Group-based optimization of potent and cell-active inhibitors of the von Hippel-Lindau (VHL) E3 ubiquitin ligase: structure-activity relationships leading to the chemical probe VH298

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Supporting Information

Table of Contents

S2 - Supplementary Figures

S11 – FP Competition Assay Data

S22 – ITC Data

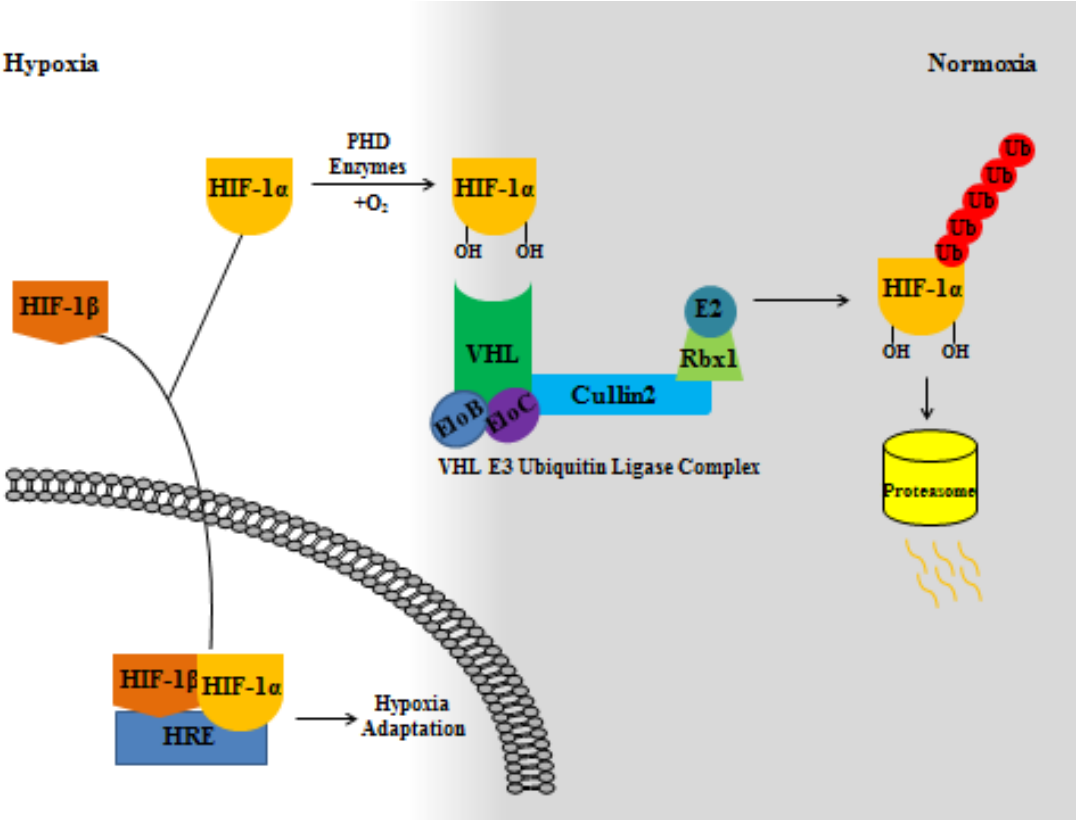
S26 – SPR Data

S30 – Evaluation of VHL inhibitors intracellular accomulation

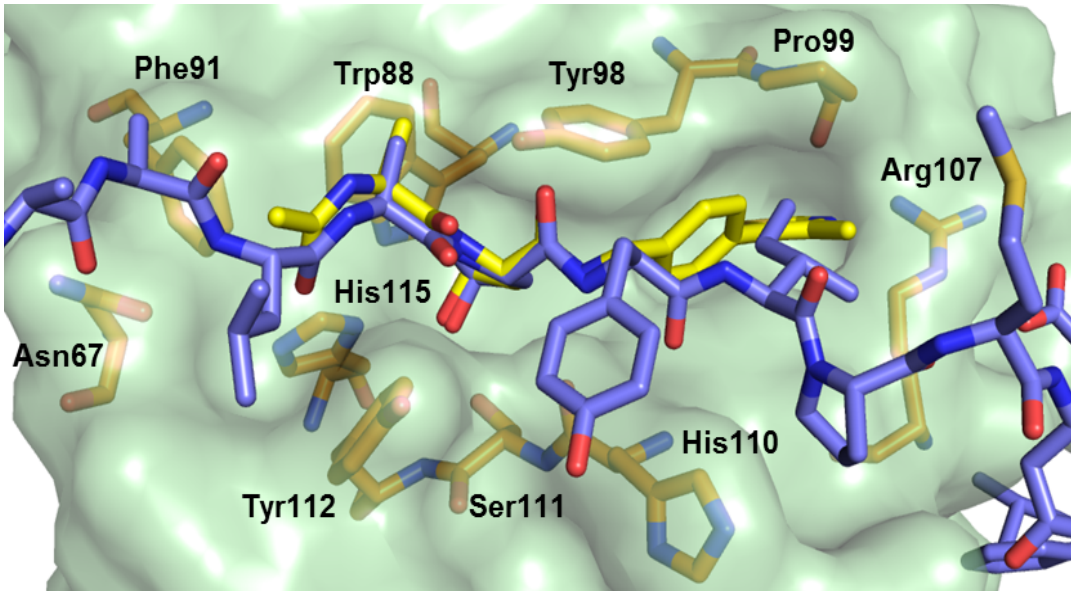
S31 – X-ray Crystallography Data Processing and Refinement Statistics

S33 – ^{1}H and ^{13}C NMR spectra

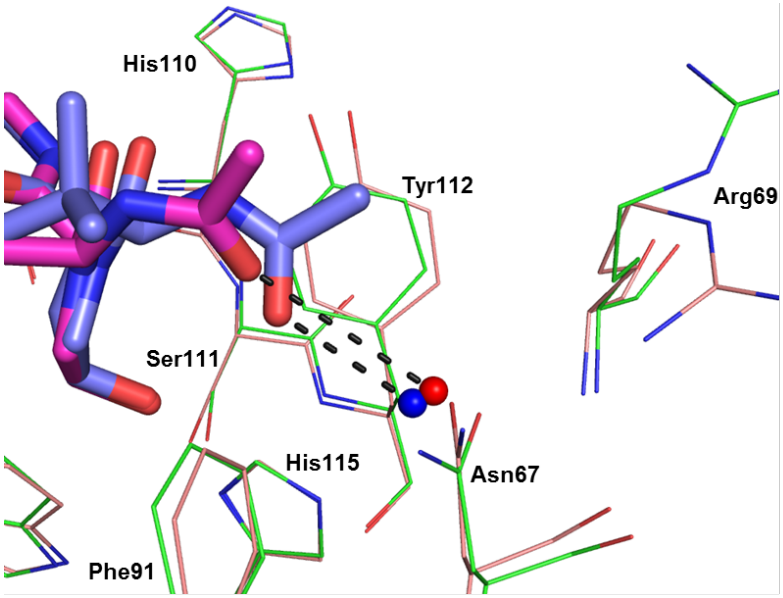
Supplementary Figures



Supplementary Figure 1. Under normoxia conditions, HIF-1α is hydroxylated, recognized by the $CRL2^{VHL}$ complex, poly ubiquitinated and target for hydrolysis by the proteasome. Under hypoxia conditions, HIF-1α dimerizes with HIF-1β forming a transcriptionally active complex that binds to Hypoxia Response Elements, promoting hypoxia adaptation.

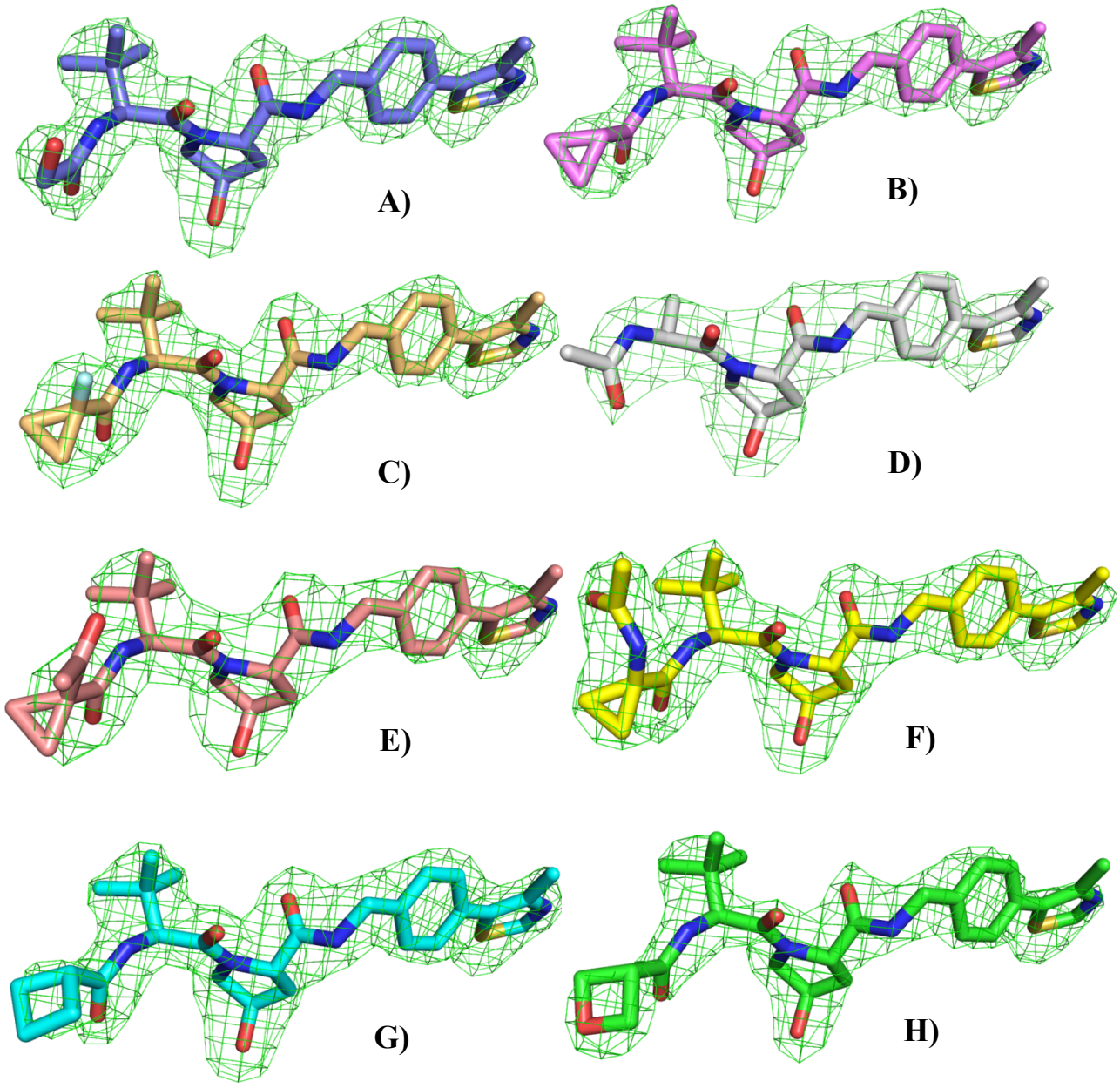


Supplementary Figure 2. Crystal structure of VBC in complex with HIF-1α peptide (purple carbons PDB 4AJY) and inhibitor **11** (yellow carbons). VHL is shown as a pale green surface and the VHL residues forming the binding pocket as orange stick representations.

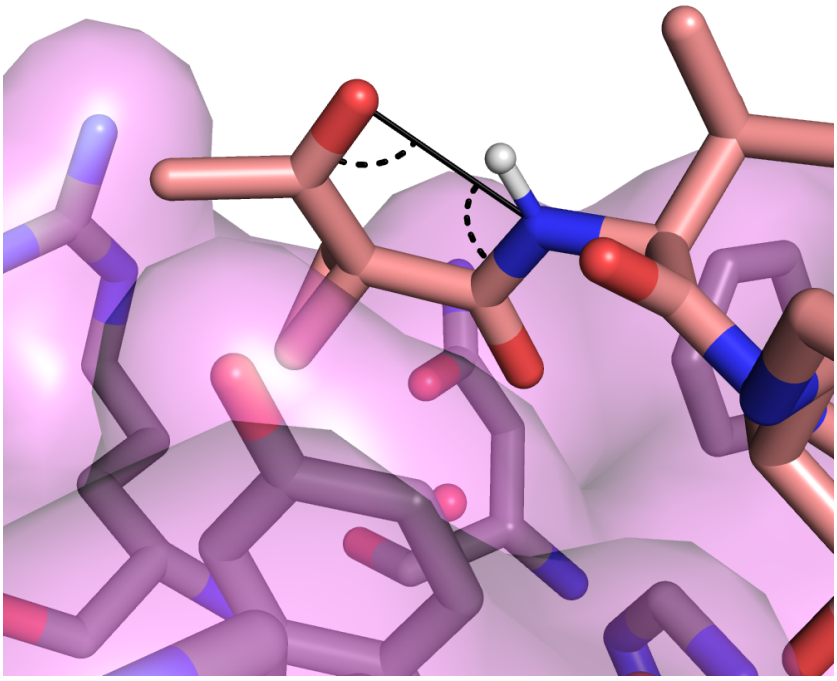


Supplementary Figure 3. Crystal structure of VBC in complex with inhibitor **VH032** (purple carbons PDB 4W9H) and inhibitor **11** (pink carbons). Residues forming the LHS of VHL pocket are shown as line representations (pink carbons **VH032** structure and green carbons inhibitor **11** structure). The structural water at the LHS is shown as a red sphere in

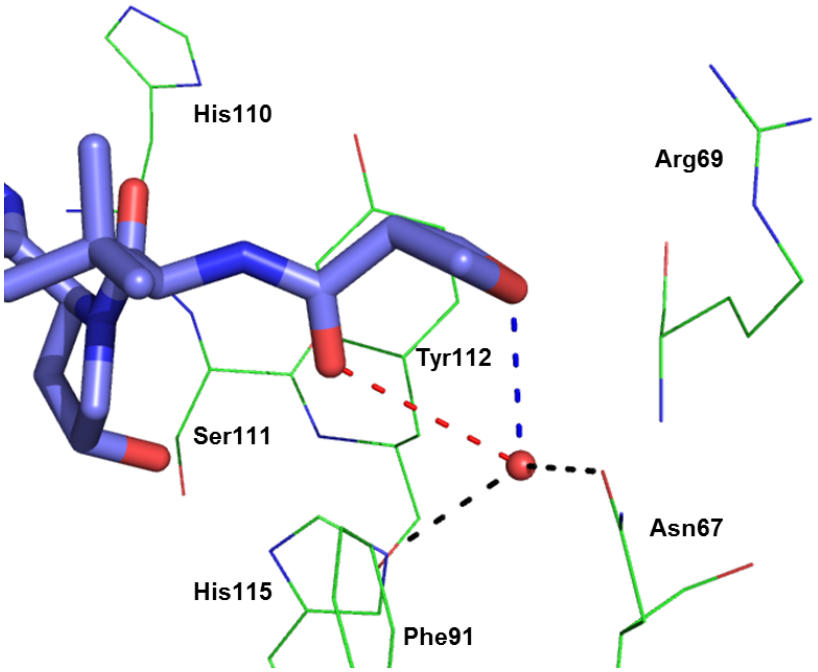
inhibitor **11** structure and as a blue sphere in **VH032** structure. Distances between inhibitors LHS carbonyl and respective structural waters are shown as black dashed lines.



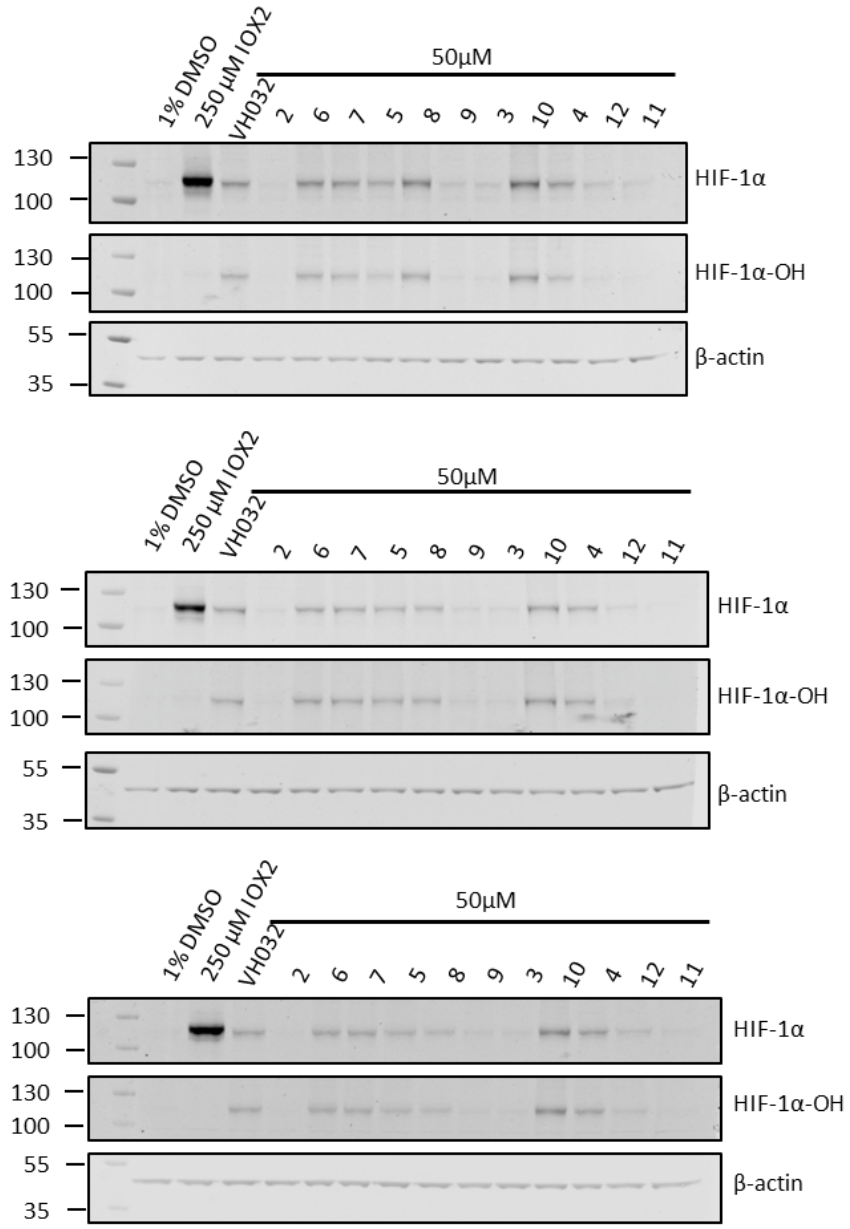
Supplementary Figure 4. Electron density at ligand binding sites. Ligands A) **3**; B) **6**; C) **10**; D) **11**; E) **16**; F) **17**; G) **18**; H) **19** shown as sticks bound to the complex of VBC (not shown). An omit map (Fo–Fc) is shown in green contoured at 3σ around each modelled ligand with a carve radius of 1.6 Å.



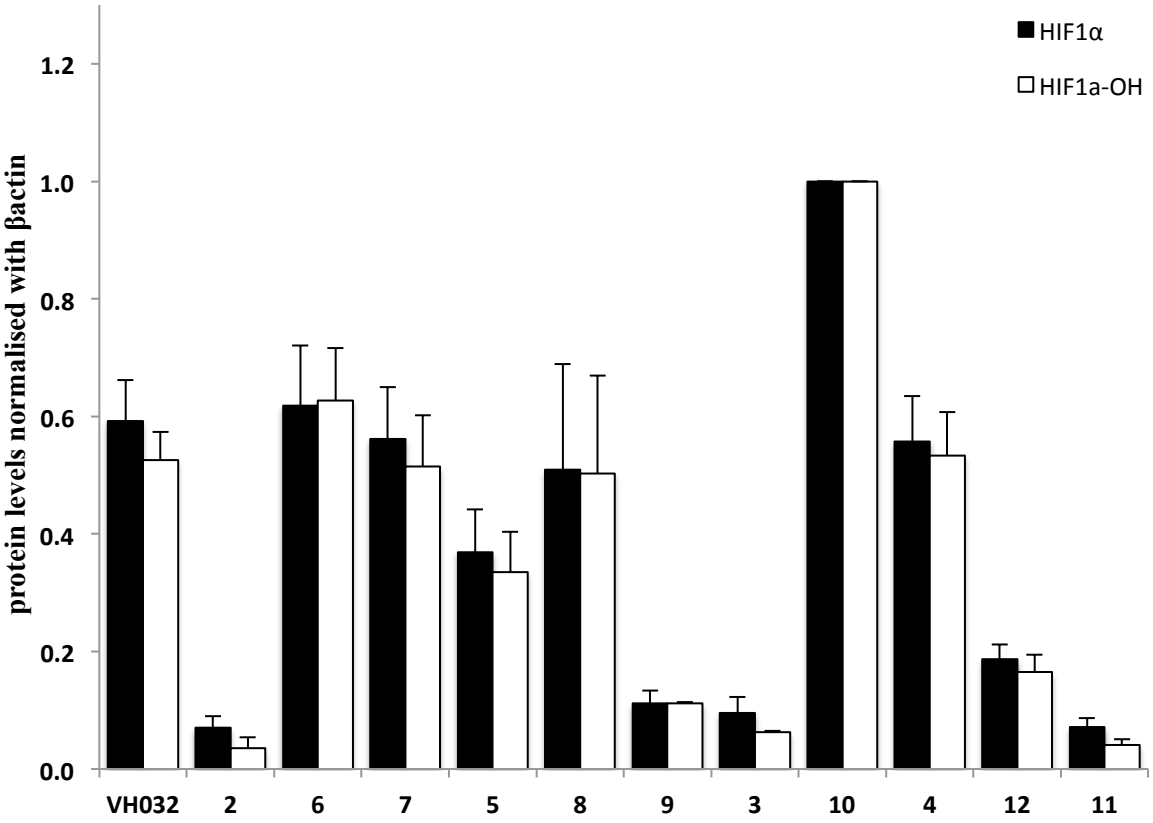
Supplementary Figure 5. Crystal structure of VBC in complex with inhibitor **16** (pink carbons). VHL is shown as purple pale surface and residues forming the LHS of VHL pocket are shown grey stick representation. Structural water is shown as a red sphere. Distance and angles are shown as black continuous and dashed lines respectively.



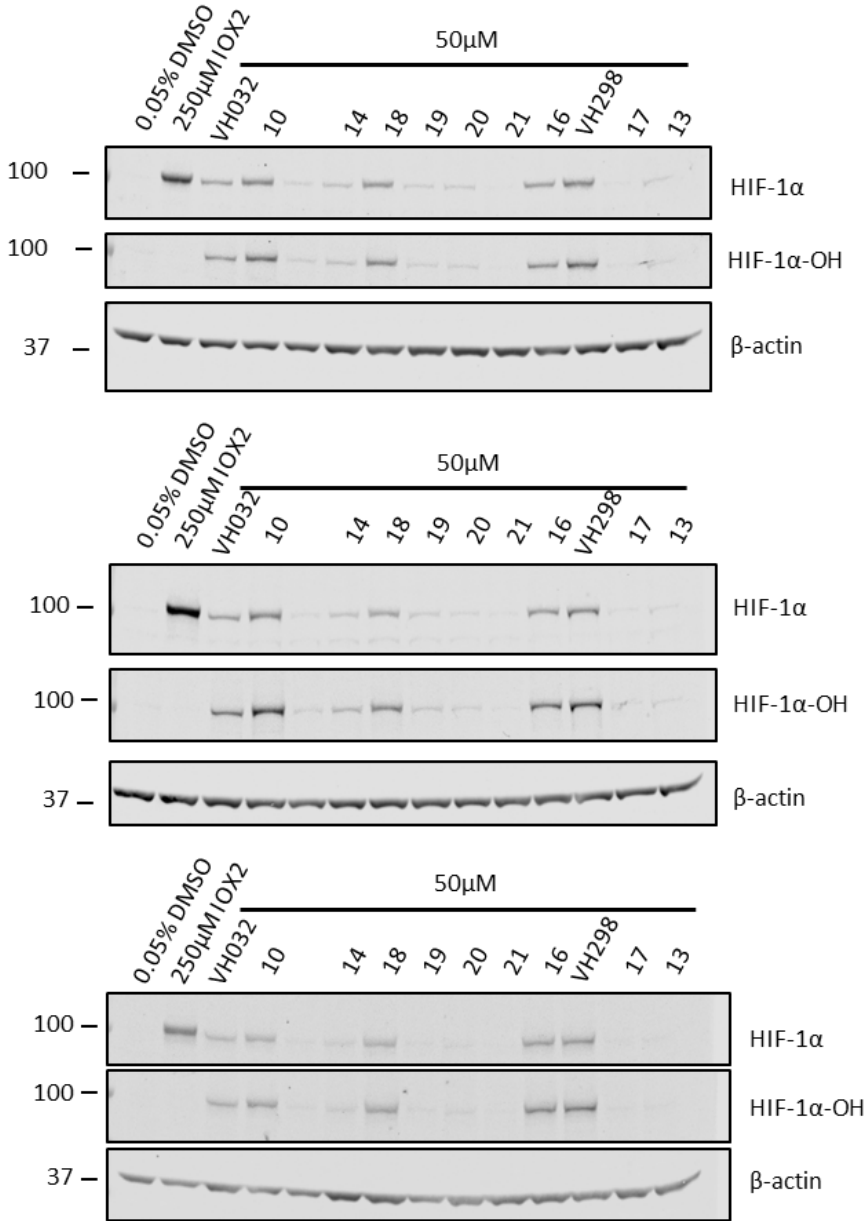
Supplementary Figure 6. Crystal structure of VBC in complex with inhibitor **19** (purple carbons). Residues forming the LHS of VHL pocket are shown as line representations (green carbons). The structural water at the LHS is shown as a red sphere. Distances between inhibitor oxygen of oxetane and inhibitor LHS amide carbonyl with structural water are shown as blue and red dashed lines respectively. Distances between structural water and residues on the LHS of the pocket are shown as black dashed lines



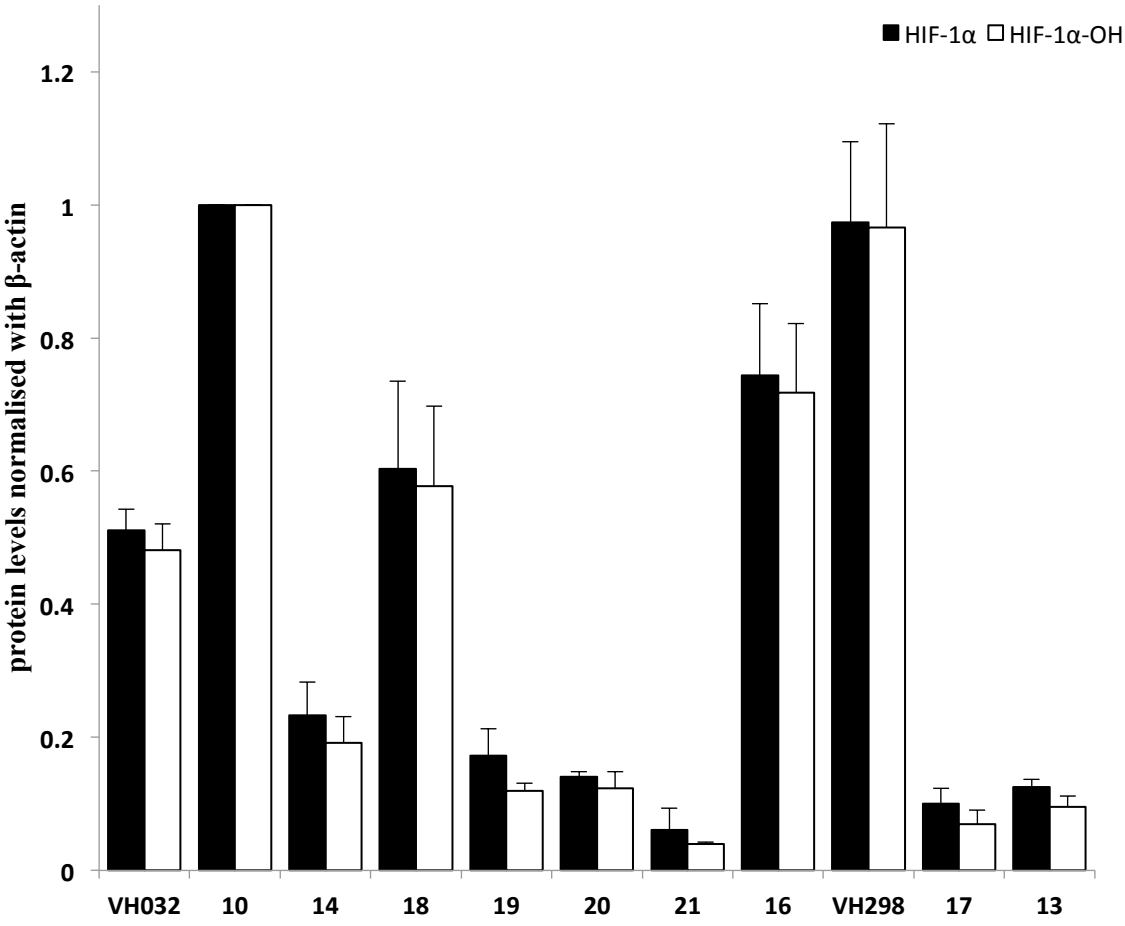
Supplementary Figure 7. Immunoblots of HIF-α subunits in HeLa cells treated with 50 µM of VHL inhibitors **VH032** and **2-12**; 1% DMSO, 250 µM IOX2 for 2h.



Supplementary Figure 8. Quantification of HIF-1α stabilization levels in HeLa cells for compounds **VH032** and **2–12** measured by Western Blot (SI Figure 7) after 2h treatment. The assay was performed in triplicate; protein levels were normalized to β-actin levels and are reported as mean ± st. dev. relative to compound **10**. IOX2 was used as positive control and cells treated with DMSO vehicle were used as negative control.

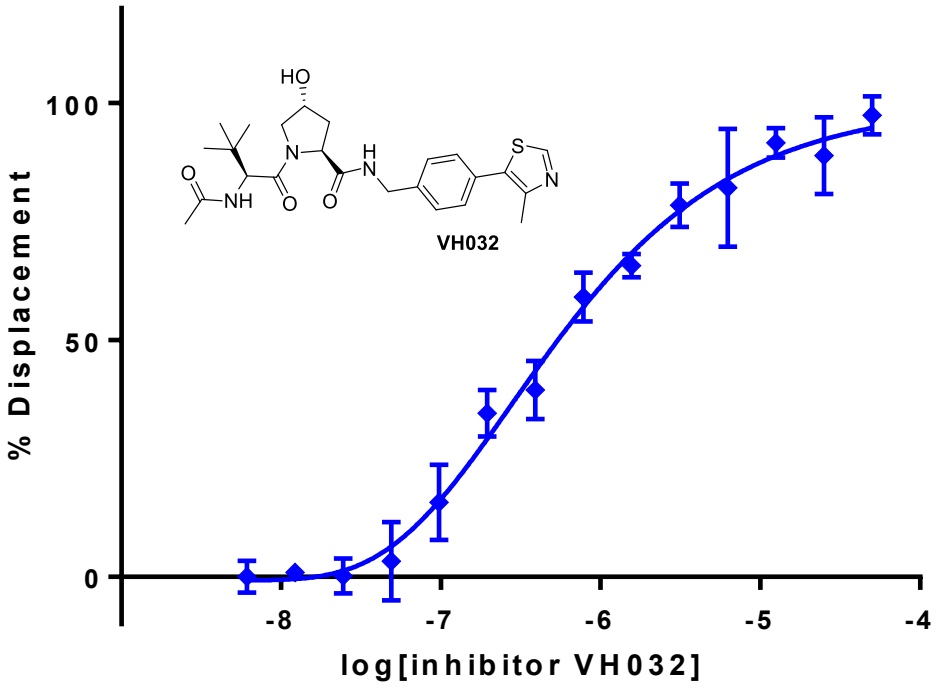


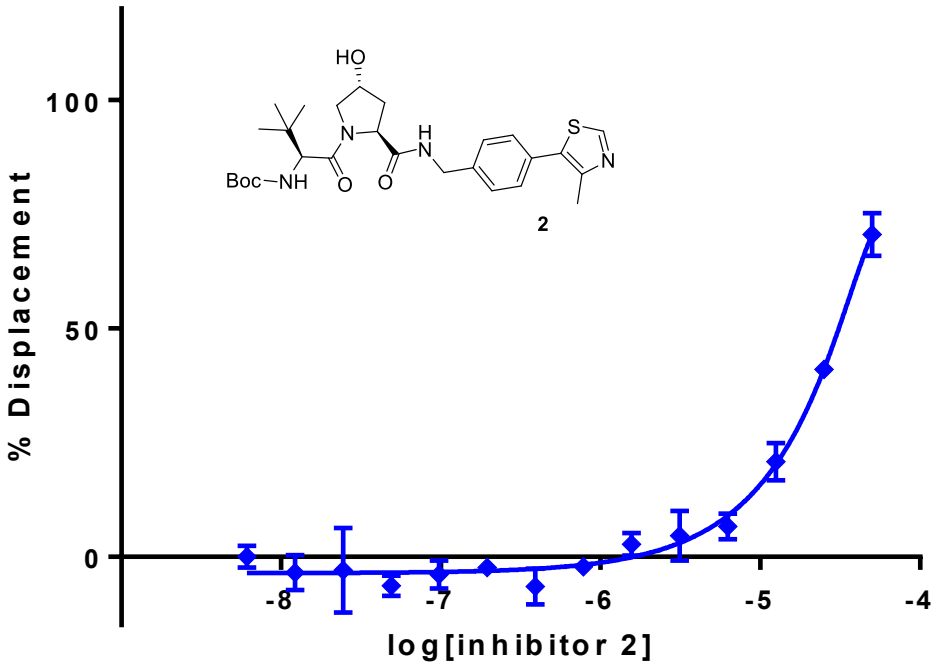
Supplementary Figure 9. Immunoblots of HIF-α subunits in HeLa cells treated with 50 μM of VHL inhibitors **VH032**, **10**, **13**, **14, VH298, 16-21**; 1% DMSO, 250 μM IOX2 for 2h.

