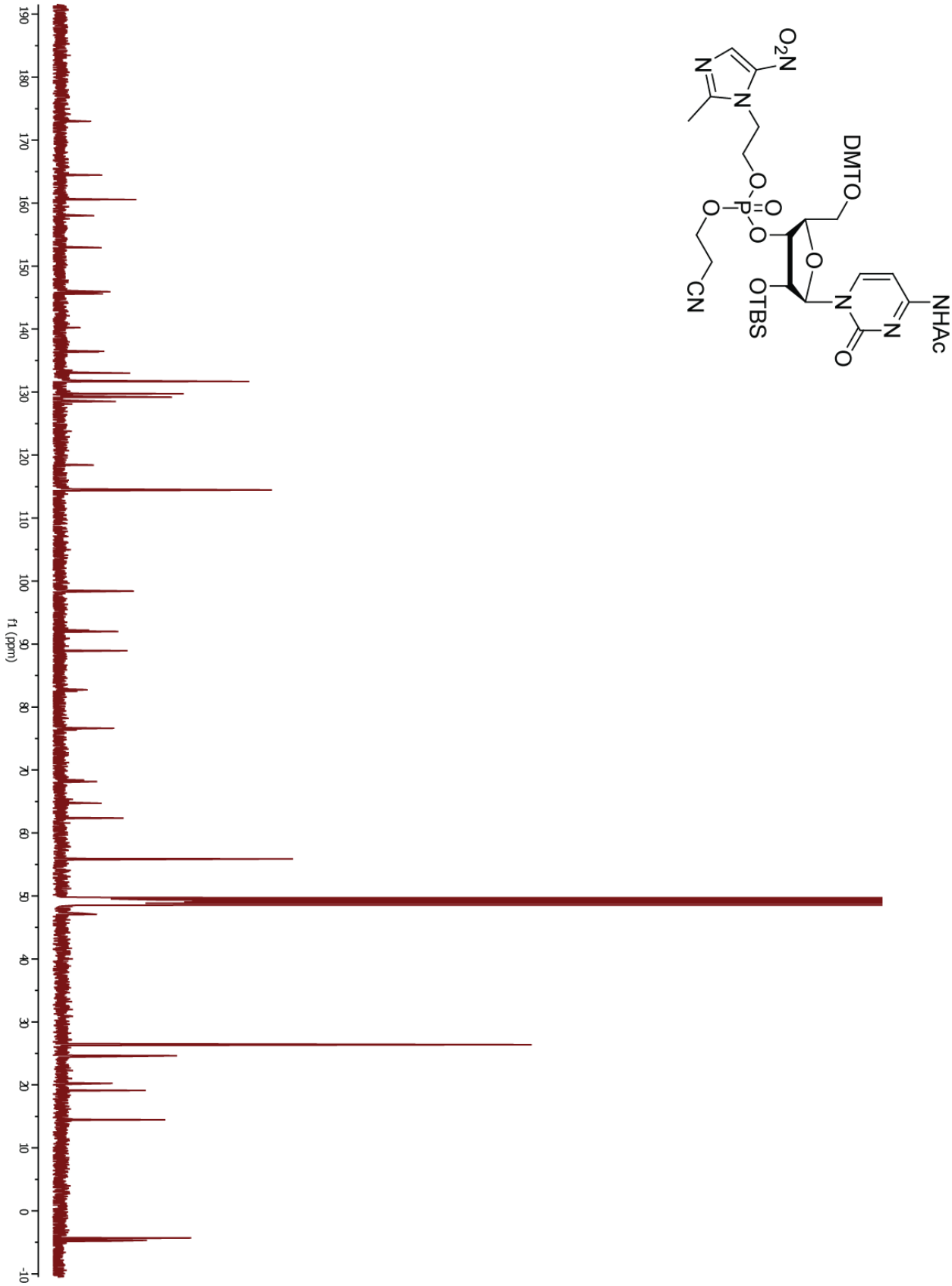
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N
O2N
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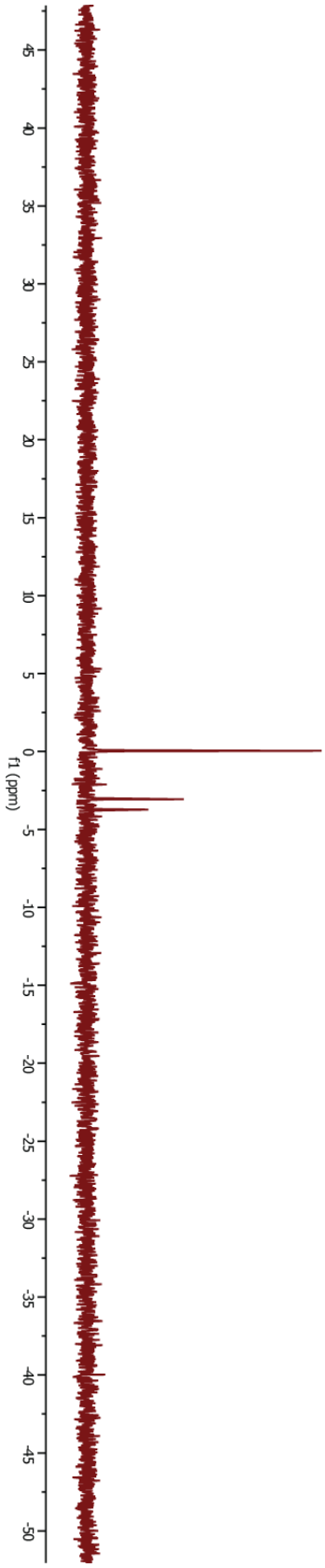
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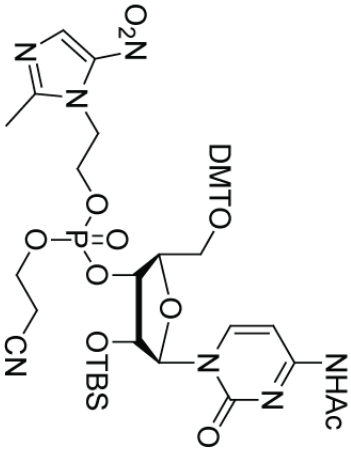
