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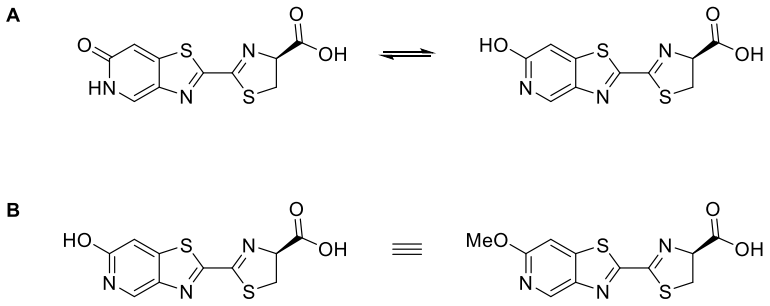
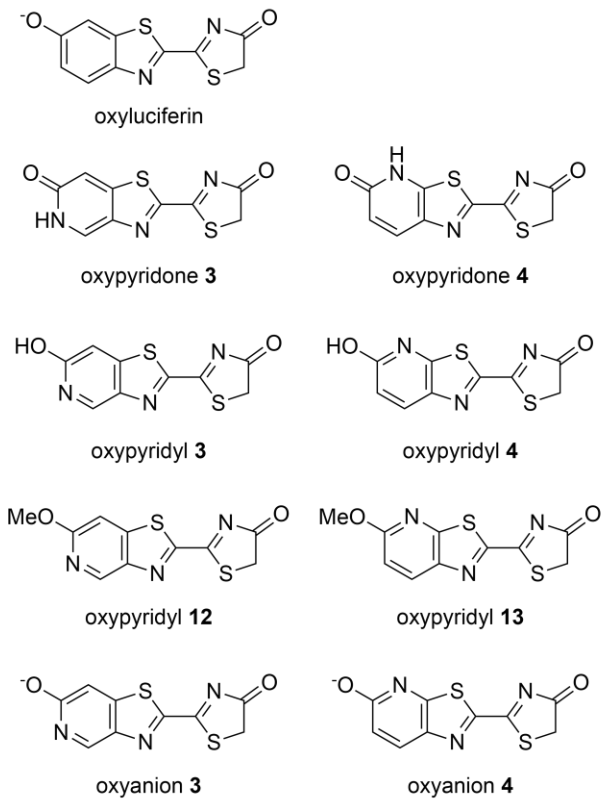


Figure S1. Pyridone luciferins and structural mimics. A) Possible tautomers for luciferin analog **3**. B) Tautomerically locked analogs mimic the hydroxypyridine isomer.

Table S1. Predicted $\Delta E_{HOMO/LUMO}$ values for luciferin analogs. $\Delta E_{HOMO-LUMO}$ values for luciferin analogs were determined using Spartan software and B3LYP 6-311 +G** level of theory.



Compound	$\Delta E_{HOMO-LUMO}$ (eV)	λ (nm)
oxyluciferin	2.19	566
oxypyridone **3**	3.08	403
oxypyridone **4**	3.55	349
oxypyridyl **3**	3.65	340
oxypyridyl **4**	3.91	317
oxypyridyl **12**	3.53	355
oxypyridyl **13**	3.84	326
oxyanion **3**	2.13	582
oxyanion **4**	2.43	510

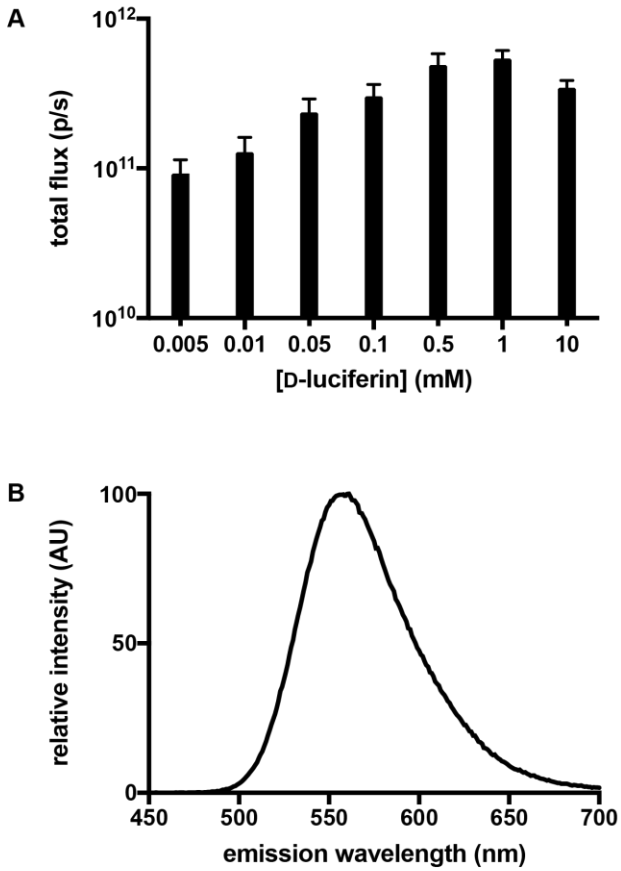


Figure S2. Recombinant Fluc is active. A) D-Luciferin (0-10 mM) was dissolved in imaging buffer, and photons were acquired one minute after Fluc (1 μg) addition. Error bars represent the standard error for the mean of n = 3 experiments. Light emission at saturating substrate conditions agrees with previous reports.[1] B) D-Luciferin exhibits maximum bioluminescence emission at 566 nm (pH 7.6), consistent with previous reports.[2]

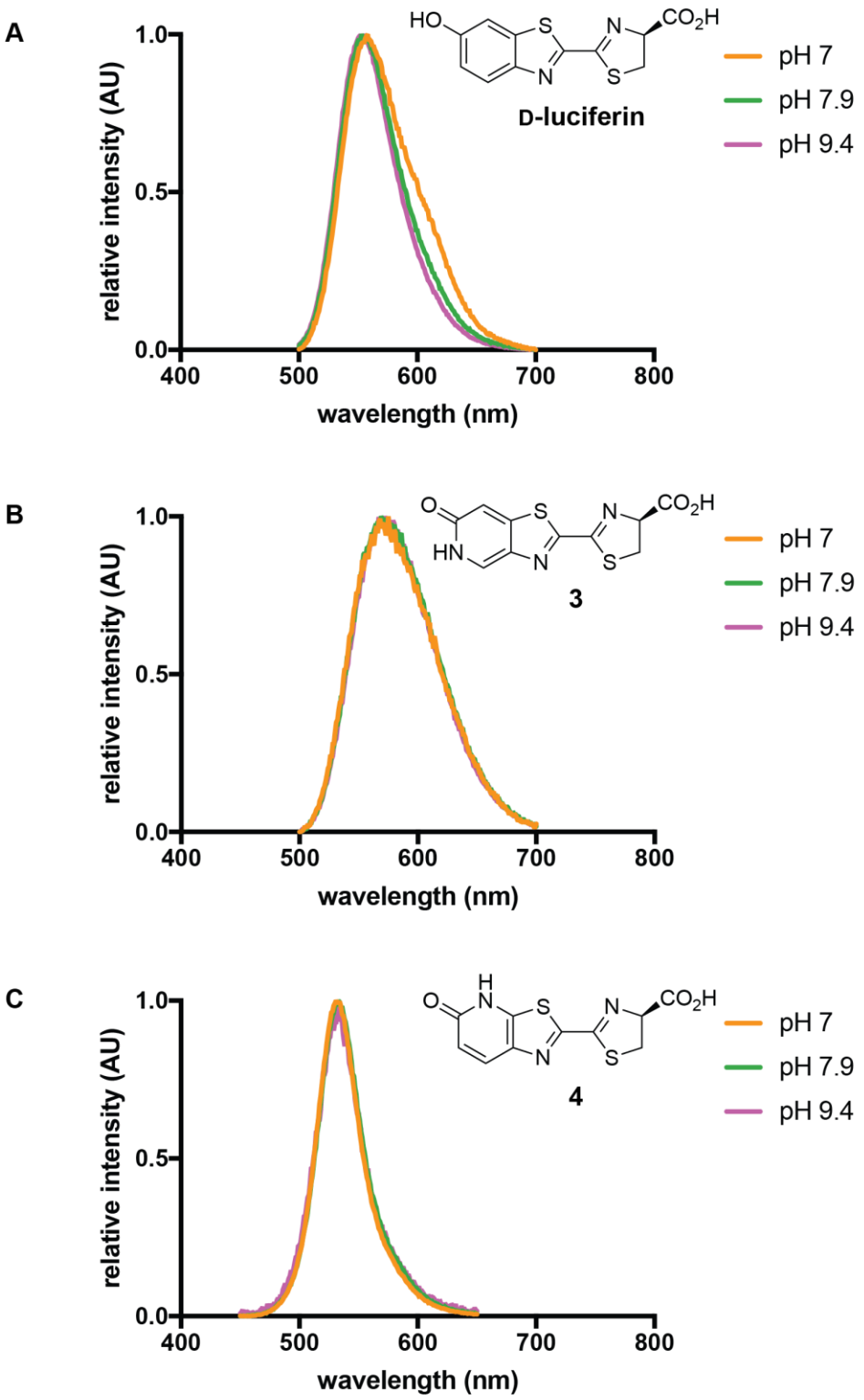
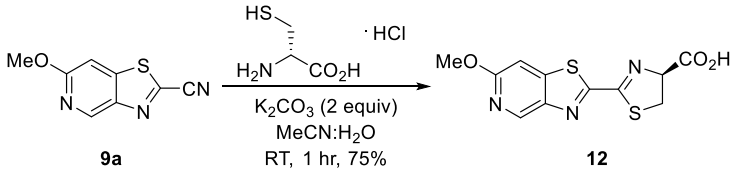


Figure S3. **Bioluminescence spectra at various pH values**. A) D-Luciferin, B) **3**, and C) **4** were dissolved in imaging buffer (50 mM Tris-HCl, 0.5 mg/ml BSA, 0.1 mM EDTA, 1 mM TCEP, 2 mM $MgSO_4$, at pH 7-9.4) with ATP (1 mM) and LiCoA (0.5 mM). Bioluminescence was initiated by the addition of Fluc (0.01 mg/mL) and spectra were acquired.

Scheme S1. Synthesis of luciferin analog **12**



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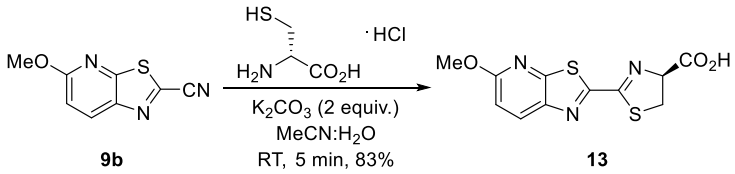
Scheme S2. Synthesis of luciferin analog **13**



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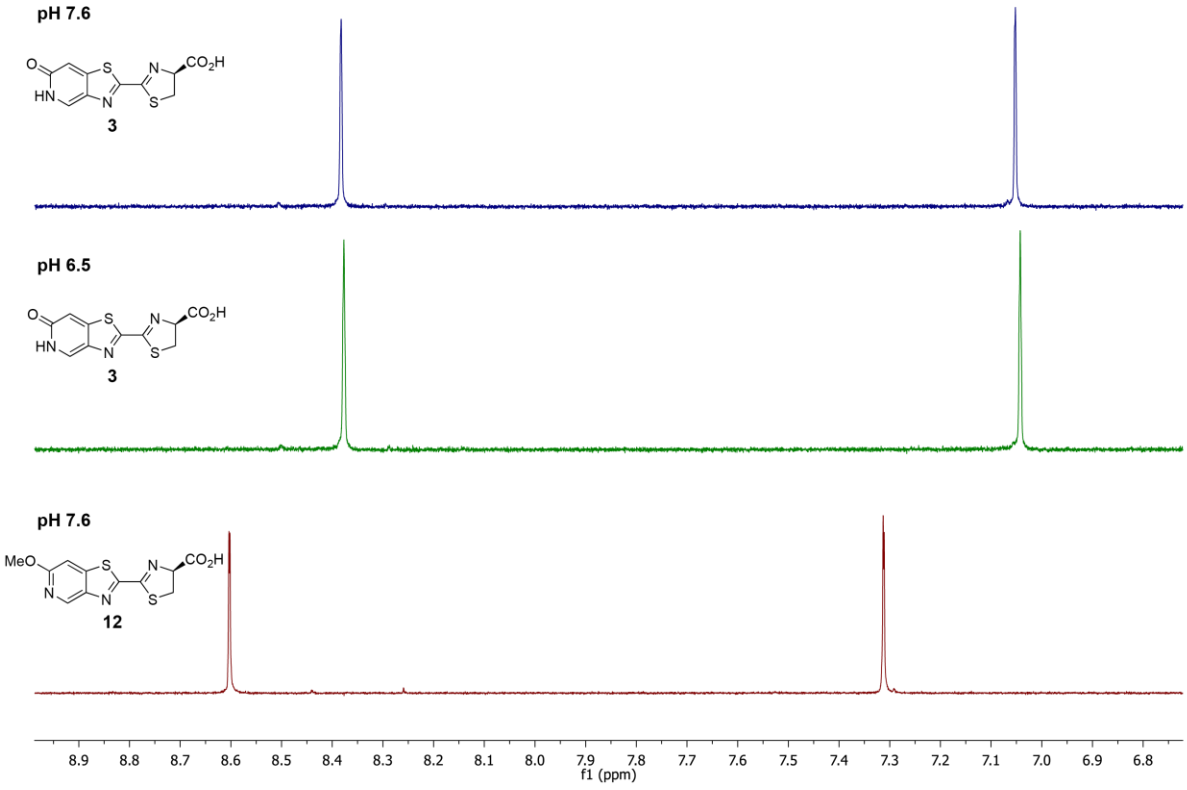


Figure S4. ^{1}H NMR spectra of luciferin analog 3 and tautomerically locked analog 12. Luciferin analogs **3** or **12** (10 mM) were dissolved in deuterated phosphate buffer (pH 6.5-7.6). NMR spectra were recorded immediately upon dissolution.