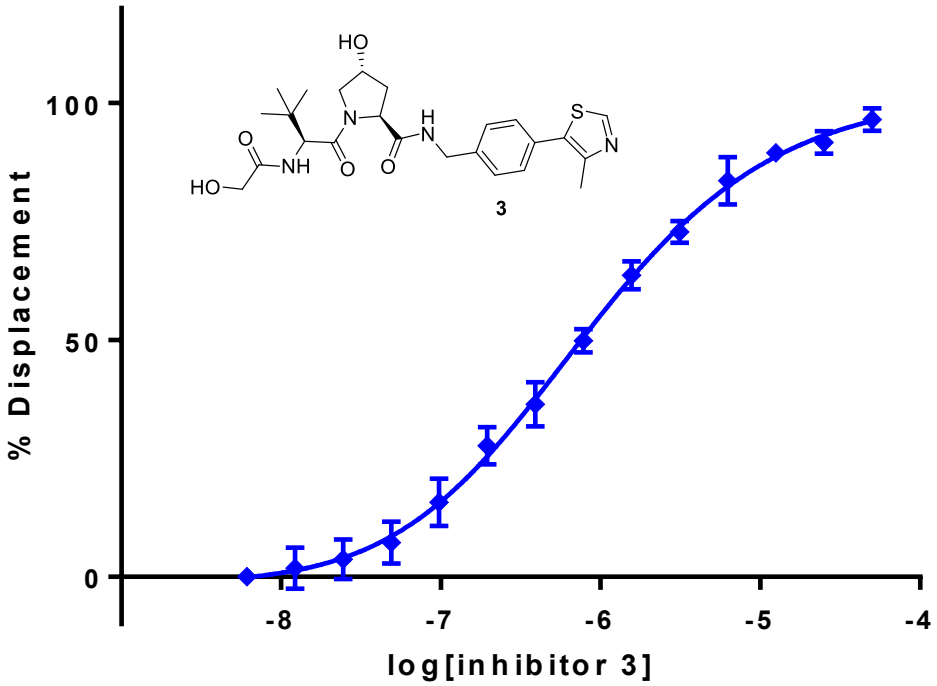


Supplementary Figure 10. Quantification of HIF-1α stabilization levels in HeLa cells for compounds VH032, VH298, 10 and 13-21 measured by Western Blot (SI Figure 9) after 2h treatment. The assay was performed in triplicate; protein levels were normalized to β-actin levels and are reported as mean ± st. dev. relative to compound **10**. IOX2 was used as positive control and cells treated with DMSO vehicle were used as negative control.

Florescence Polarization Competition Assay Graphs



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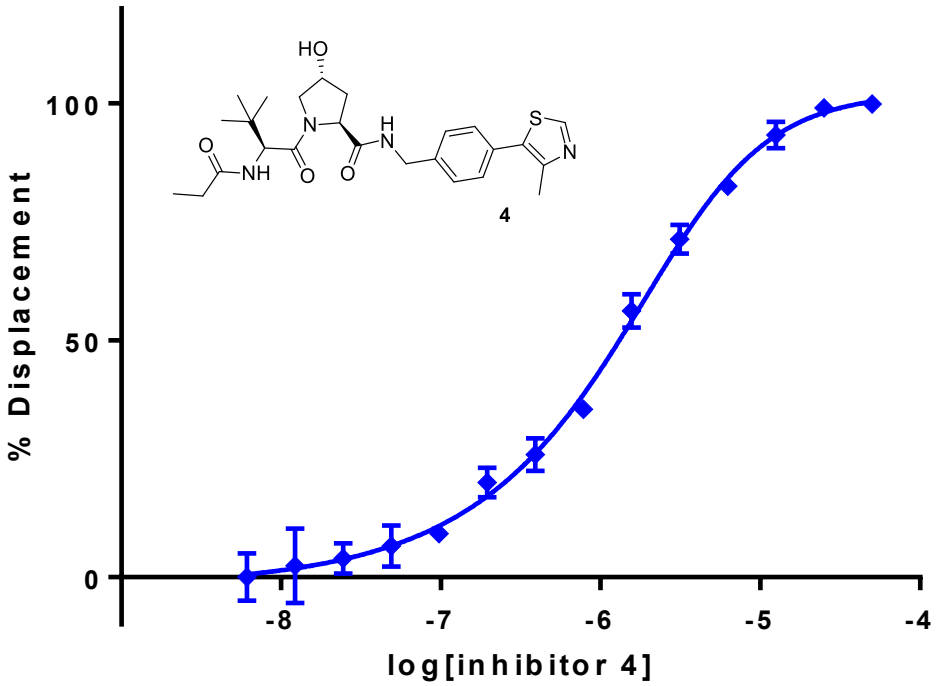
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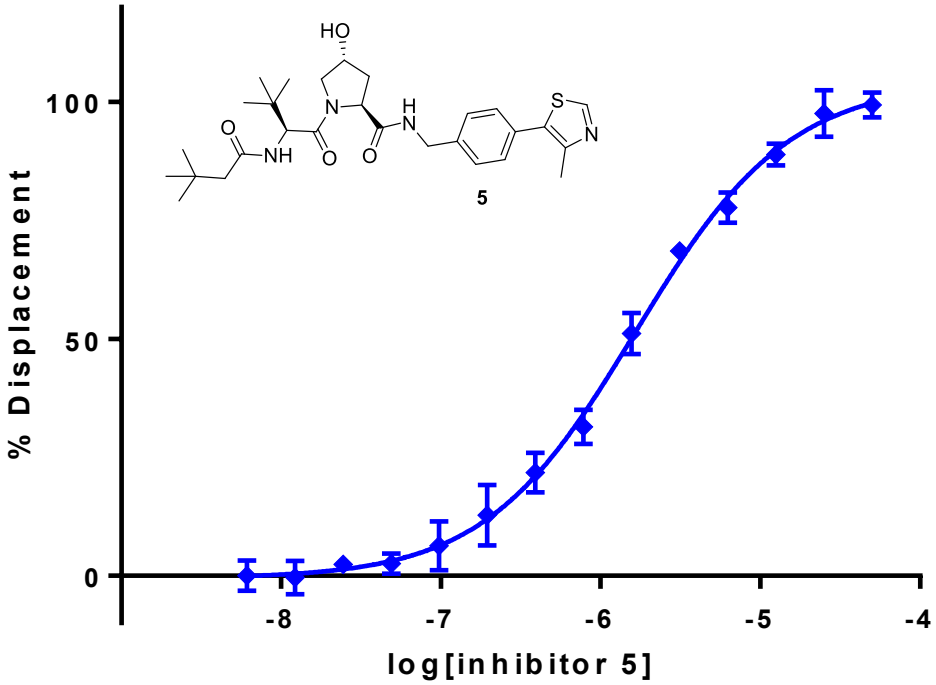
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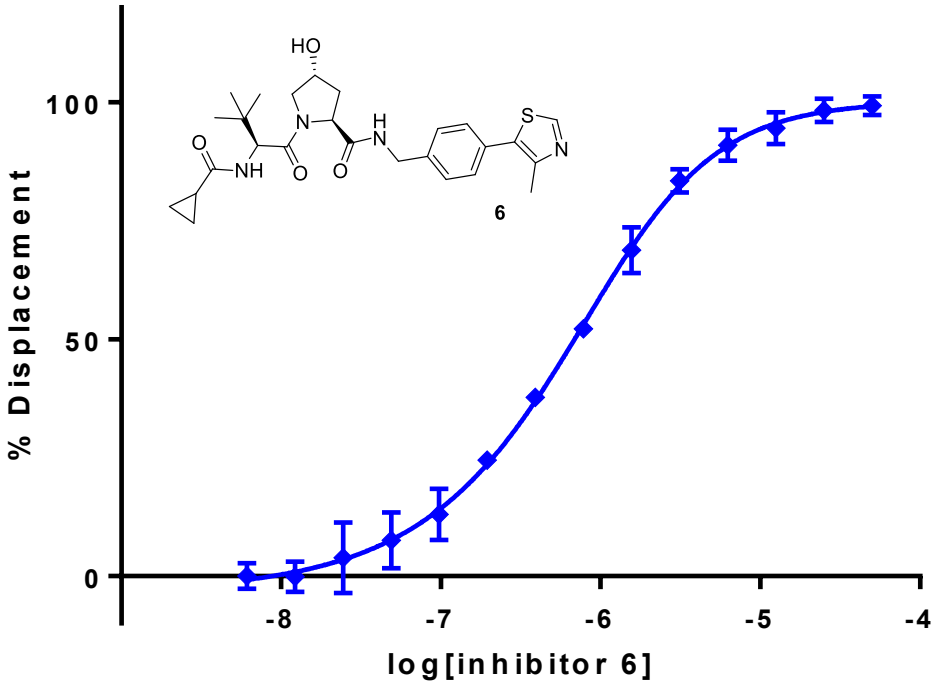
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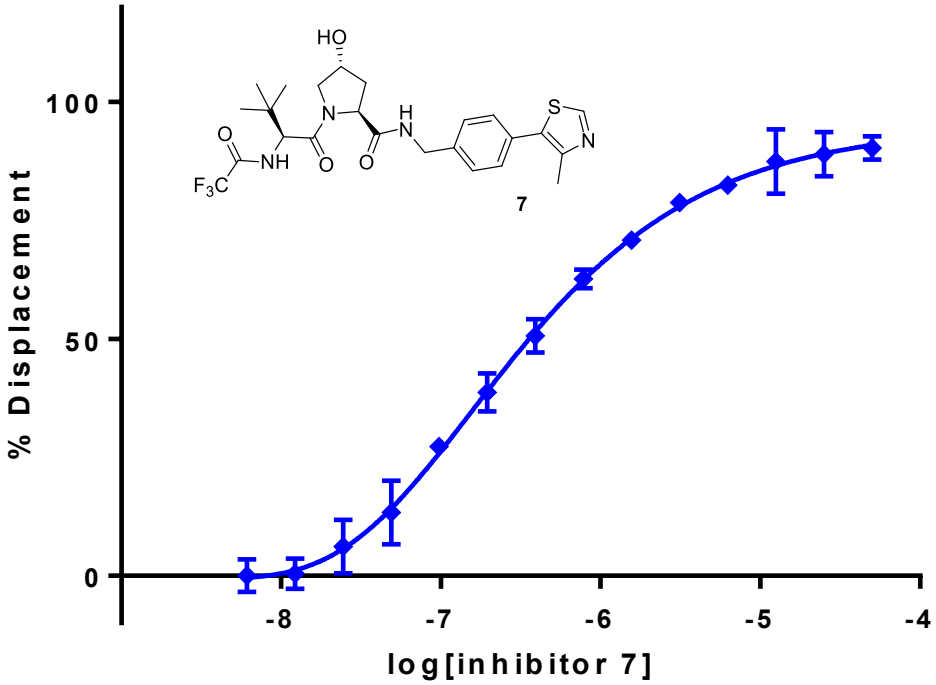
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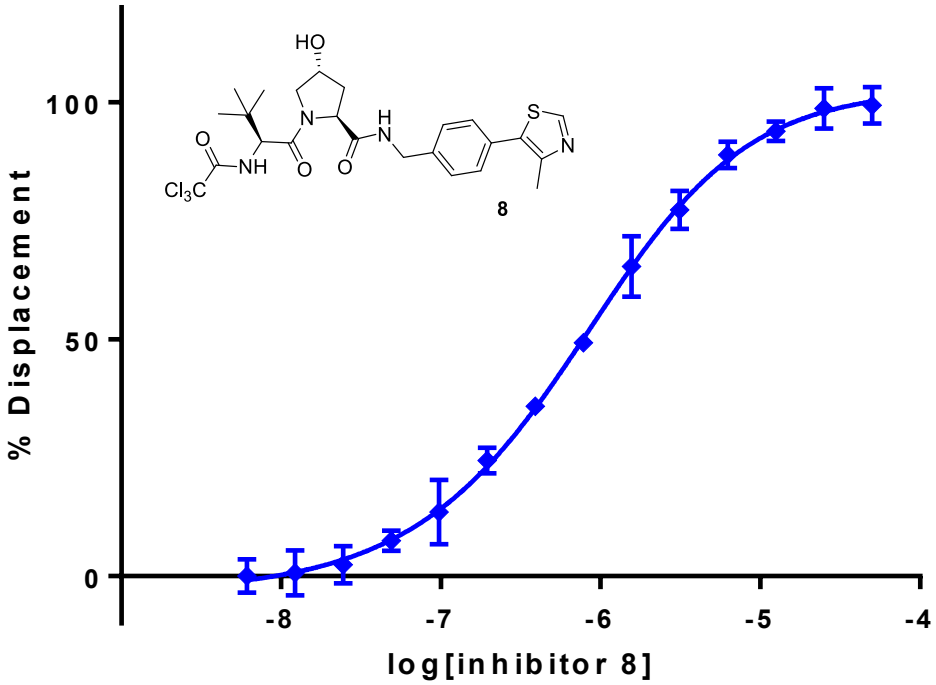
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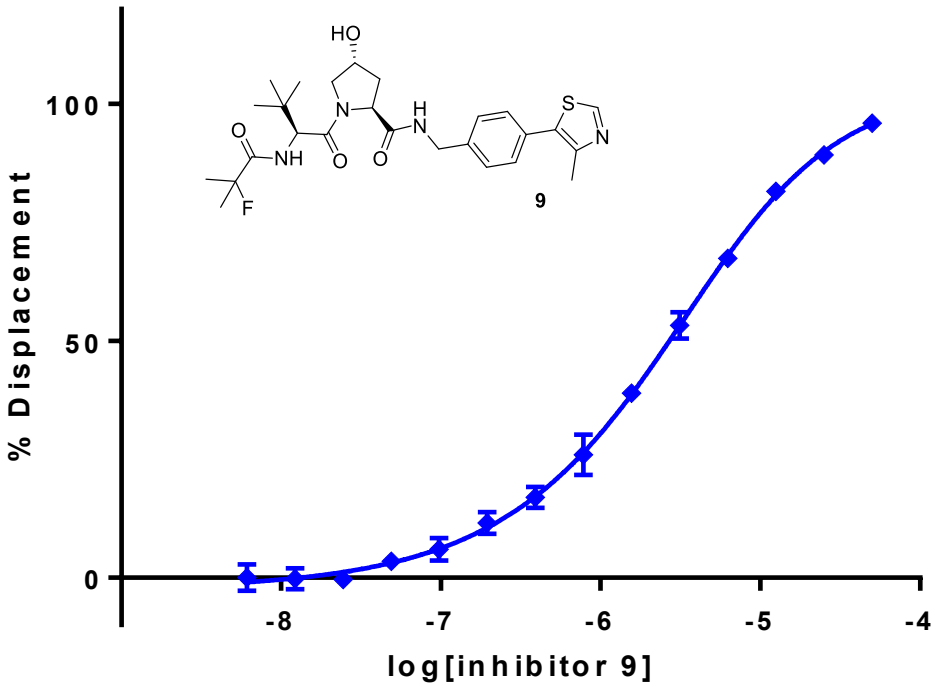
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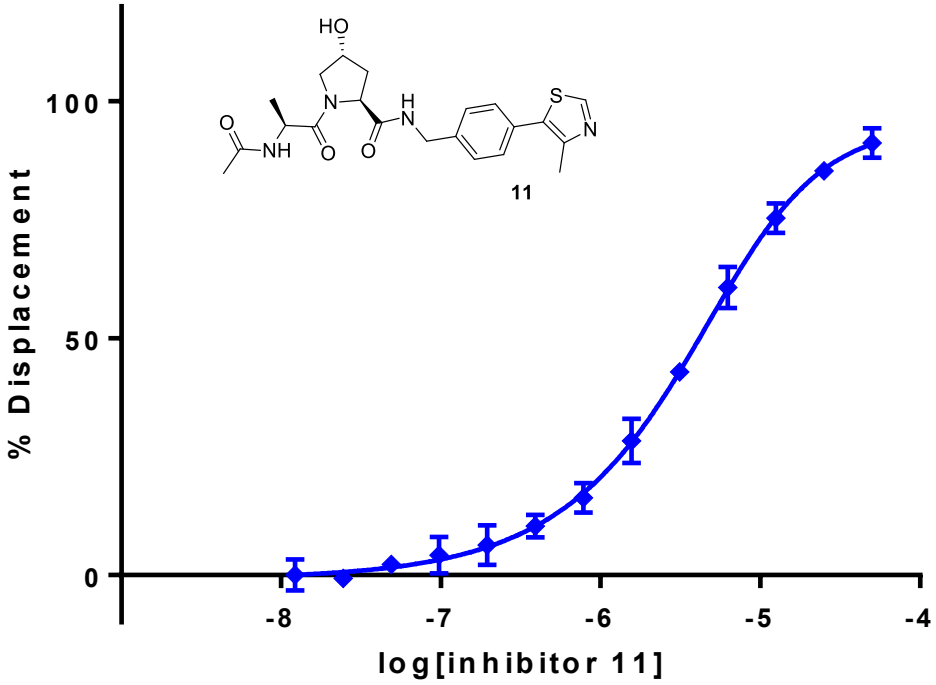
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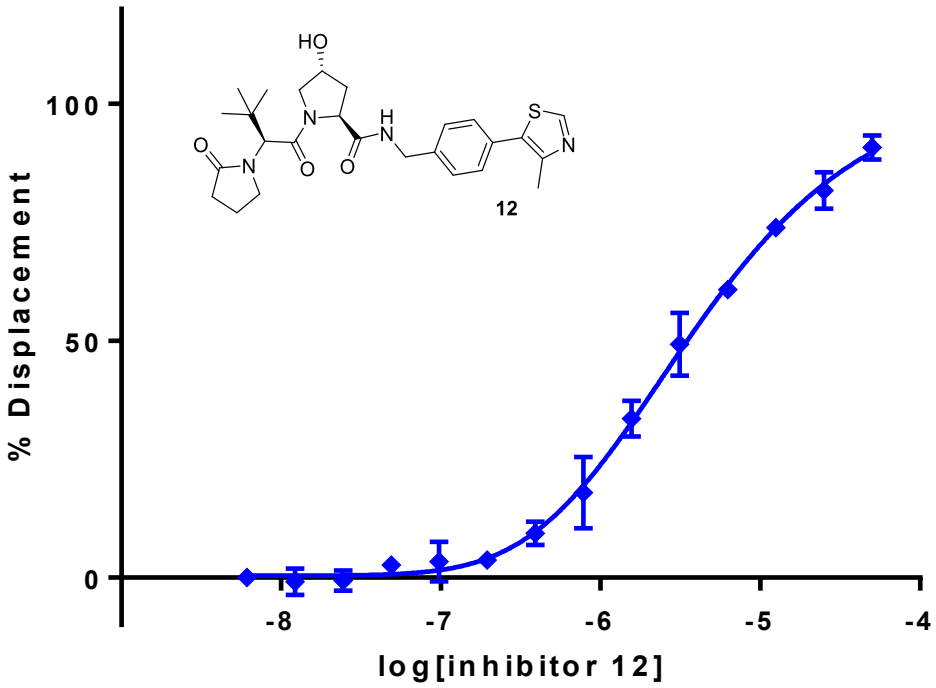
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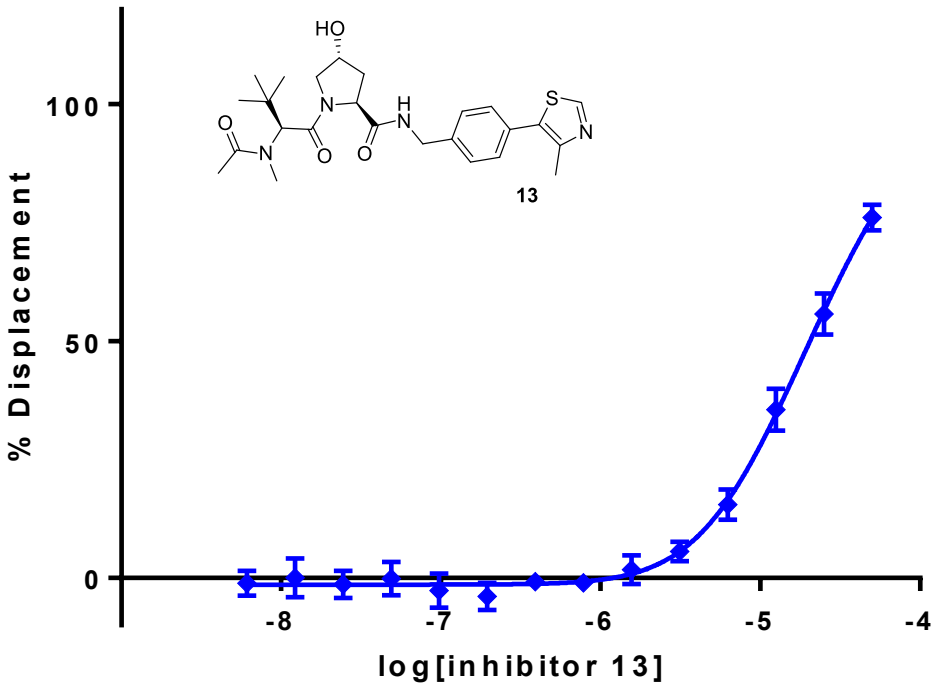
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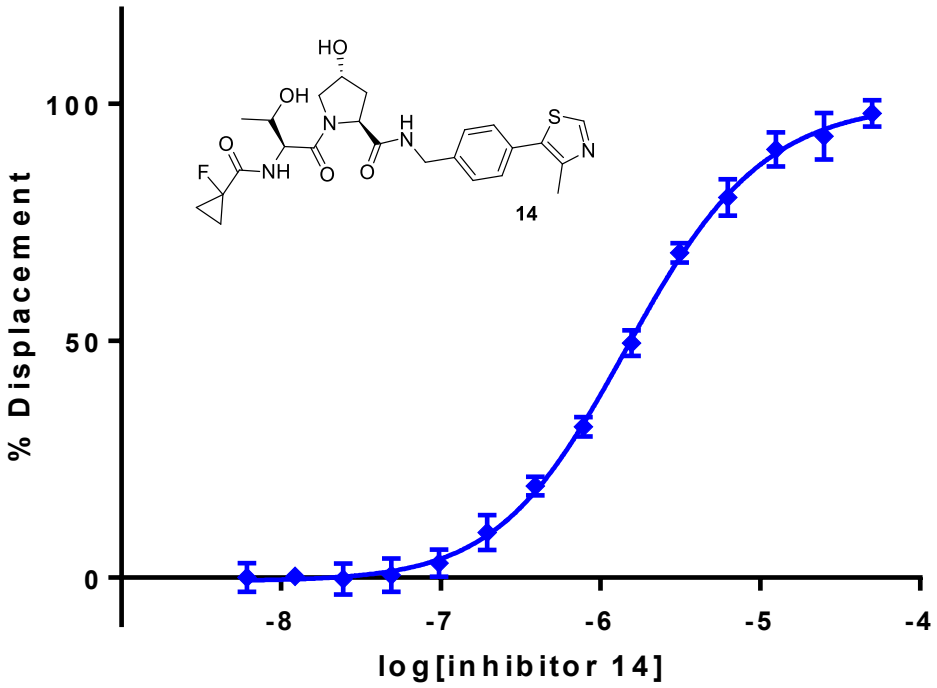
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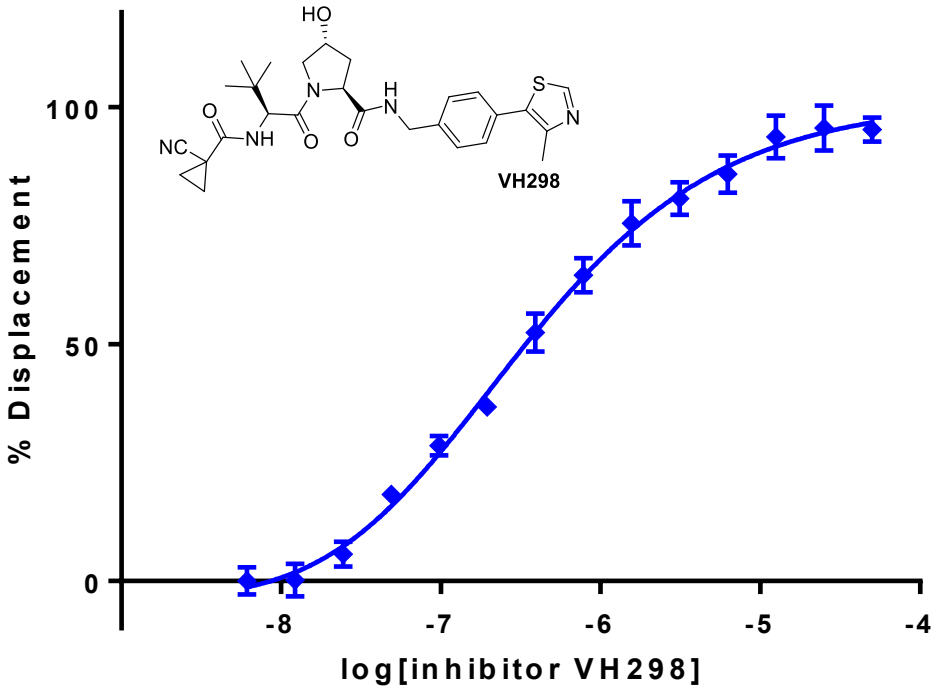
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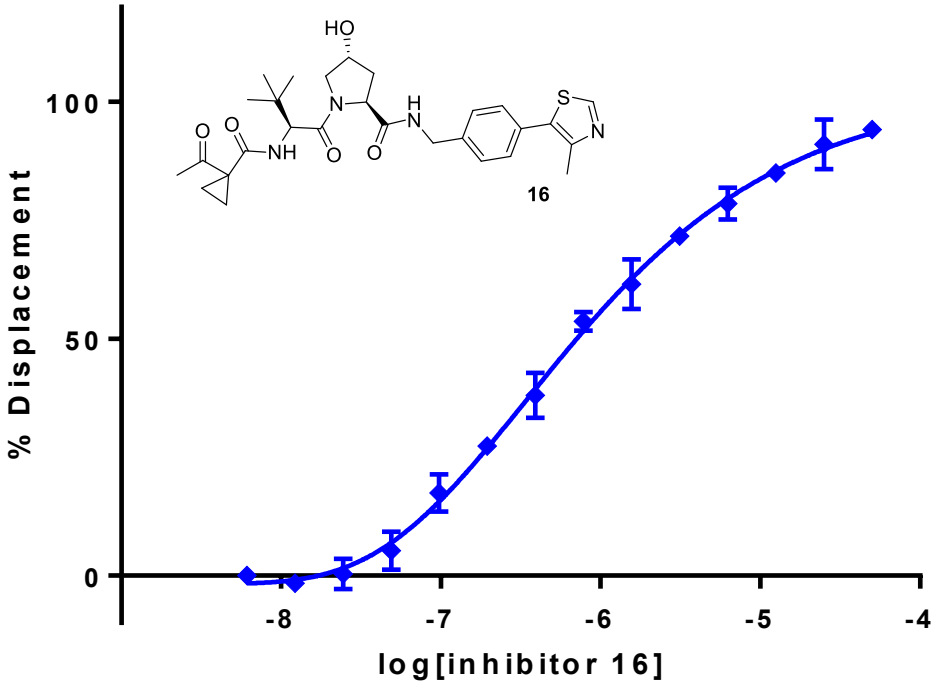
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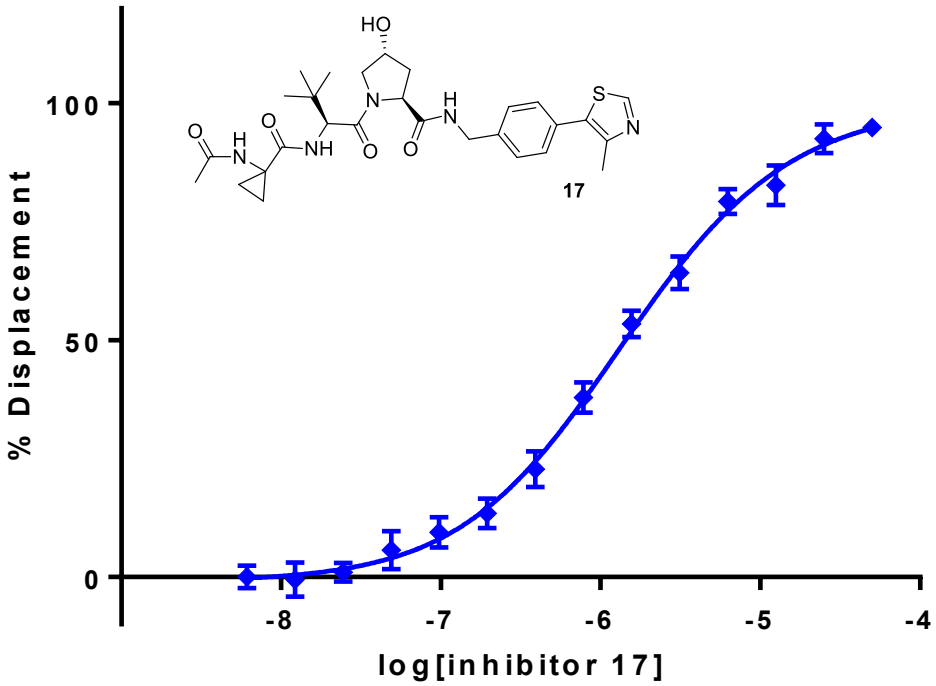
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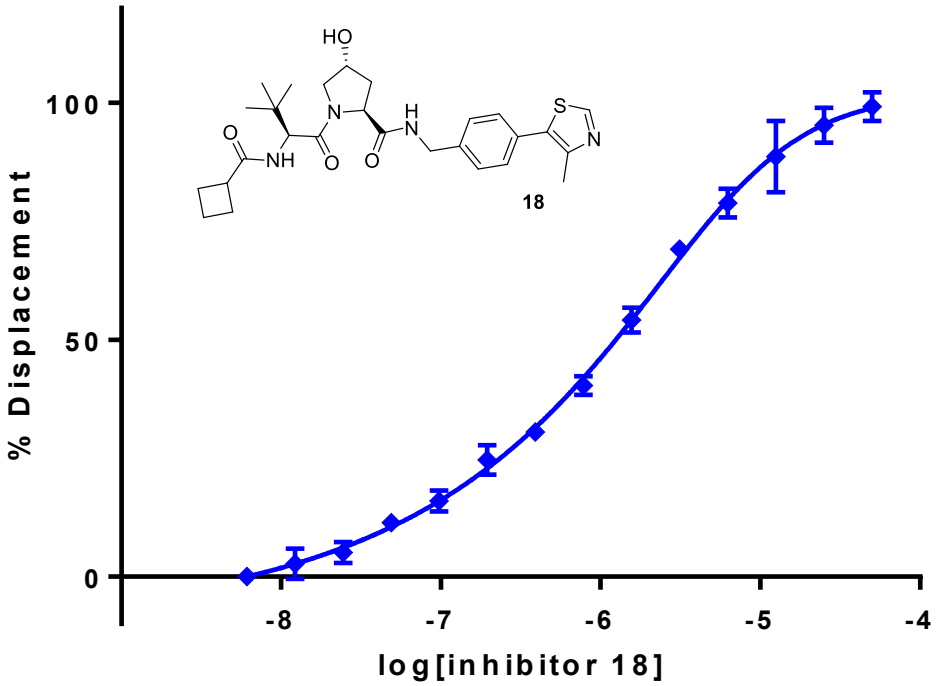
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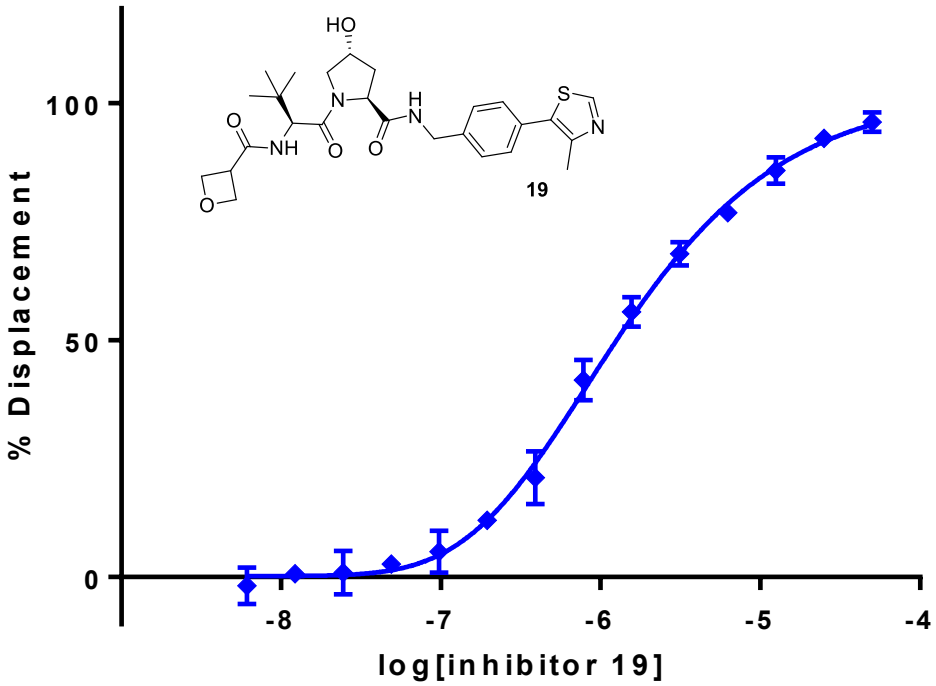
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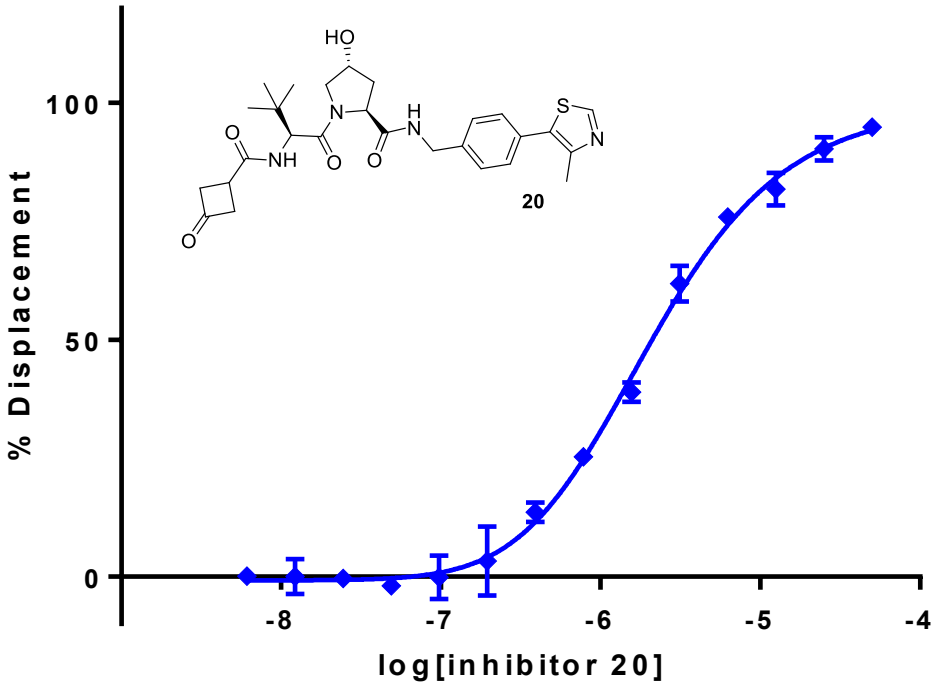
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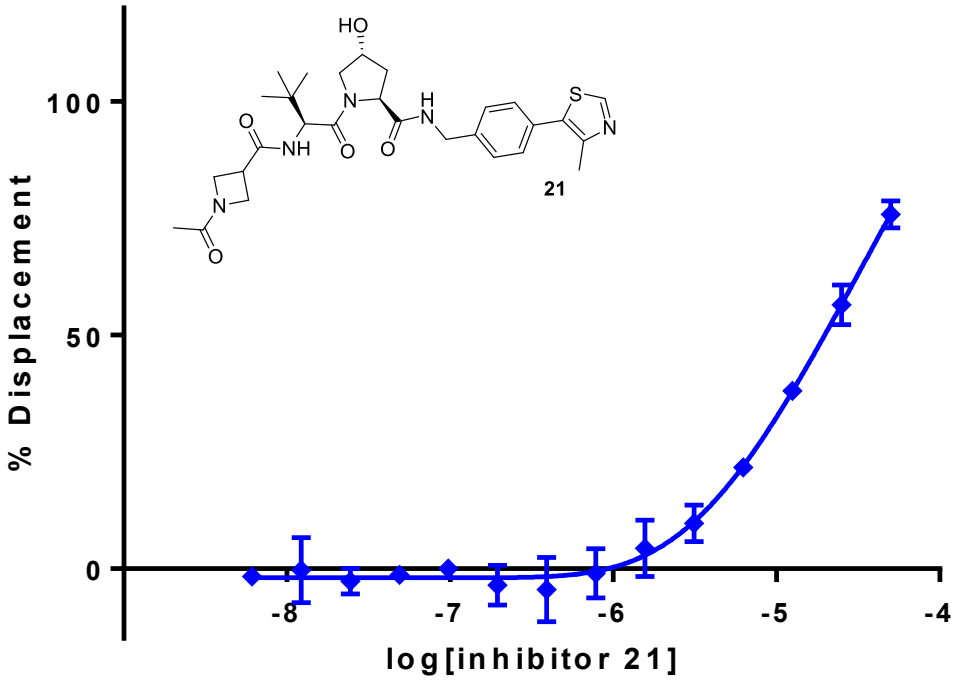
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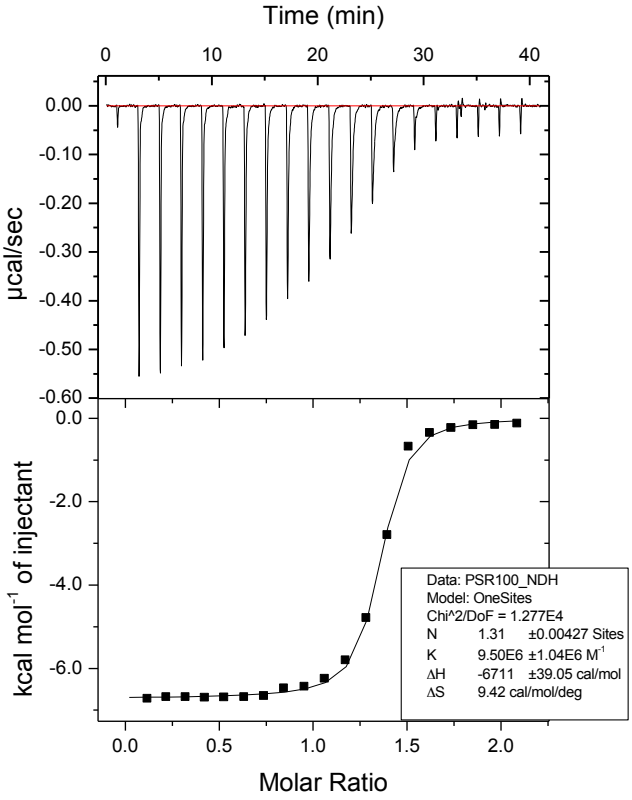