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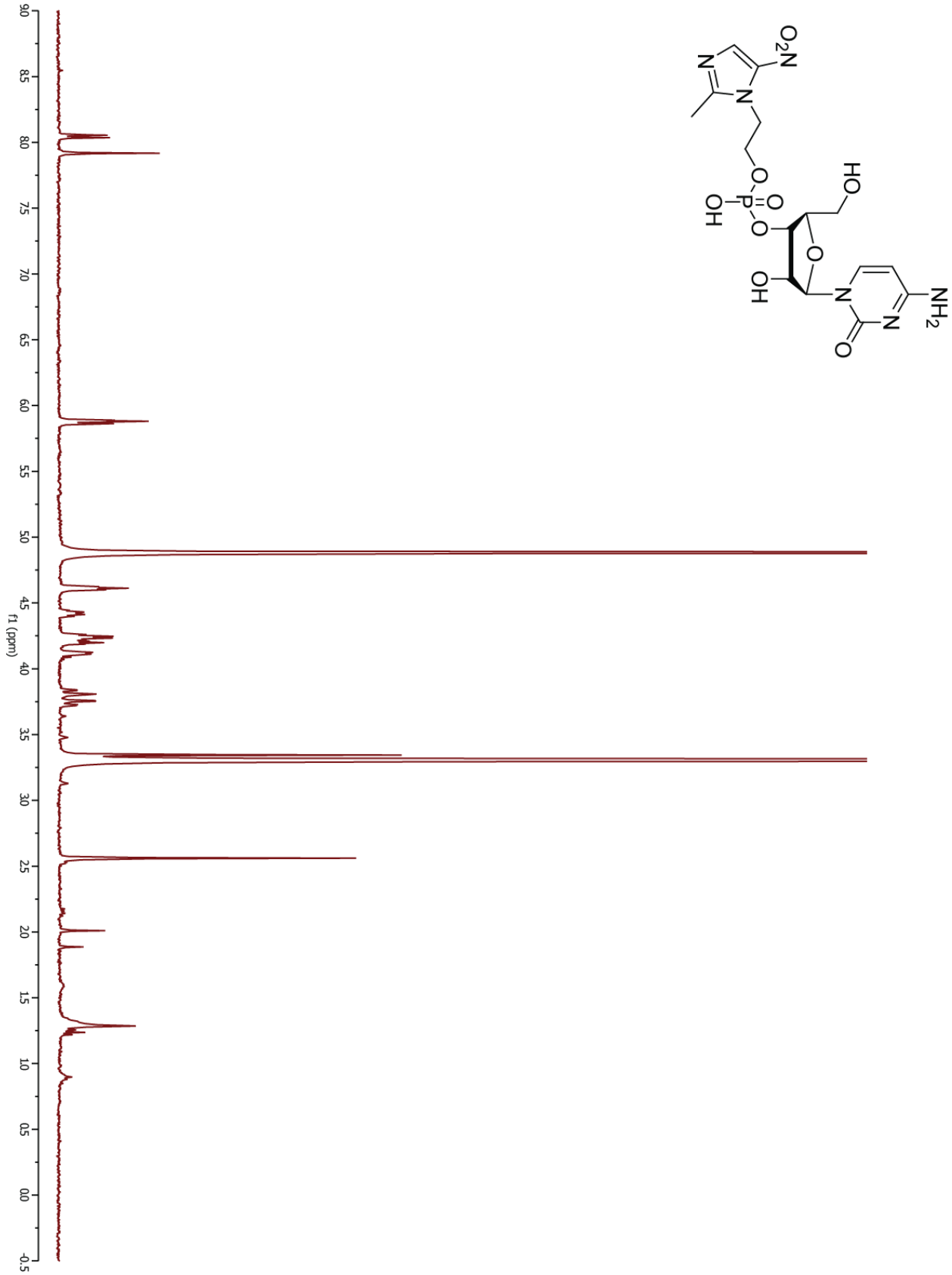
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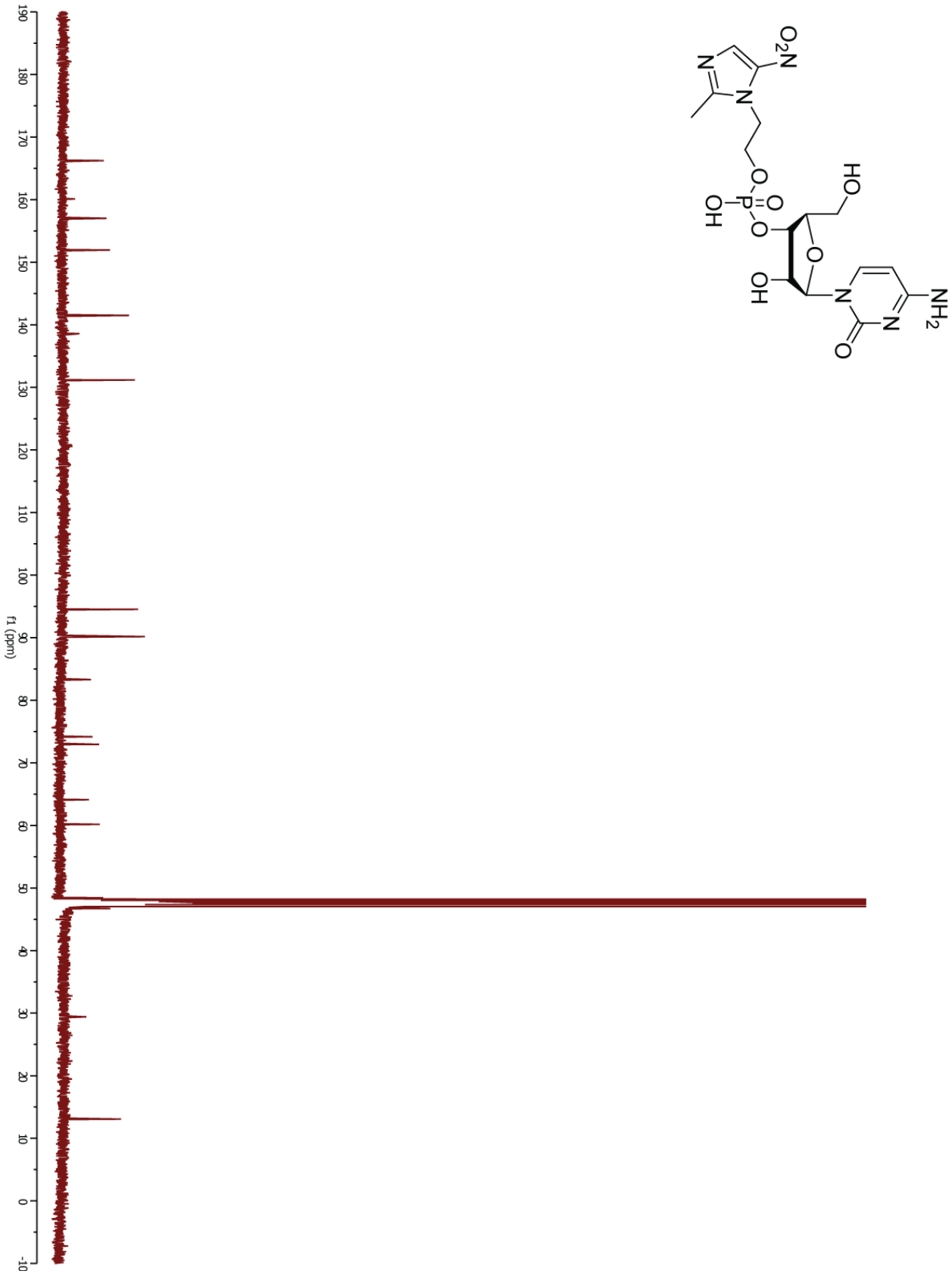
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N