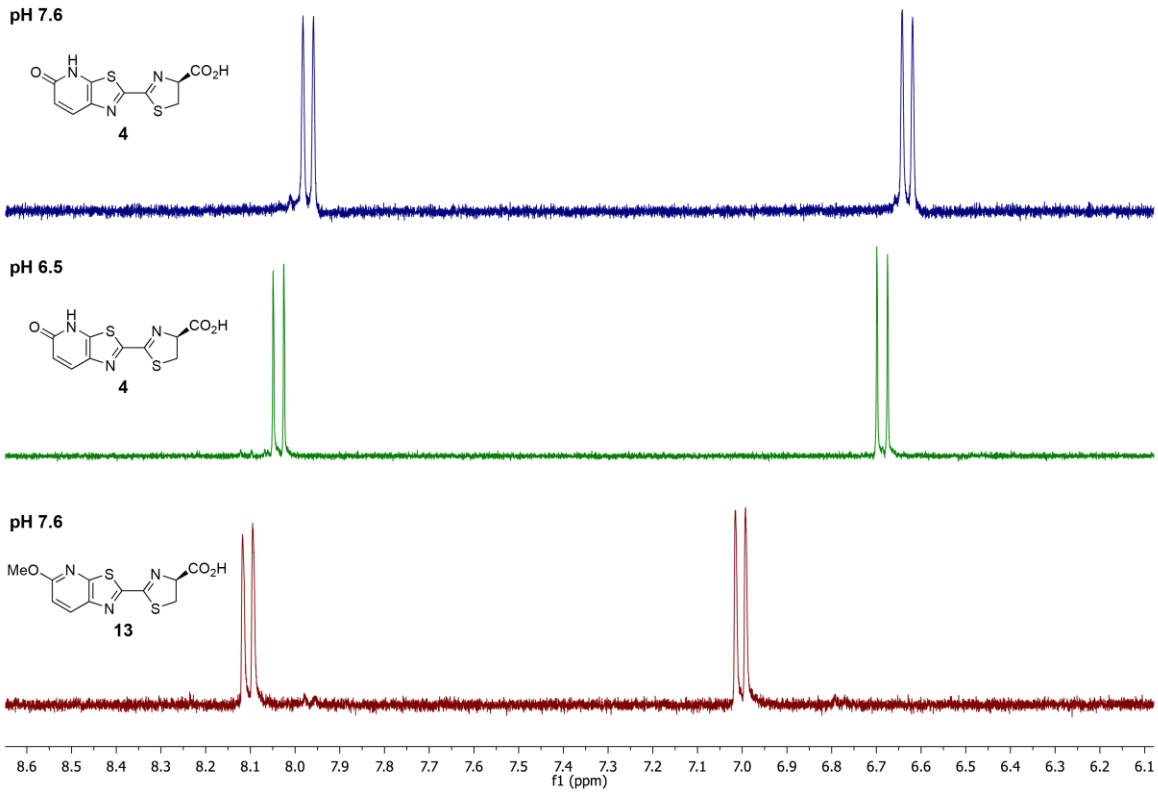


Figure S5. ^{1}H NMR spectra of luciferin analog 4 and tautomerically locked analog 13. Luciferin analogs **4** or **13** (10 mM) were dissolved in deuterated phosphate buffer (pH 6.5 or 7.6). NMR spectra were recorded immediately upon dissolution.

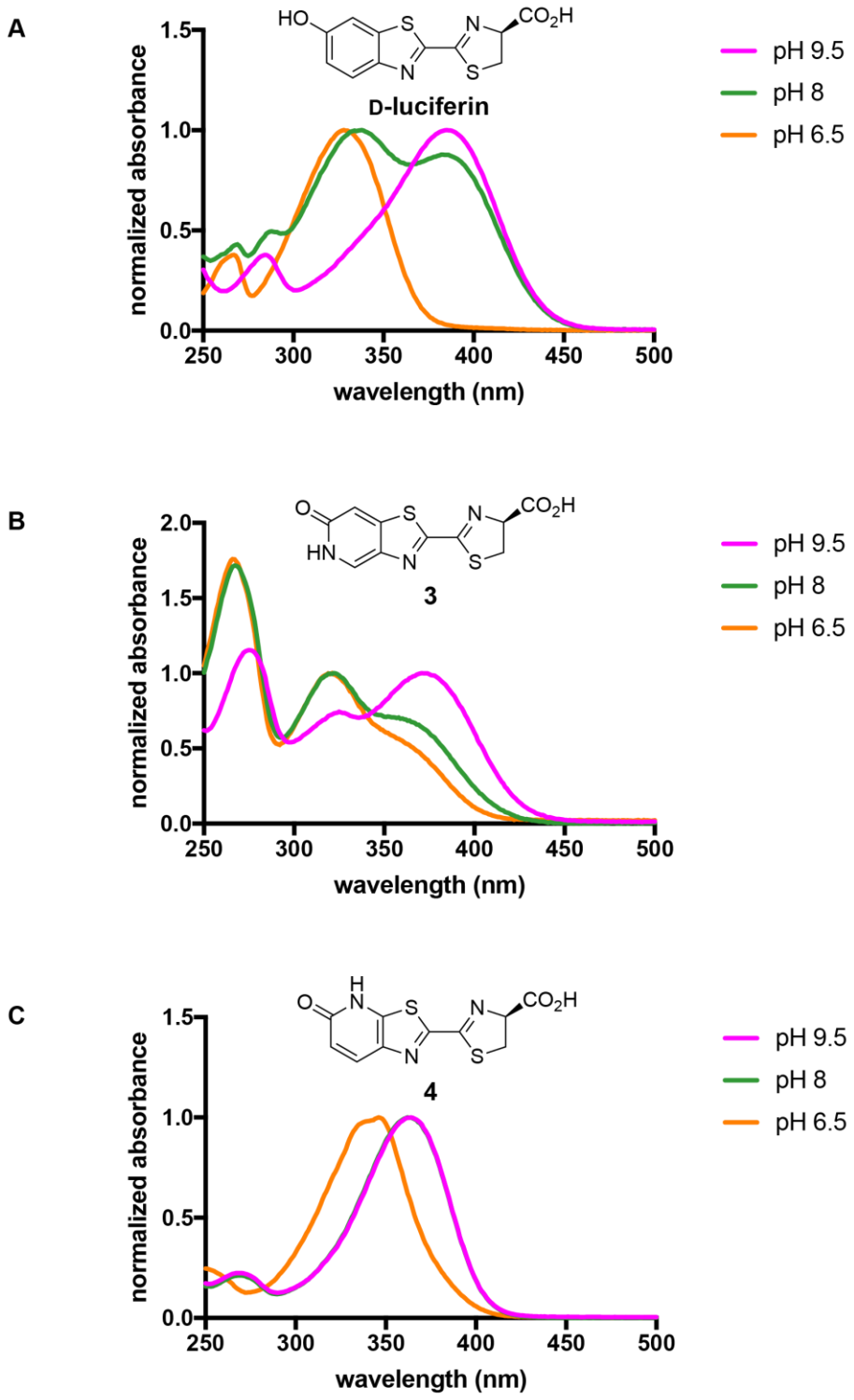


Figure S6. Absorbance data for luciferins. A) D-Luciferin and luciferin analogs B) **3** and C) **4** were dissolved in buffer (bis-tris propane 20 mM, pH 6.5-9.5). Absorbance spectra were normalized.

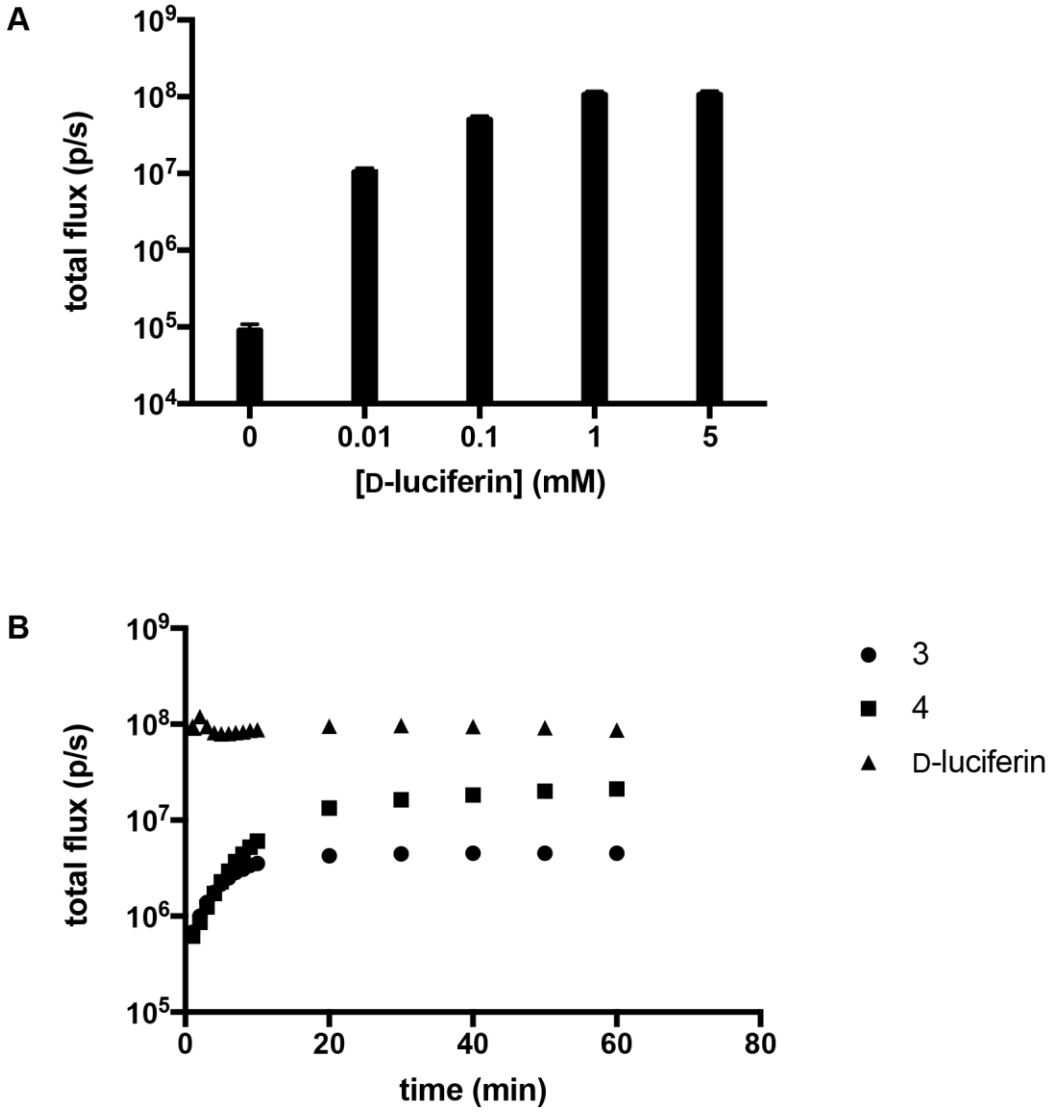


Figure S7. Bioluminescence imaging with mammalian cells. A) HEK293 cells expressing Fluc were treated with D-luciferin (0-10 mM) in PBS and imaged after 10 minutes. Error bars represent the standard error of the mean for n = 3 experiments. B) HEK293 cells expressing luciferase were treated with D-luciferin, **3** or **4** (5 mM) in PBS and photon flux was measured over 60 minutes. Error bars represent the standard error of the mean for n = 3 experiments.