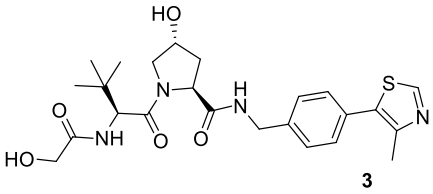

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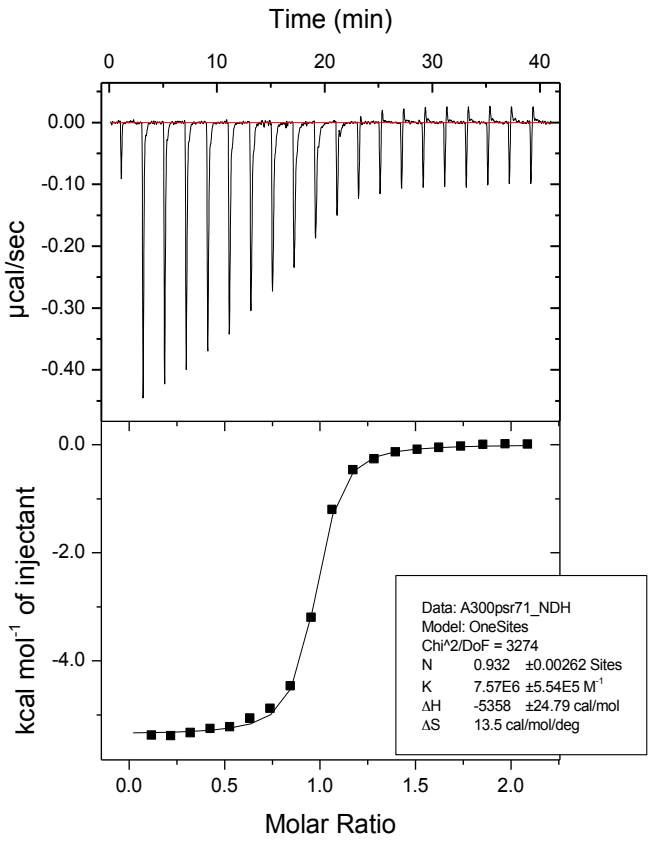

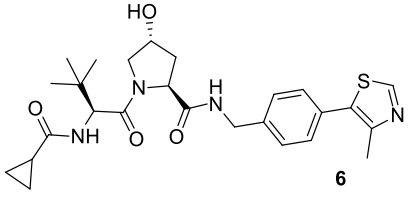

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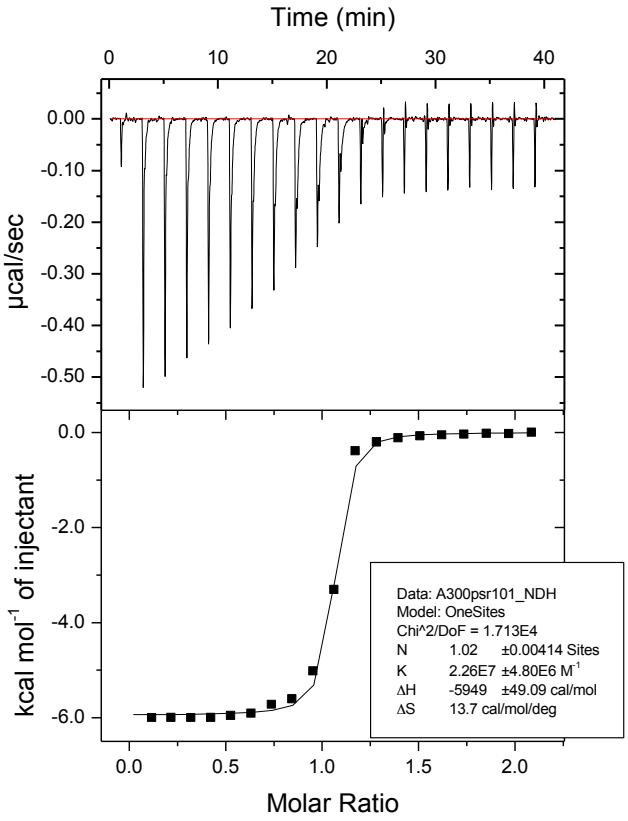
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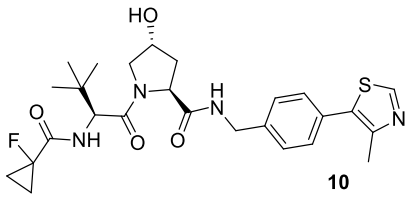
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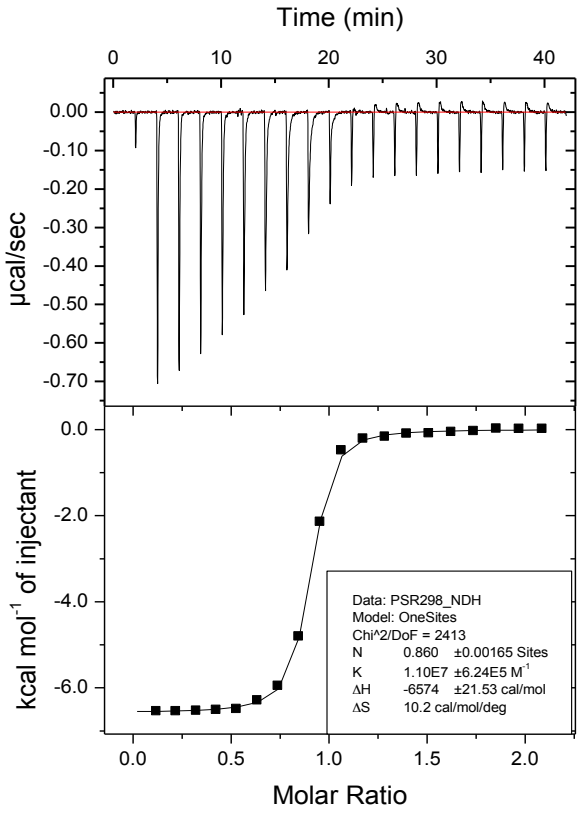
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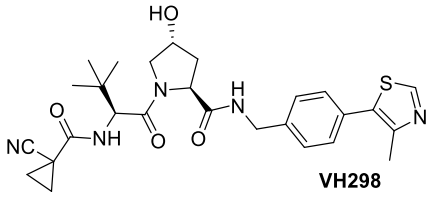
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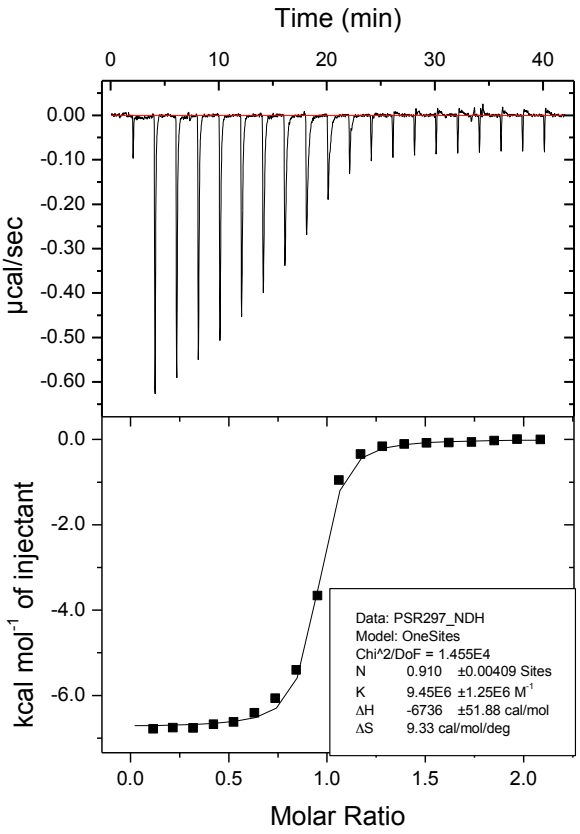
VH298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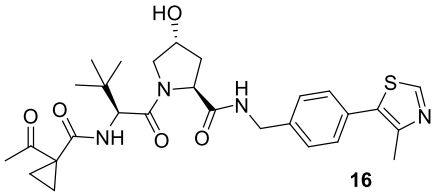
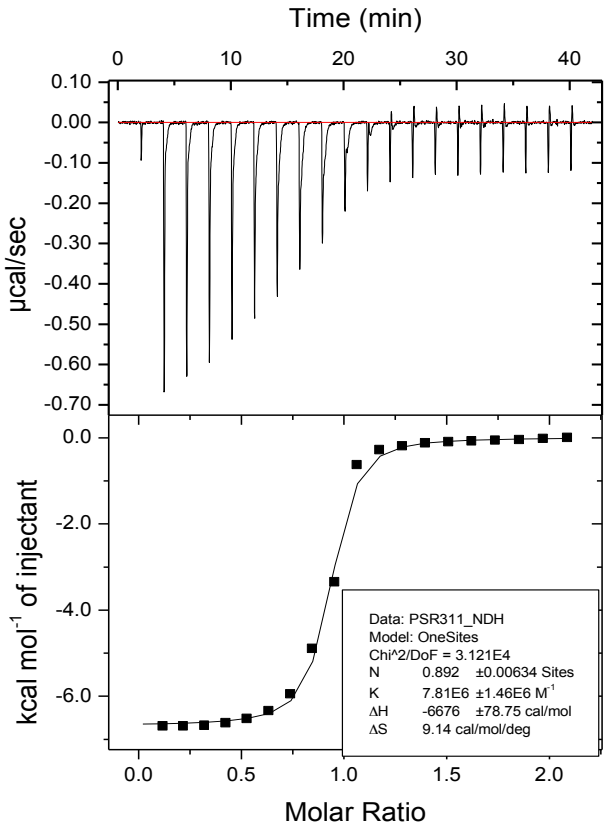
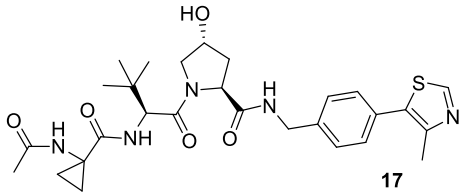
µcal/sec
kcal mol-1 of injectant
Molar Ratio
Data: PSR297_NDH
Model: OneSites
Chi^2/DoF = 1.455E4
N 0.910 ±0.00409 Sites
K 9.45E6 ±1.25E6 M-1
ΔH -6736 ±51.88 cal/mol
ΔS 9.33 cal/mol/deg



16


Time (min)
µcal/sec
kcal mol-1 of injectant
Molar Ratio
Data: PSR311_NDH
Model: OneSites
Chi^2/DoF = 3.121E4
N 0.892 ±0.00634 Sites
K 7.81E6 ±1.46E6 M-1
ΔH -6676 ±78.75 cal/mol
ΔS 9.14 cal/mol/deg



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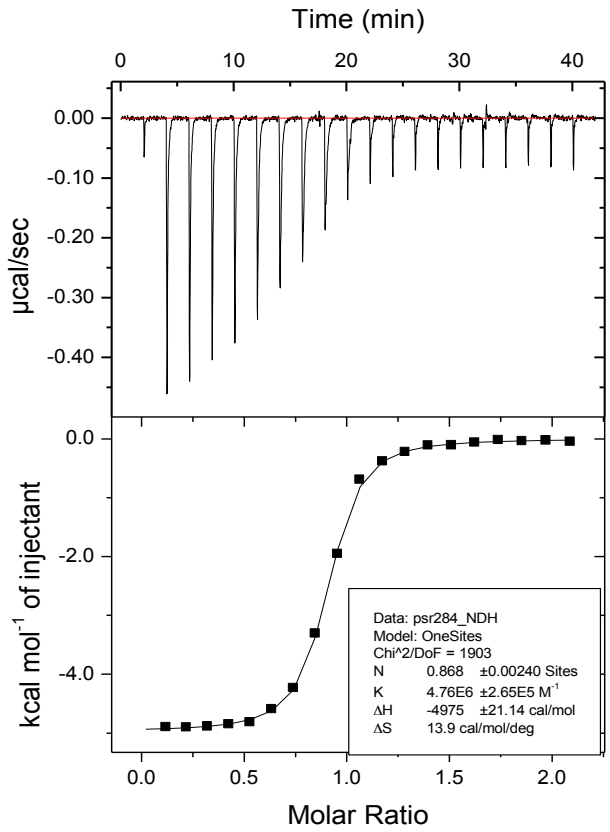
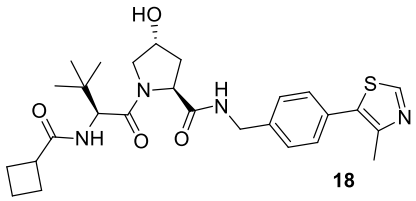

Time (min)
µcal/sec
kcal mol-1 of injectant
Molar Ratio
Data: psr284_NDH
Model: OneSites
Chi^2/DoF = 1903
N 0.868 ±0.00240 Sites
K 4.76E6 ±2.65E5 M-1
ΔH -4975 ±21.14 cal/mol
ΔS 13.9 cal/mol/deg



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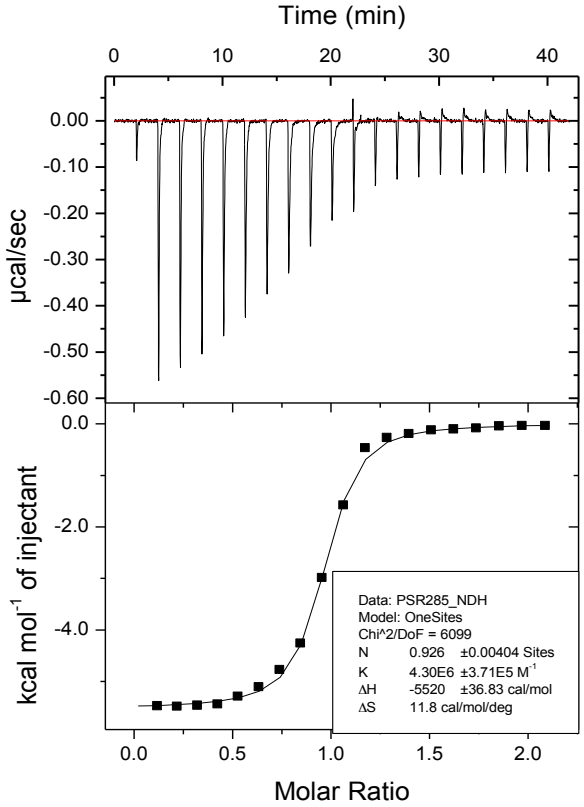
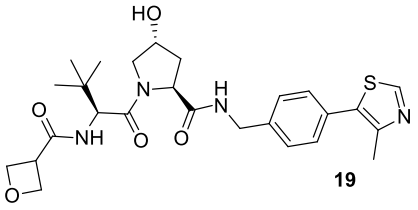
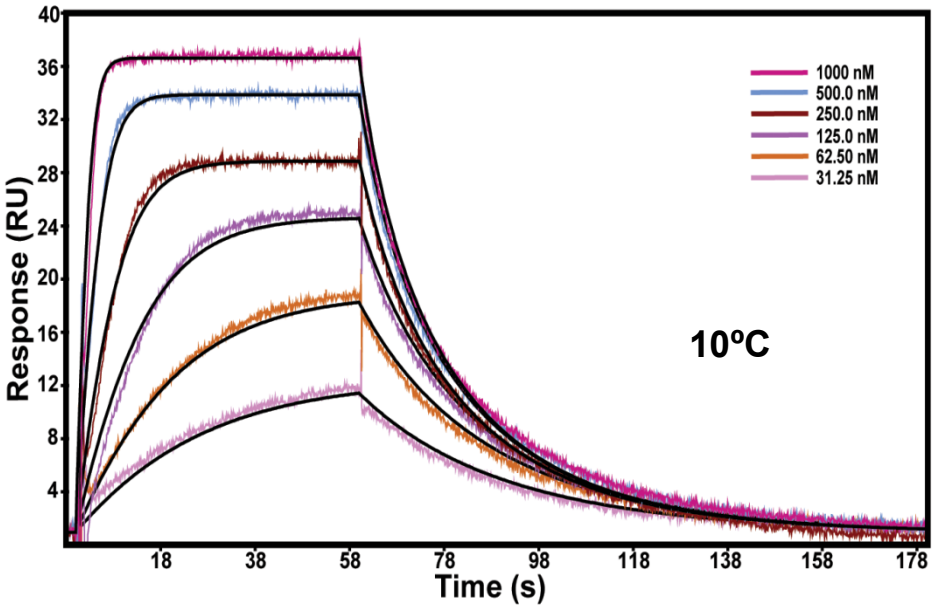
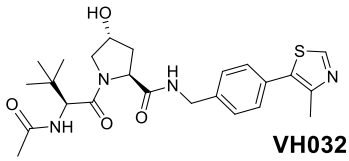

Time (min)
µcal/sec
kcal mol-1 of injectant
Molar Ratio
Data: PSR285_NDH
Model: OneSites
Chi^2/DoF = 6099
N 0.926 ±0.00404 Sites
K 4.30E6 ±3.71E5 M-1
ΔH -5520 ±36.83 cal/mol
ΔS 11.8 cal/mol/deg

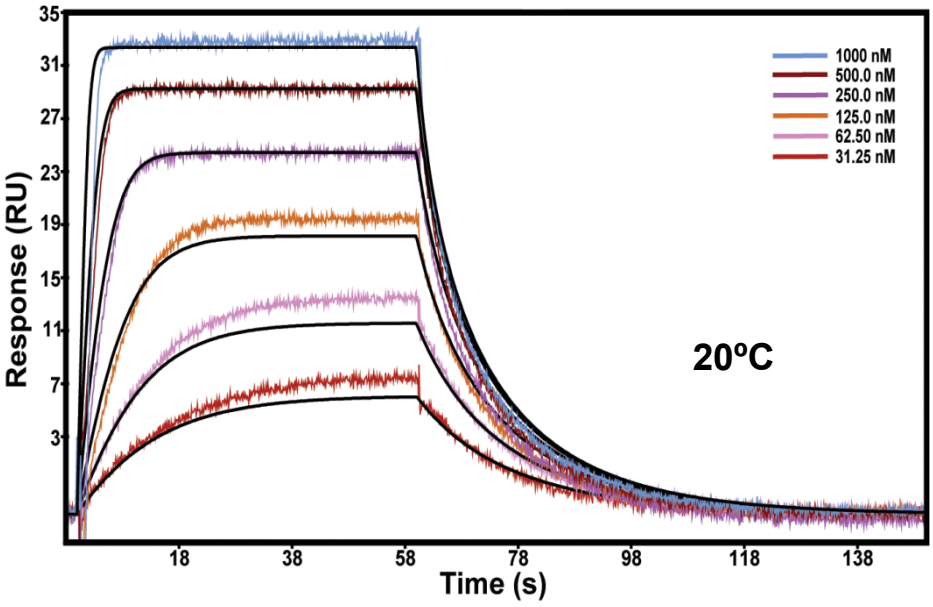


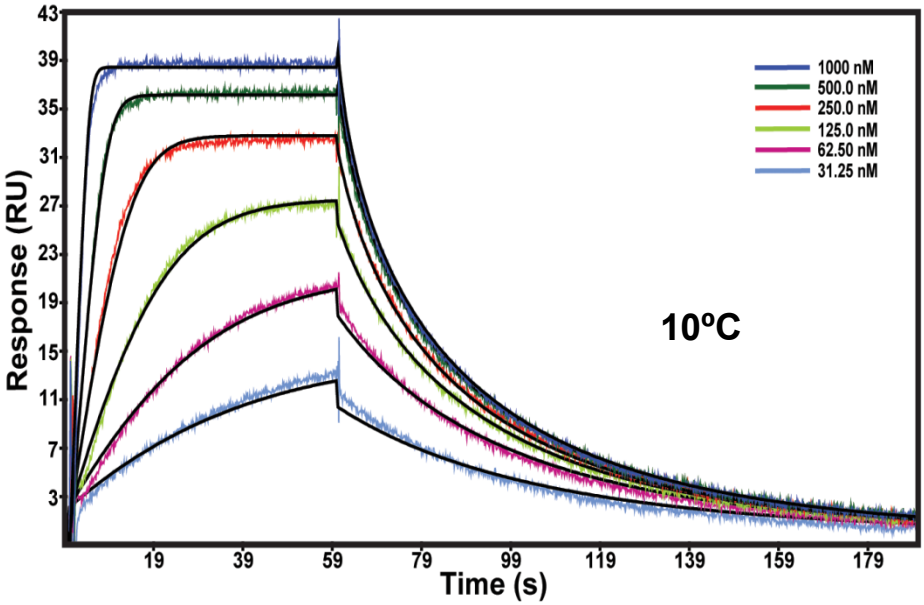
19

SPR Sensograms





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Time (s)
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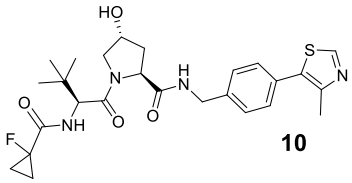
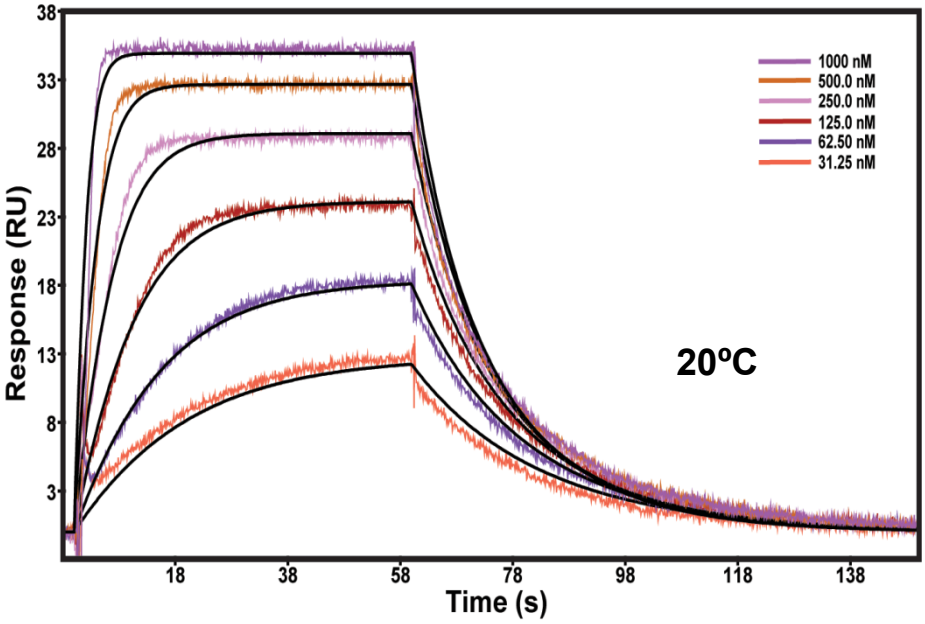
250.0 nM
125.0 nM
62.50 nM
31.25 nM
10ºC


HO
S
N
H
O
NH
F

10


Response (RU)
Time (s)
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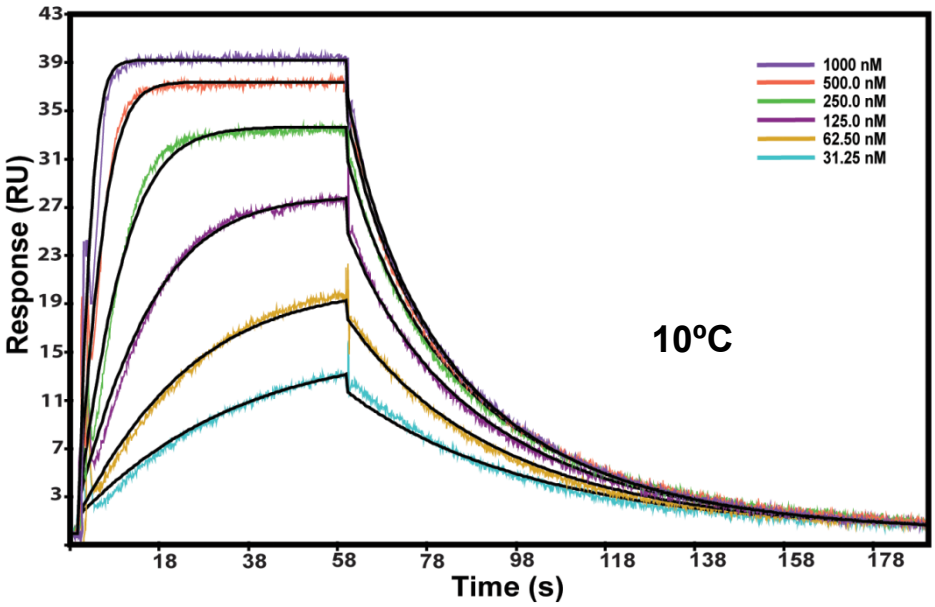
500.0 nM
250.0 nM
125.0 nM
62.50 nM
31.25 nM
20ºC


Response (RU)
Time (s)
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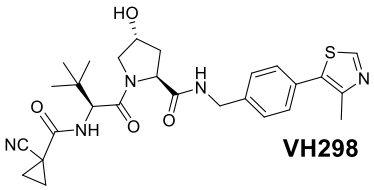
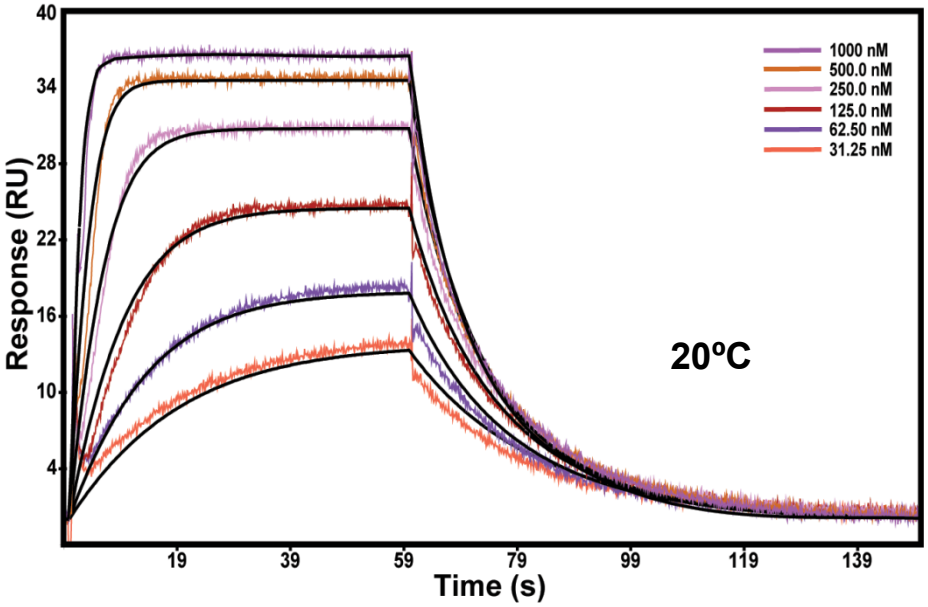
500.0 nM
250.0 nM
125.0 nM
62.50 nM
31.25 nM
10ºC