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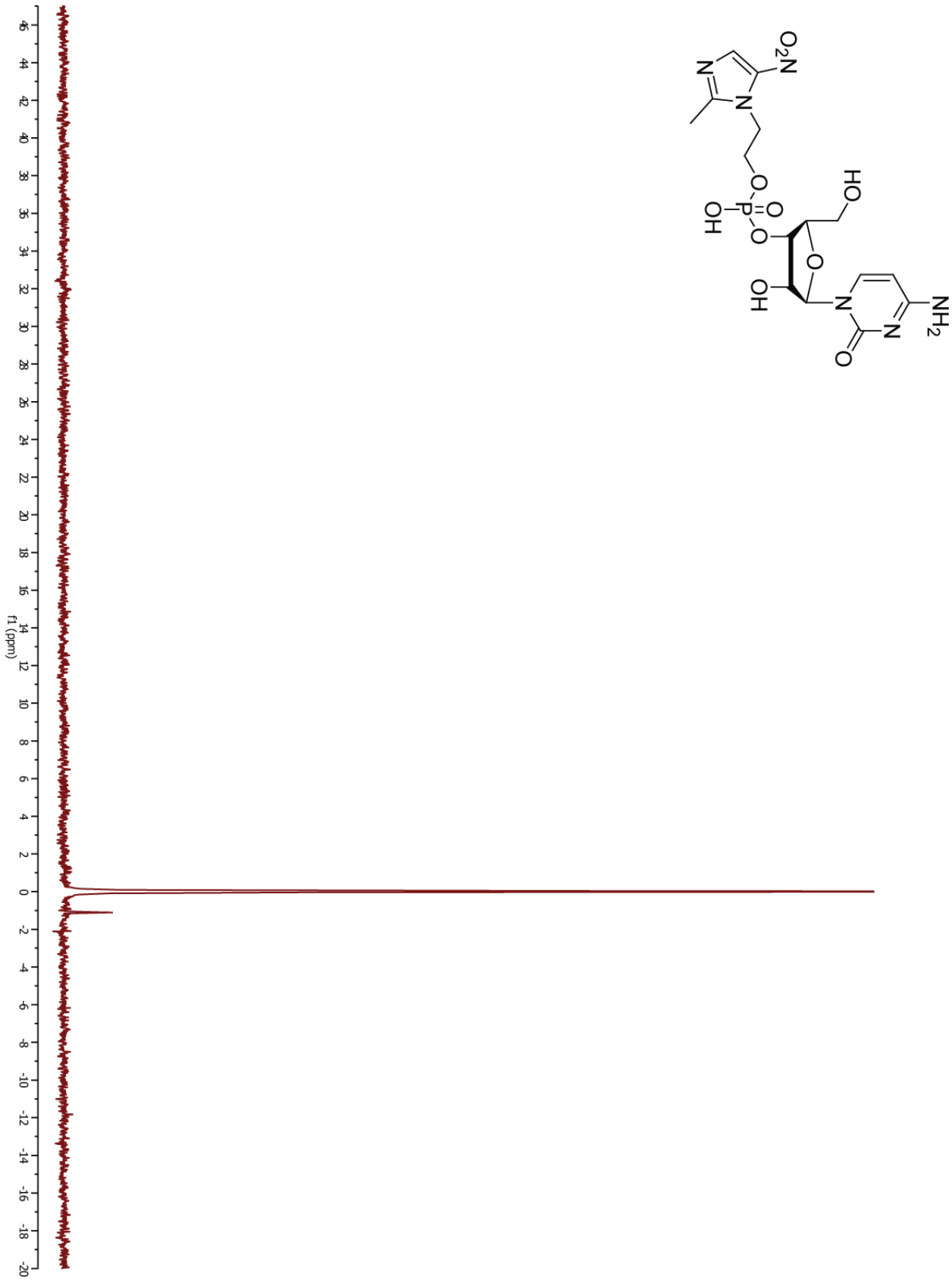
NH2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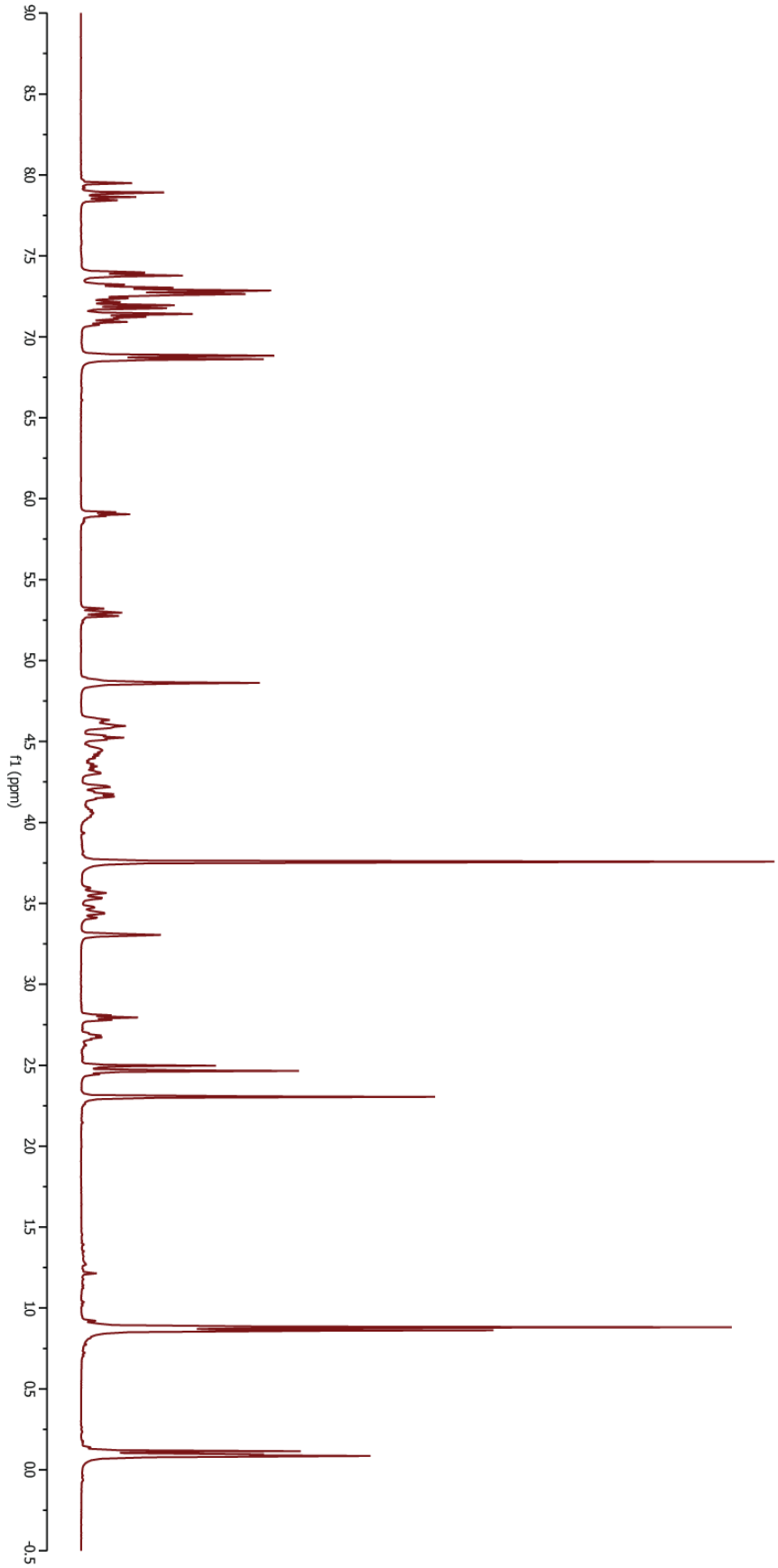
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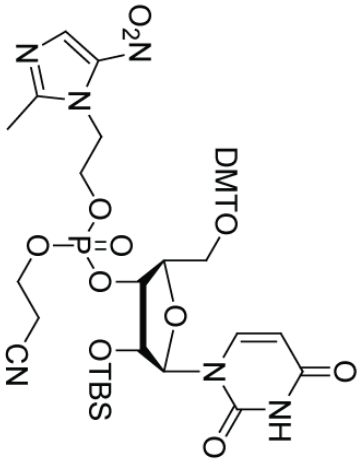