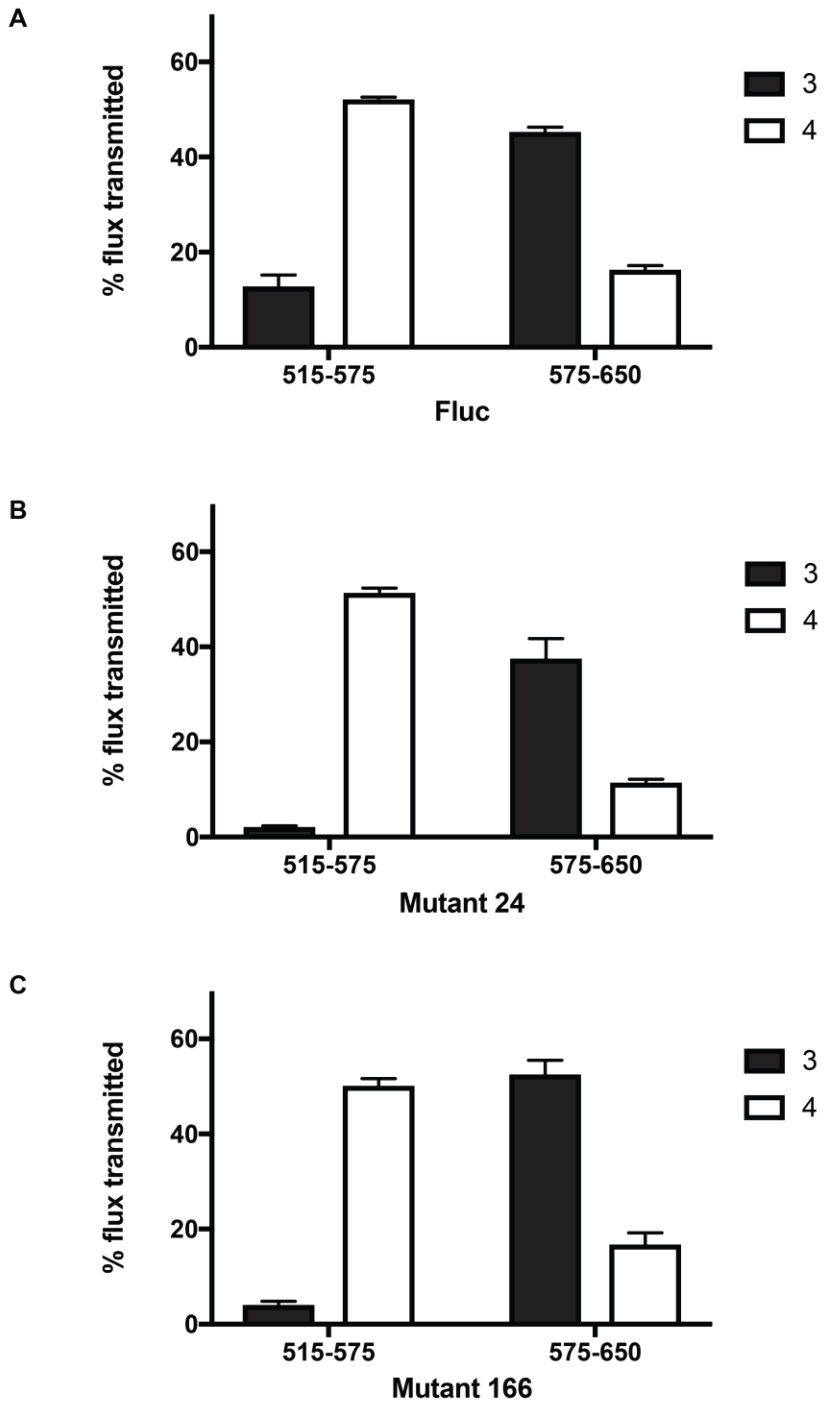


Figure S8. Spectral resolution in mammalian cells. HEK293 cells expressing A) Fluc, B) mutant 24, or C) mutant 166, were imaged upon incubation with **3** or **4** (1 mM). Optical filters (515-575 nm or 575-650 nm) were used during the acquisition. Photon outputs acquired with the filters were divided by the total luminescence (no filter) to afford "% flux transmitted". Error bars represent the standard error of the mean for $n \geq 3$ experiments. Bioluminescent signals were also normalized to luciferase expression levels (measured via flow cytometry).

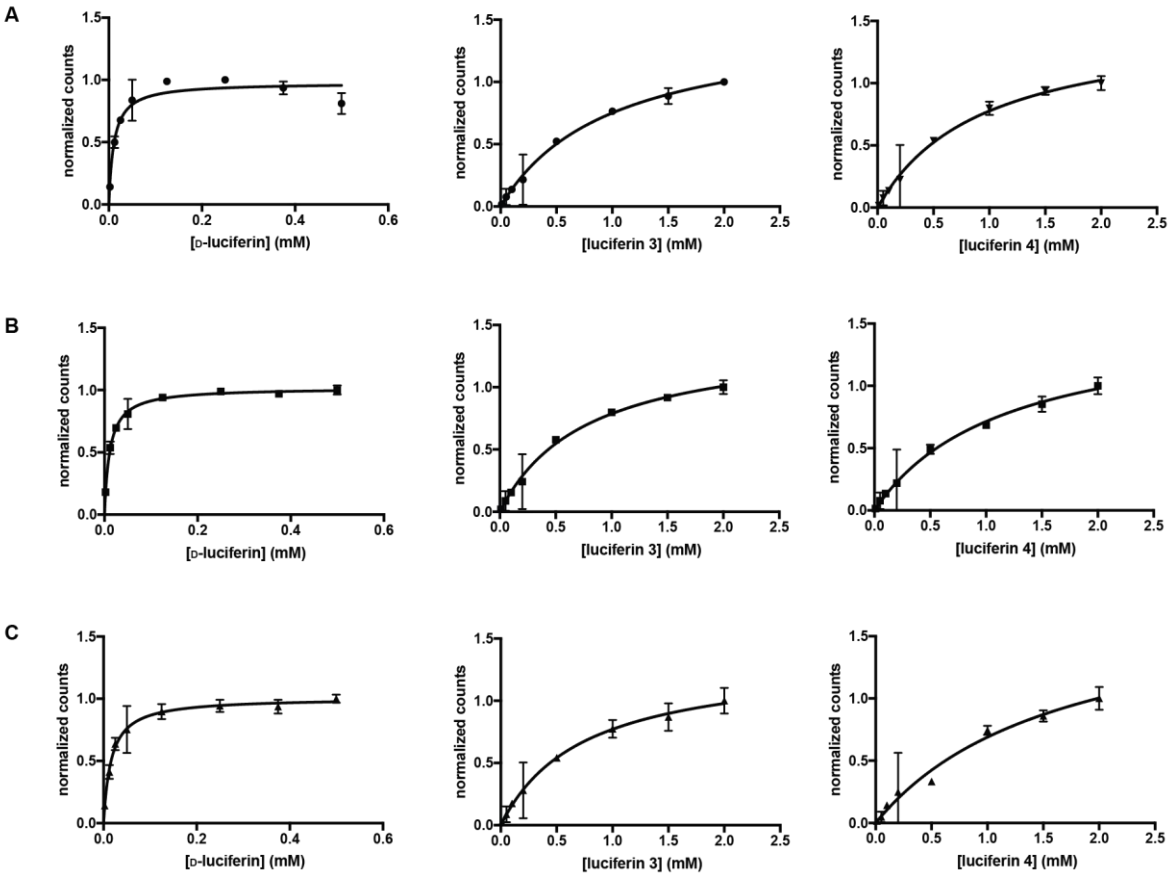


Figure S9. Initial rates of light emission for Fluc or mutant luciferases with D-luciferin or luciferin analogs. A) Fluc, B) mutant 24, and C) mutant 166 were incubated with D-luciferin or luciferin analogs **3** or **4** (0.01 – 2 mM) and light emission values were recorded. Data were then fit according to Michaelis-Menten kinetics and binding constants (K_m) were obtained. Error bars represent the standard error of the mean for n = 9 experiments.

Table S2. Enzymatic parameters for Fluc.

Km (mM)[a]	Compound
0.011 ± 0.003	**D-luc**
0.98 ± 0.09	**3**
0.94 ± 0.10	**4**

[a] Kinetic constants are apparent values, determined via measurements of initial rates of light emission. Apparent K_m values for D-luciferin and Fluc agree with reported values.[3]

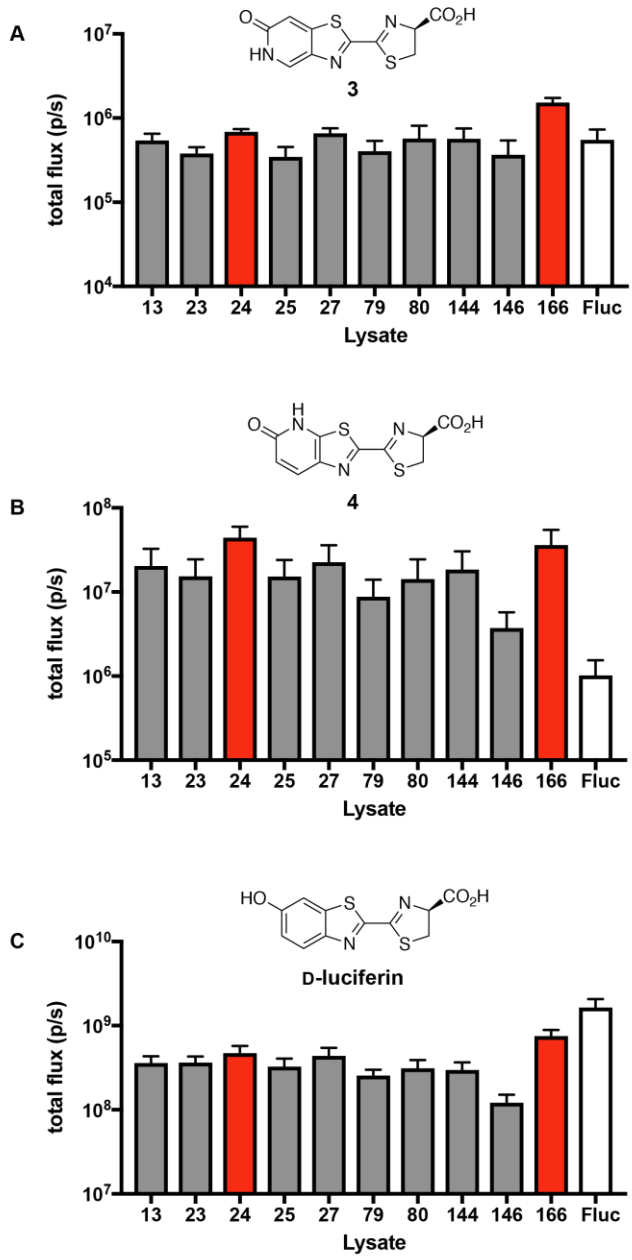


Figure S10. Mutant enzymes provide greater photon outputs with analog 4 in bacterial lysate. Bacteria expressing mutant 24 or 166 (red bars), other mutant enzymes (grey bars) or Fluc (white bars) were selected from primary screens, expanded, and lysed. Lysates were imaged with the luciferins shown. Error bars represent the standard error of the mean for n = 3 experiments.

Table S3. Sequencing analysis of "bright" mutants.

Mutant	Sequence
13	M249F, T252S, F295L, S314T, G316T, A326V, P334S
23	V240A, M249L, L264F, S314T, G316T, K321R
24	M249L, Q283R, S314T, G316T
25	I226V, V240A, M249L, I282T, F295L, S314T, G316T
27	I232T, M249L, S314T, G316T
79	M249L, S314T, G316S
80	M249L, S314T, G316T
144	V241A, F247Y, S314T, G316T
146	V241A, F247L, S314T, G316T
166	M249L, I257F, F295L, S314T, G316T, A326V, P334S

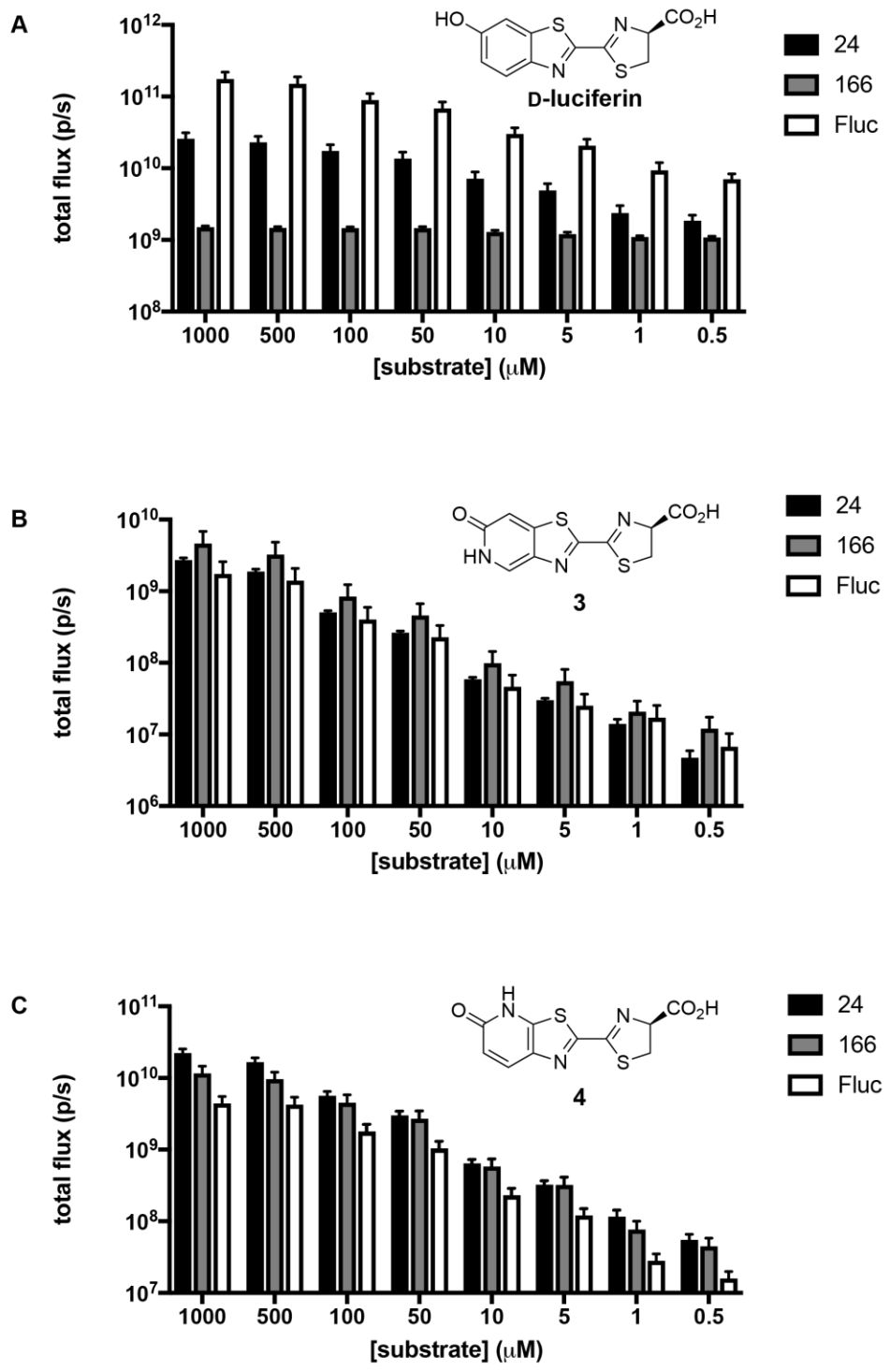


Figure S11. Bioluminescent photon production from "bright" luciferase enzymes and luciferin analogs. A) D-Luciferin or luciferin analogs B) **3** or C) **4** (0.5 – 1000 μM) were incubated with purified mutant luciferases (24 and 166) or native Fluc (and ATP) and light emission was quantified. Error bars represent the standard error of the mean for n ≥ 3 experiments.