HO
S
N
H
N
O
NH
O
O
NC

VH298


Response (RU)
Time (s)
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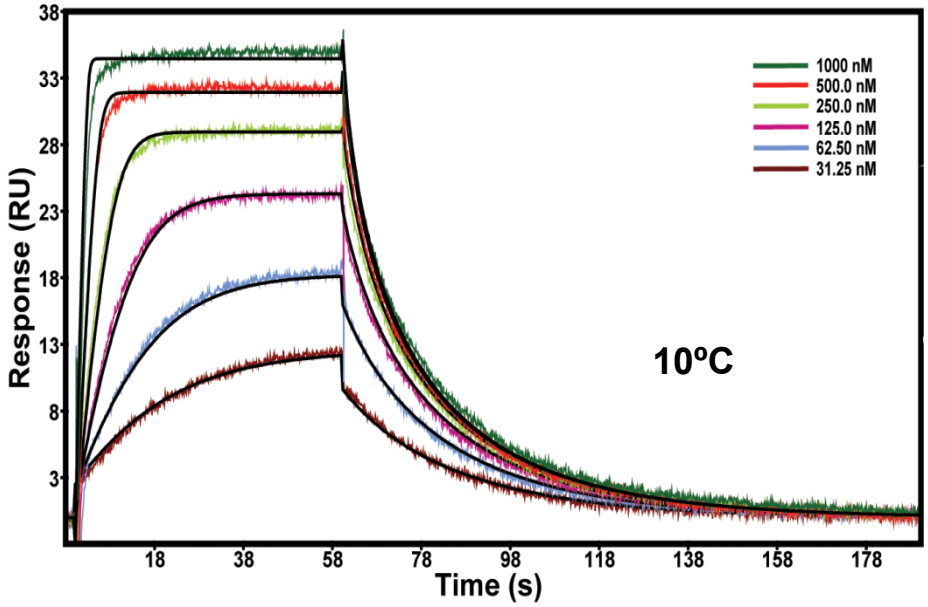
125.0 nM
62.50 nM
31.25 nM
20ºC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Time (s)
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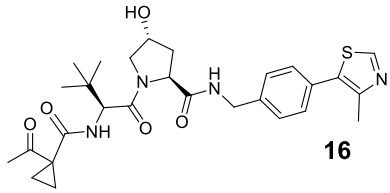
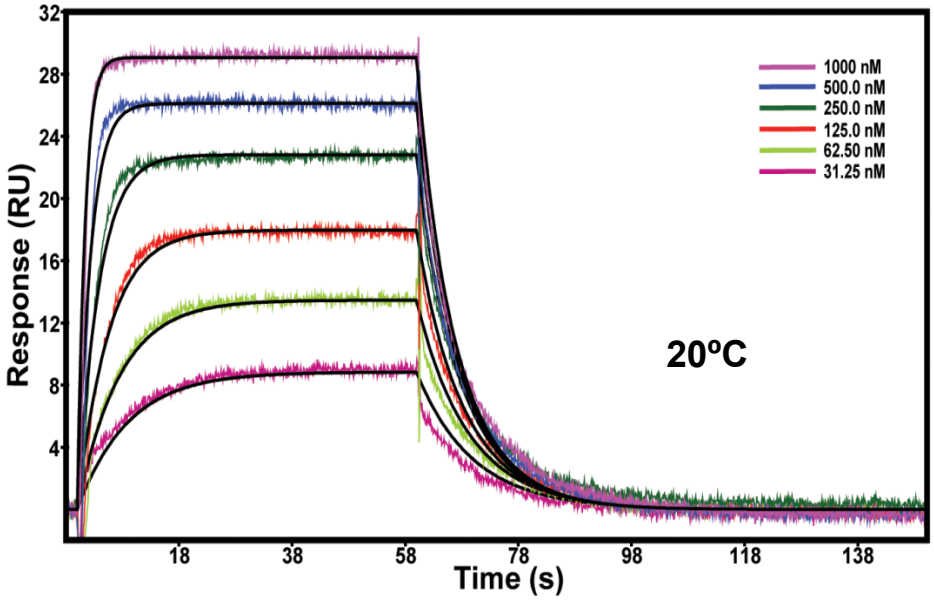
125.0 nM
62.50 nM
31.25 nM
10ºC


HO
S
N
H
N
O
NH
O
O
O

16


Response (RU)
Time (s)
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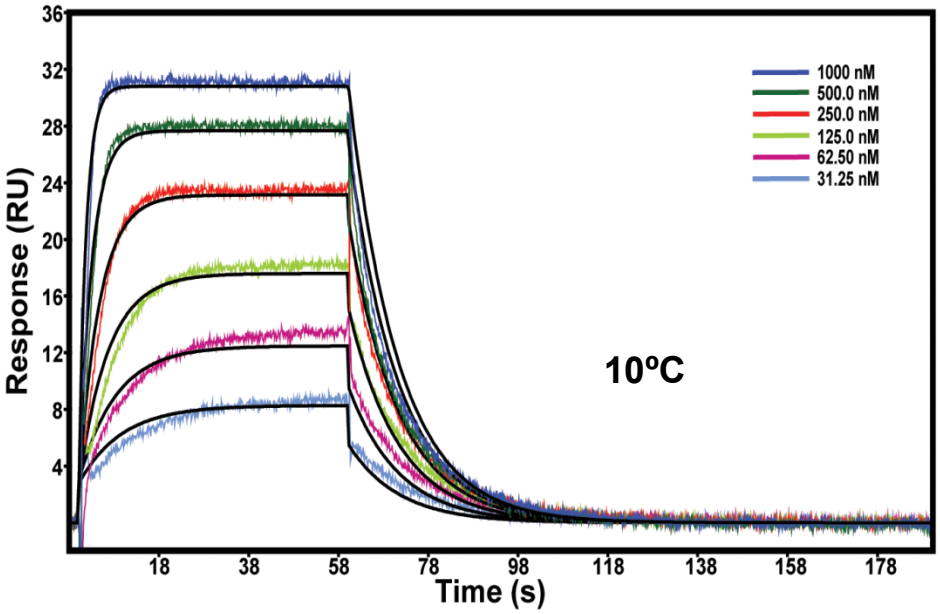
250.0 nM
125.0 nM
62.50 nM
31.25 nM
20ºC


Response (RU)
Time (s)
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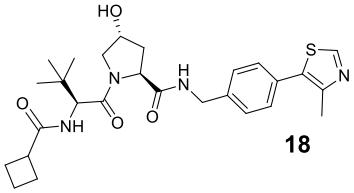
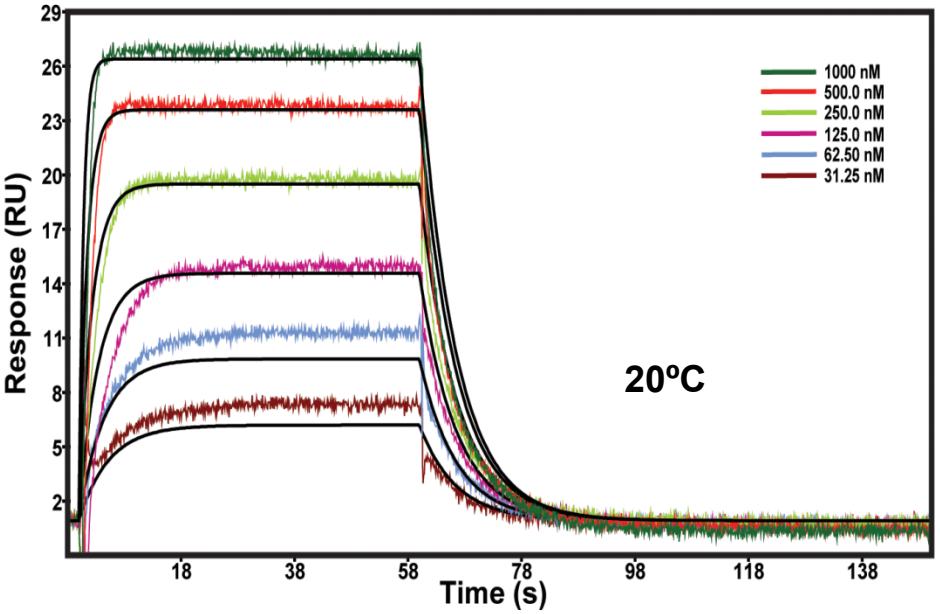
250.0 nM
125.0 nM
62.50 nM
31.25 nM
10ºC


HO
S
N
H
N
O
NH
O
O

18


Response (RU)
Time (s)
1000 nM
500.0 nM
250.0 nM
125.0 nM
62.50 nM
31.25 nM
20ºC

X-ray Crystallography

Supplementary Table 1
Evaluation of intracellular accumulation of VHL inhibitors **VH032**, **10**, **VH298**, **16** and **18** on HeLa cells after 2 h treatment with compounds.

Inhibitor	Compound associated with cells (μM)	Fraction unbound (fu)	Final free compound concentration in cells (μM)
VH032	463.8	0.0016	0.742
10	383.6	0.0002	0.071
VH298	150.0	0.0007	0.058
16	100.0	0.0008	0.046
18	204.0	0.0008	0.093

Propanolol compound with high concentration associated with cells (2679.0 μM) and Metoprolol compound with high fraction unbound (0.0050) were used as controls for the experiment.

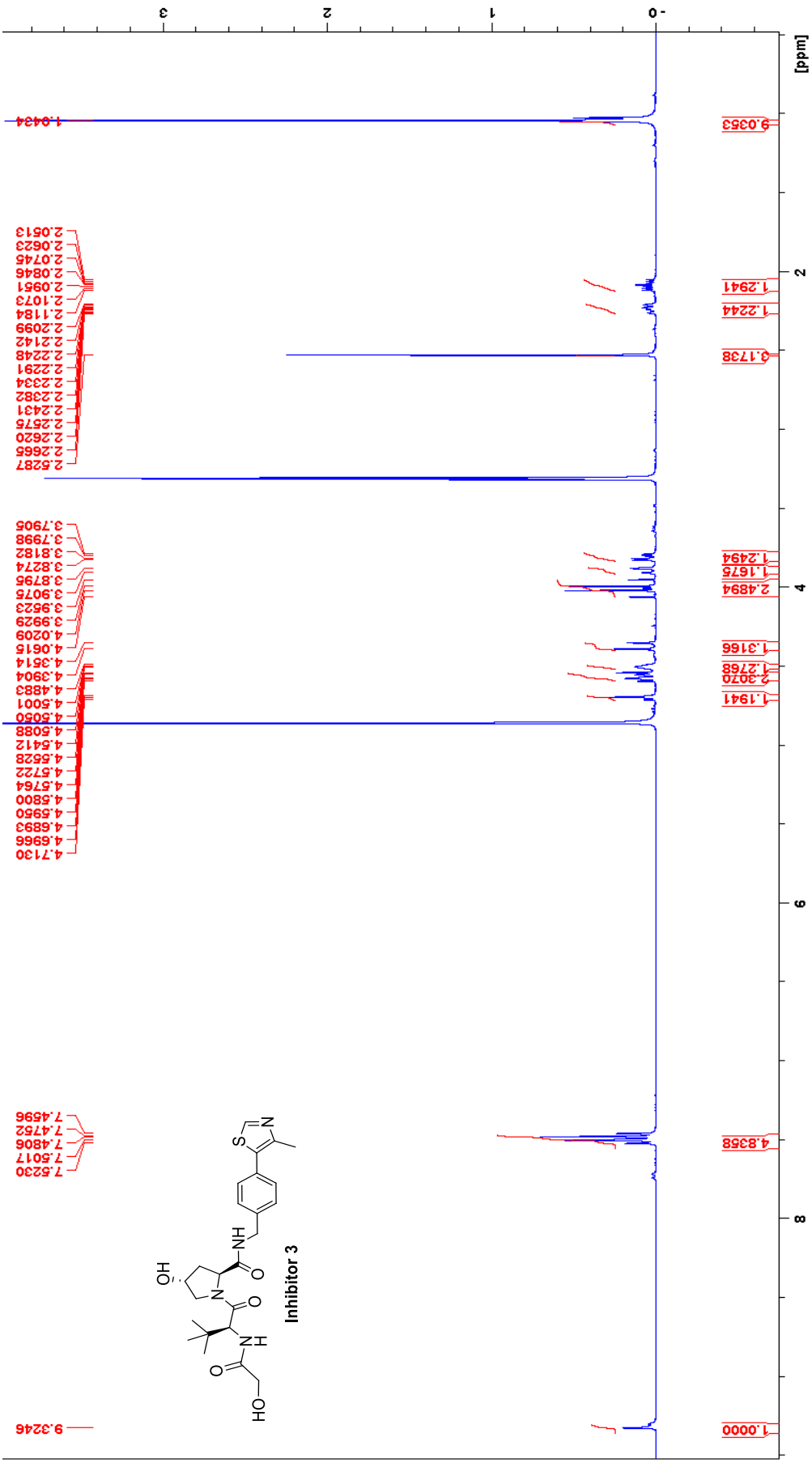
Supplementary Table 2.
Crystallographic data processing and refinement statistics.

Values in parentheses are for the highest resolution shell.

Dataset	Inhibitor 3	Inhibitor 6	Inhibitor 10	Inhibitor 11
Synchrotron	Diamond	Diamond	Diamond	Diamond
Wavelength (Å)	0.9174	0.9174	0.9174	0.9282
Processing statistics				
Space group	$P4_122$	$P4_122$	$P4_122$	$P4_122$
Unit cell parameters				
a,*b* (Å)	93.3	93.2	93.4	93.1
c (Å)	365.0	365.1	365.2	363.0
Resolution limits (Å)	48.9–2.10 (2.14–2.10)	48.9–2.20 (2.24–2.20)	49.0–2.20 (2.24–2.20)	48.8–2.90 (3.03–2.90)
Total reflections	849102 (41946)	556127 (31559)	540233 (25019)	311841 (39080)
Unique reflections	95188 (4654)	82957 (4452)	83072 (4446)	36661 (4375)
Completeness (%)	99.8 (99.9)	99.8 (100)	99.6 (99.0)	100 (100)
Multiplicity	8.9 (9.0)	6.7 (7.1)	6.5 (5.6)	8.5 (8.9)
R_{merge} (%)	14.2 (98.0)	14.1 (85.7)	10.4 (72.2)	21.2 (89)
$I/\sigma(I)$	9.4 (2.1)	8.6 (2.2)	10.8 (2.0)	7.3 (2.2)
$CC_{1/2}$ (%)	99.8 (84.1)	99.7 (83.7)	99.9 (73.8)	99.0 (85.2)
Wilson *B* factor (Å^2)	23.5	23.2	27.5	26.2
Mosaicity (°)	0.05	0.05	0.13	0.26
Refinement statistics				
Resolution limits (Å)	93.3–2.10 (2.15–2.10)	93.2–2.20 (2.26–2.20)	93.4–2.20 (2.26–2.20)	93.08–2.90 (2.97–2.90)
R_{work} (%)	20.3 (25.1)	20.1 (24.9)	19.7 (27.7)	20.2 (28.5)
R_{free} (%)	24.5 (30.2)	24.9 (30.8)	24.5 (32.9)	26.8 (32.5)
No. reflections	90242 (6564)	78650 (5687)	78759 (5677)	34701 (1893)
No. test reflections	4830 (370)	4192 (307)	4203 (309)	2505 (137)
Model atoms	11635	11675	11642	11274
Protein *B* factor (Å^2)	37.2	37.4	40.3	62.3
Ligand *B* factor (Å^2)	26.9	24.6	27.9	41.2
r.m.s.d. bonds (Å)	0.007	0.008	0.008	0.007
r.m.s.d. angles (°)	1.27	1.29	1.28	1.25
Ramachandran plot				
Favoured (%)	97.6	97.4	97.4	96.0
Allowed (%)	2.3	2.5	2.5	3.2
Disallowed (%)	0.1	0.1	0.1	0.8
PDB code	5NVV	5NVW	5NVX	5NVY

Values in parentheses are for the highest resolution shell.

Dataset	Inhibitor 16	Inhibitor 17	Inhibitor 18	Inhibitor 19
Synchrotron	Diamond	Diamond	Diamond	Diamond
Wavelength (Å)	0.9282	0.9282	0.9282	0.9795
Processing statistics				
Space group	$P4_122$	$P4_122$	$P4_122$	$P4_122$
Unit cell parameters				
a,*b* (Å)	94.8	94.8	94.6	94.6
c (Å)	368.1	368.4	368.1	368.6
Resolution limits (Å)	49.6–2.70 (2.79–2.70)	49.6–2.30 (2.35–2.30)	49.5–2.10 (2.14–2.10)	49.5–2.20 (2.24–2.20)
Total reflections	355433 (35459)	580756 (35833)	626378 (25675)	772384 (41366)
Unique reflections	47321 (4567)	75814 (4421)	98140 (4785)	86260 (4448)
Completeness (%)	99.8 (99.9)	99.8 (99.9)	99.4 (99.9)	100 (100)
Multiplicity	7.5 (7.8)	7.7 (8.1)	6.4 (5.4)	9.0 (9.3)
R_{merge} (%)	13.2 (100.6)	8.8 (80.2)	6.6 (61.3)	12.0 (70.9)
$I/\sigma(I)$	11.9 (2.2)	15.0 (2.8)	14.8 (2.5)	11.0 (3.1)
$CC_{1/2}$ (%)	99.9 (75.9)	99.9 (85.8)	99.9 (81.4)	99.8 (91.6)
Wilson *B* factor (Å^2)	36.0	34.6	33.2	31.1
Mosaicity (°)	0.18	0.06	0.05	0.05
Refinement statistics				
Resolution limits (Å)	94.8–2.70 (2.77–2.70)	94.8–2.30 (2.36–2.30)	94.6–2.10 (2.15–2.10)	94.6–2.20 (2.26–2.20)
R_{work} (%)	21.1 (31.4)	19.7 (22.9)	19.6 (25.4)	19.5 (22.6)
R_{free} (%)	27.0 (35.7)	23.8 (29.7)	23.8 (28.7)	24.2 (26.8)
No. reflections	44695 (3222)	71853 (5225)	93055 (6804)	81807 (5917)
No. test reflections	2419 (172)	3819 (286)	4964 (366)	4366 (321)
Model atoms	11069	11603	11697	11707
Protein *B* factor (Å^2)	52.8	47.7	44.5	42.4
Ligand *B* factor (Å^2)	41.3	39.2	33.5	29.8
r.m.s.d. bonds (Å)	0.007	0.007	0.007	0.008
r.m.s.d. angles (°)	1.22	1.21	1.25	1.27
Ramachandran plot				
Favoured (%)	96.1	97.6	96.1	96.1
Allowed (%)	3.9	2.3	3.6	3.9
Disallowed (%)	0	0.1	0	0
PDB code	5NVZ	5NW0	5NW1	5NW2


Inhibitor 3
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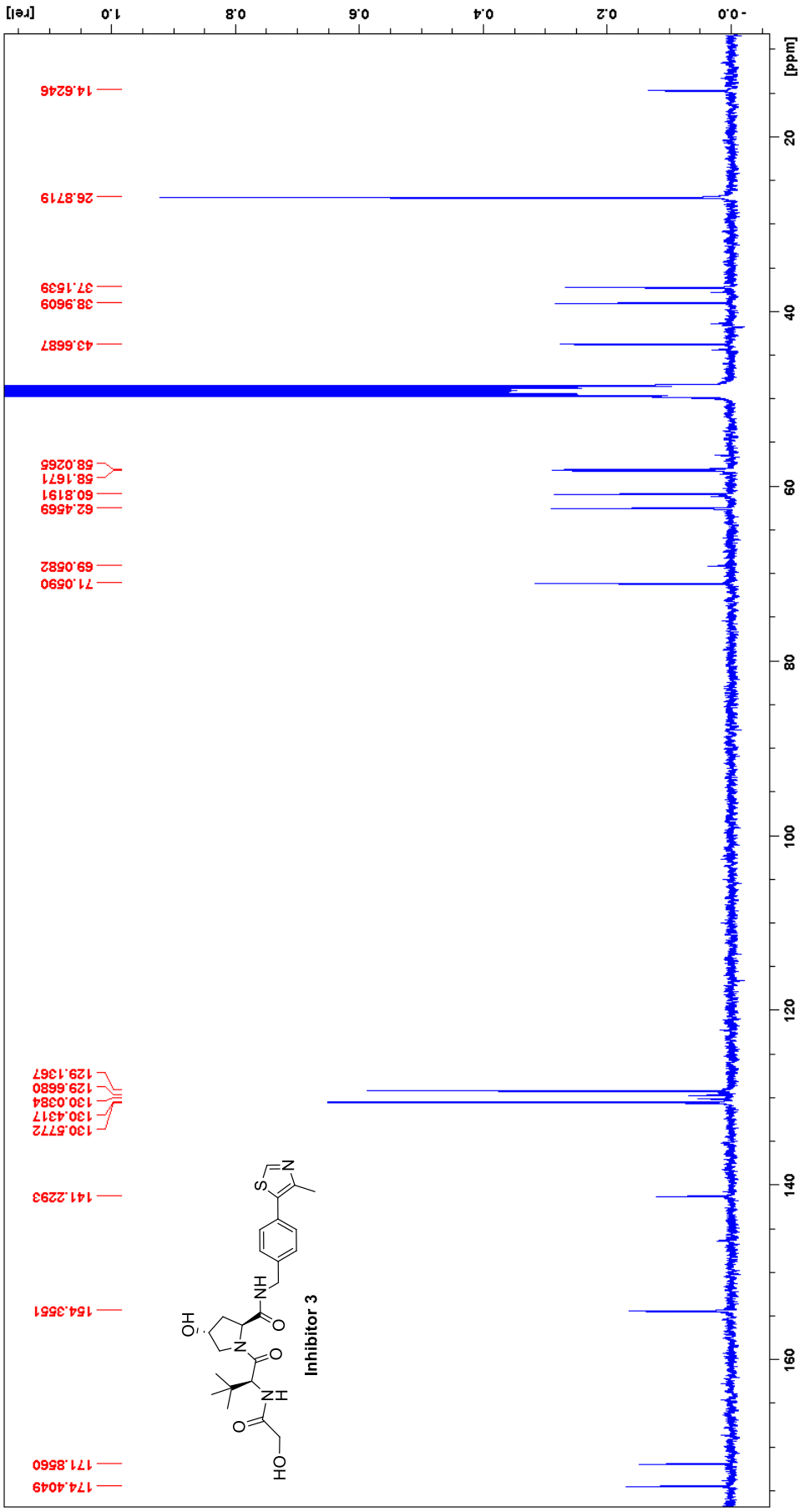
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0.2
0.4
0.6
0.8
1.0
[ppm]
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60
80
100
120
140
160
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26.8719
37.1539
38.9609
43.6687
58.0265
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60.8191
62.4569
69.0582
71.0590
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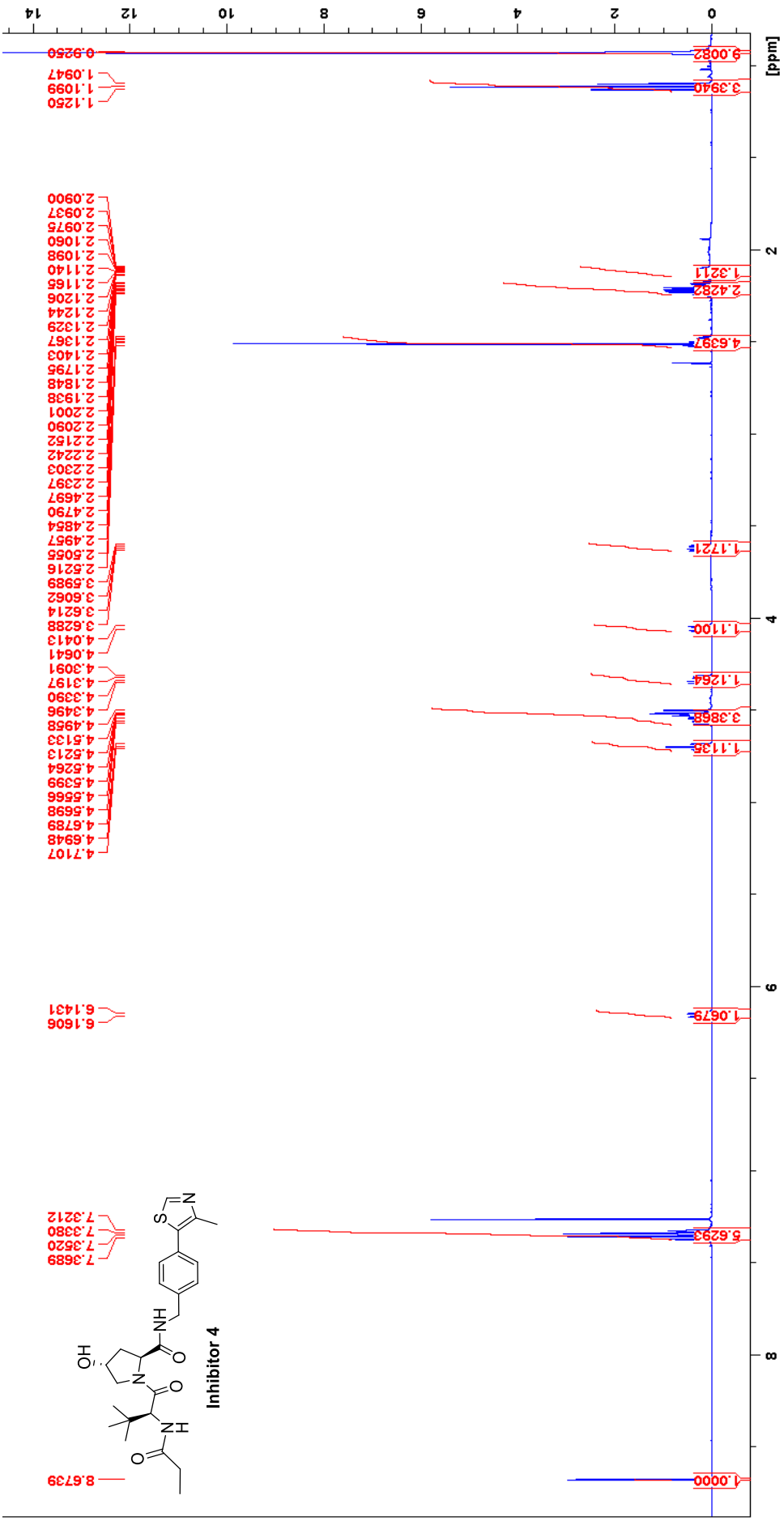
174.4049
Inhibitor 3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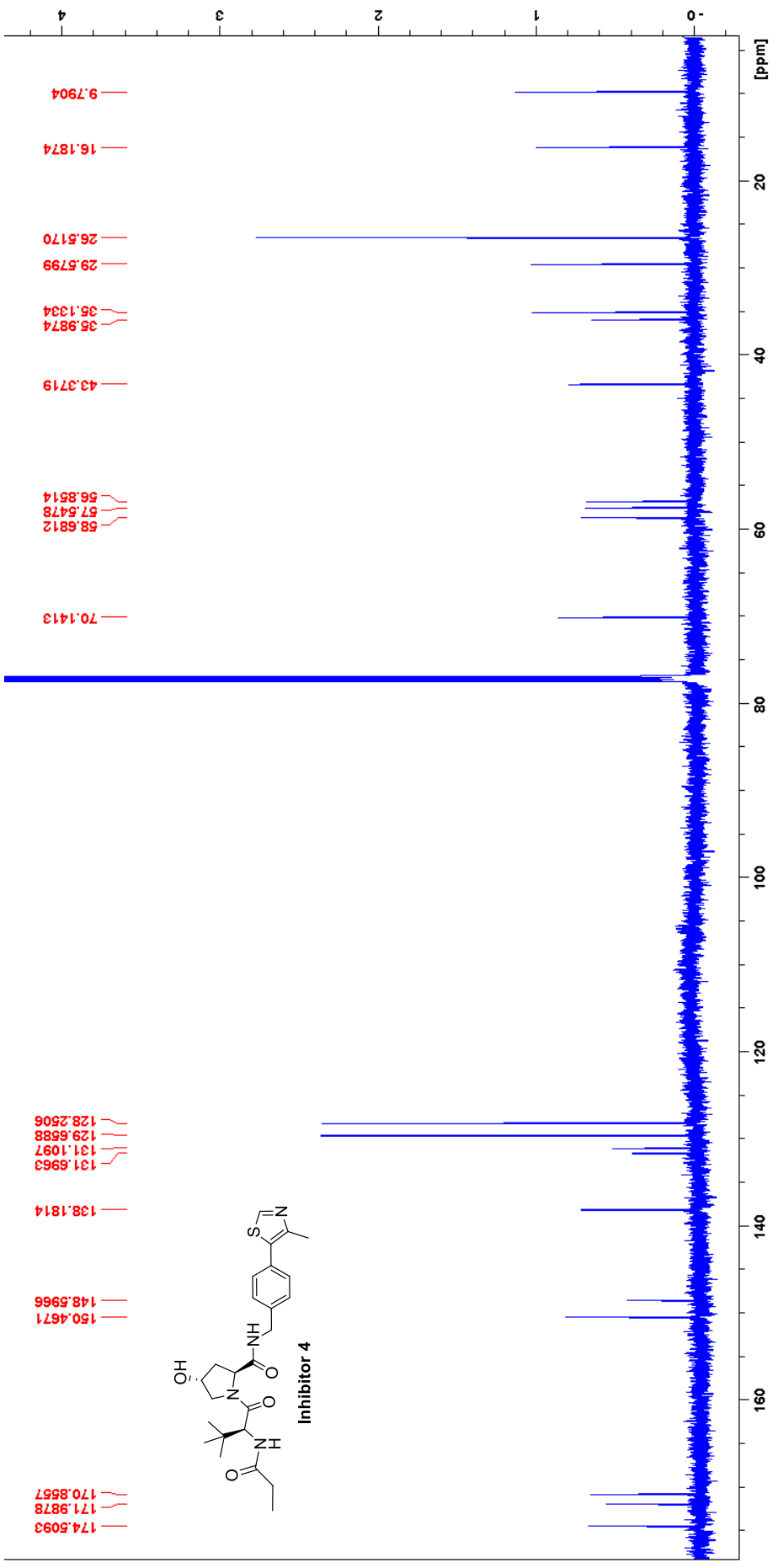
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29.5799
35.1334
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43.3719
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57.6478
58.6812
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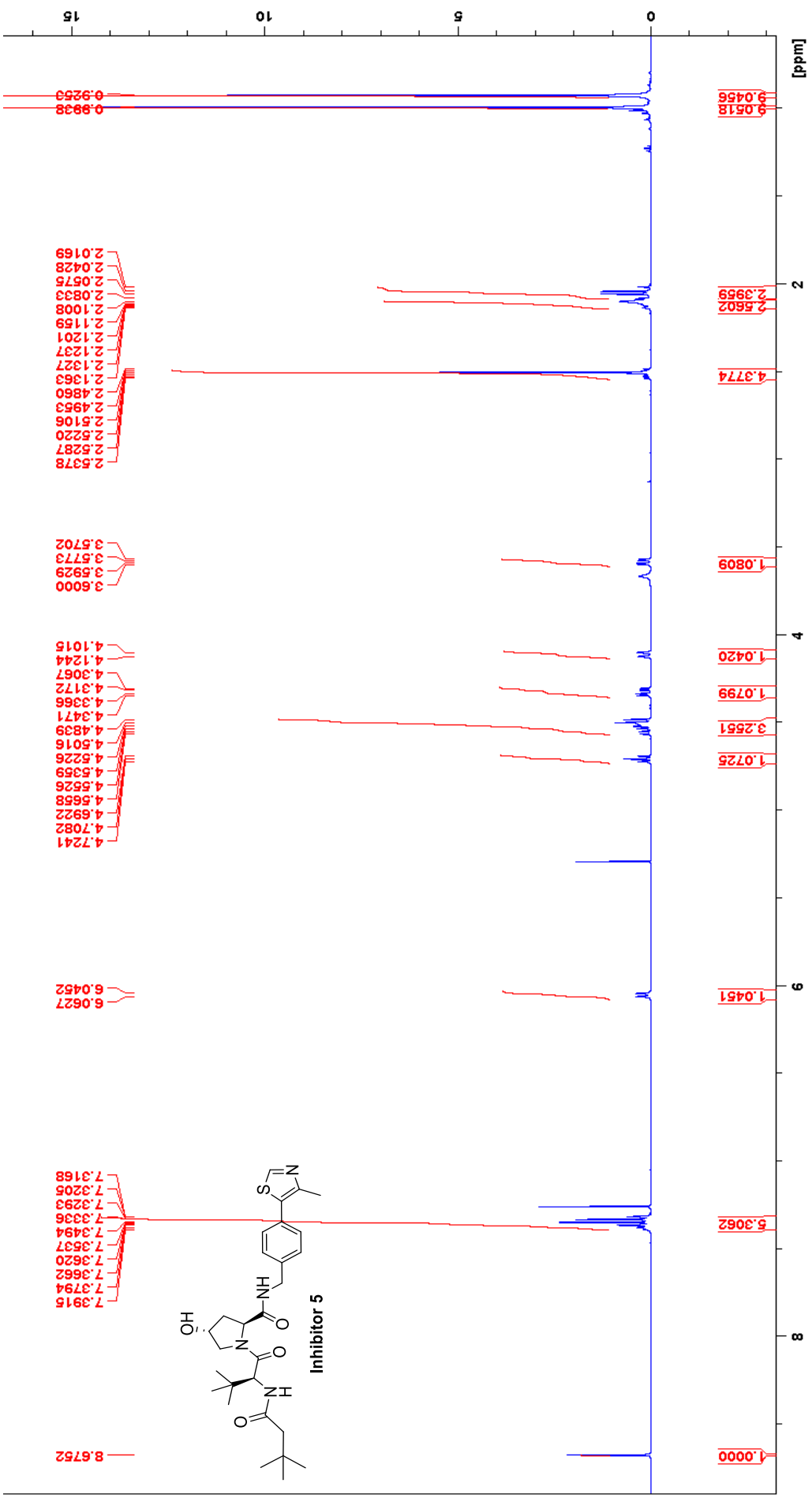
128.2506
129.6588
131.1097
131.6963
138.1814
148.5966
150.4671
170.8557
171.9878
174.5093
[ppm]
Inhibitor 4