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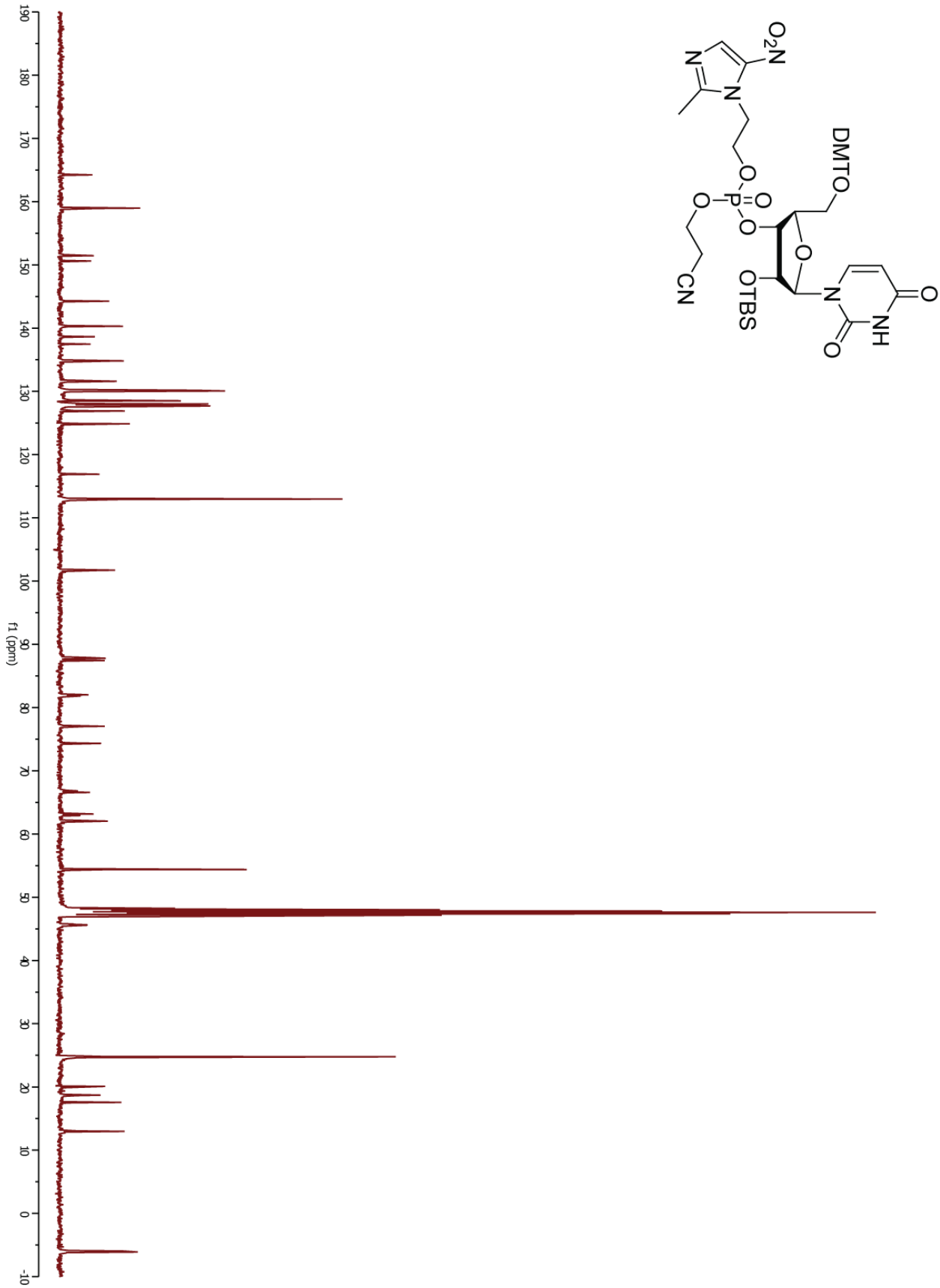
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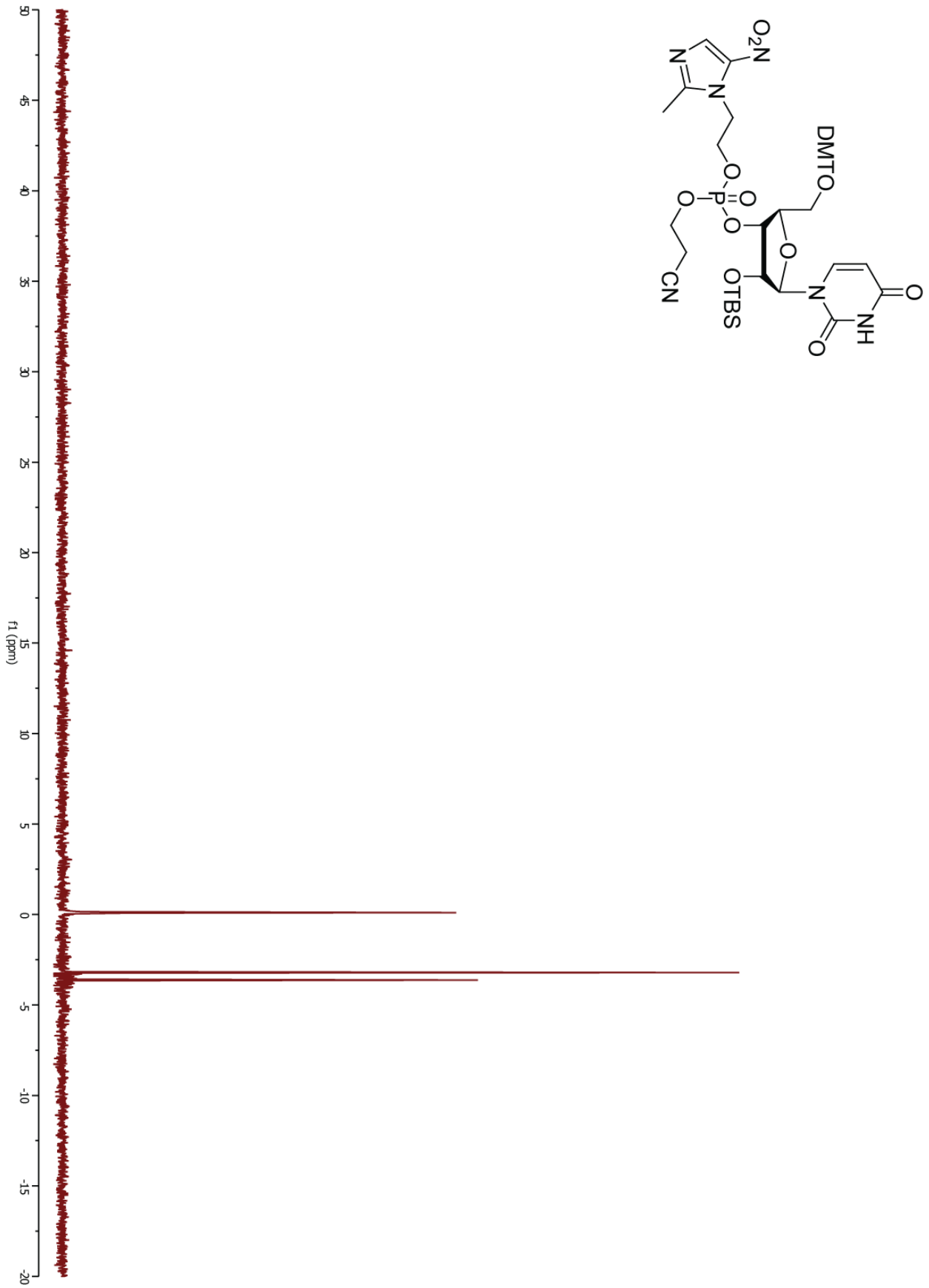
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