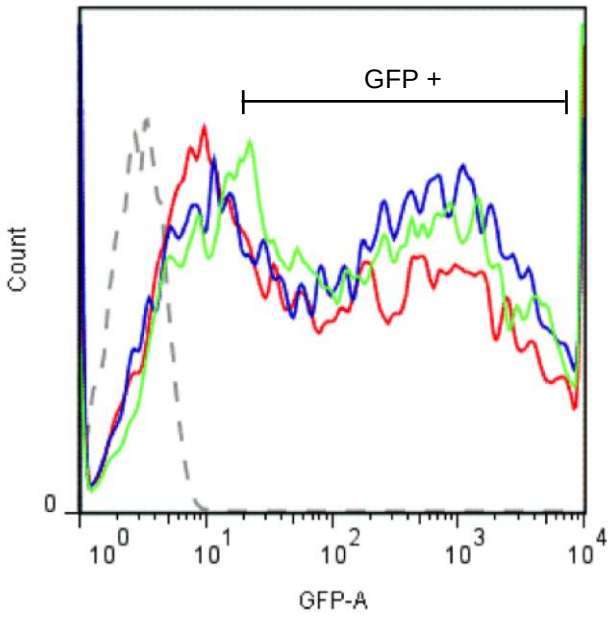
Table S4. Enzymatic parameters for mutant 24.

Km (mM)[a]	Compound
0.0116 ± 0.0005	D-luc
0.78 ± 0.08	**3**
1.17 ± 0.13	**4**

Table S5. Enzymatic parameters for mutant 166.

Km (mM)[a]	Compound
0.0163 ± 0.0011	D-luc
0.74 ± 0.05	**3**
1.7 ± 0.5	**4**

[a] Kinetic constants are apparent values, determined via measurements of initial rates of light emission. Apparent Km values for D-luciferin and Fluc agree with reported values.[3]



	Sample name	Percent GFP Positive
	Mock	0.01
	Fluc	65.13
	mutant 24	69.37
	mutant 166	71.22

Figure S12. Luciferase expression was measured in cellular samples. HEK293 cells transiently expressing luciferase (as an IRES-GFP fusion) were analyzed by flow cytometry. Cells exhibiting ≥ 10 fold higher GFP fluorescence intensity (GFP-A) than mock cells were considered GFP positive (GFP +). GFP positive cells were counted and compared to the entire population to determine percent positive values for normalization. A representative plot is shown.

References

1. Jones, K. A.; Porterfield, W. B.; Rathbun, C. M.; McCutcheon, D. C.; Paley, M. A.; Prescher, J. A., Orthogonal Luciferase–Luciferin Pairs for Bioluminescence Imaging. *J Am Chem Soc* **2017,** *139* (6), 2351-2358.
2. Branchini, B. R.; Murtiashaw, M. H.; Magyar, R. A.; Portier, N. C.; Ruggiero, M. C.; Stroh, J. G., Yellow-green and red firefly bioluminescence from 5,5-dimethyloxyluciferin. *J Am Chem Soc* **2002,** *124* (10), 2112-2113.
3. Branchini, B. R.; Southworth, T. L.; Murtiashaw, M. H.; Boije, H.; Fleet, S. E., A mutagenesis study of the putative luciferin binding site residues of firefly luciferase. *Biochemistry* **2003,** *42* (35), 10429-10436.