[ppm]
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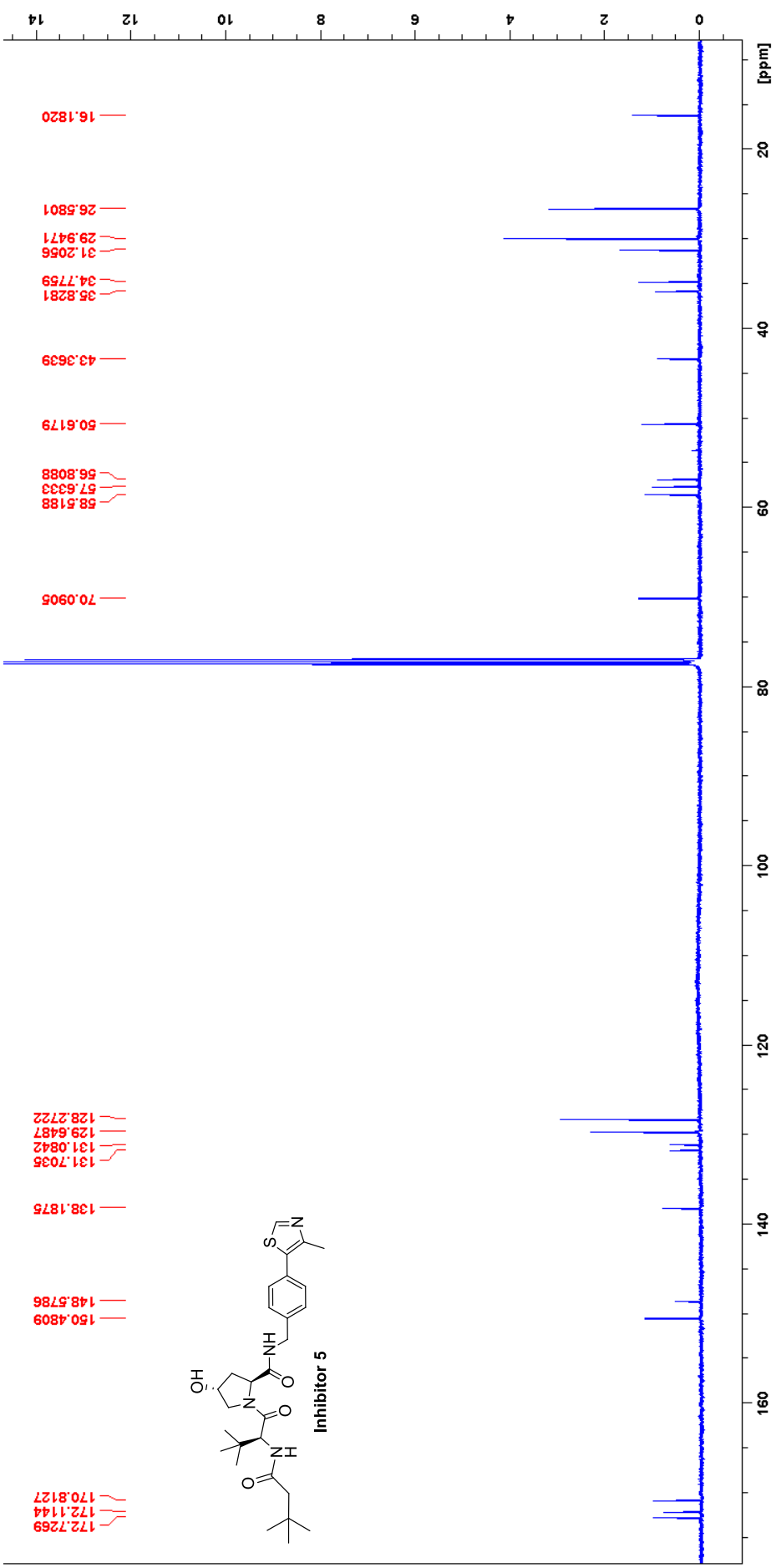
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29.9471
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129.6487
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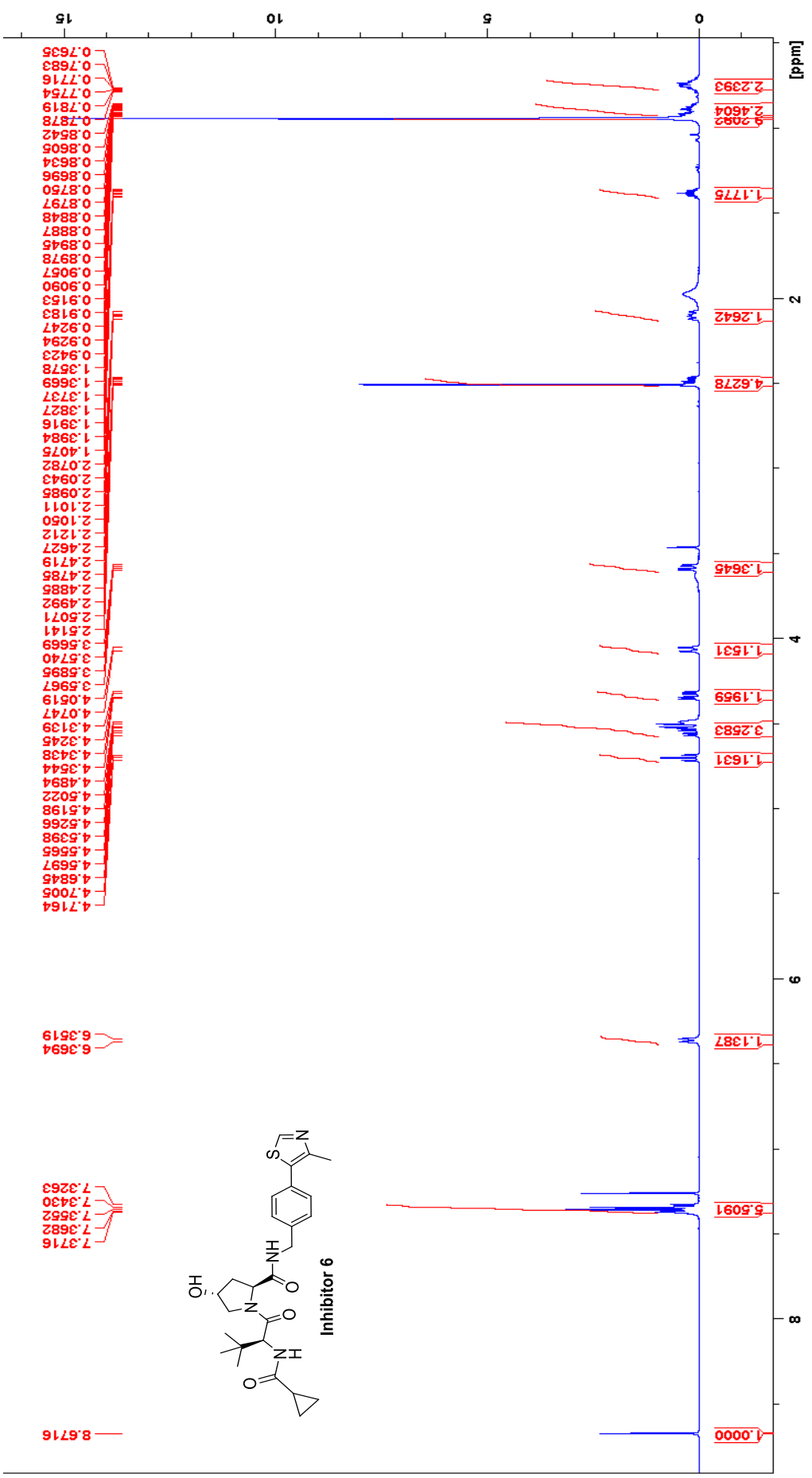
Inhibitor 5
[ppm]


Inhibitor 6
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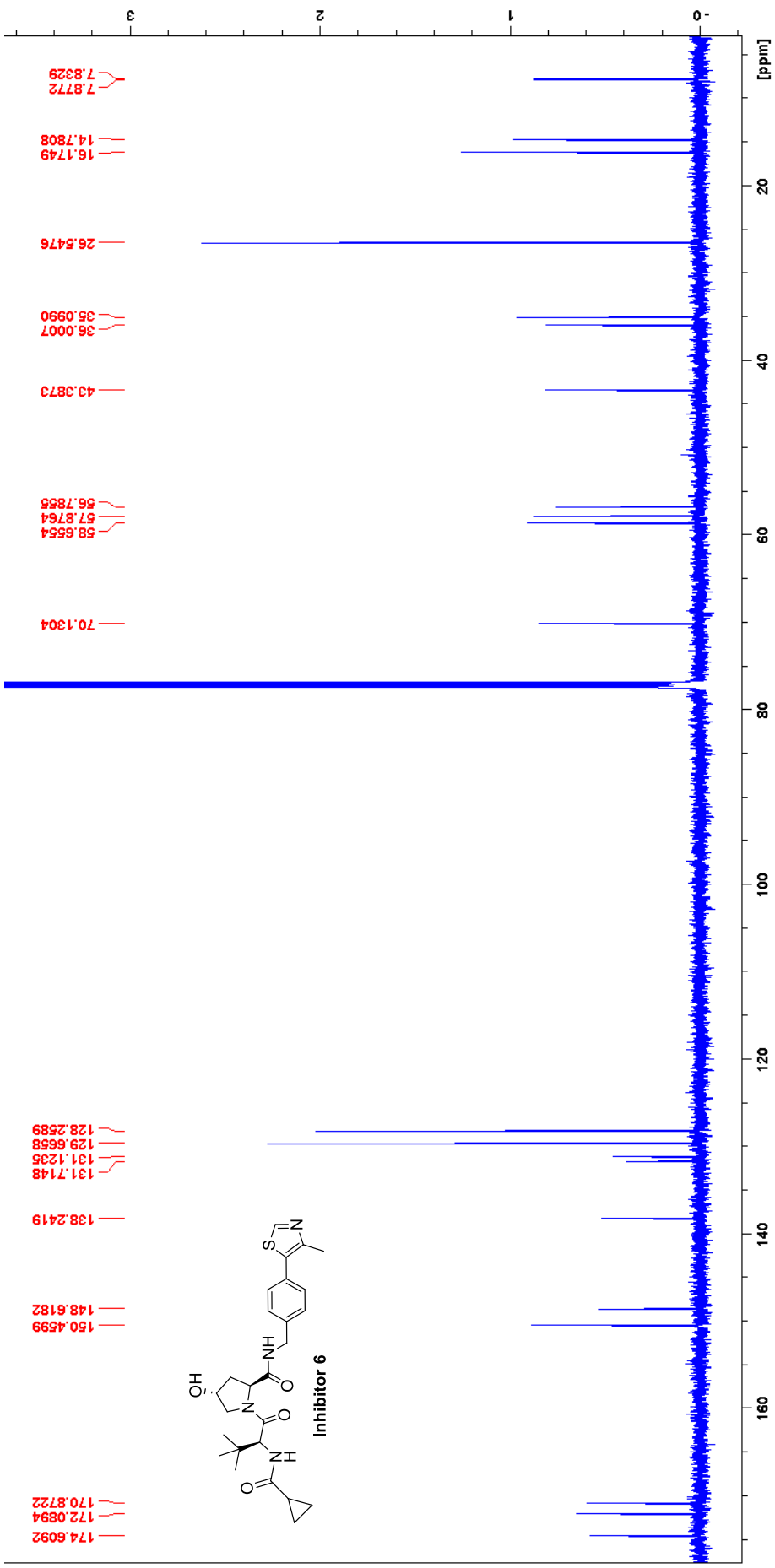
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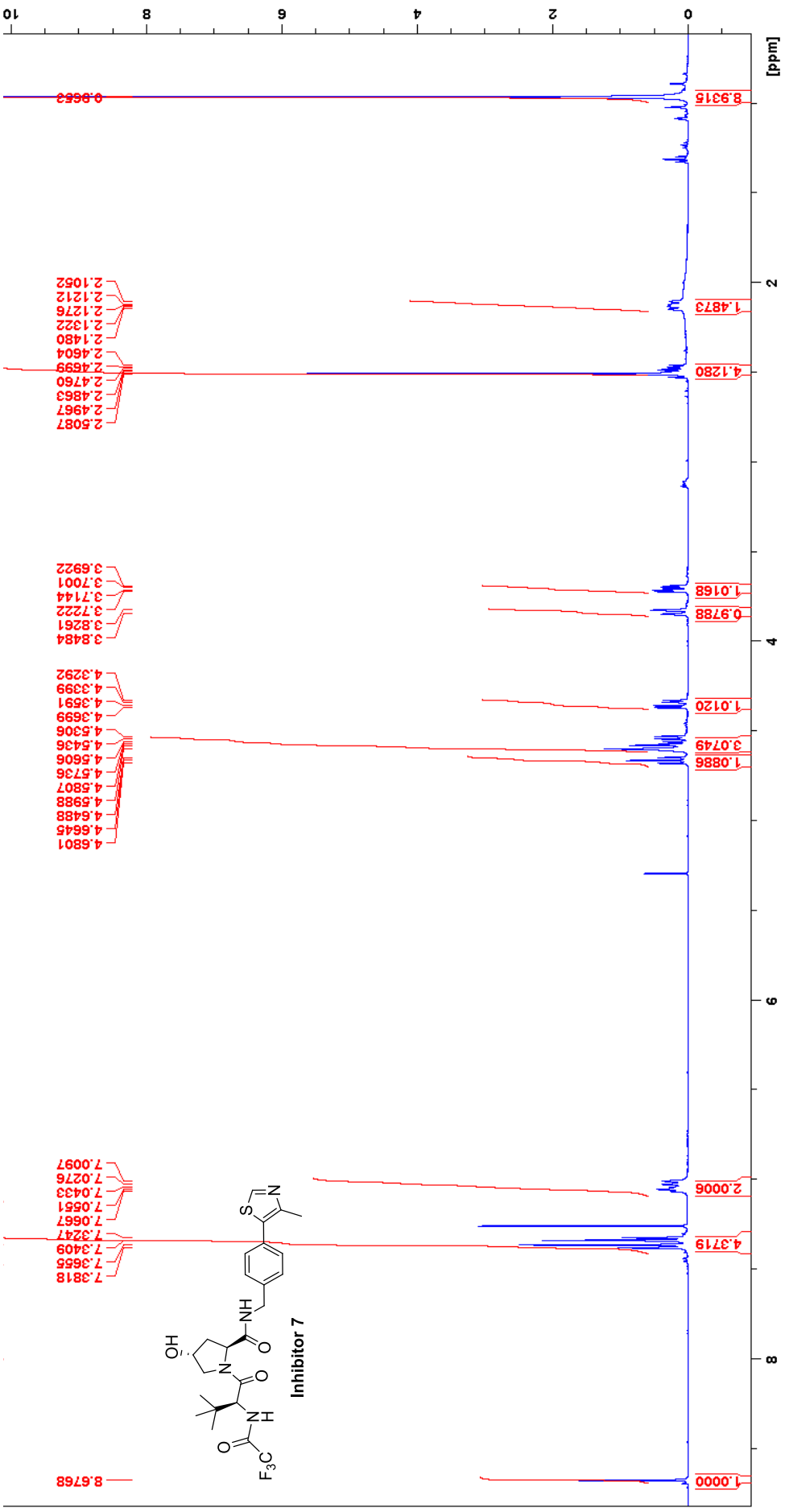
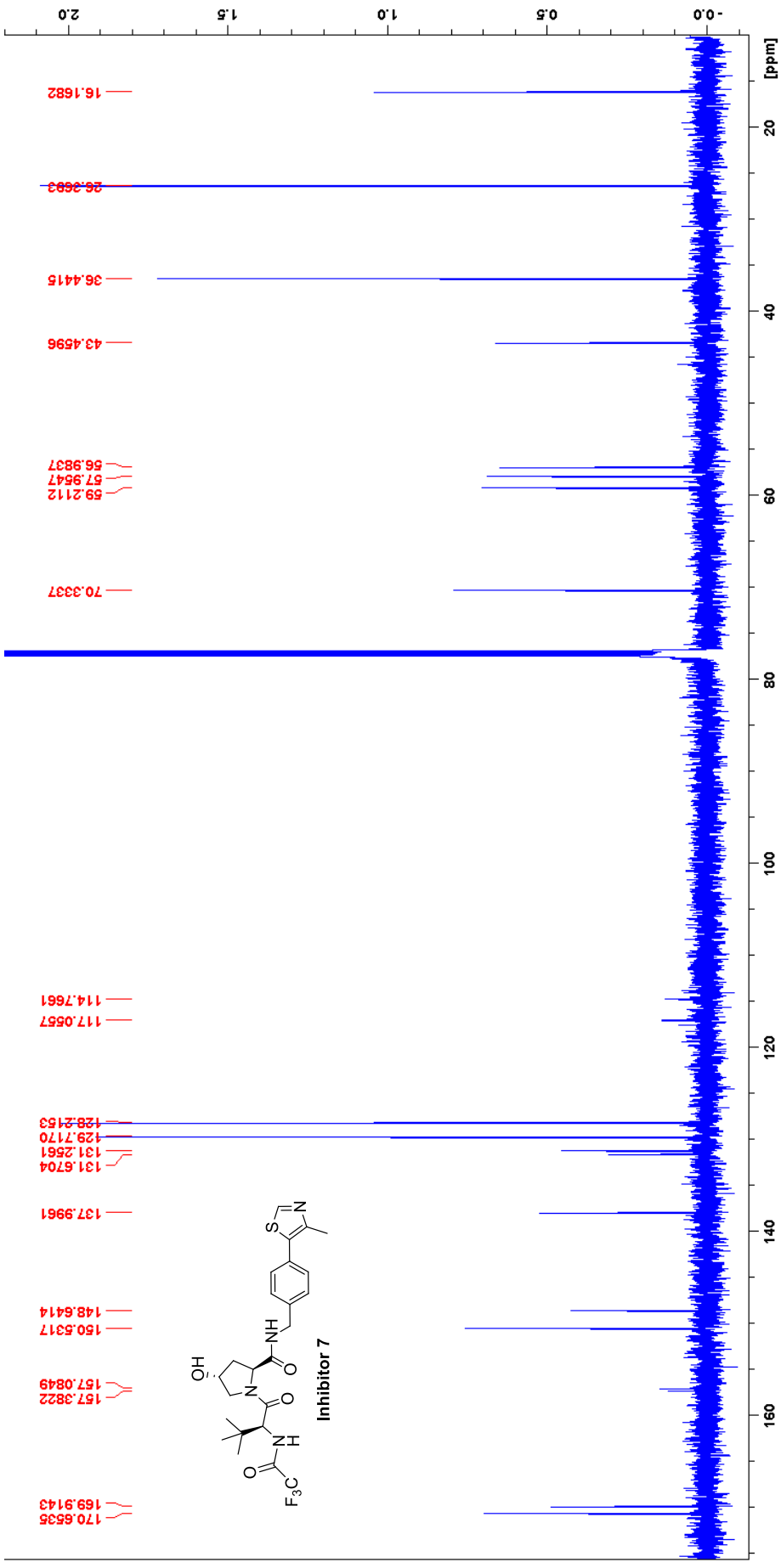
Inhibitor 6
[ppm]