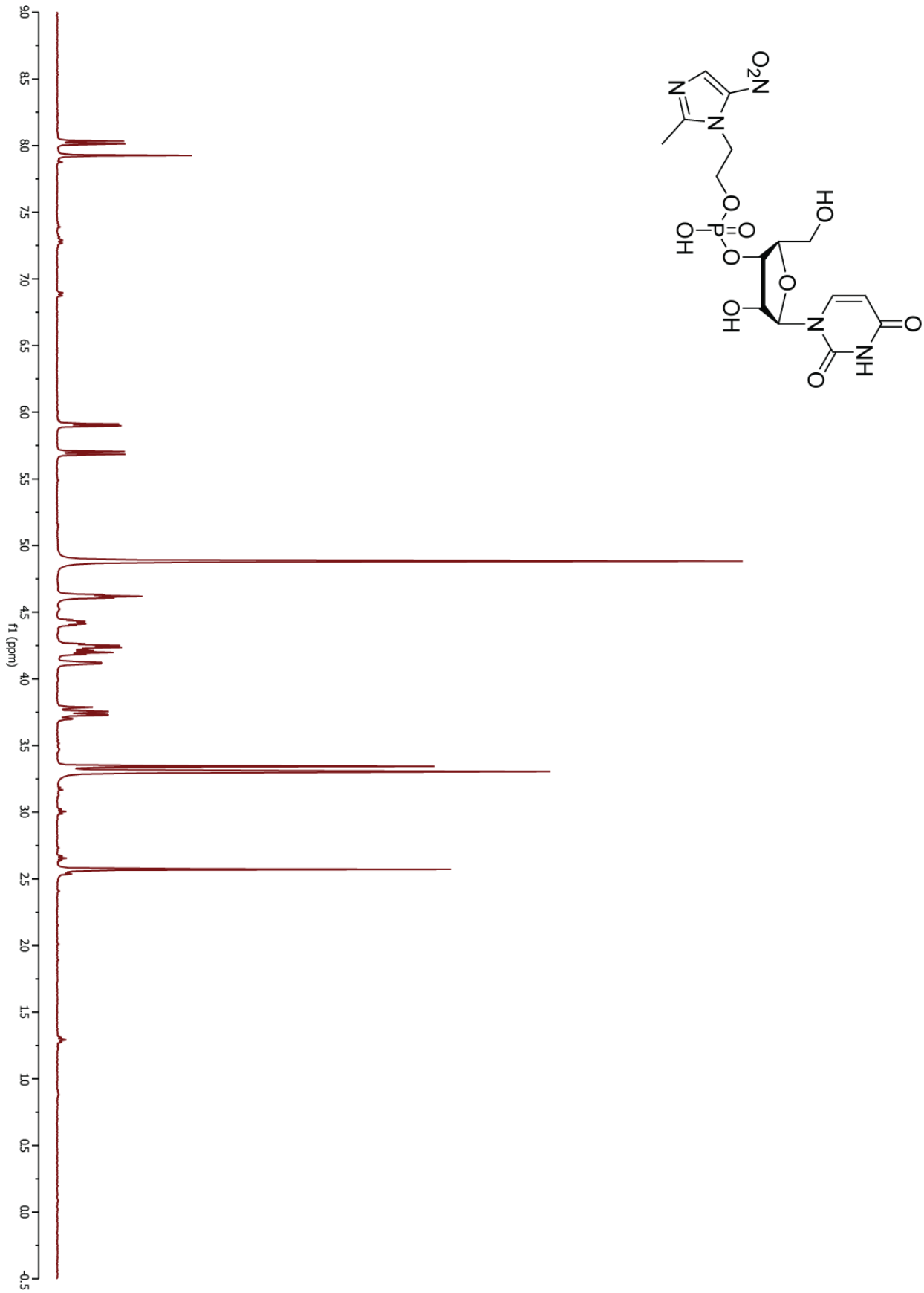
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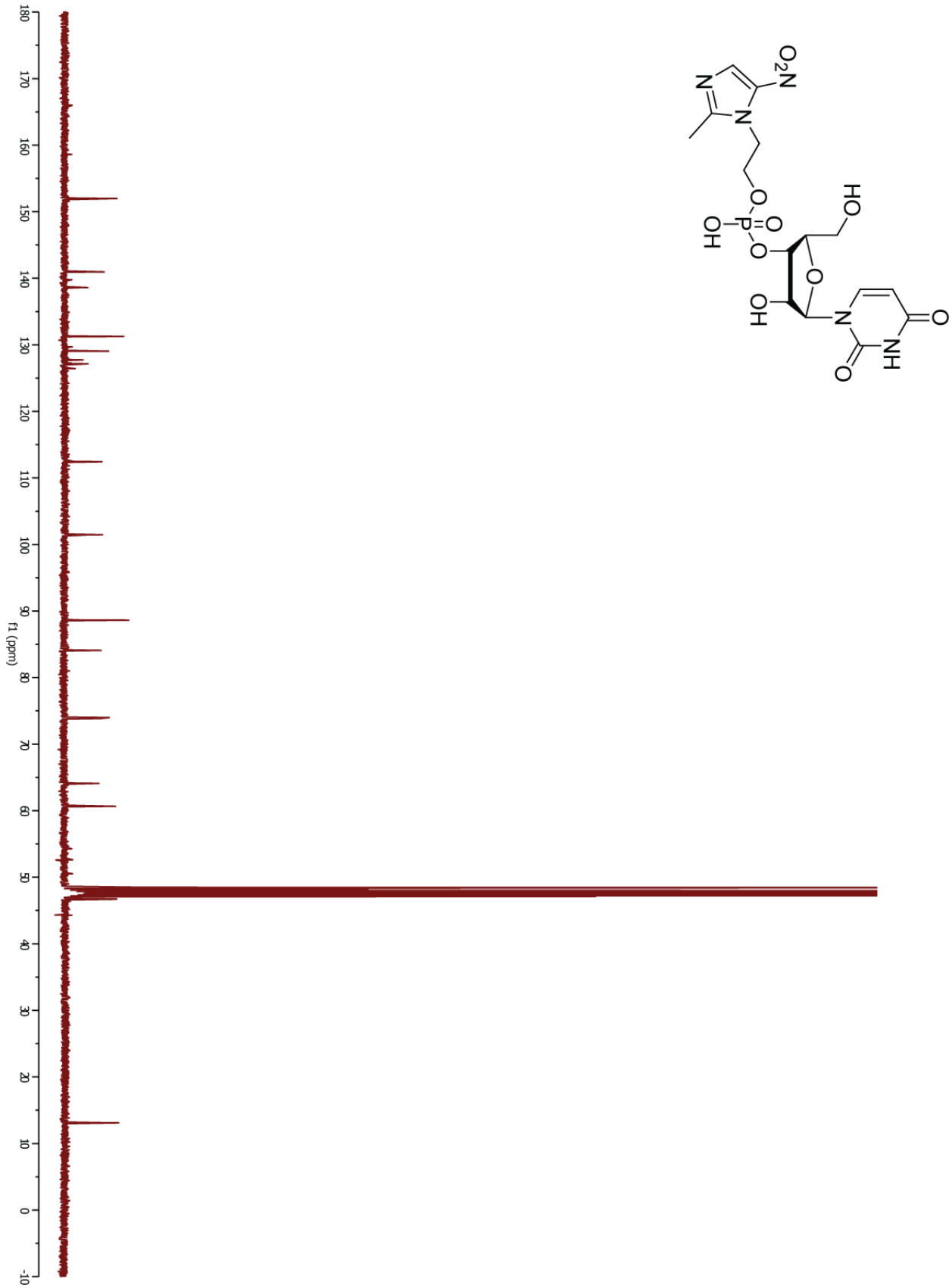
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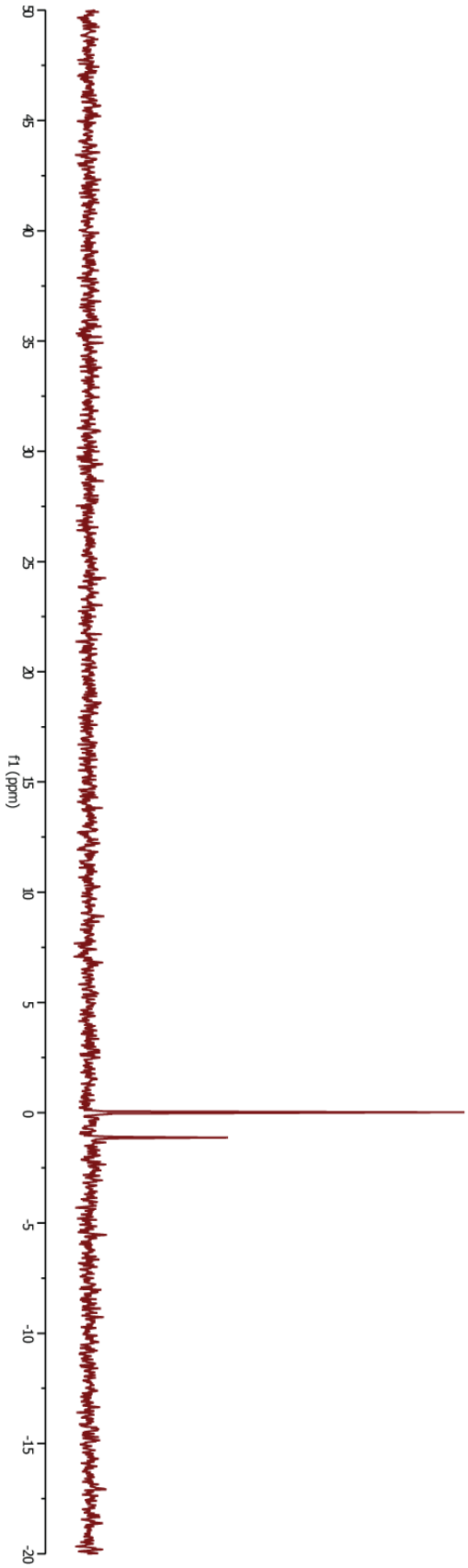