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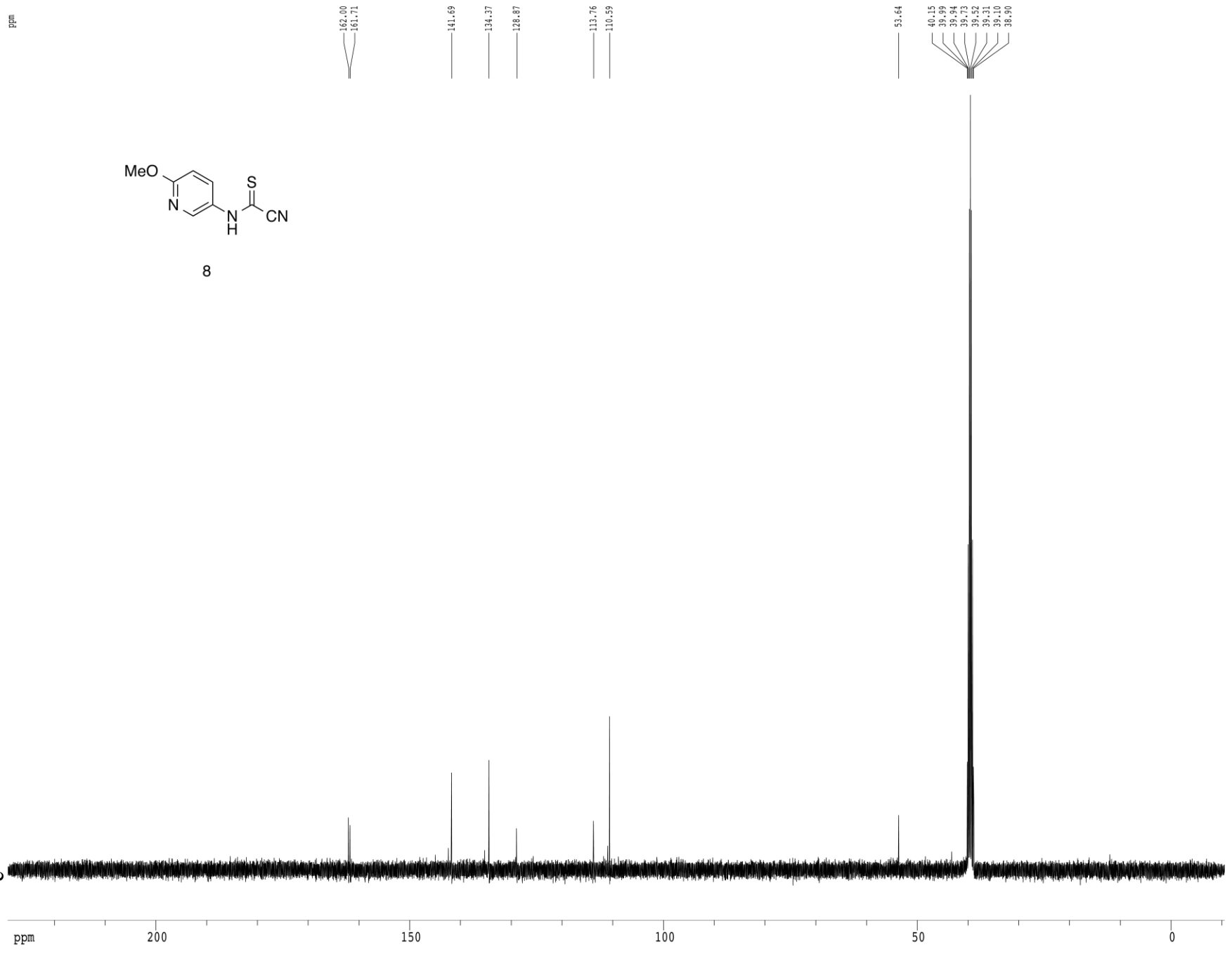
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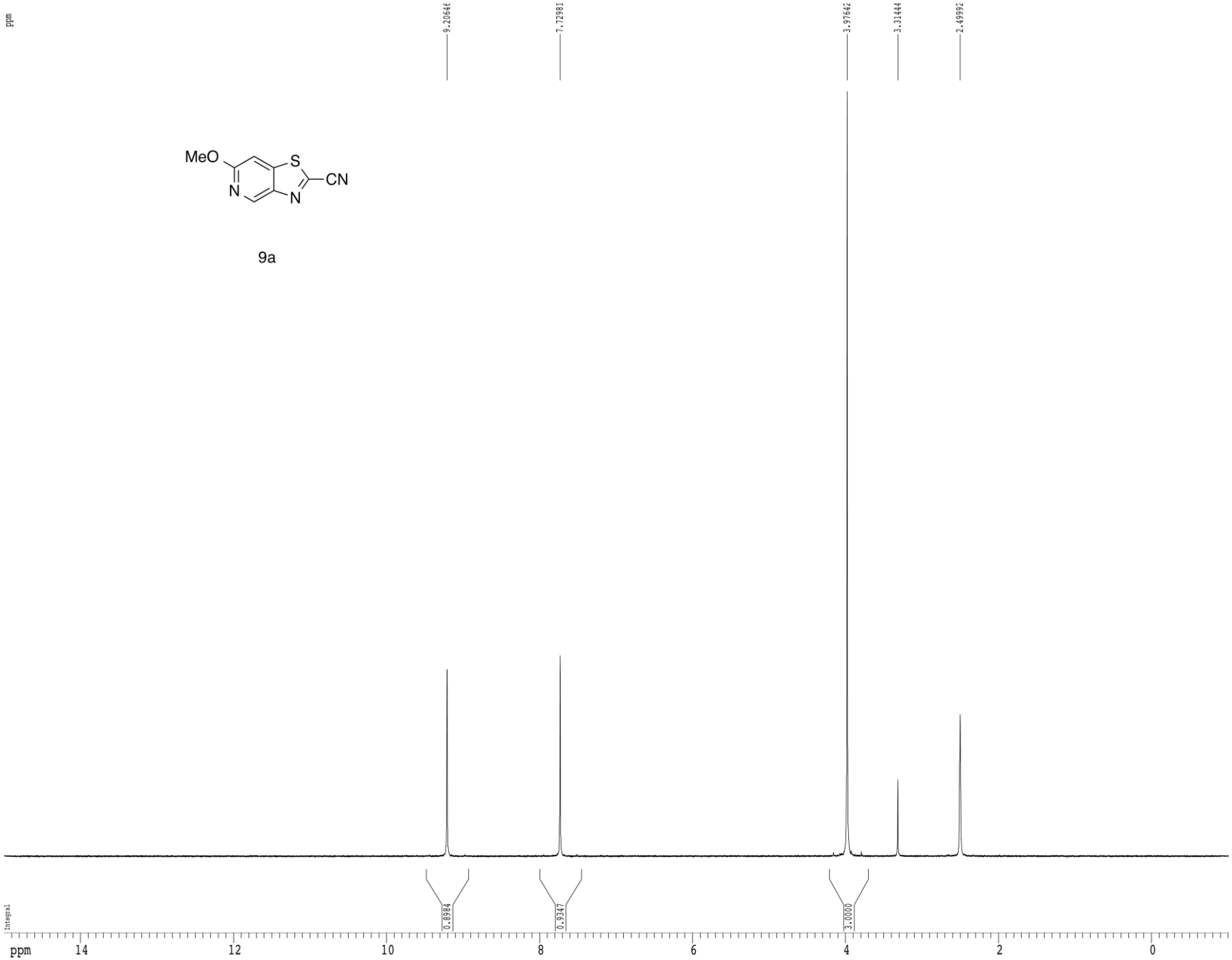
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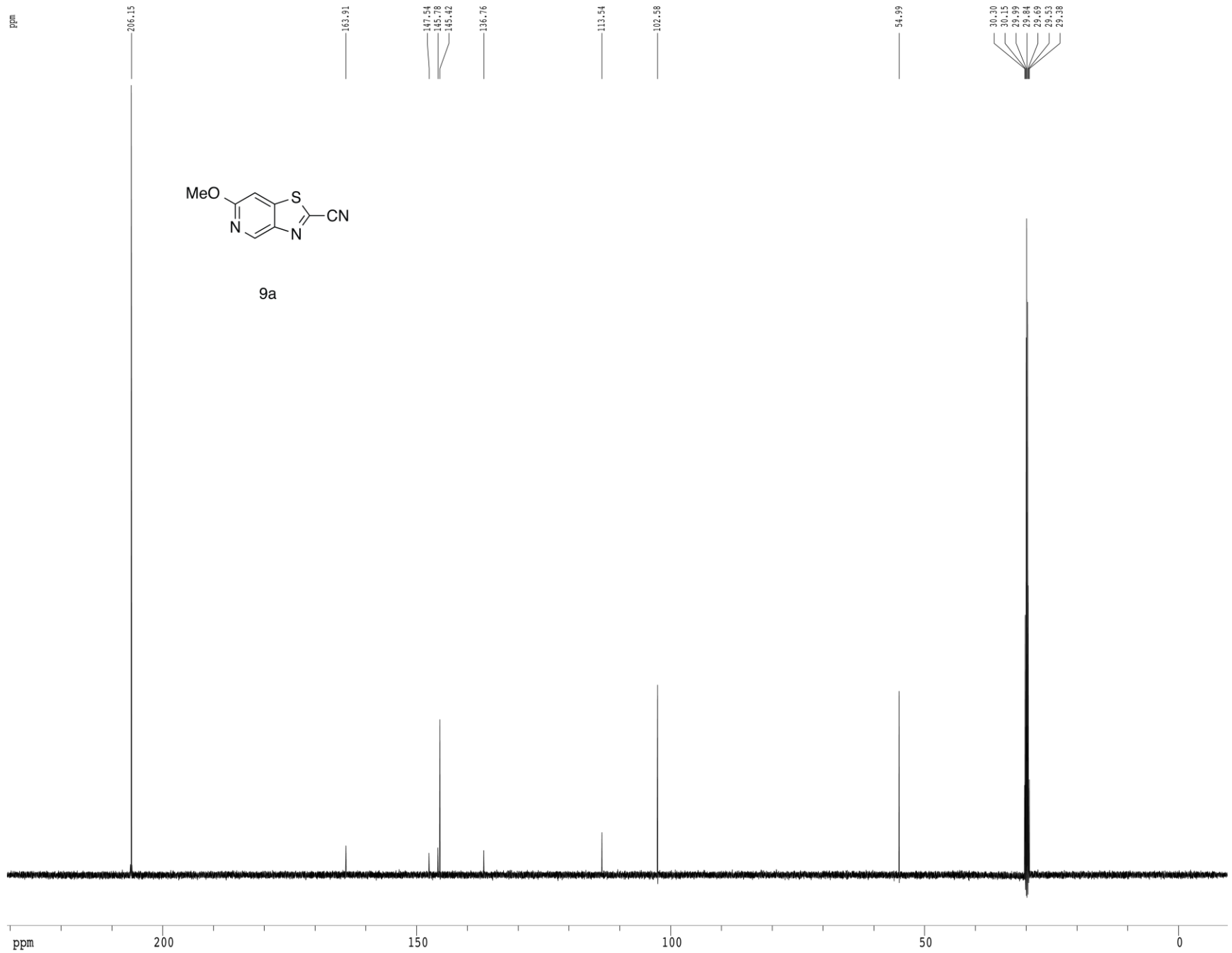
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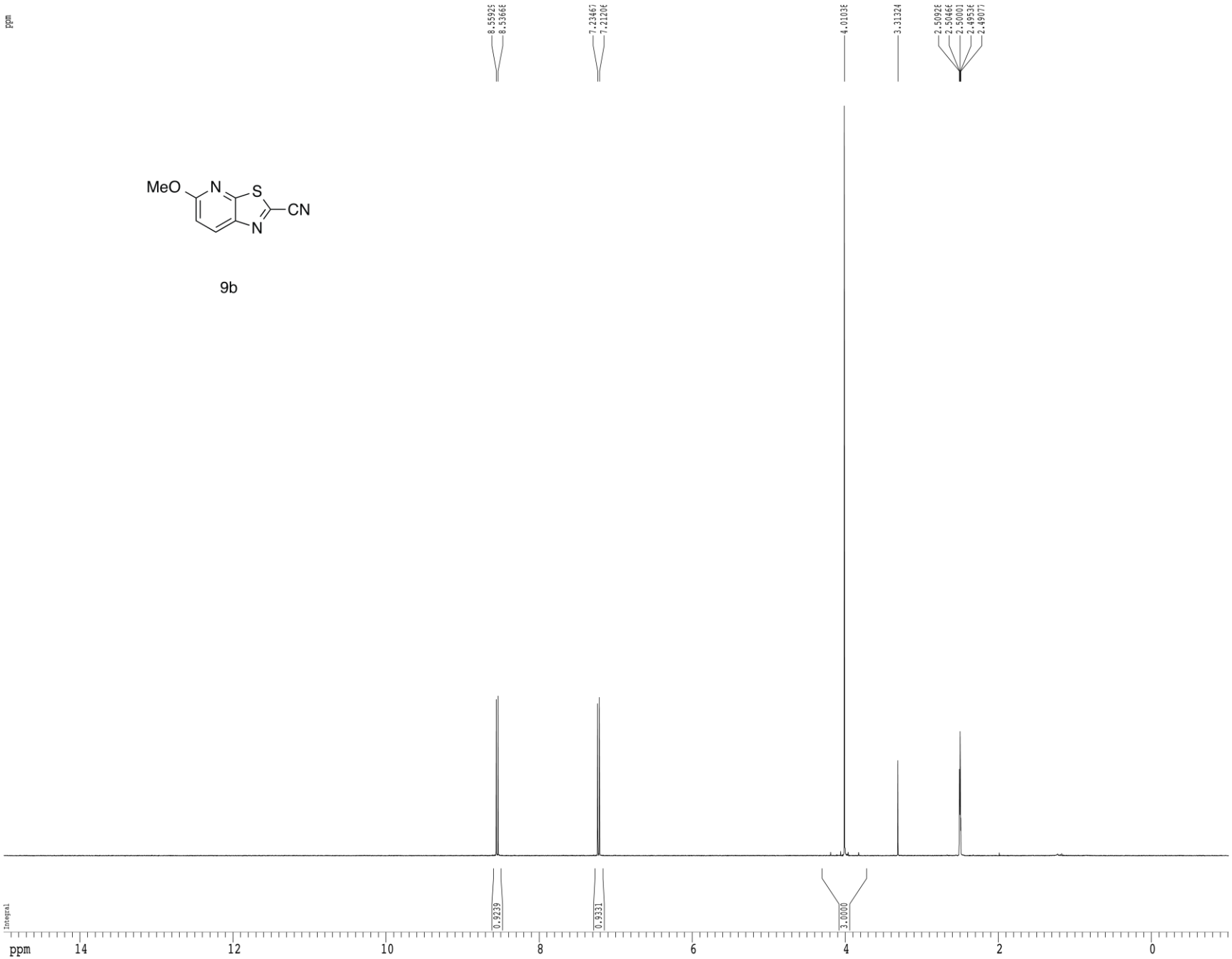
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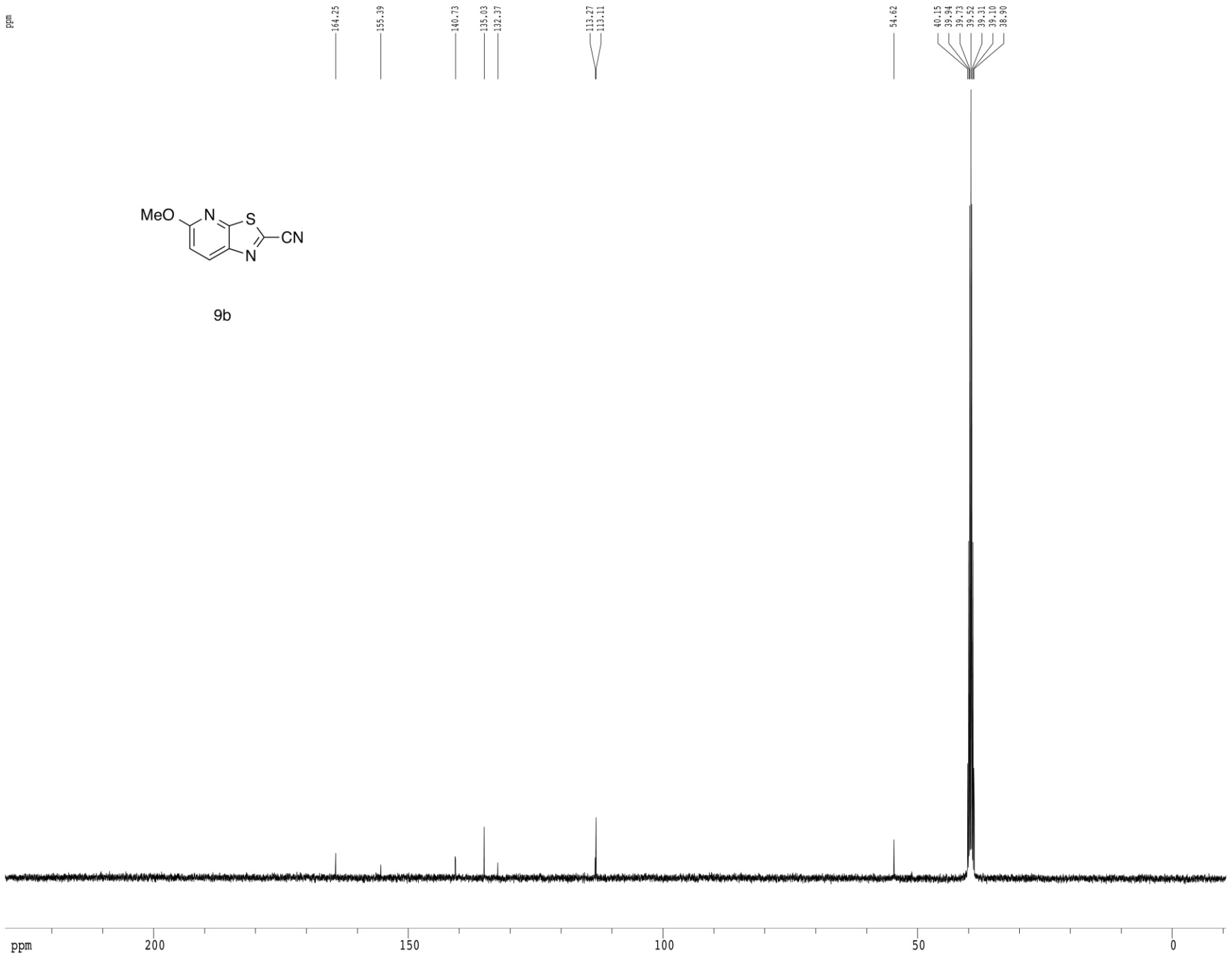