Inhibitor 7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131.2561
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157.3822
169.9143
170.6535
Inhibitor 7
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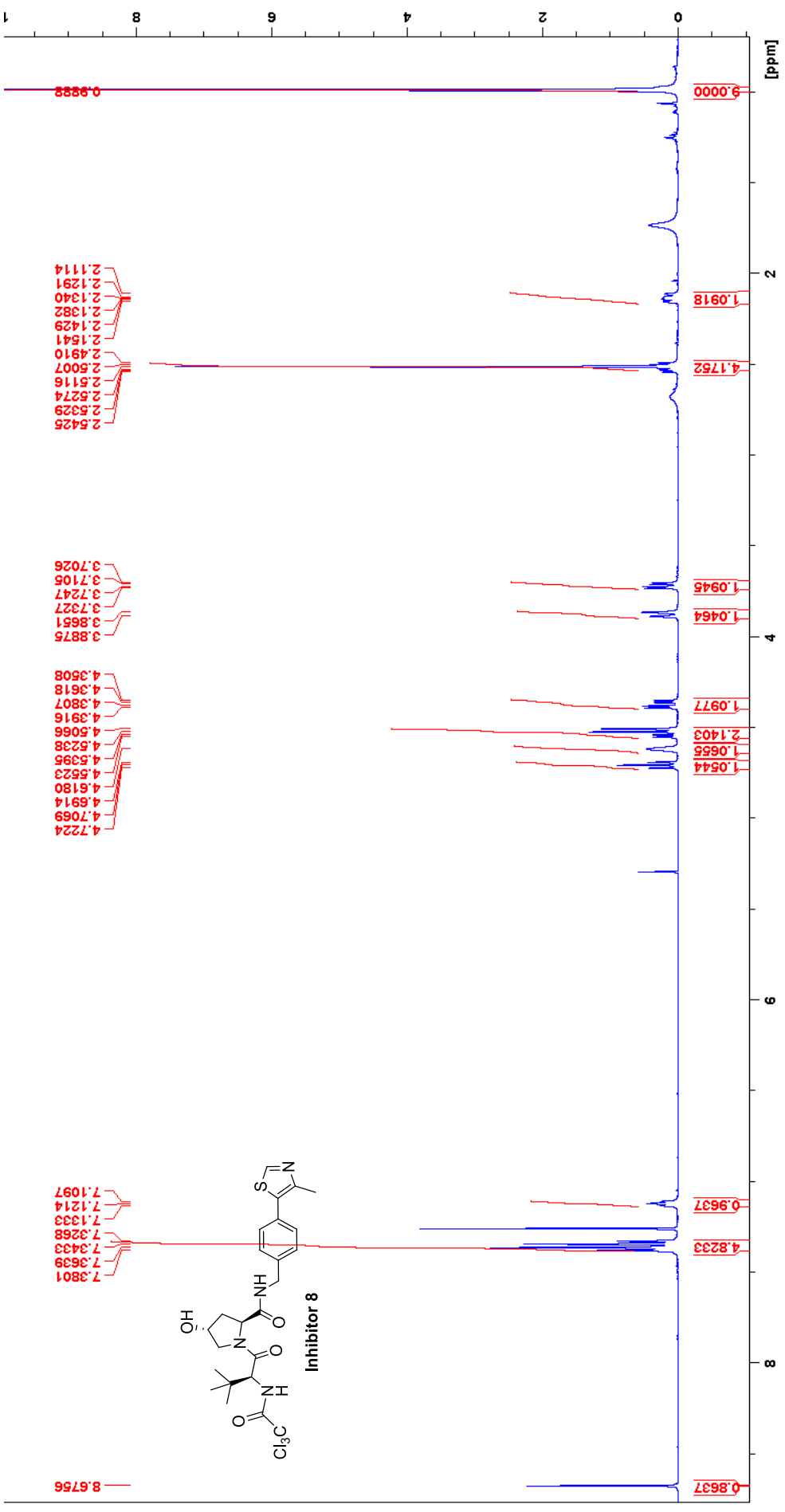
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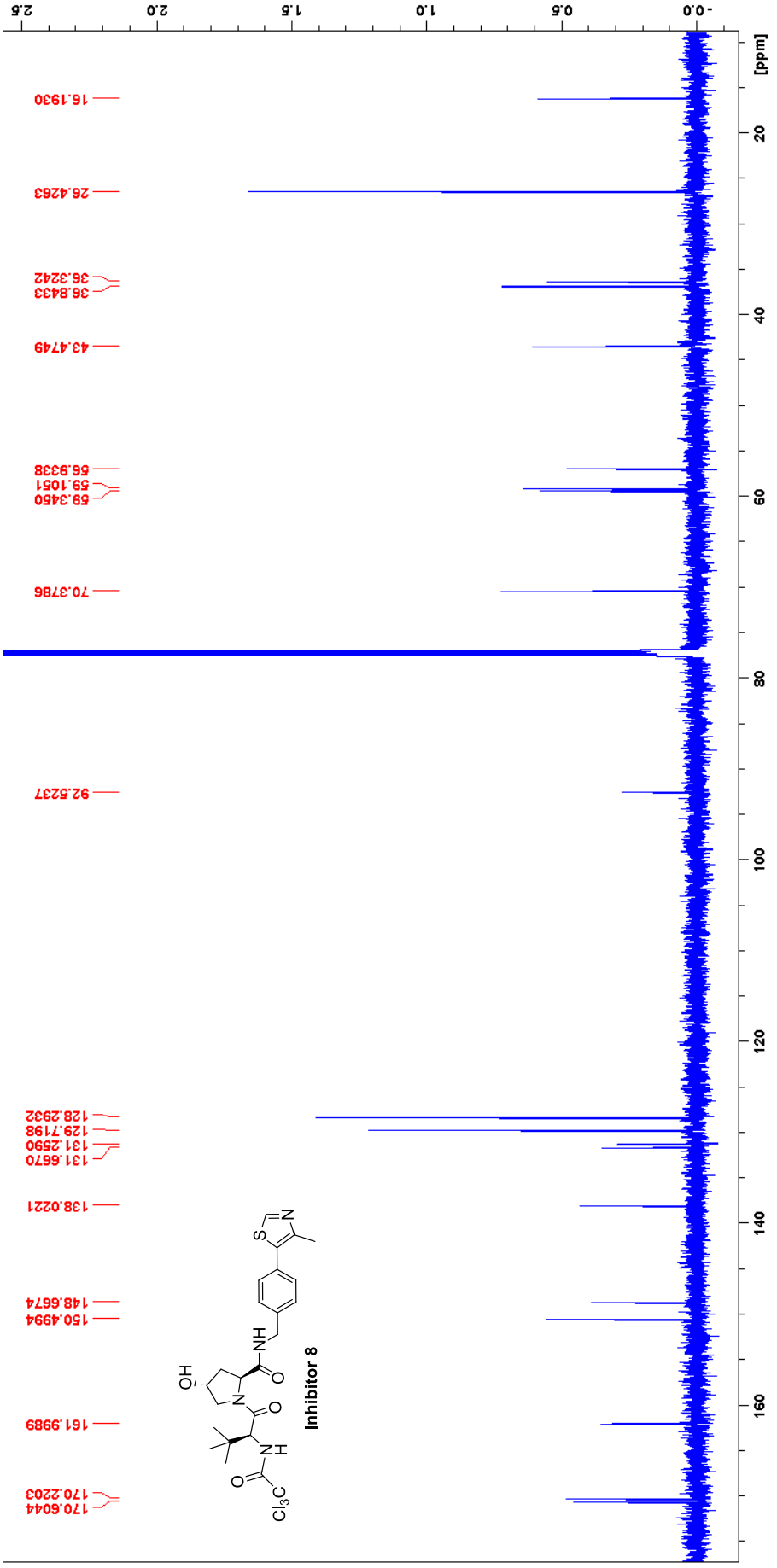
Inhibitor 8
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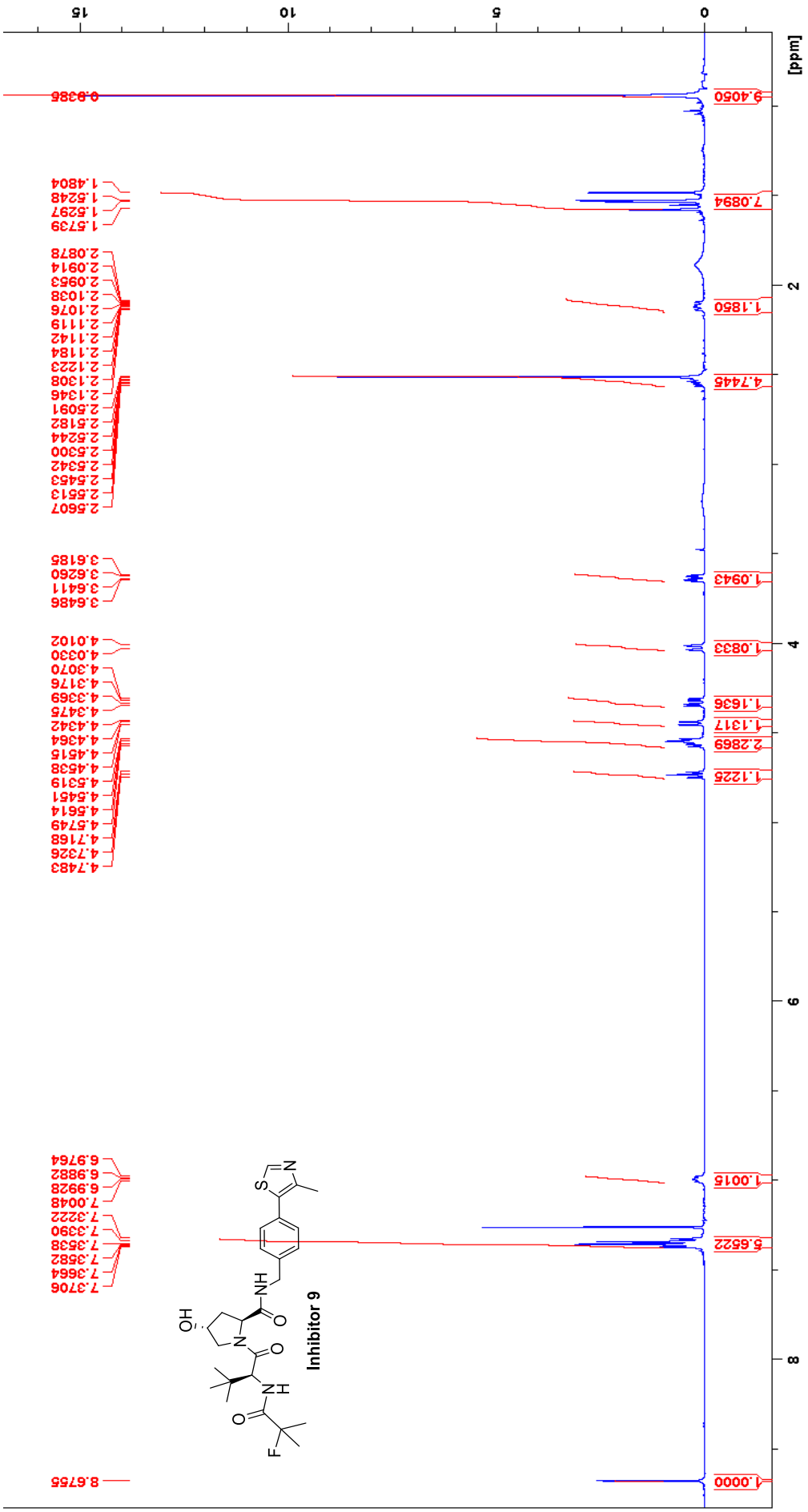
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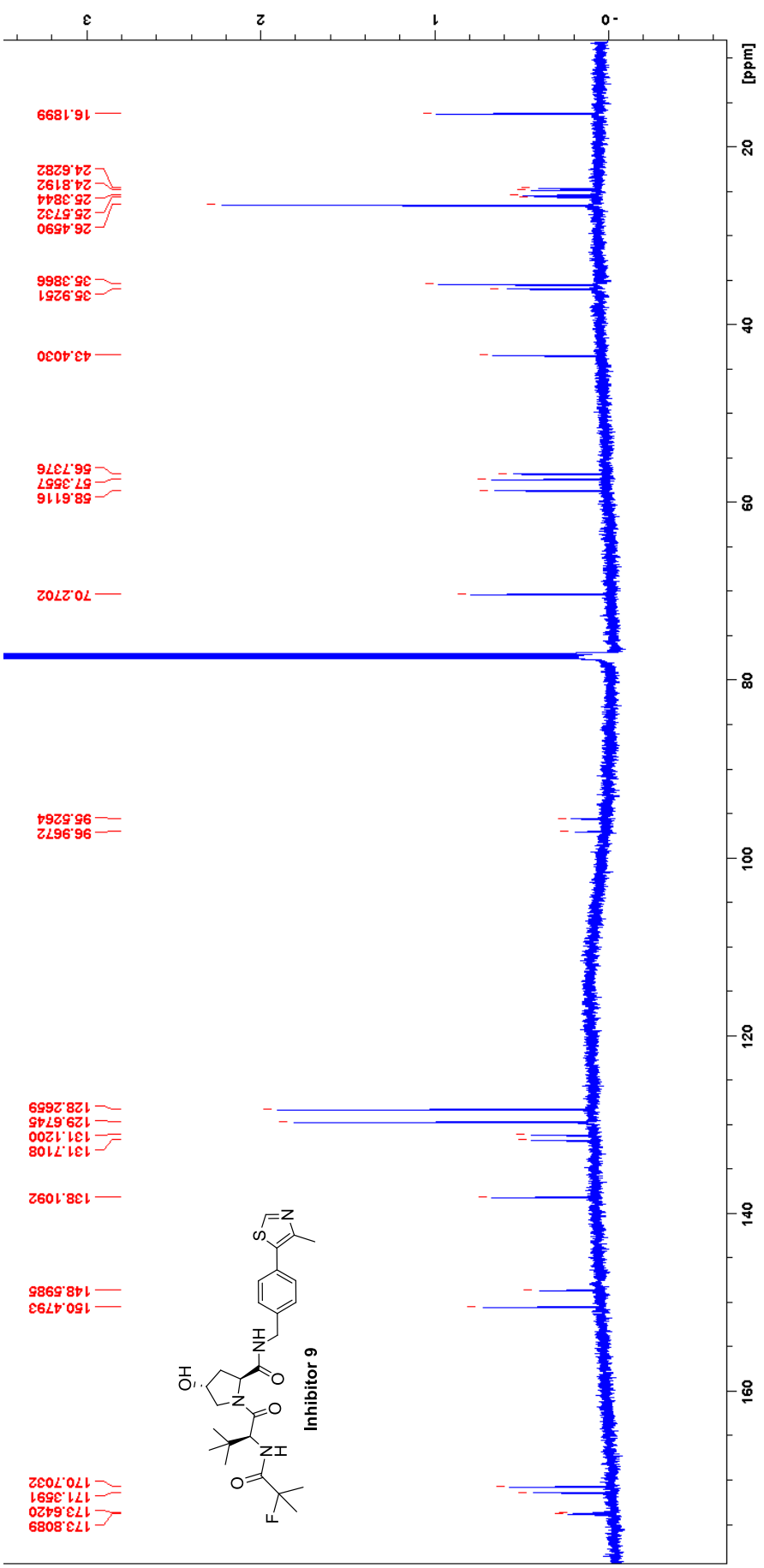
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Inhibitor 9
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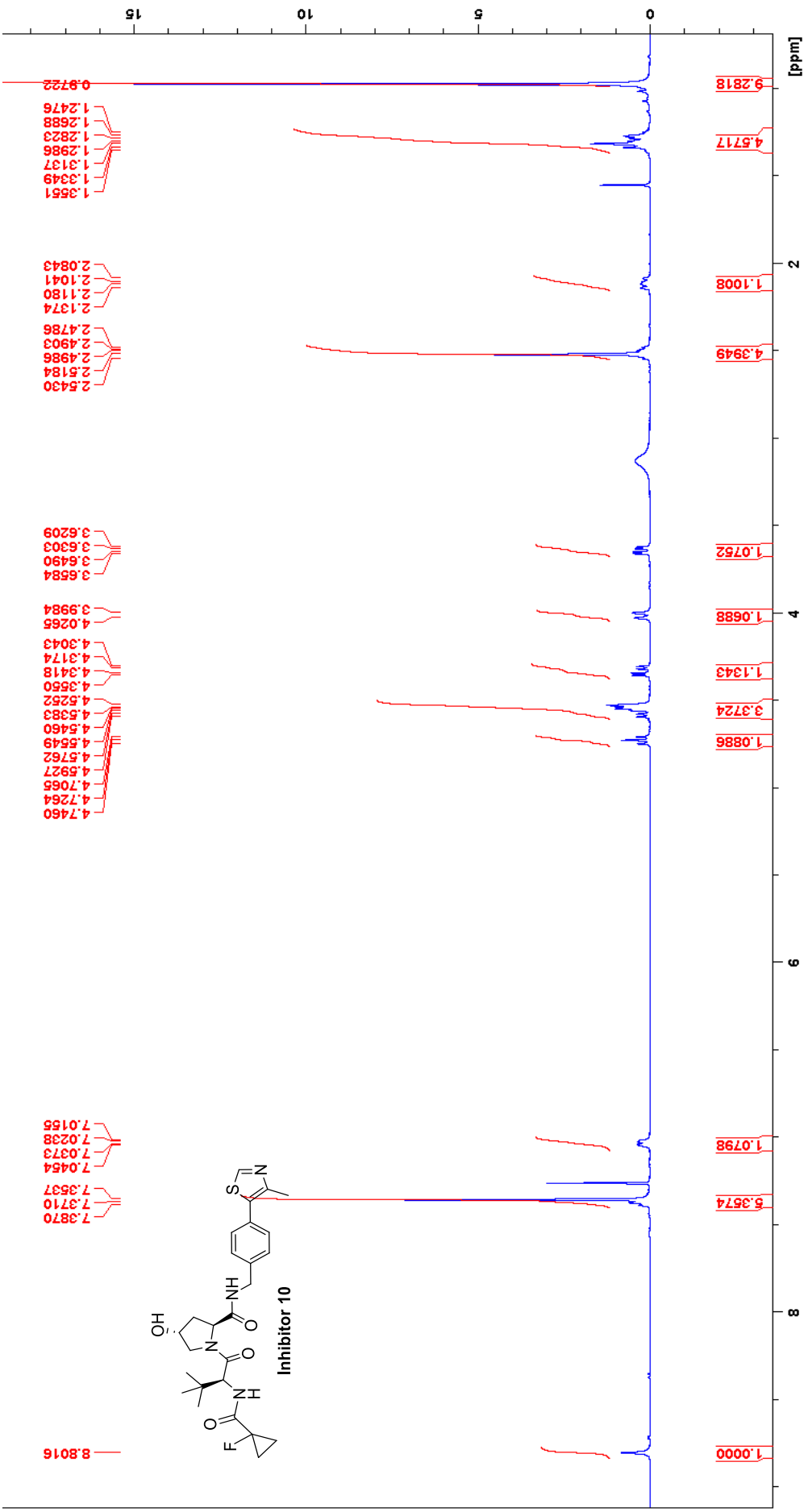
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1.2823
1.2986
1.3137
1.3349
1.3551
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4.3174
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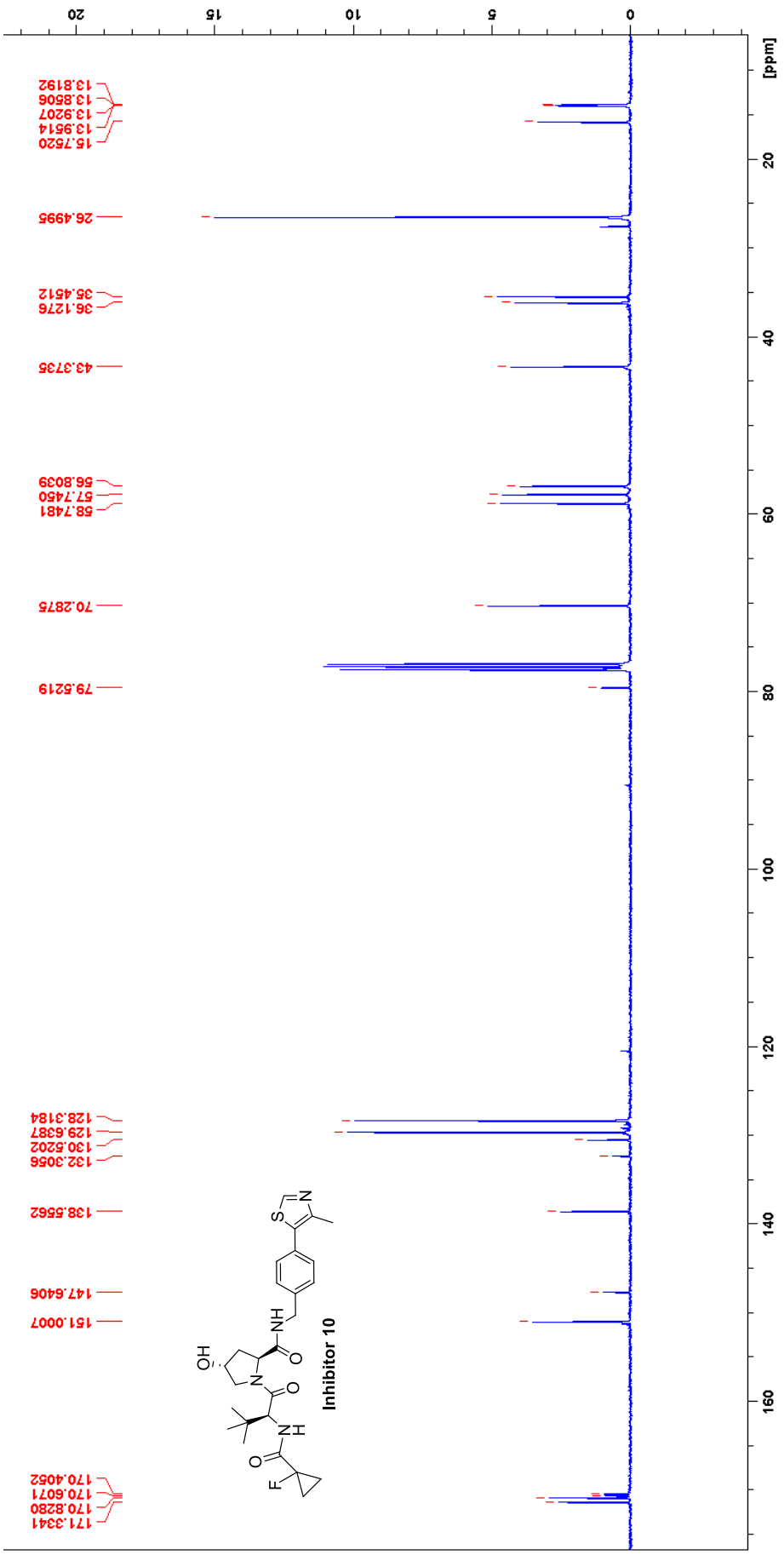
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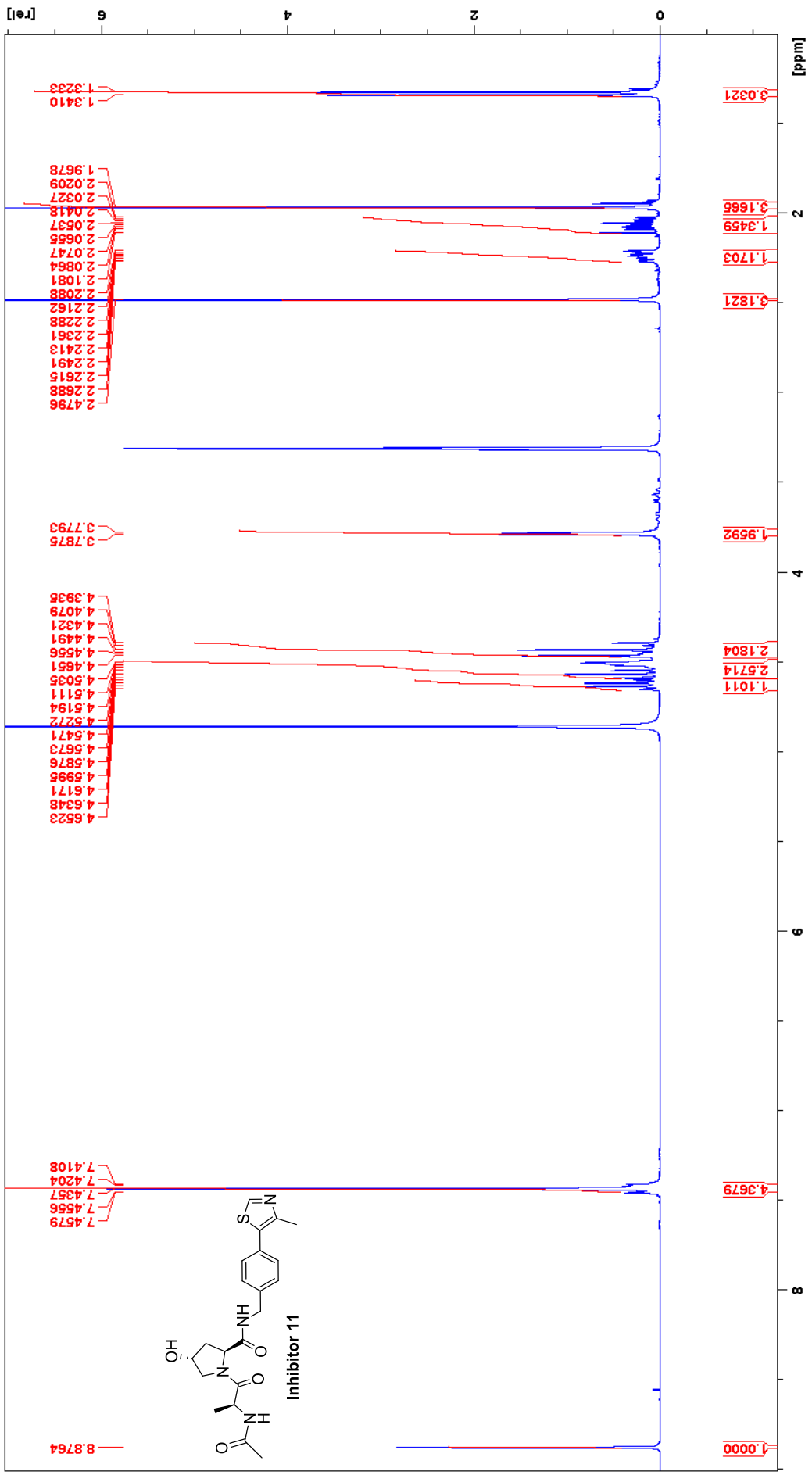
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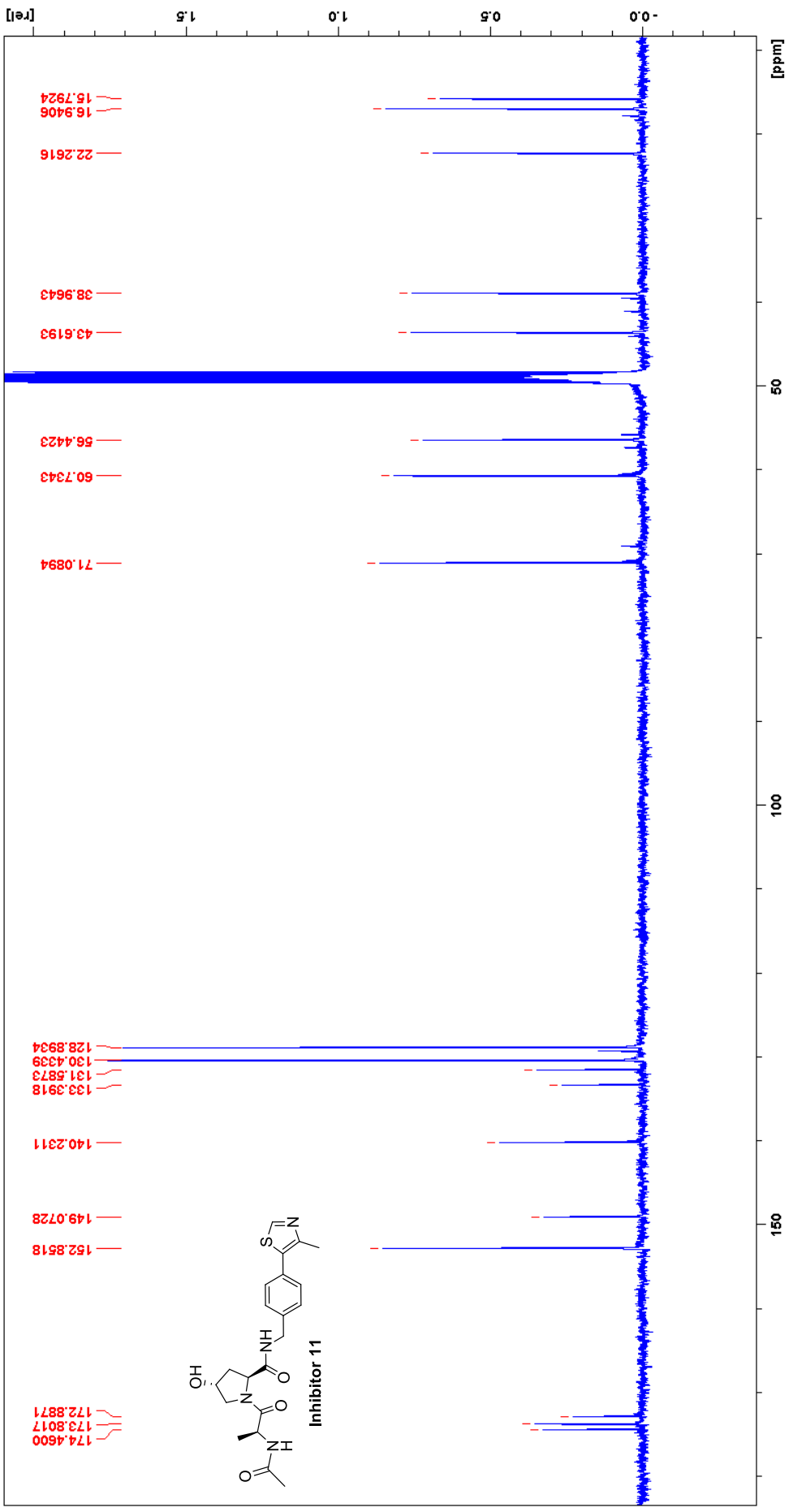
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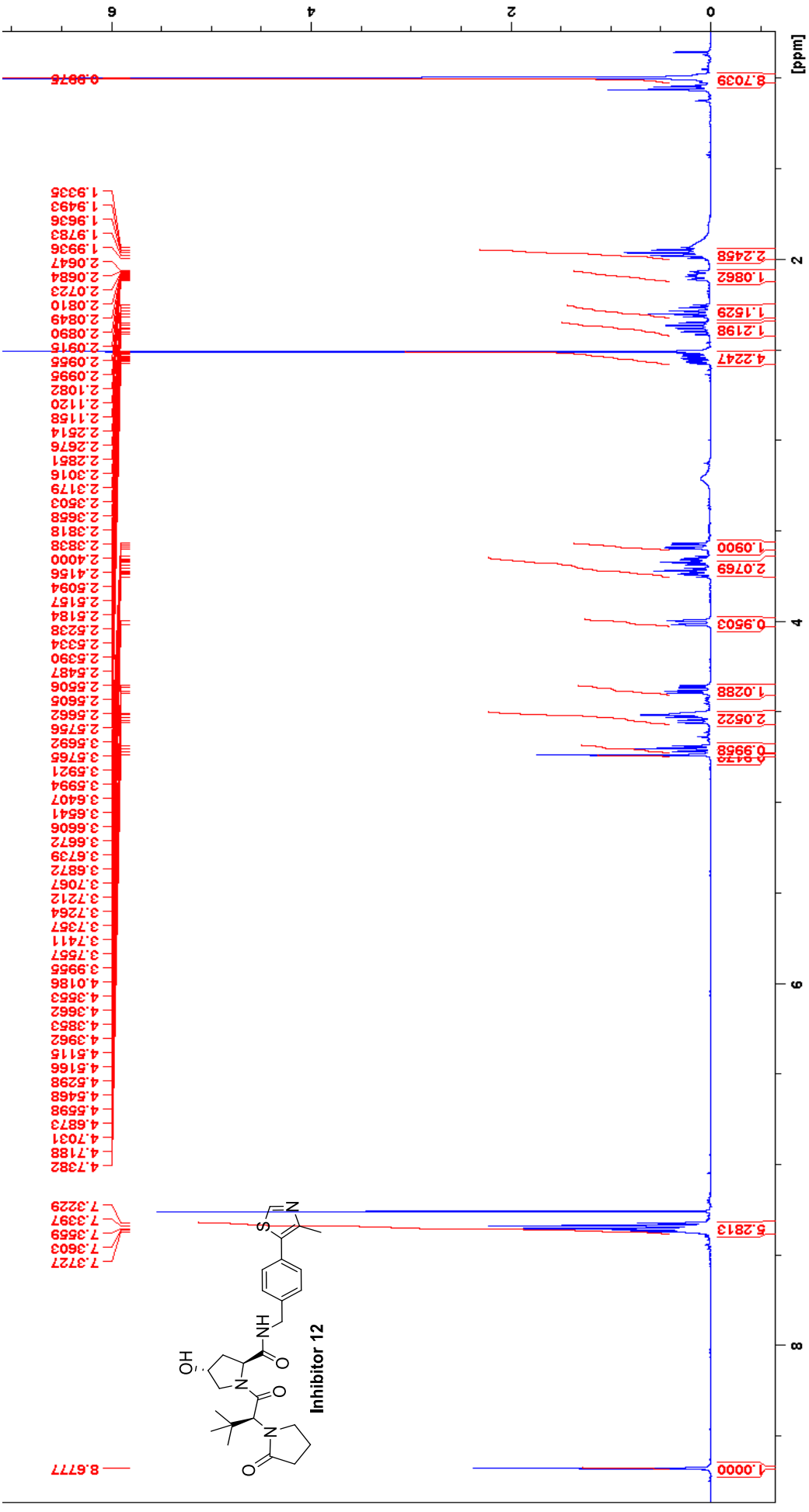
Inhibitor 11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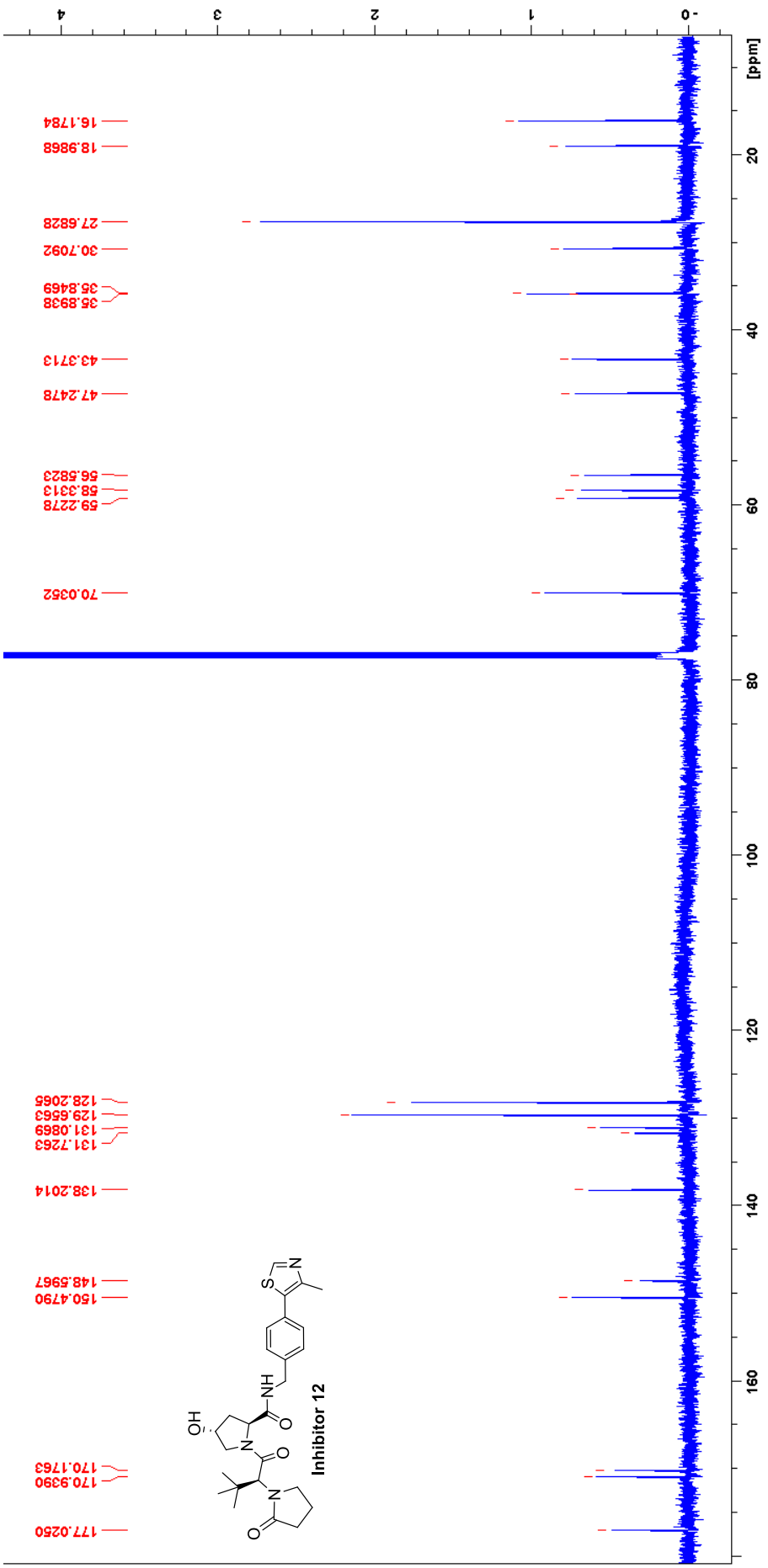