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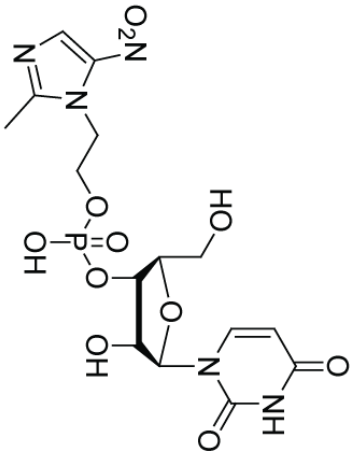
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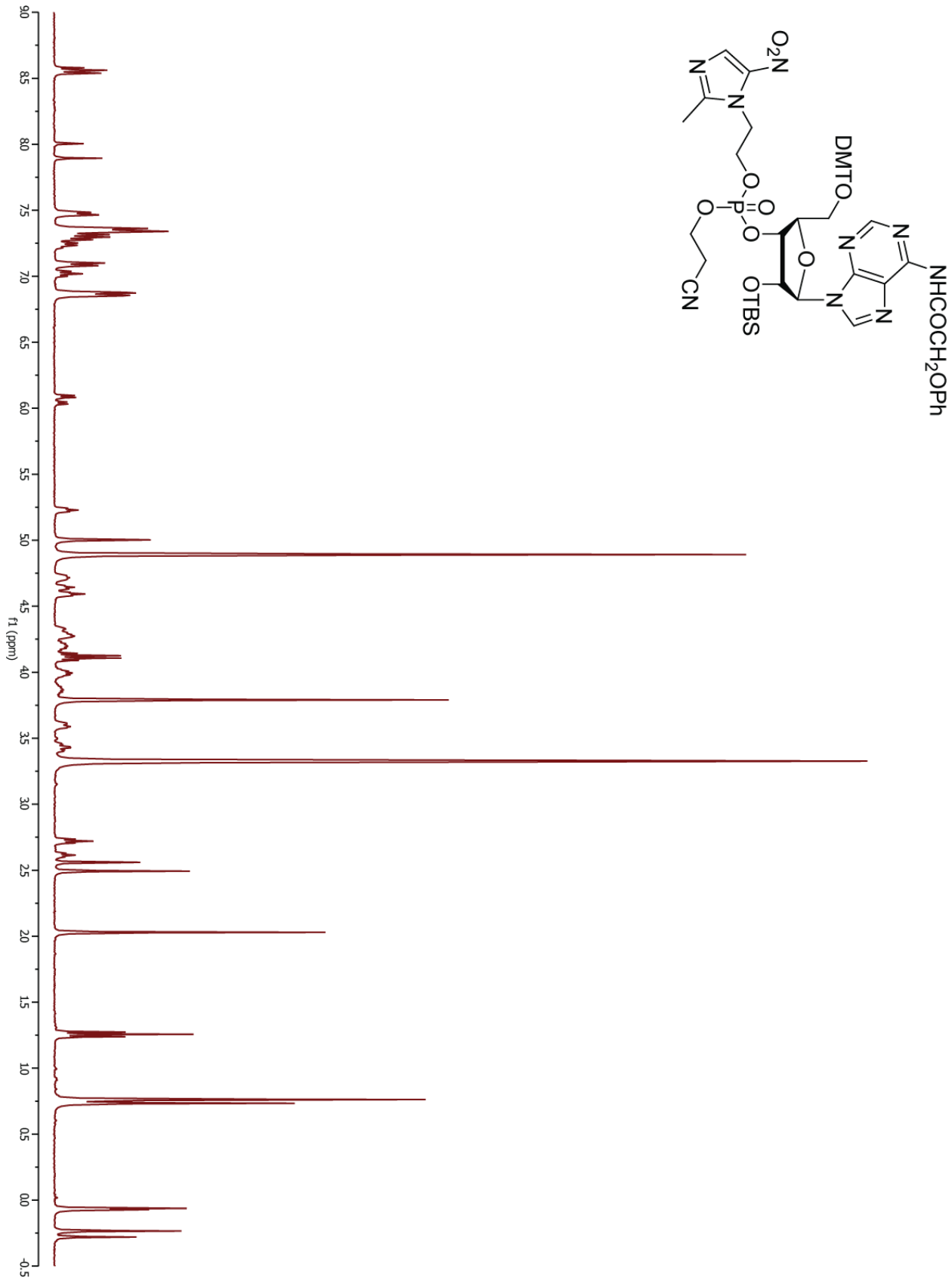
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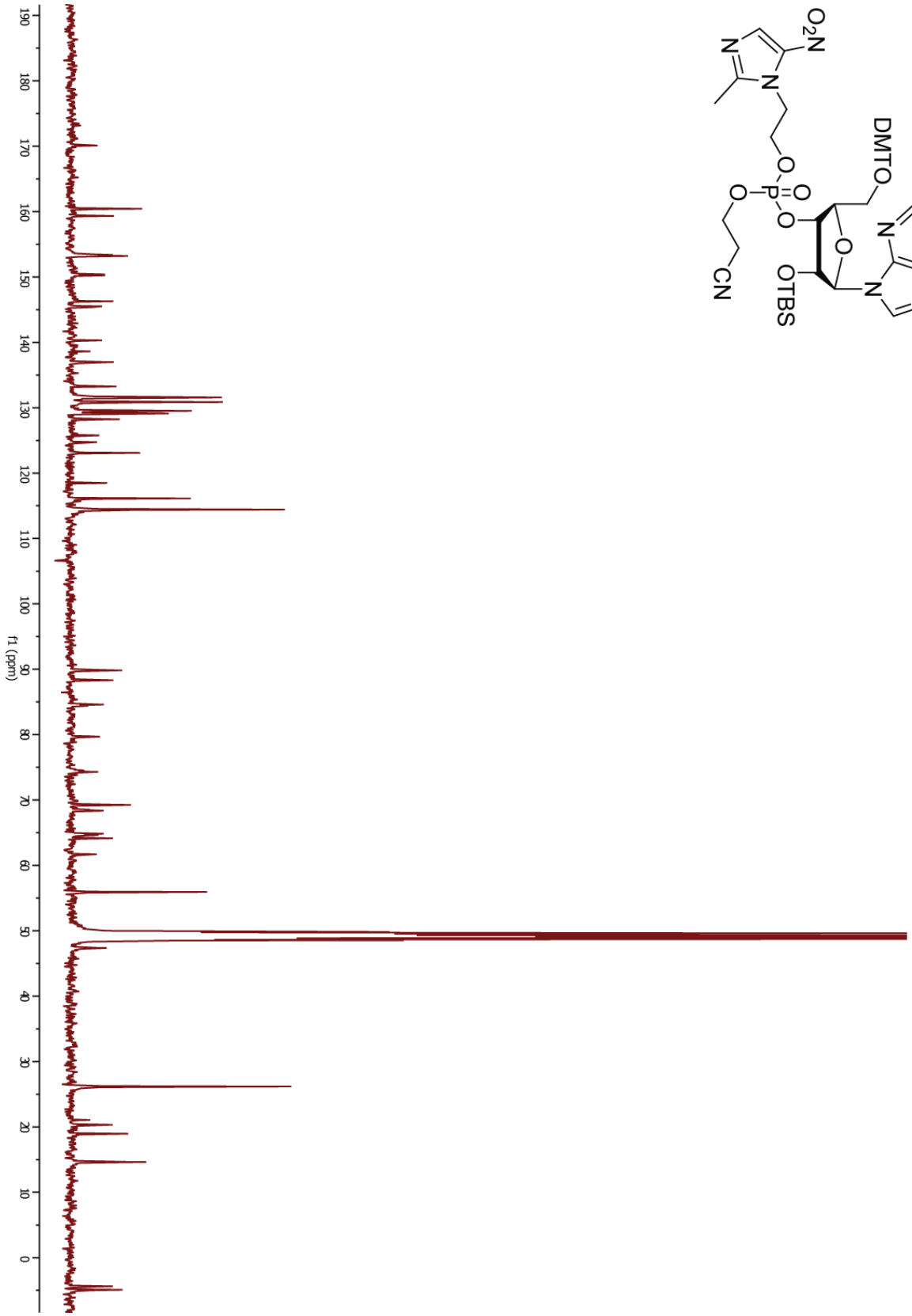