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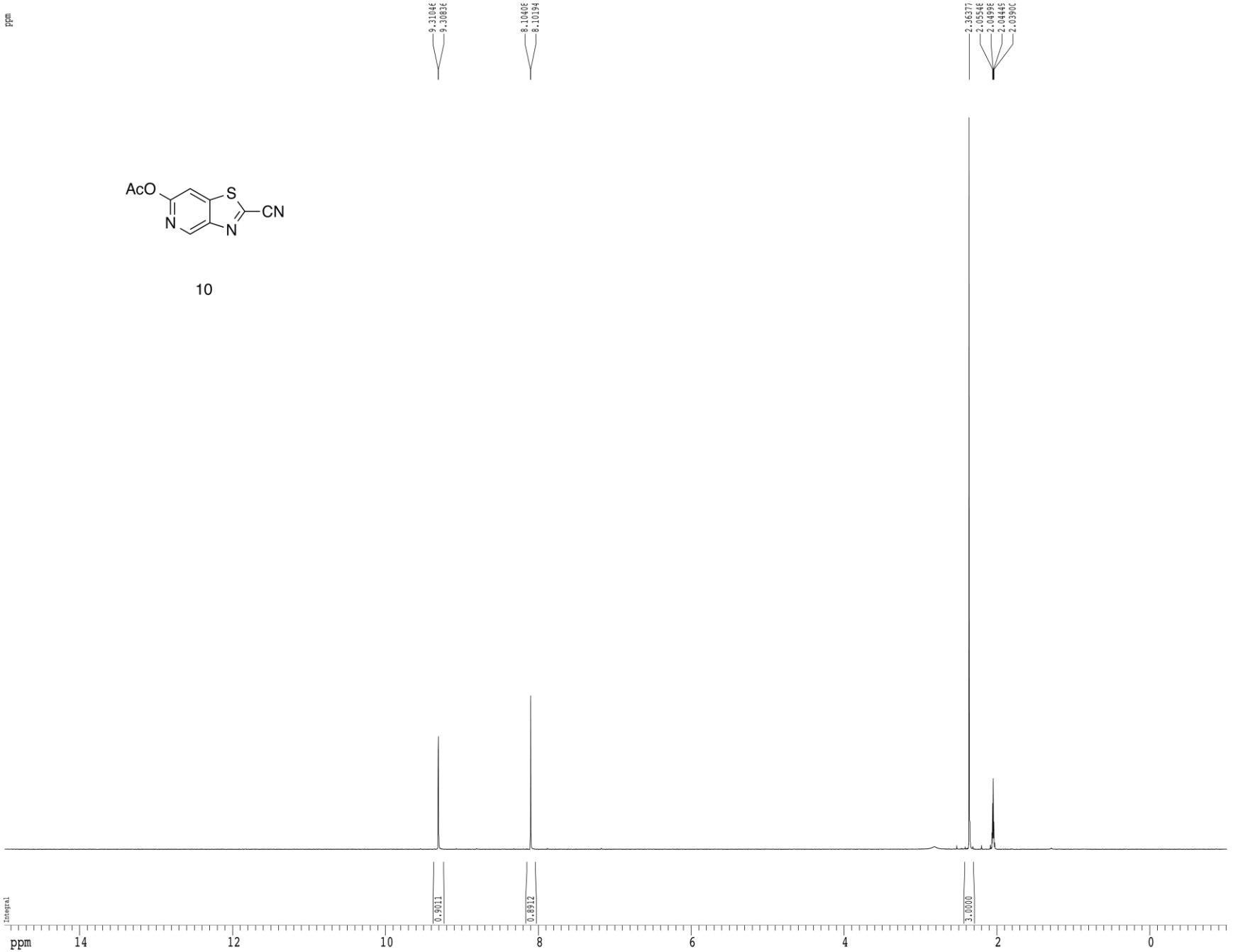
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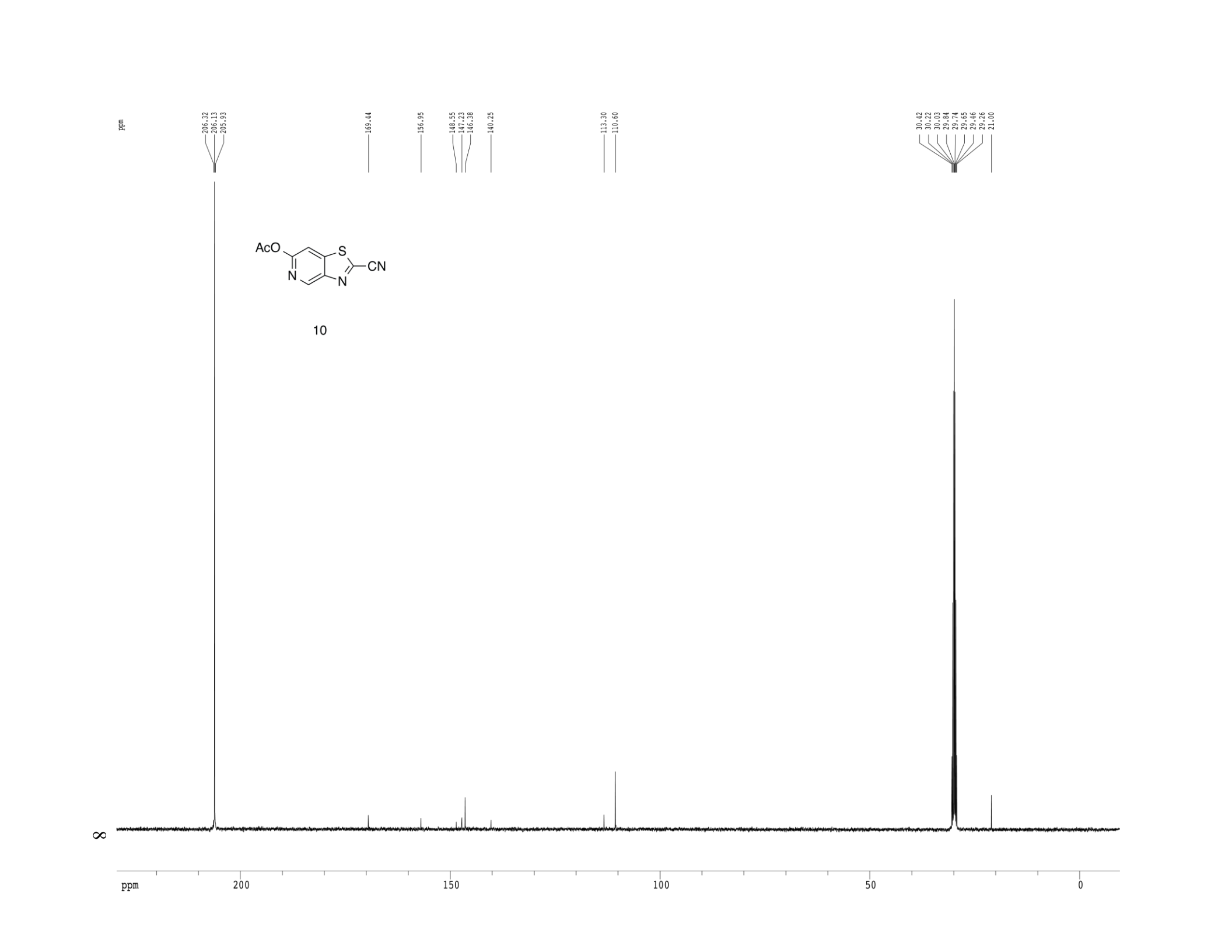
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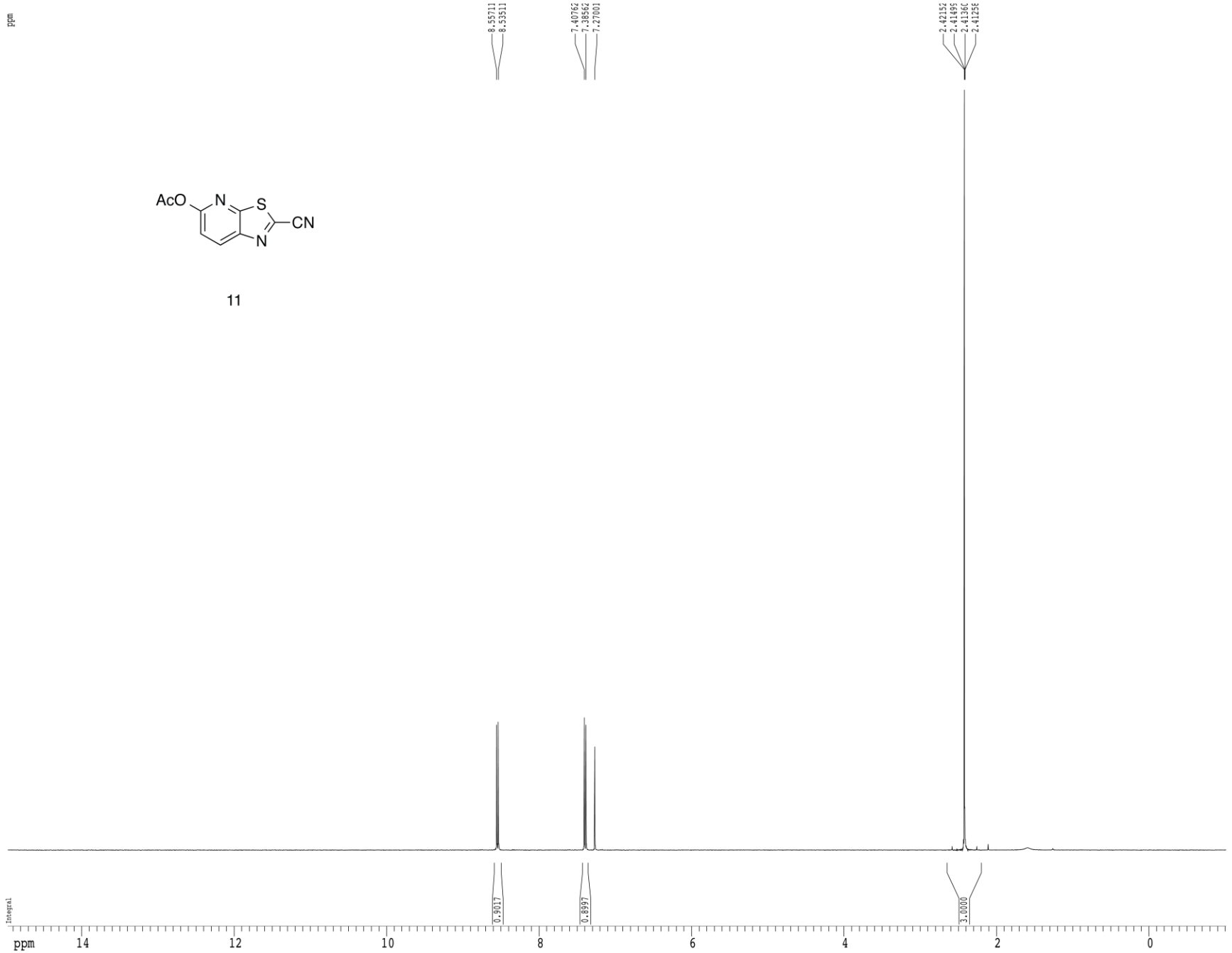
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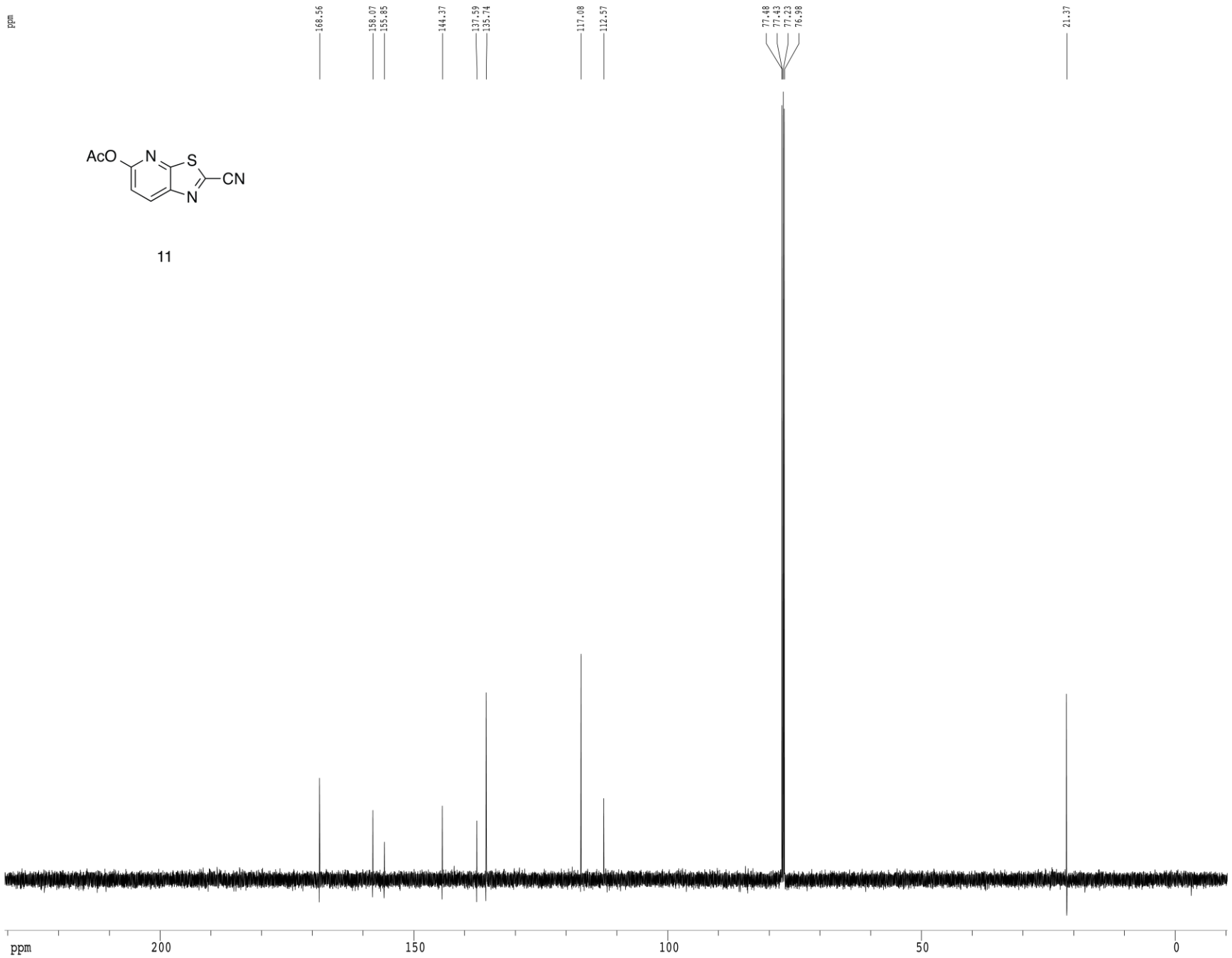