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Inhibitor 12
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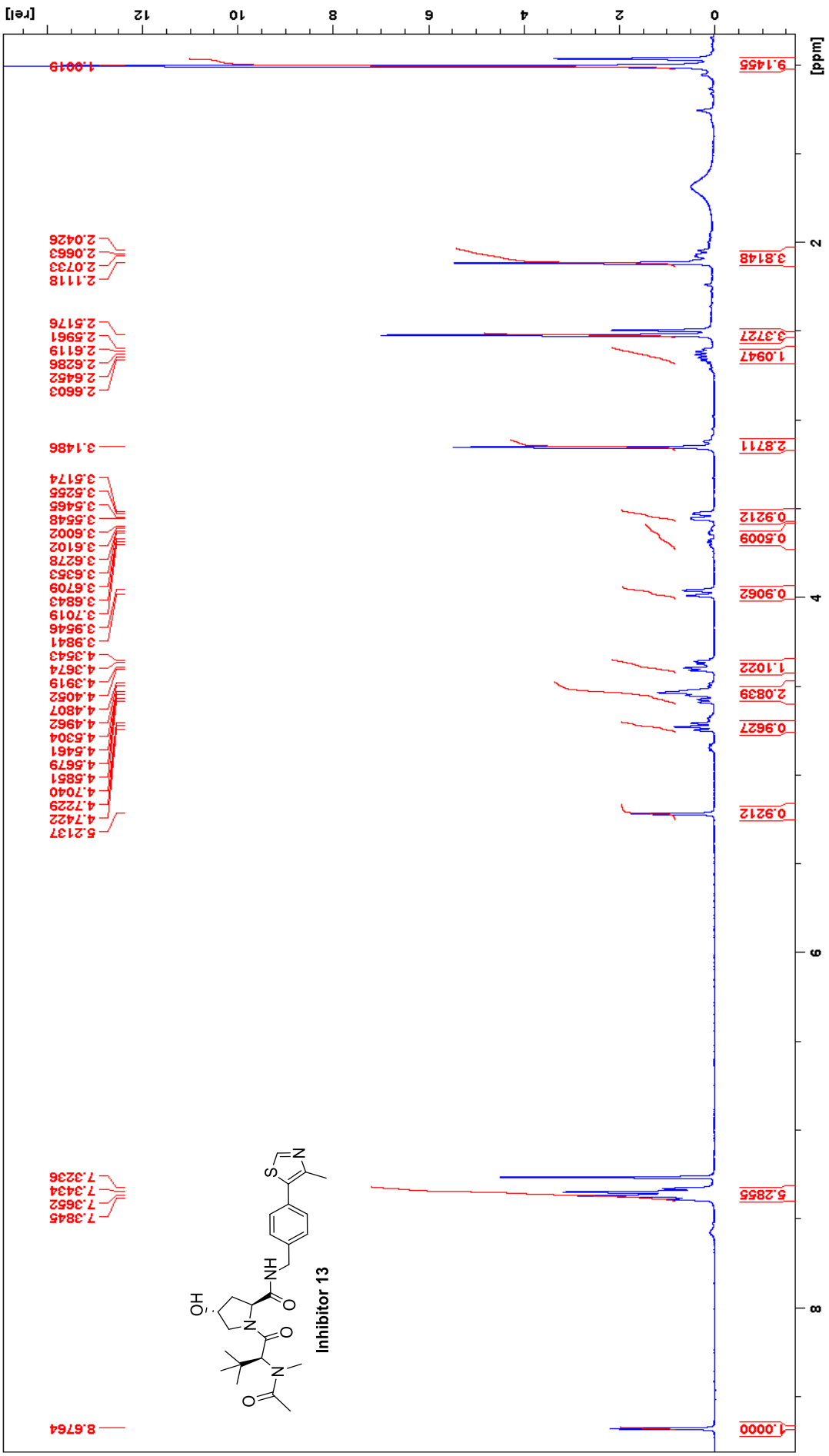
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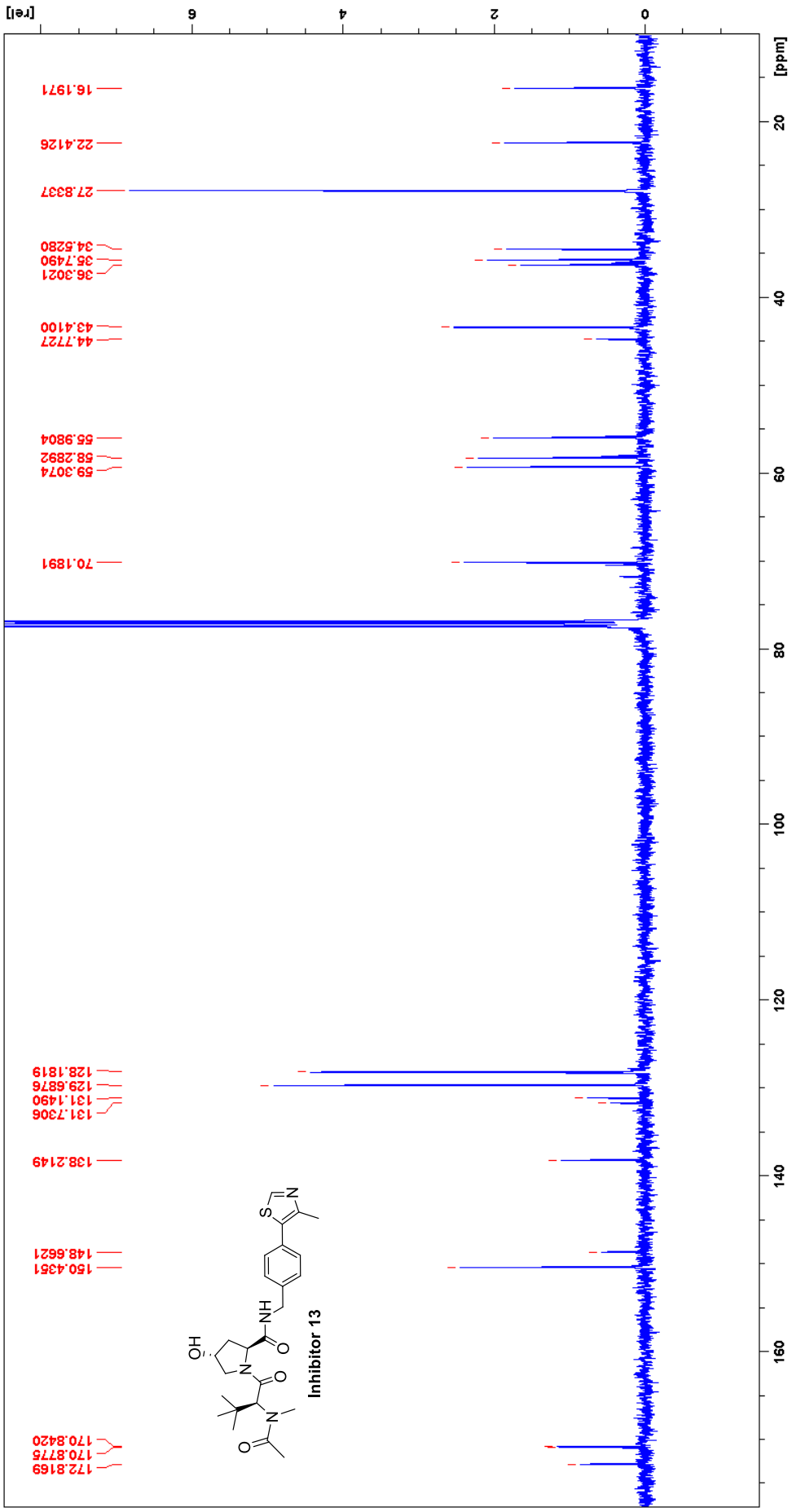
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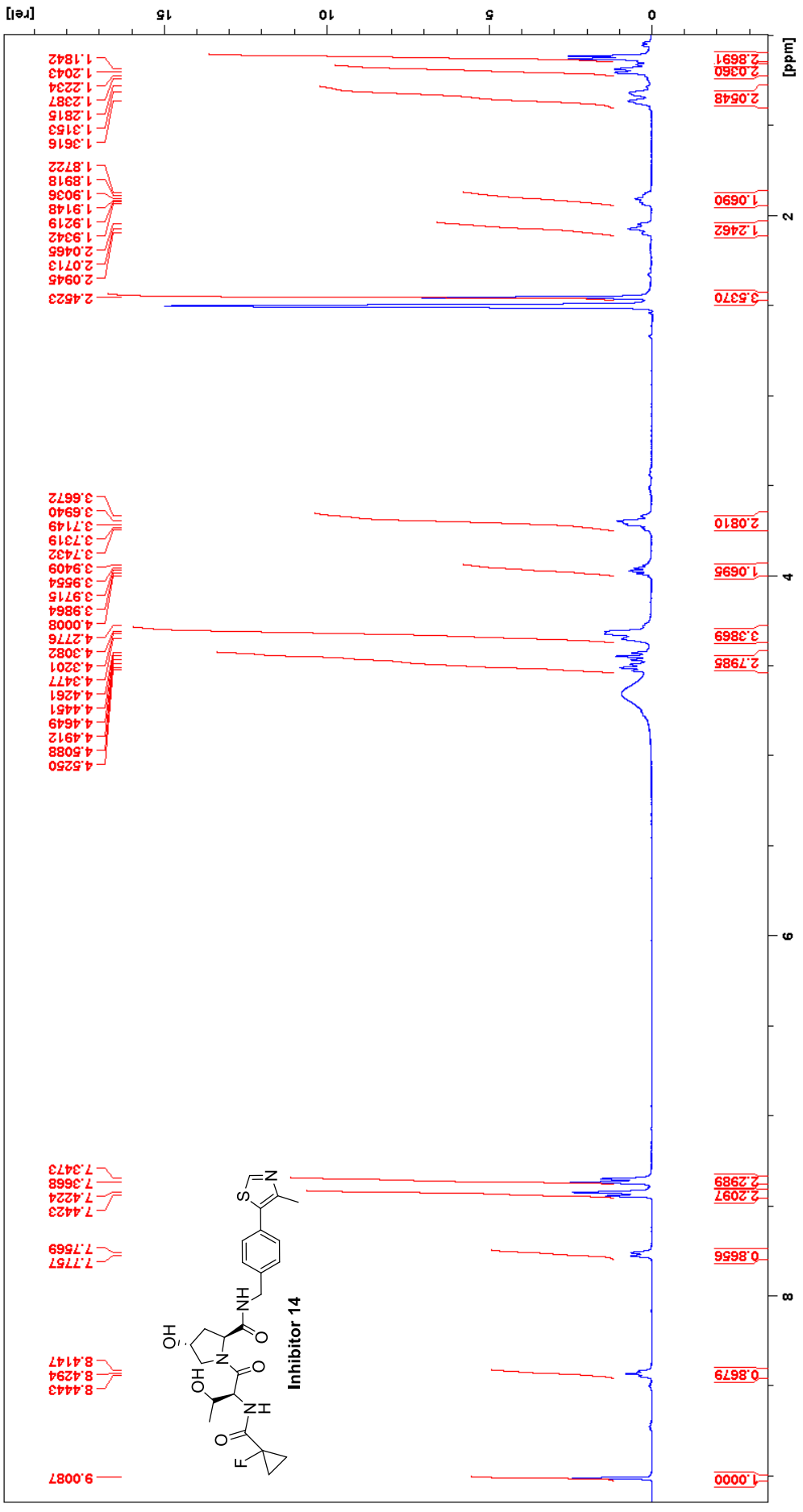
Inhibitor 13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8.4147
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7.7569
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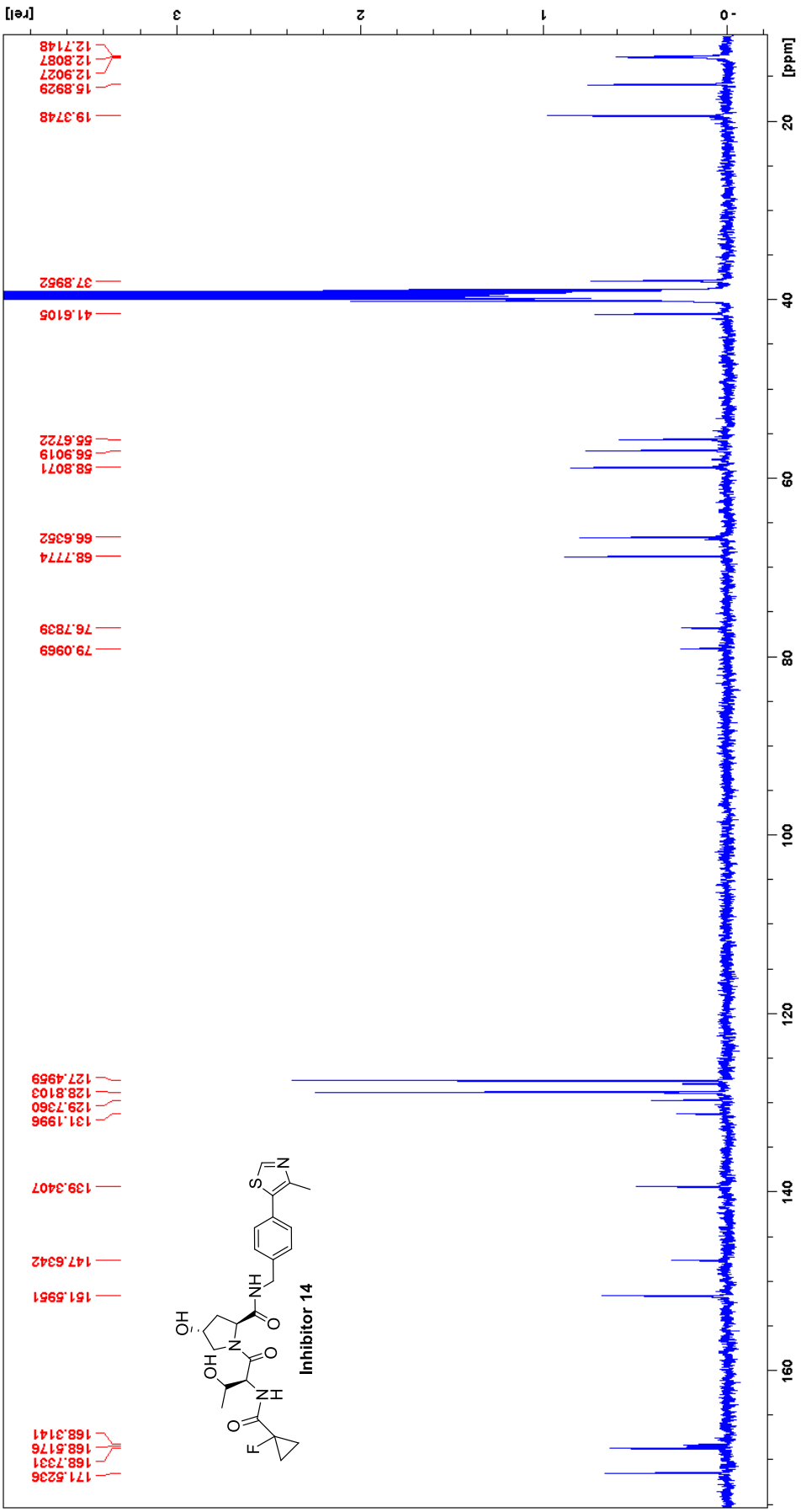
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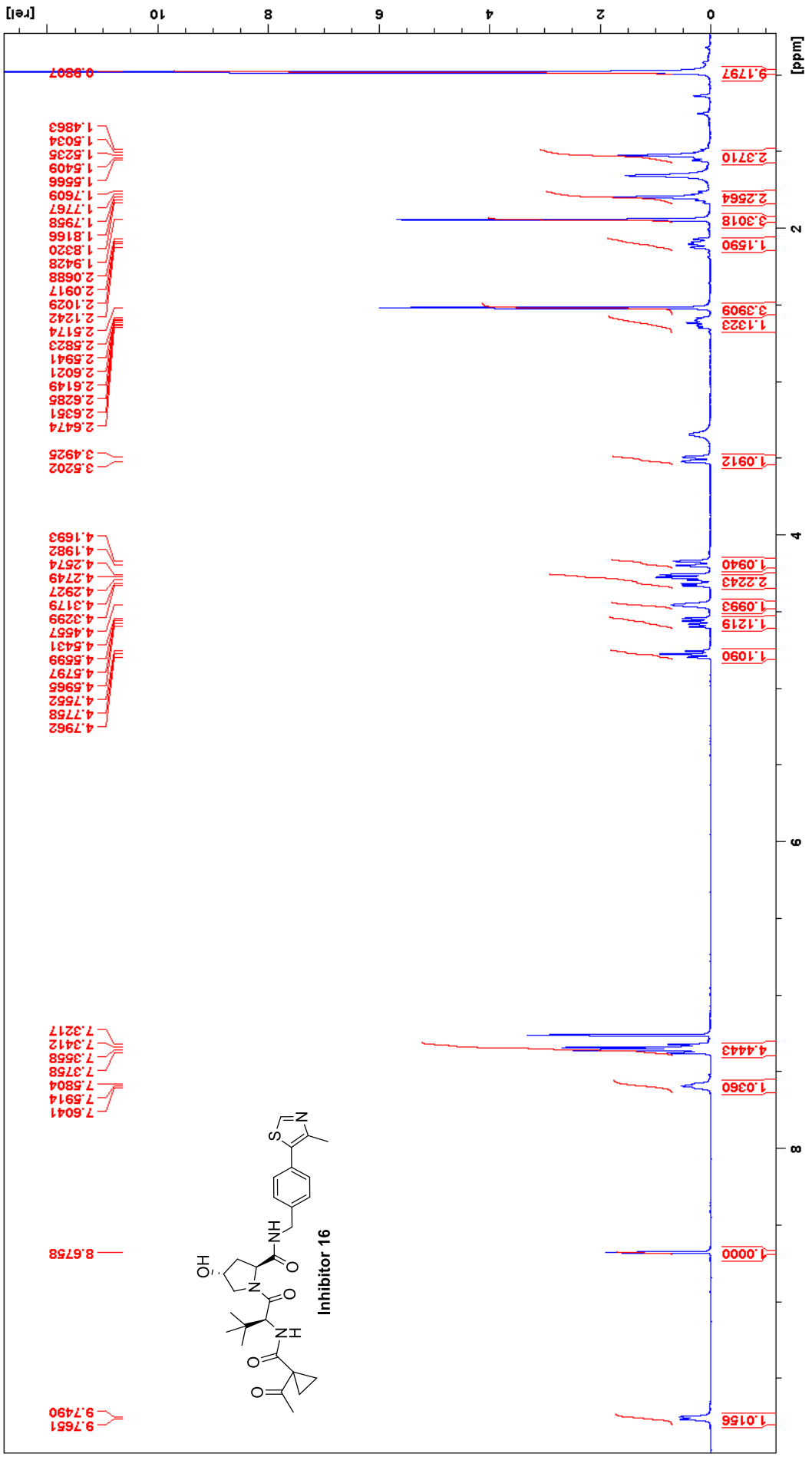
Inhibitor 14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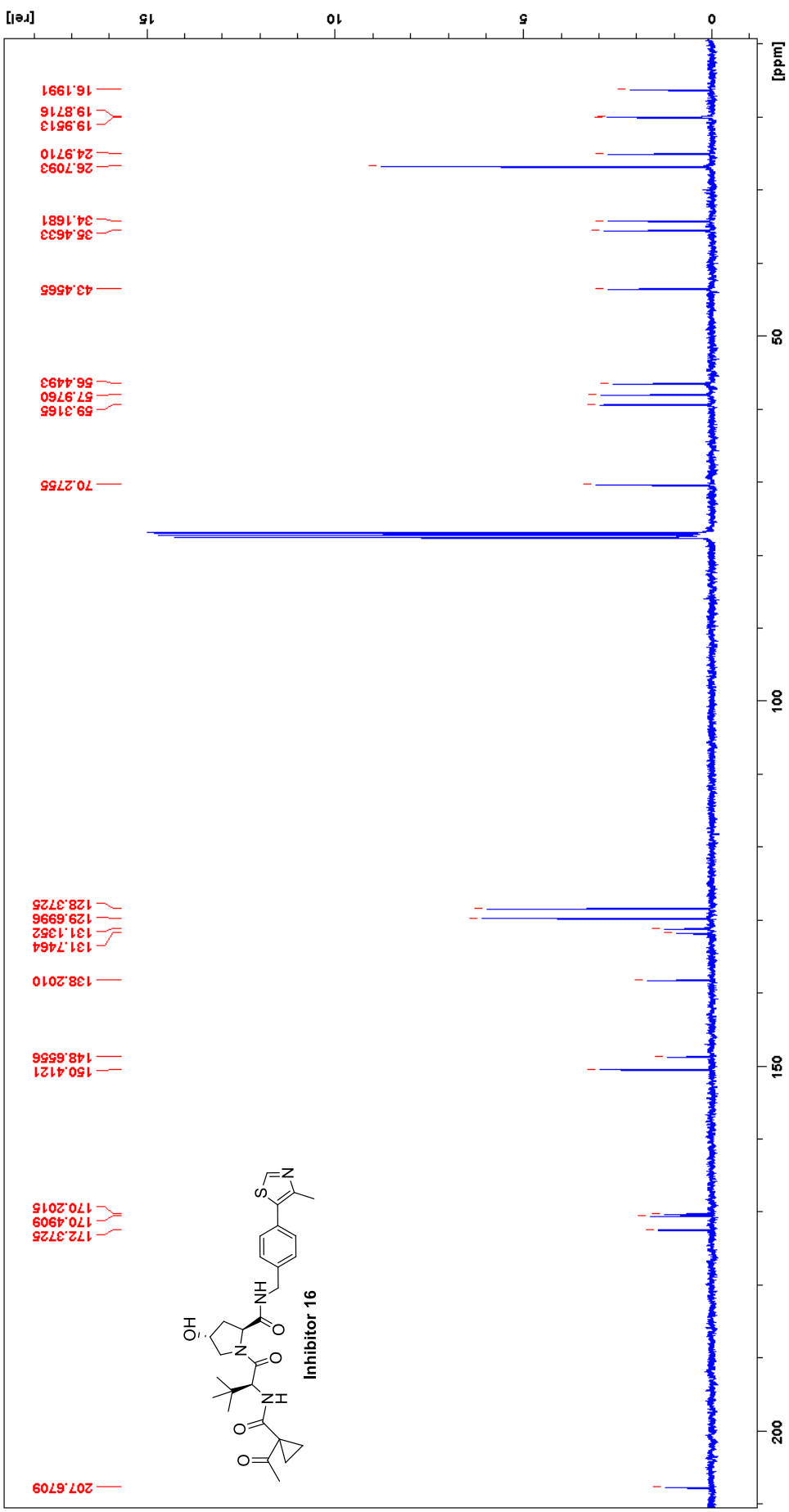
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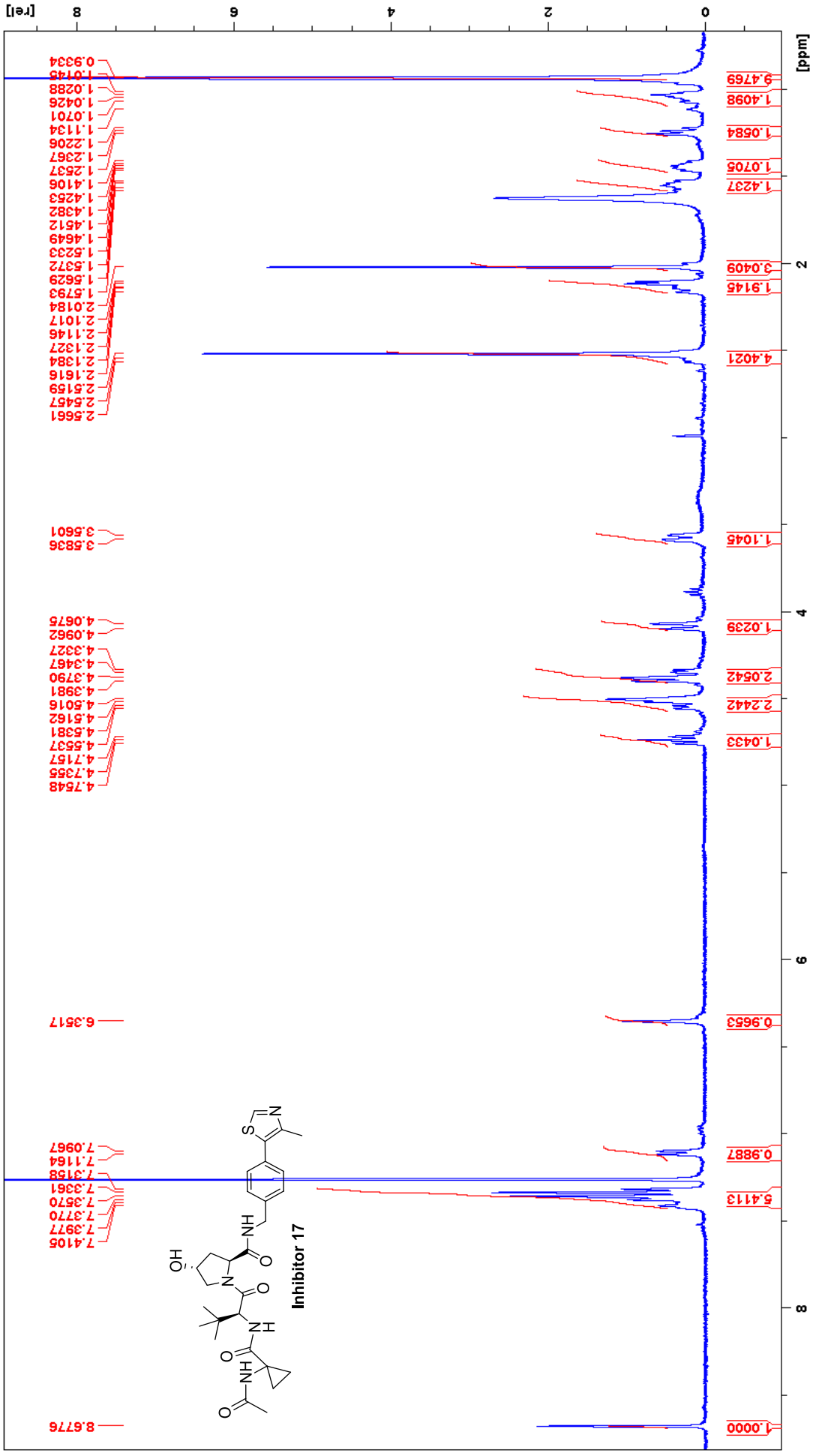
Inhibitor 16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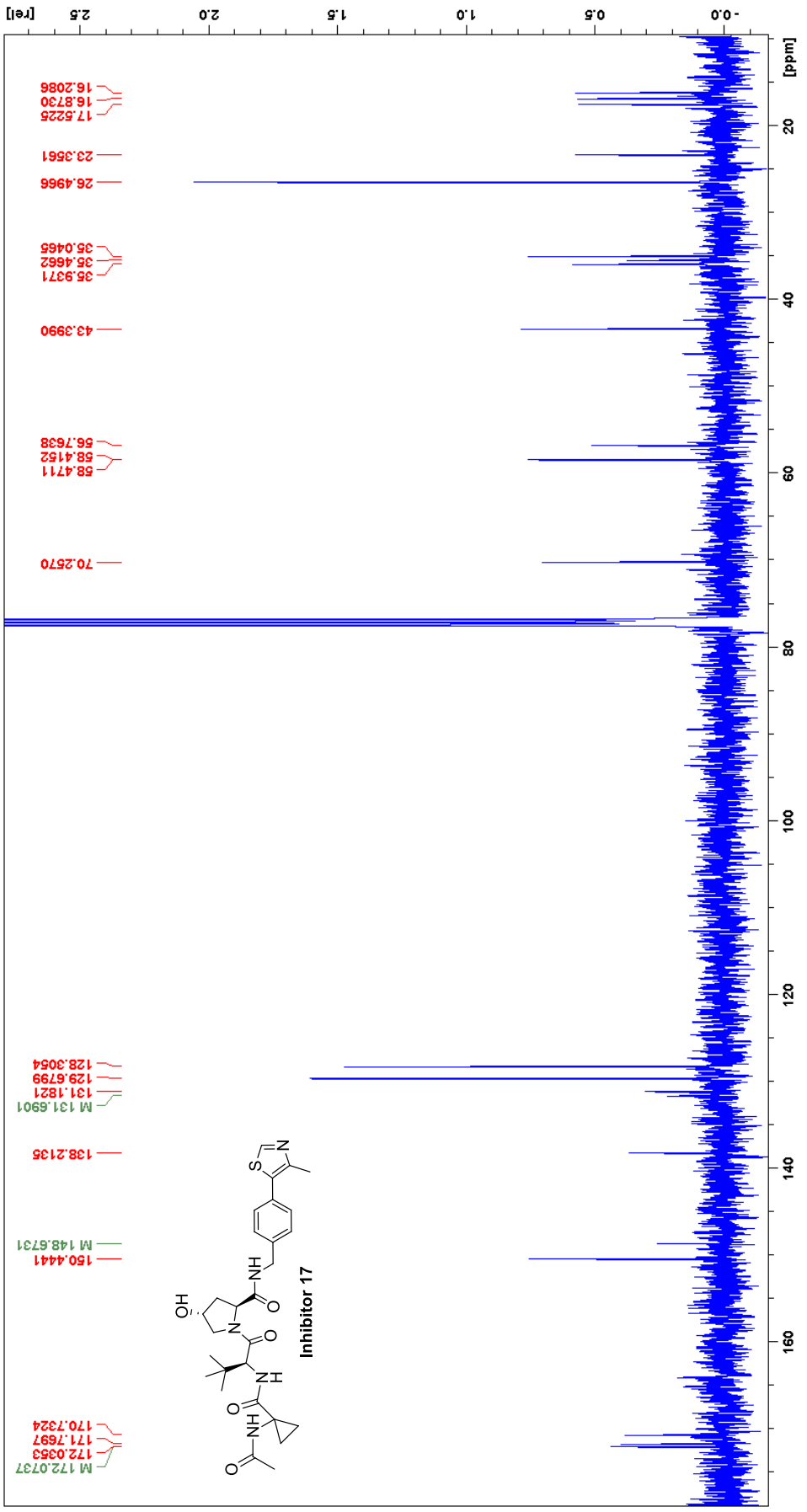
Inhibitor 17
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M 148.6731
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M 131.6901
131.1821
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128.3054
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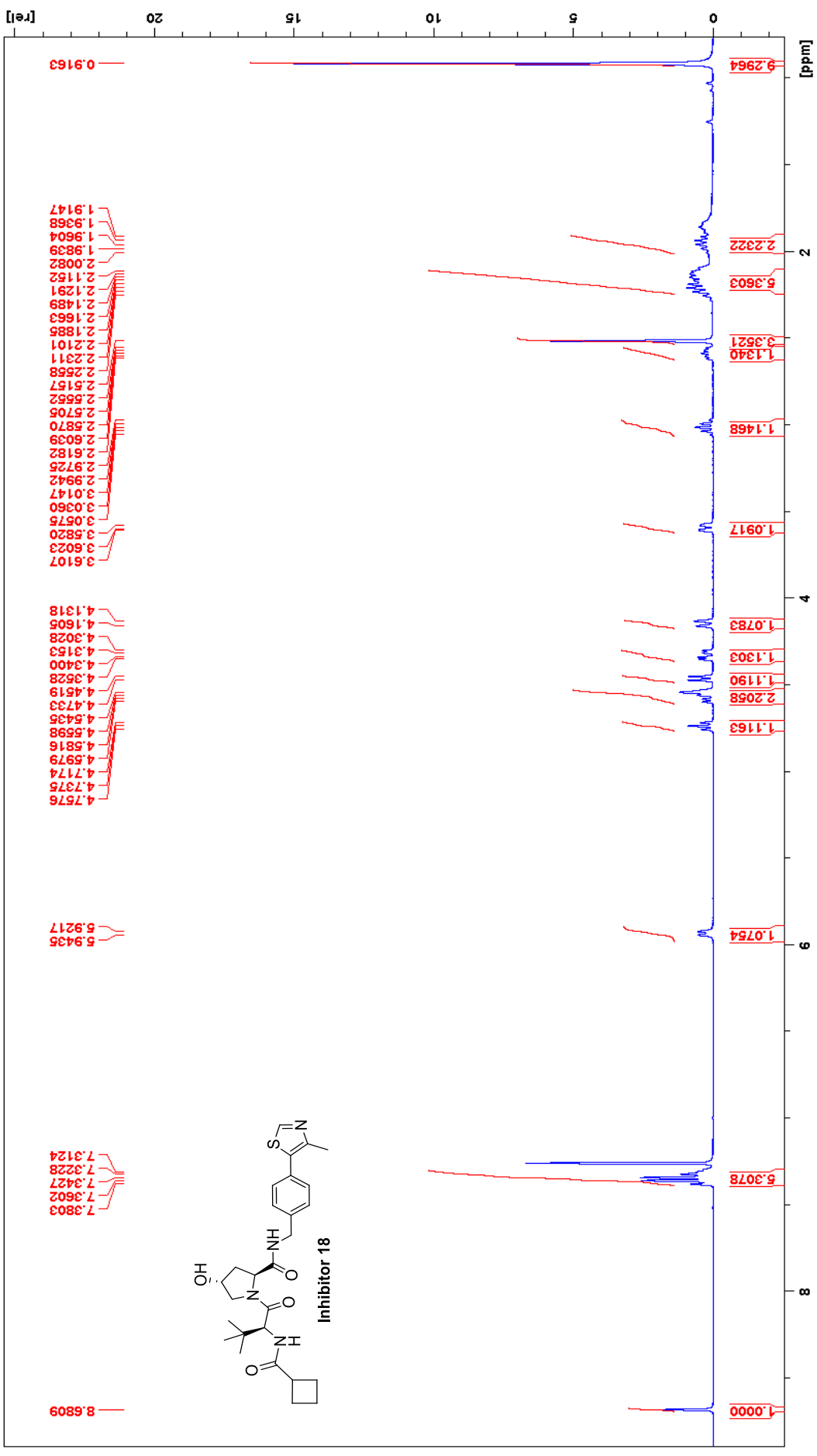