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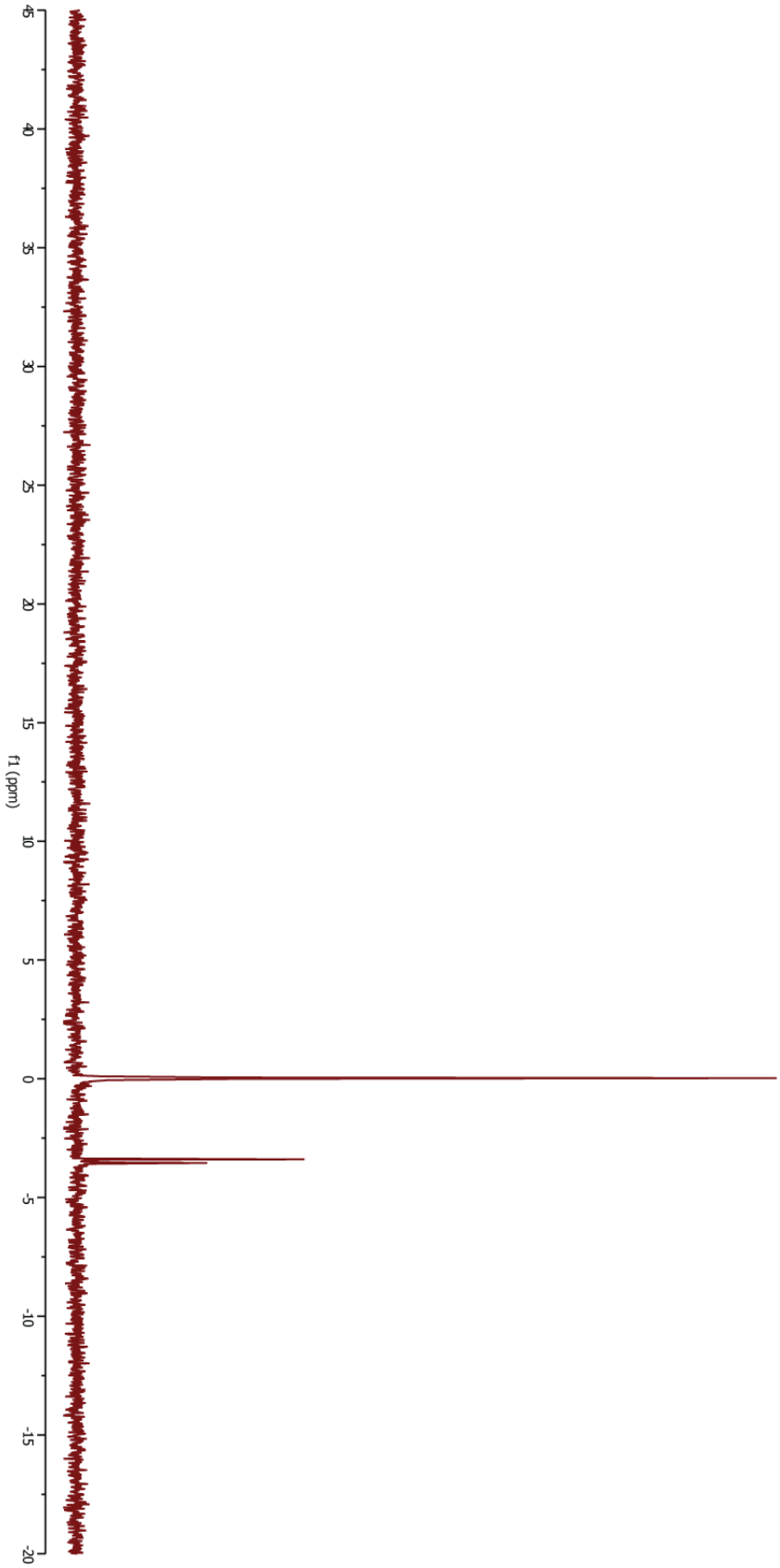
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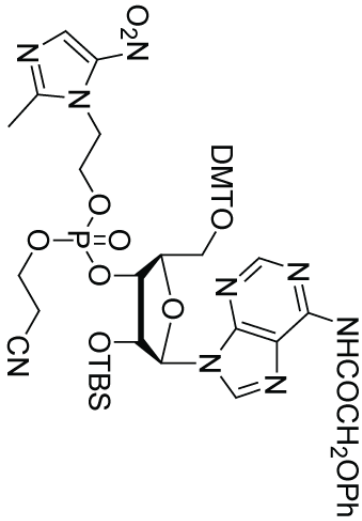
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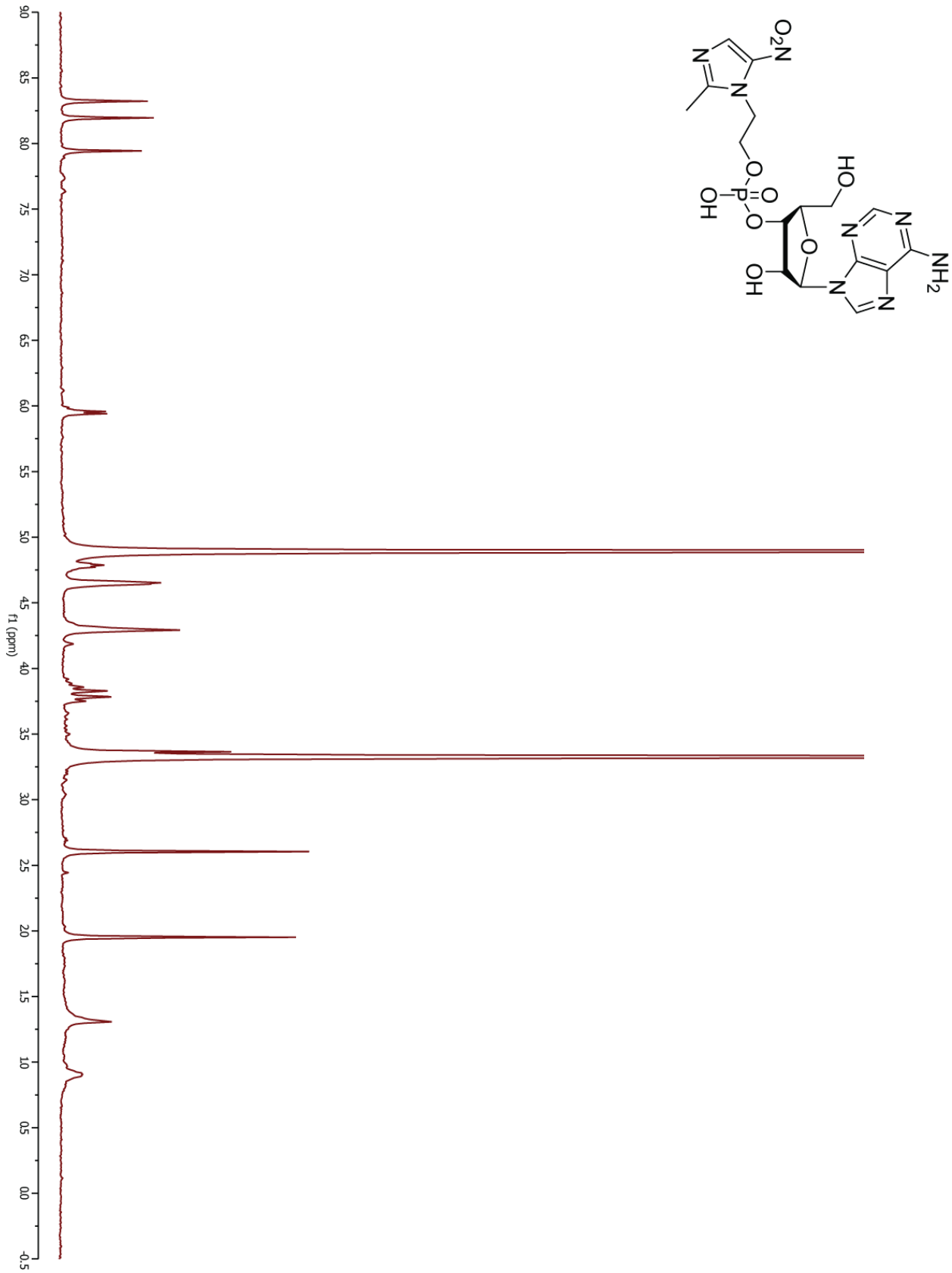