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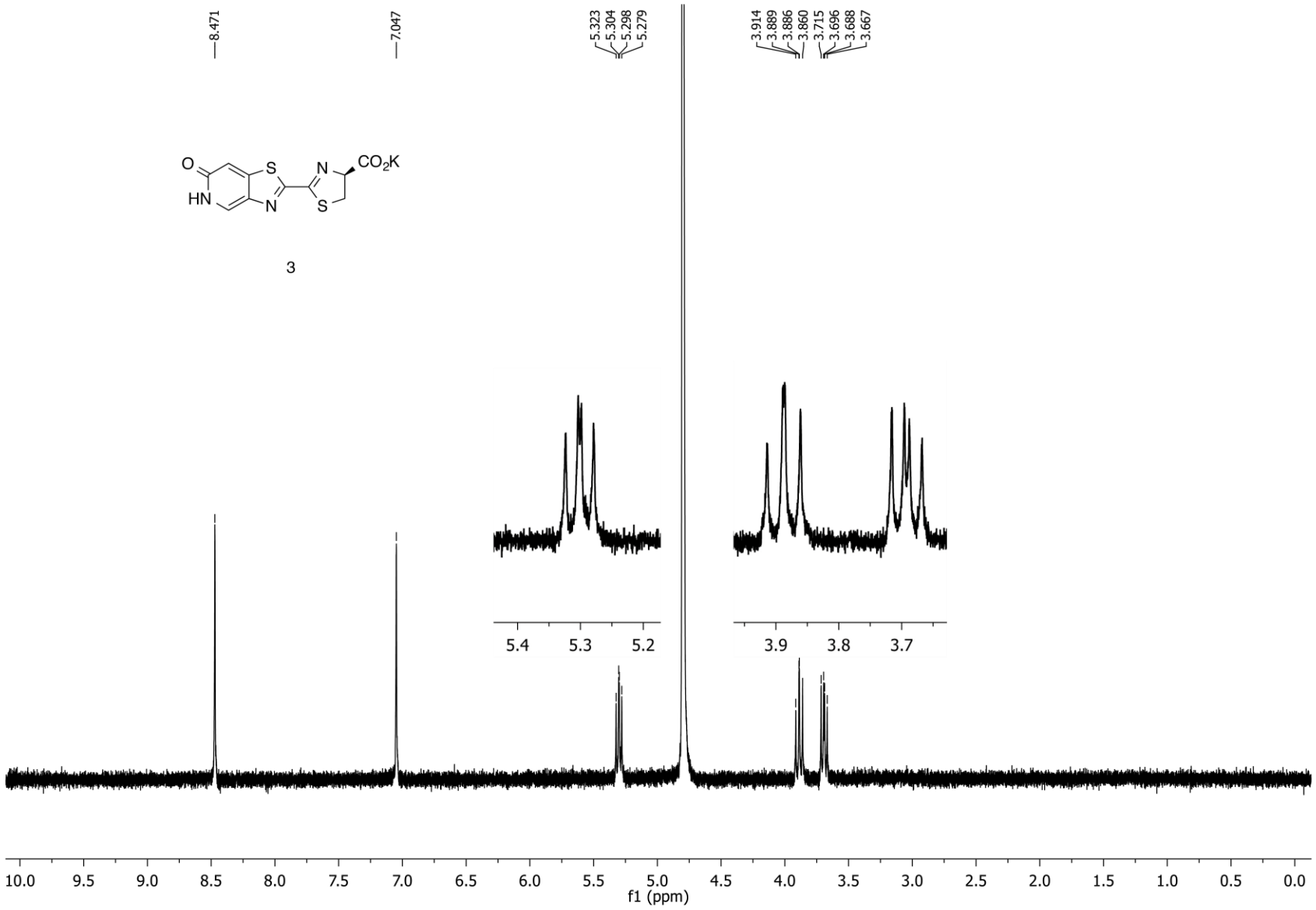
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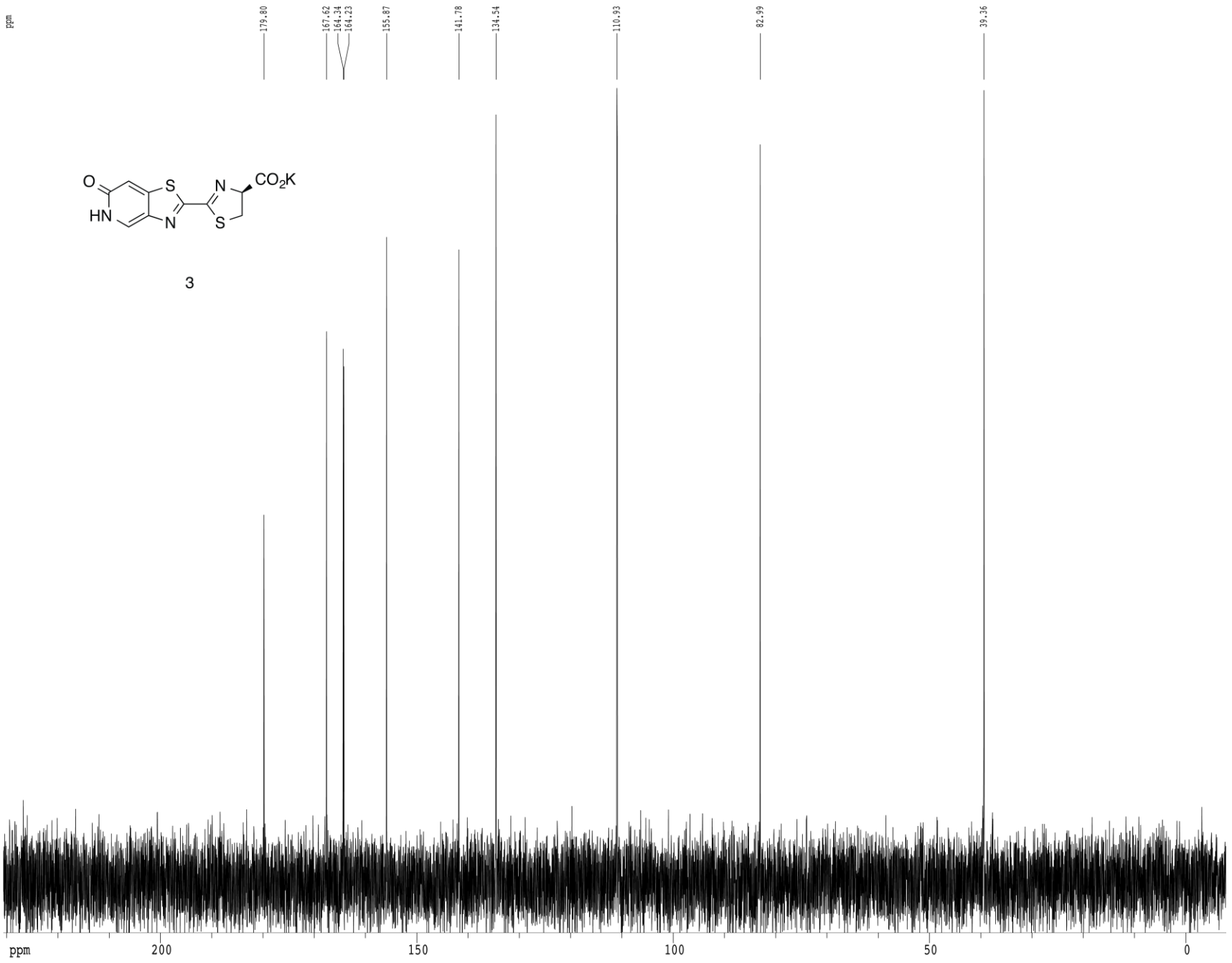
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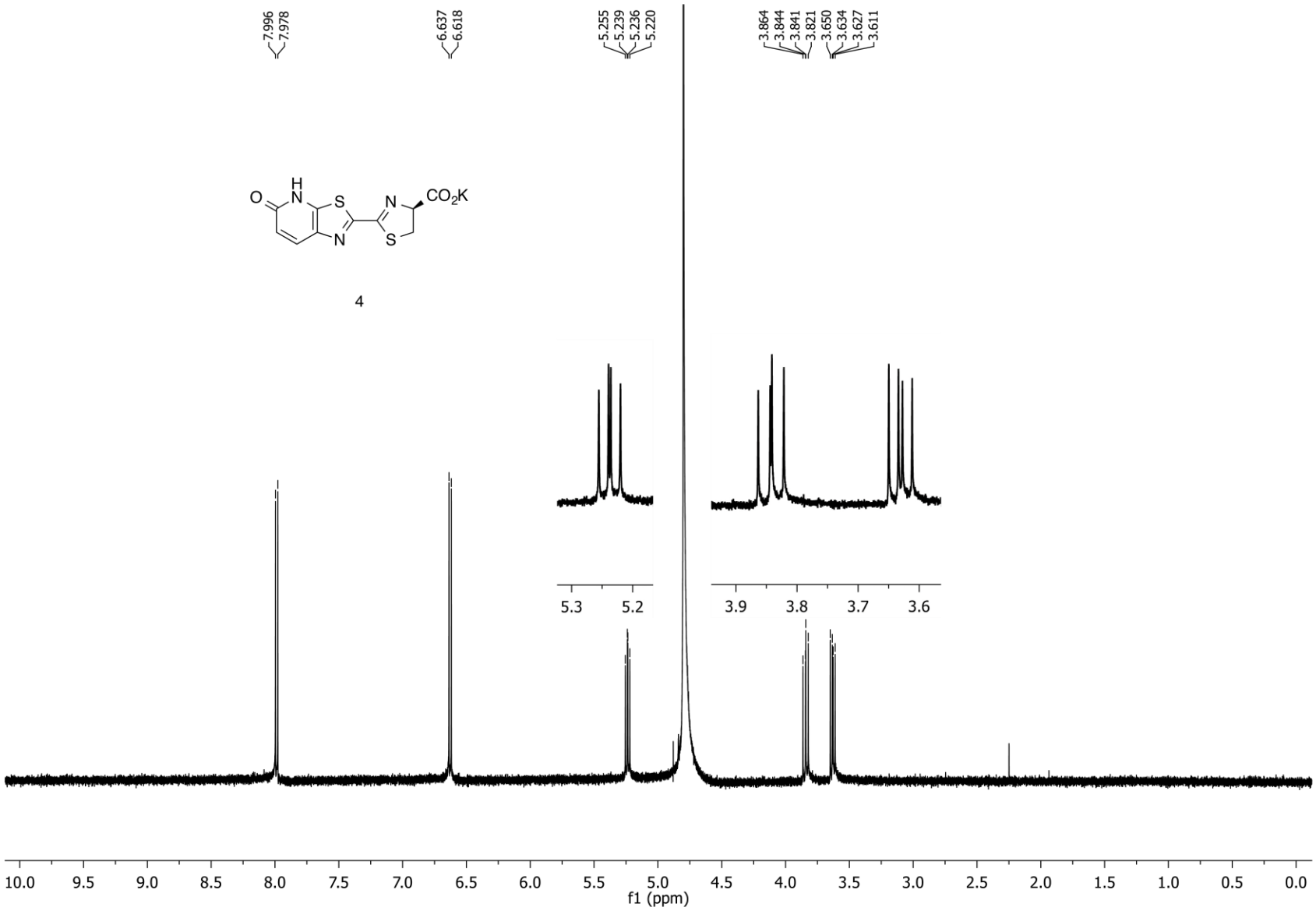
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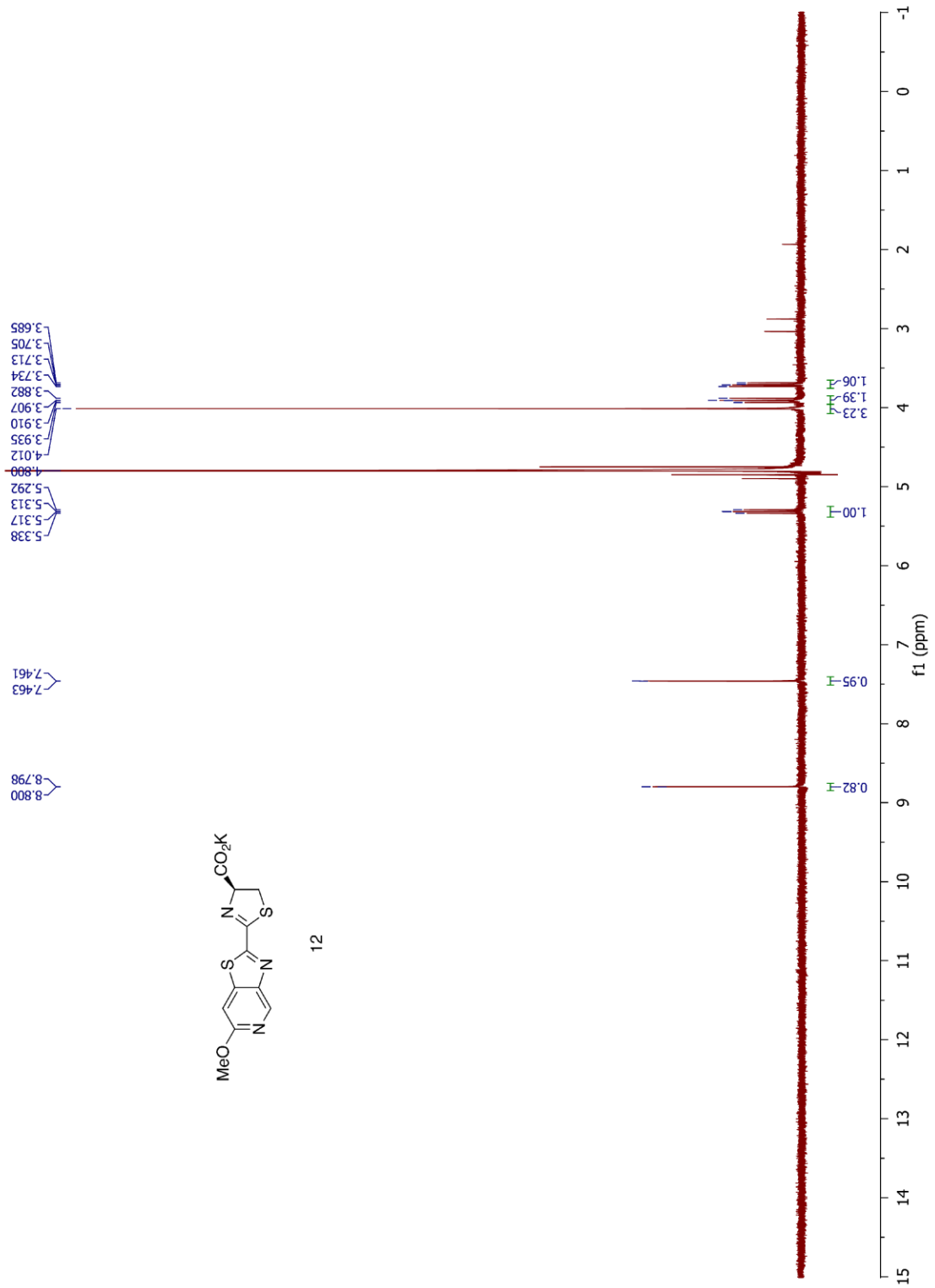
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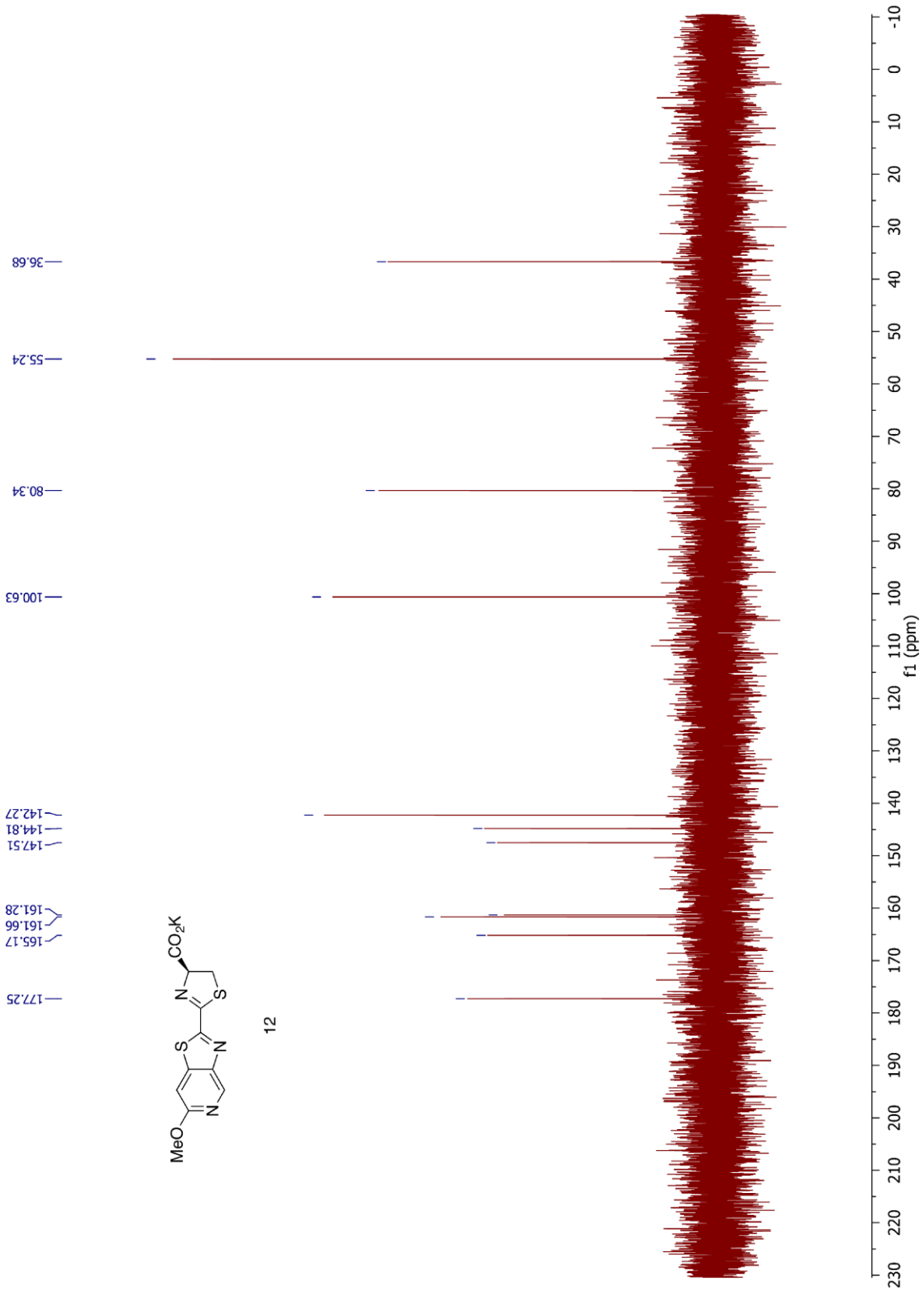
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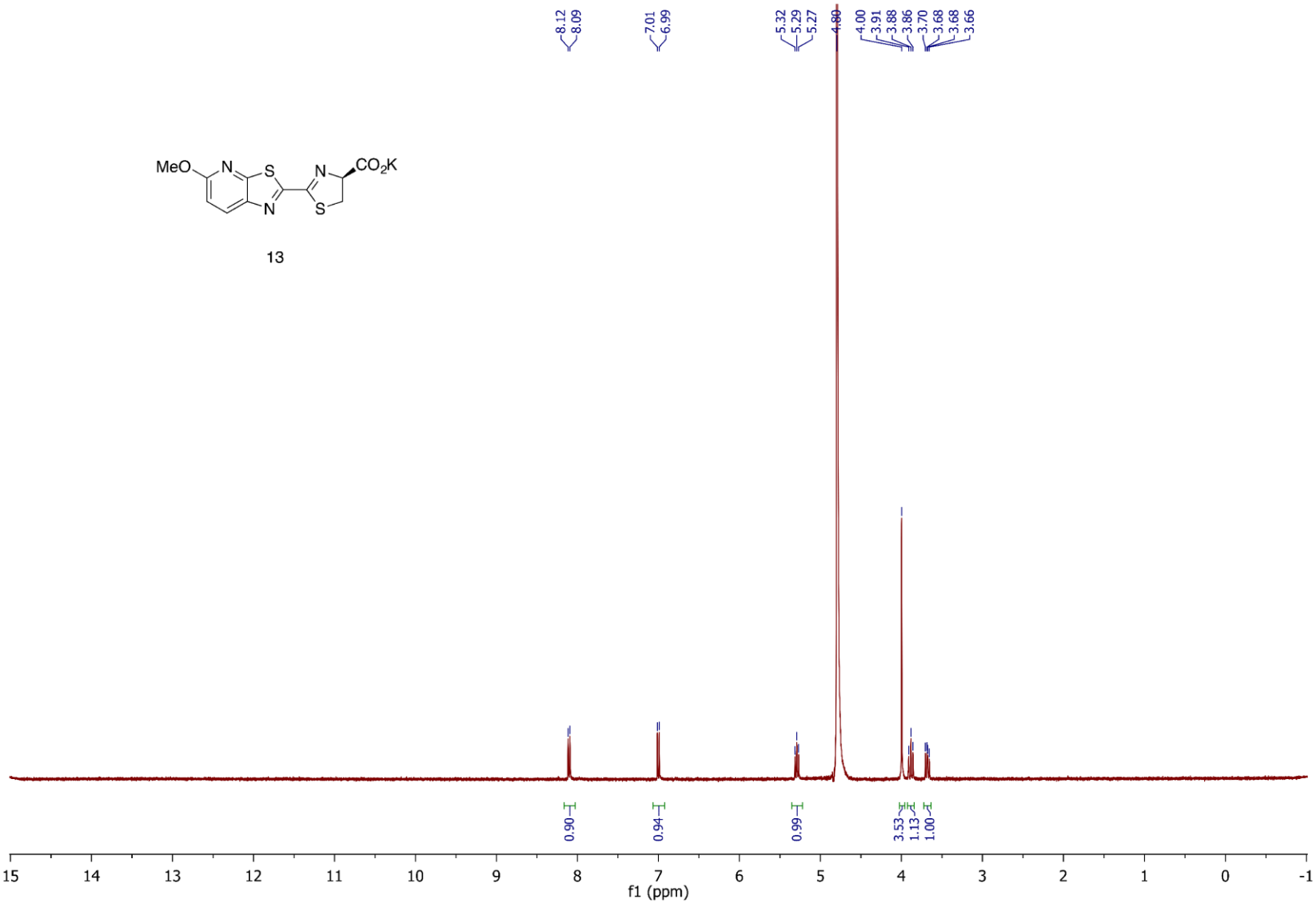
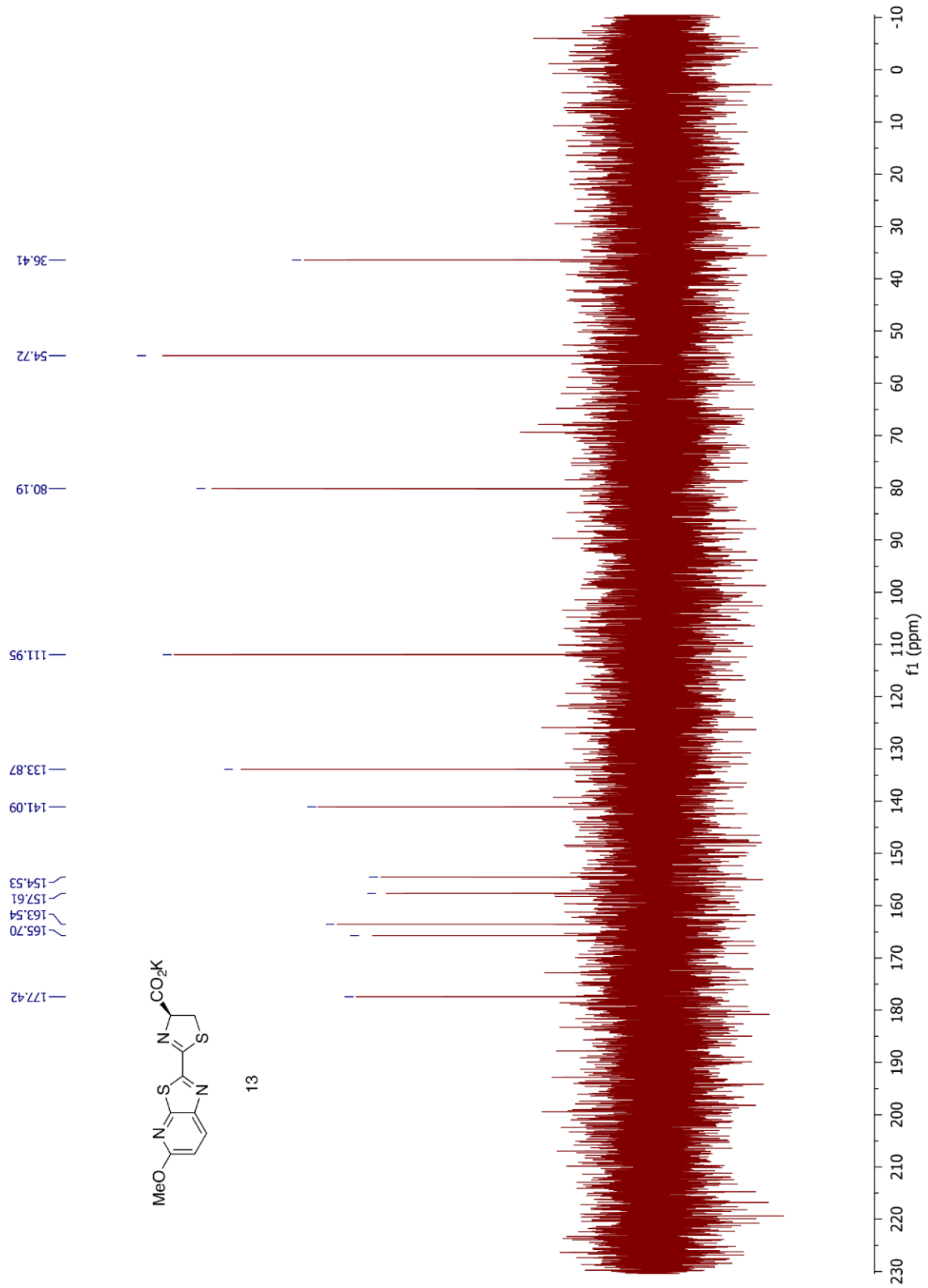
80.34
55.24
36.68
MeO
CO_2K
12
f1 (ppm)


MeO
N
S
N
N
S
CO2K
13
8.12
8.09
7.01
6.99
5.32
5.29
5.27
4.80
4.00
3.91
3.88
3.86
3.70
3.68
3.68
3.66
0.90
0.94
0.99
3.53
1.13
1.00
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
f1 (ppm)


177.42
165.70
163.54
157.61
154.53
141.09
133.87
111.95
80.19
54.72
36.41
MeO
13
f1 (ppm)