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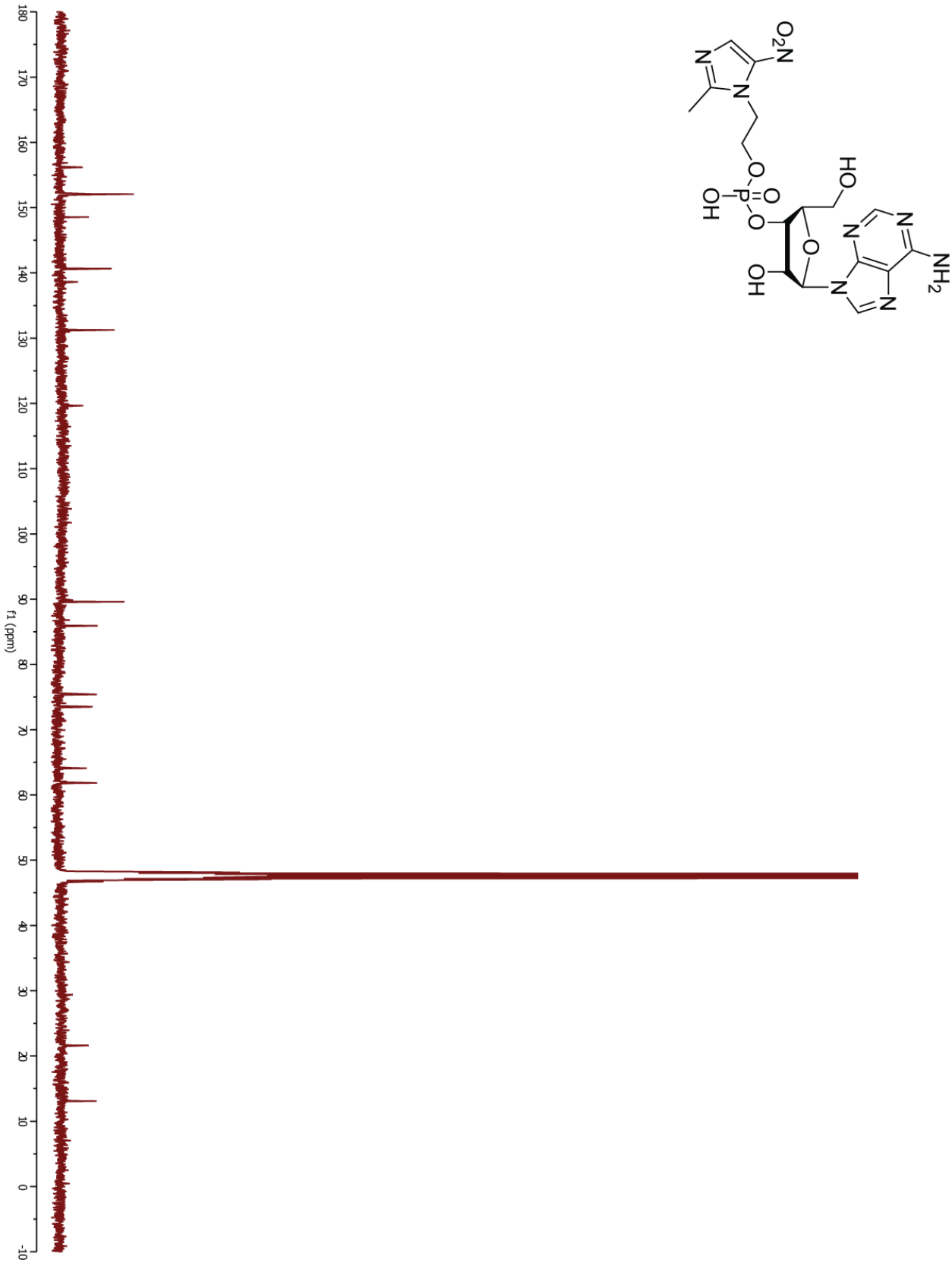
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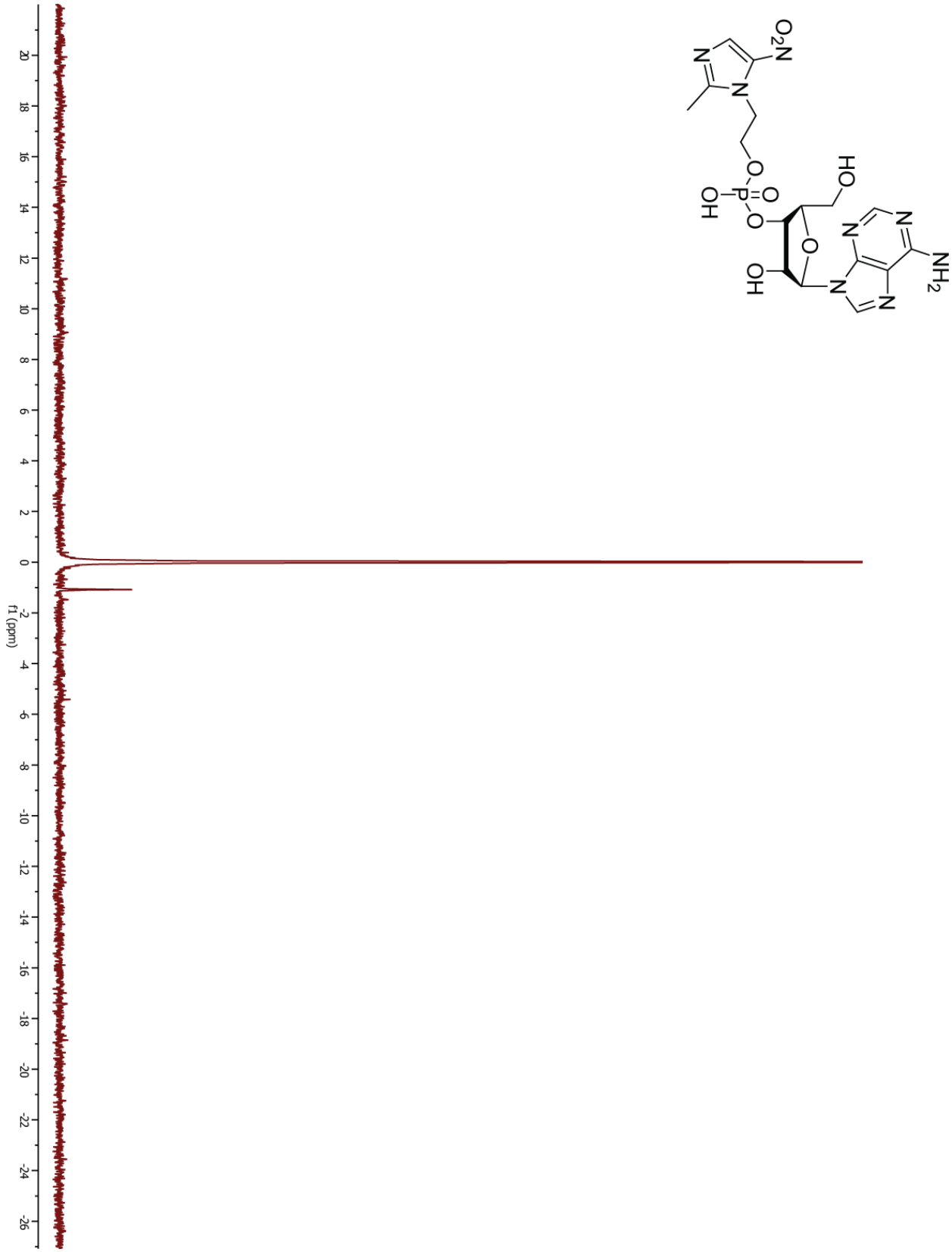
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