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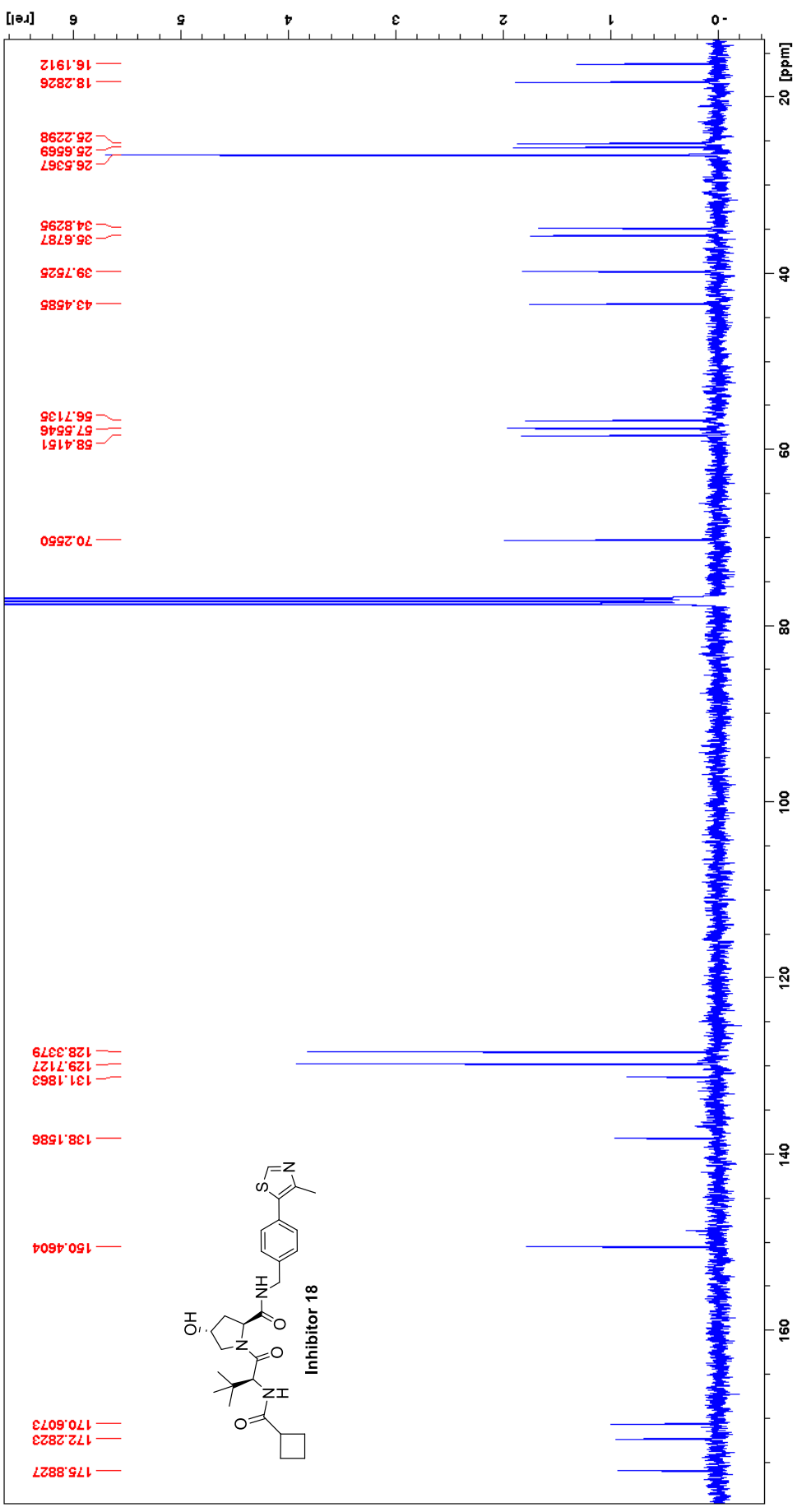
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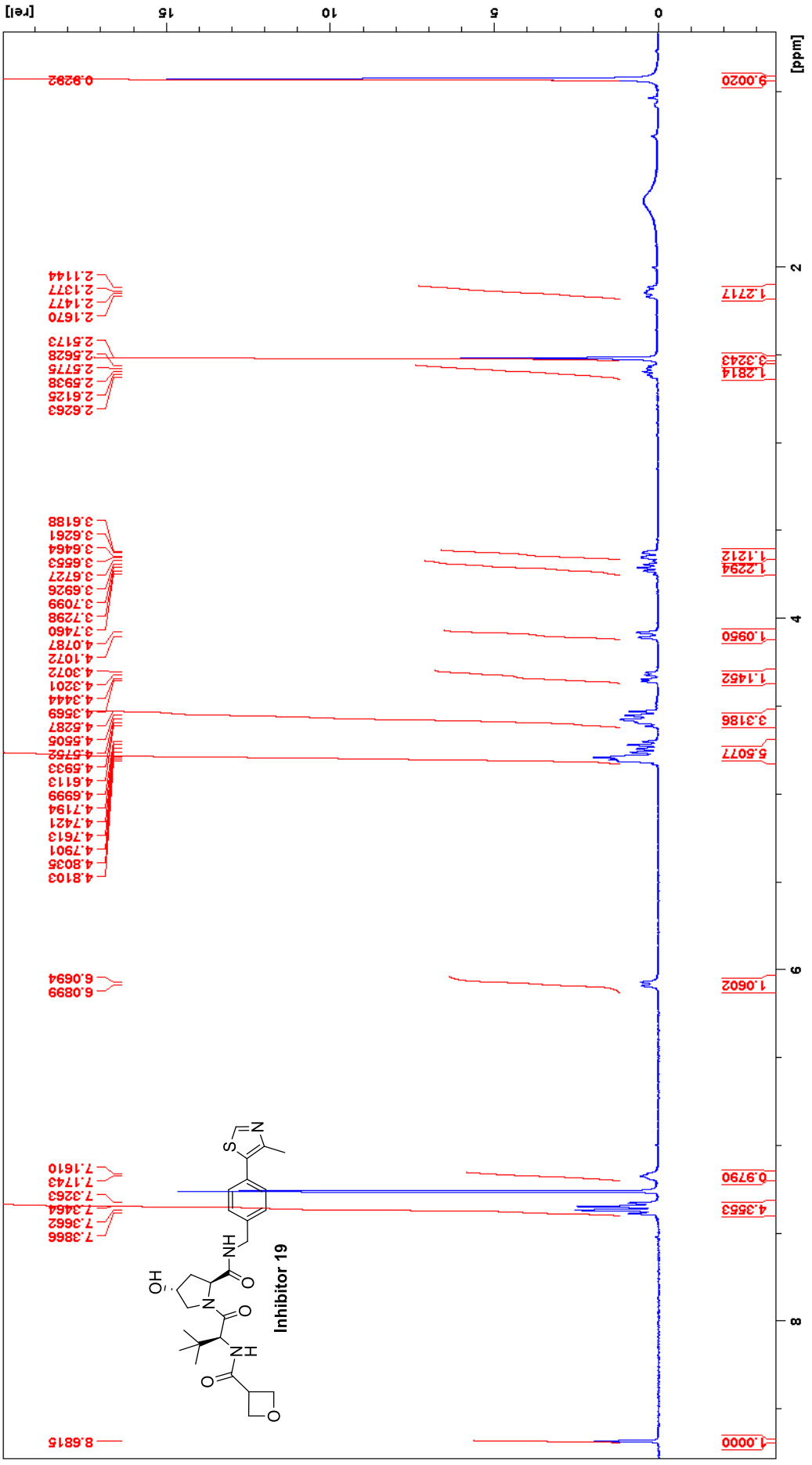
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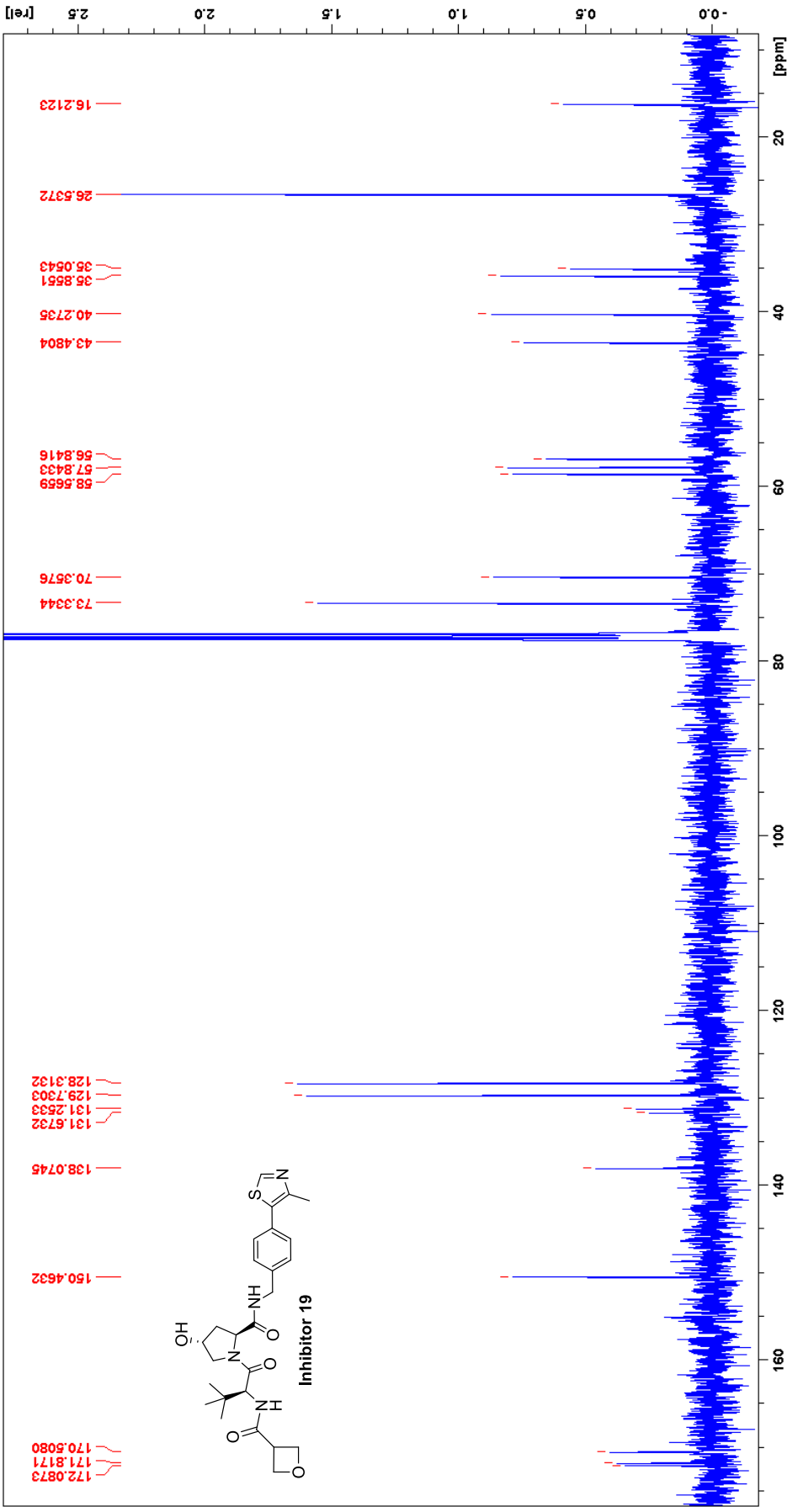
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Inhibitor 19
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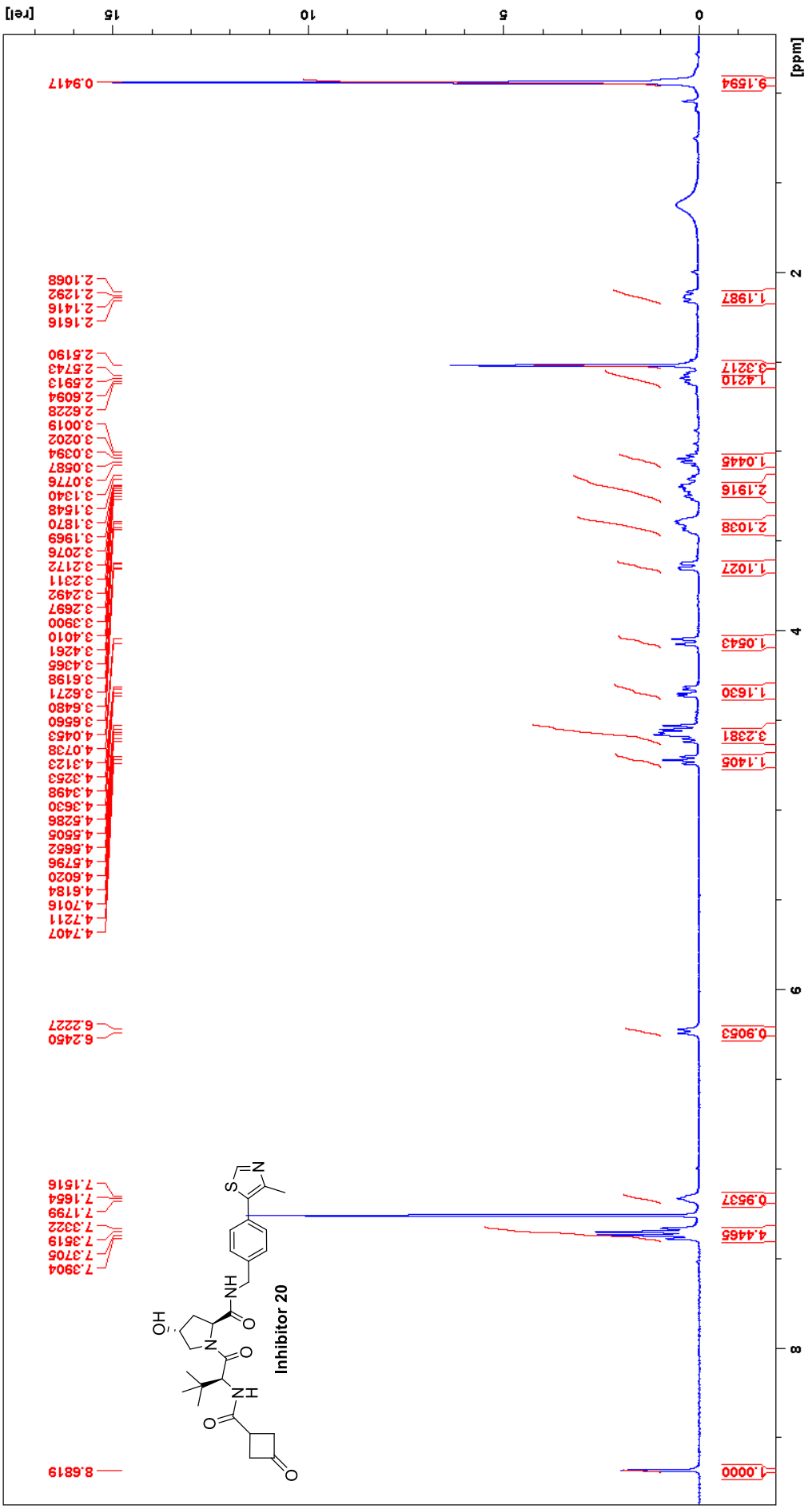
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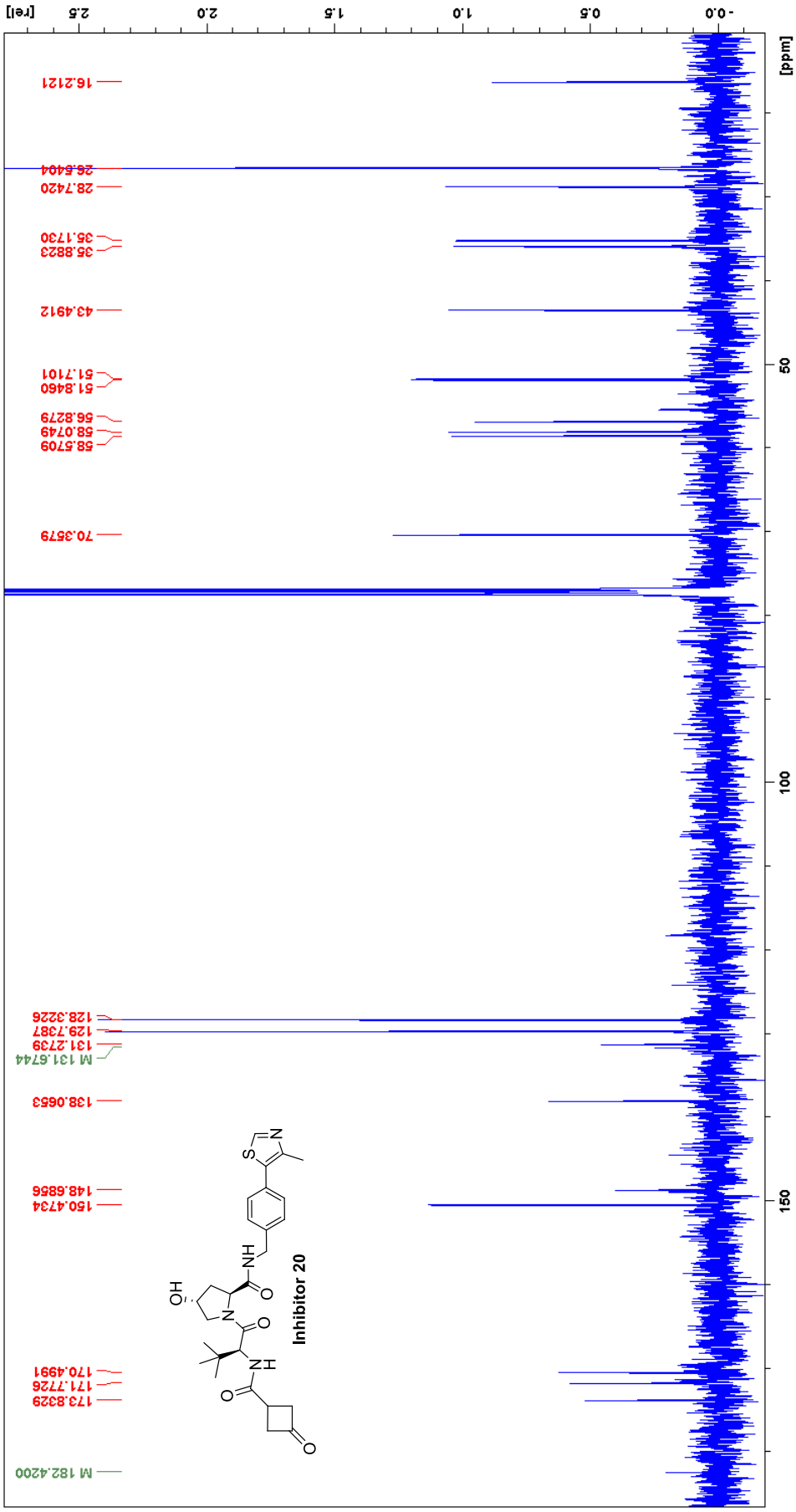
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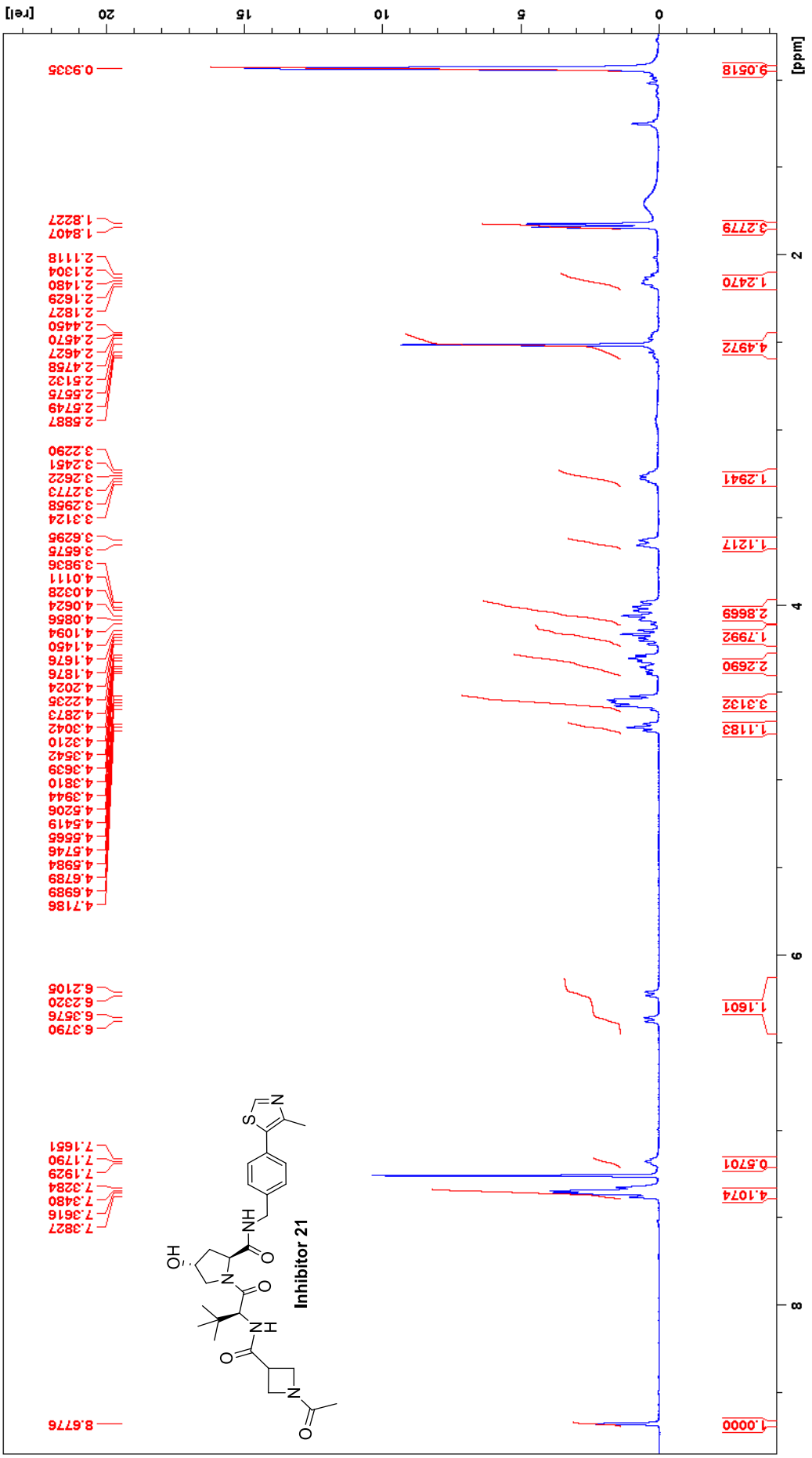
Inhibitor 20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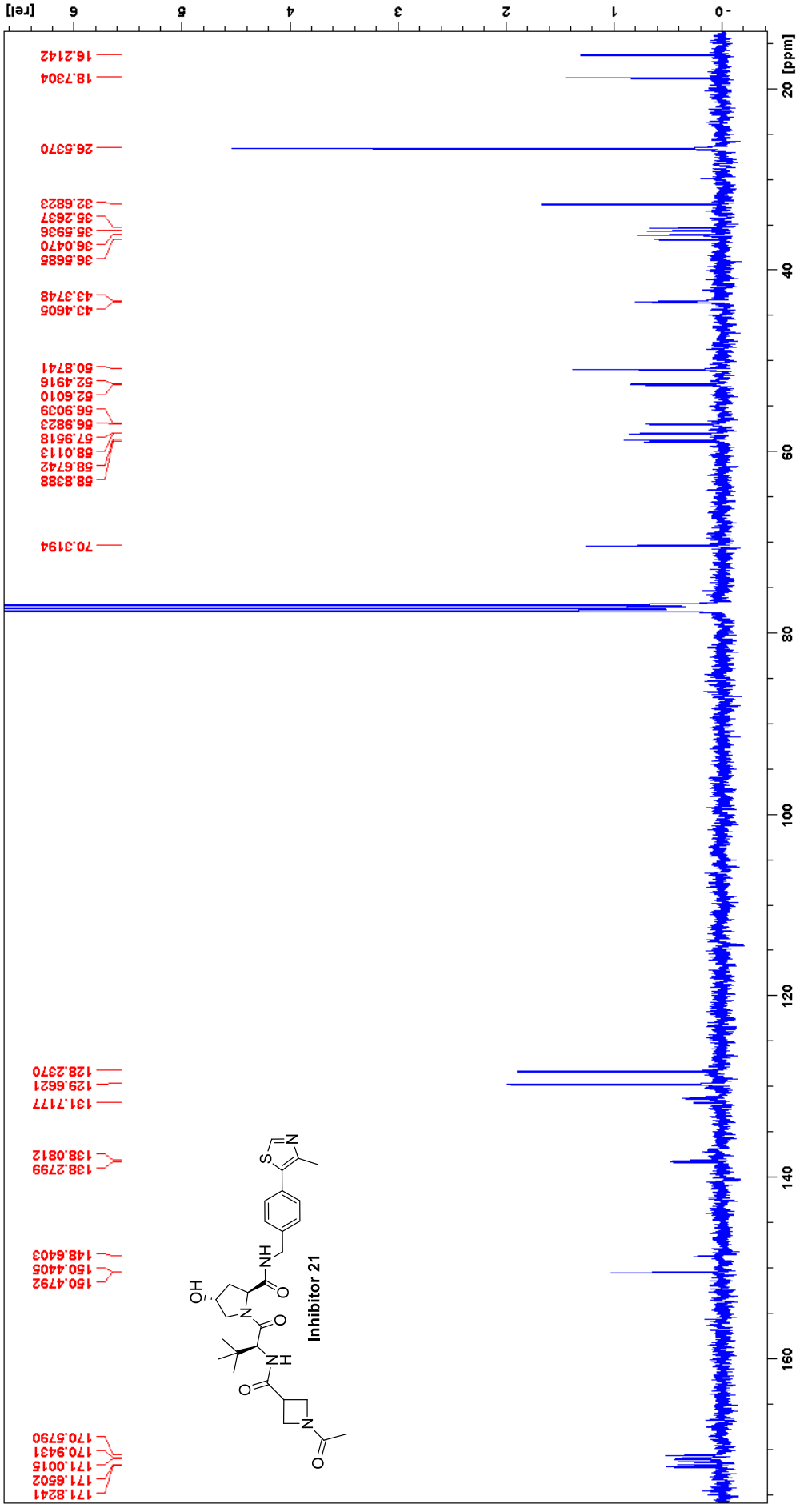
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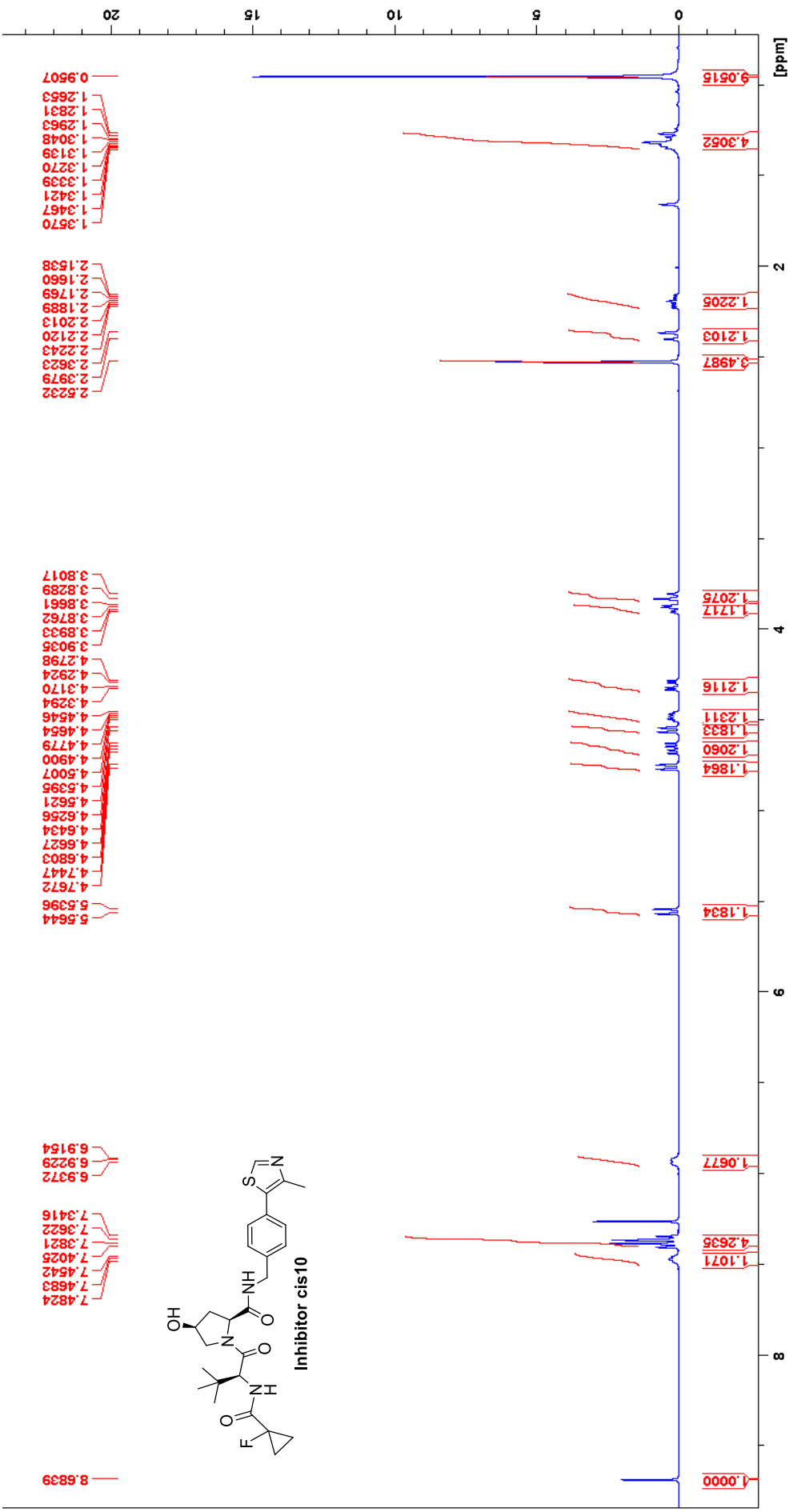
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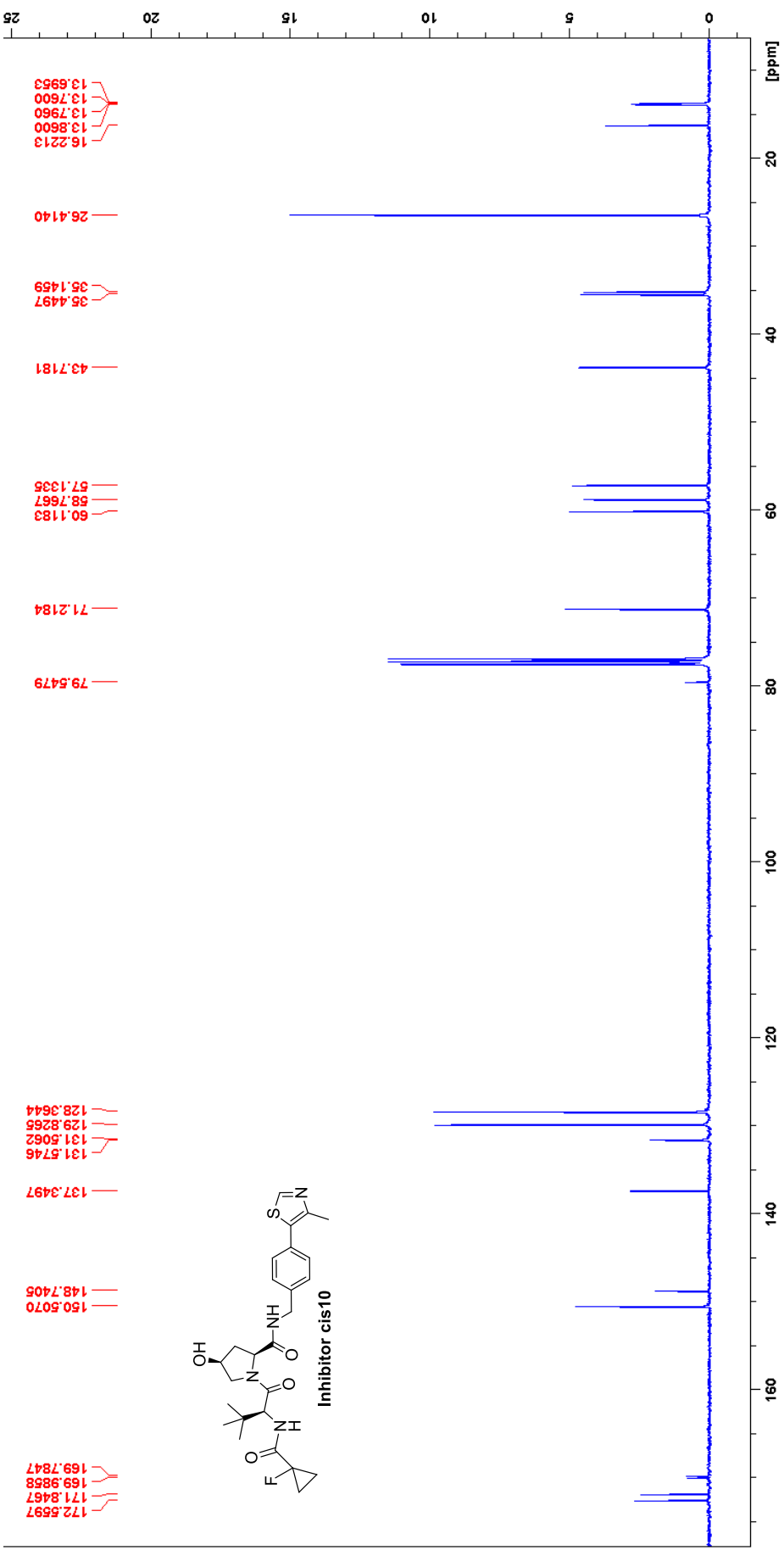
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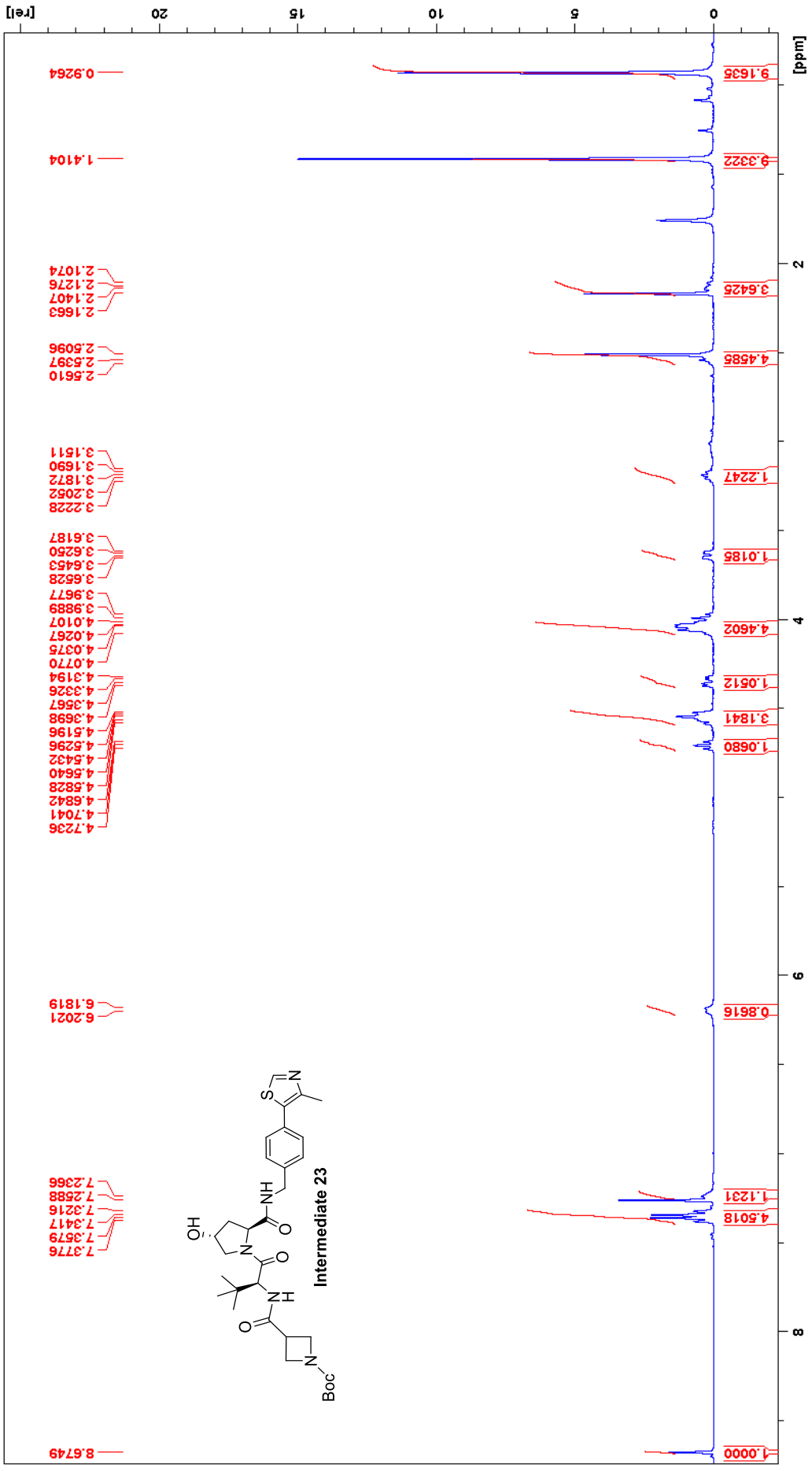
Intermediate 23
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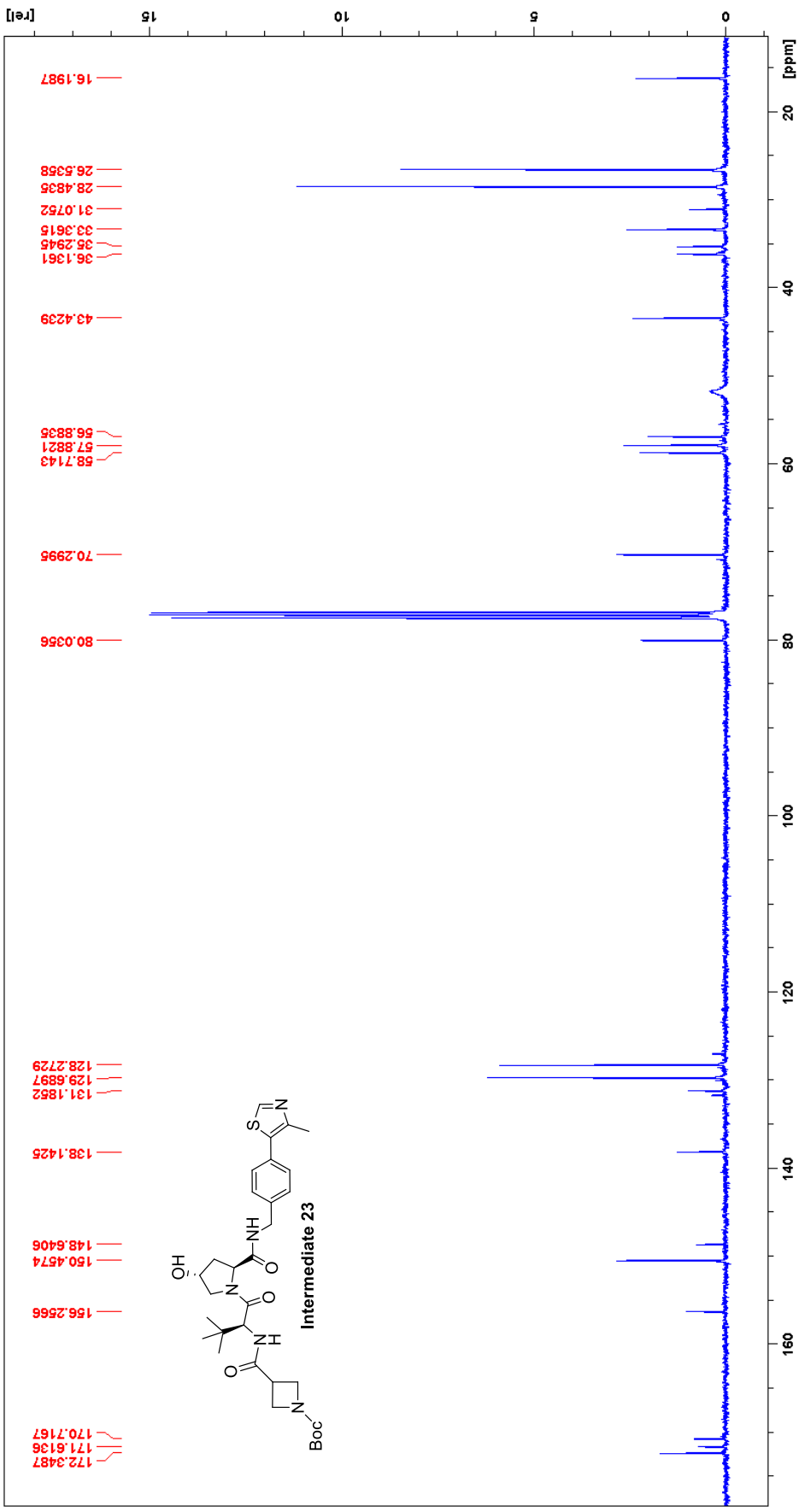
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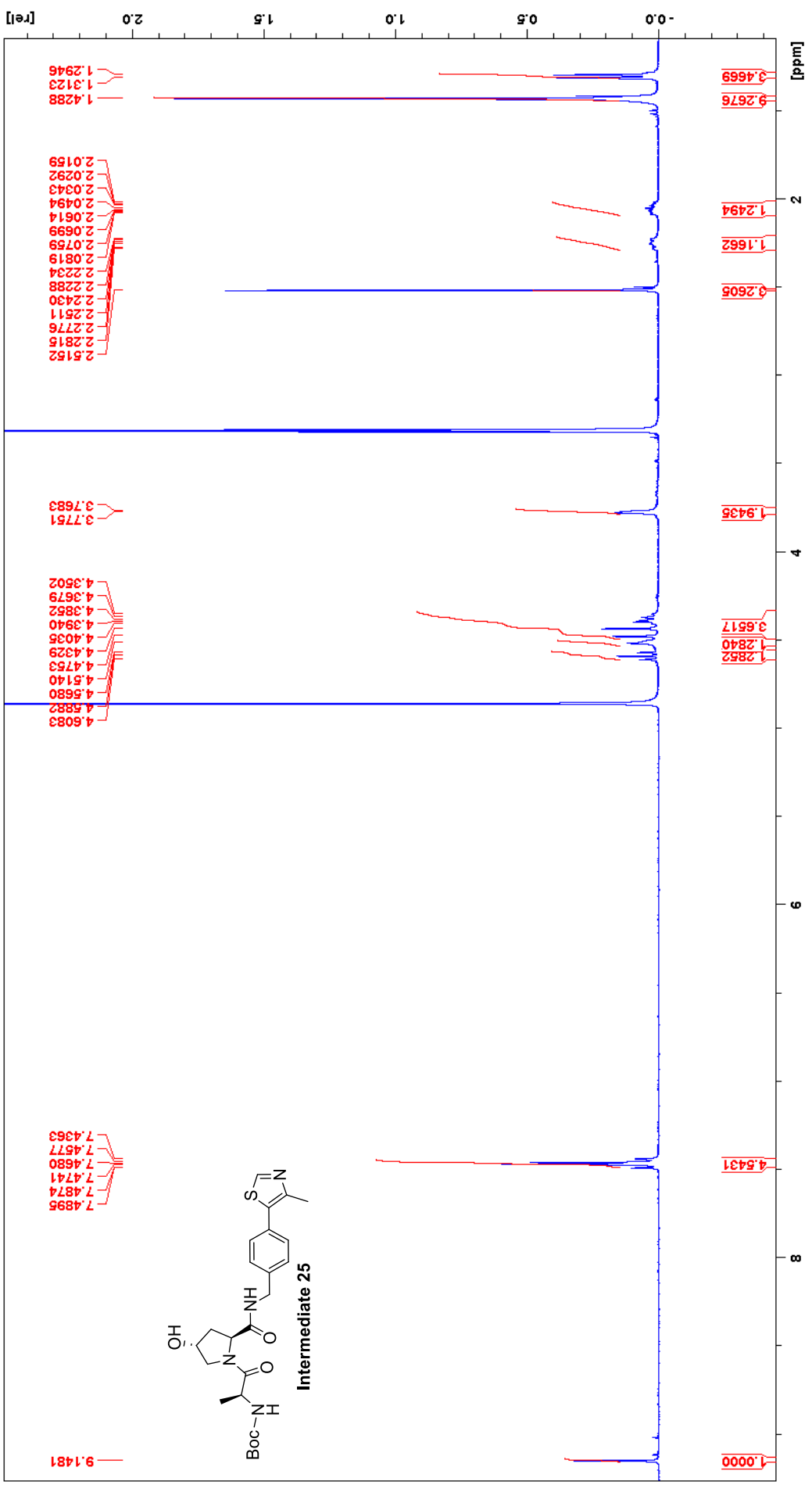
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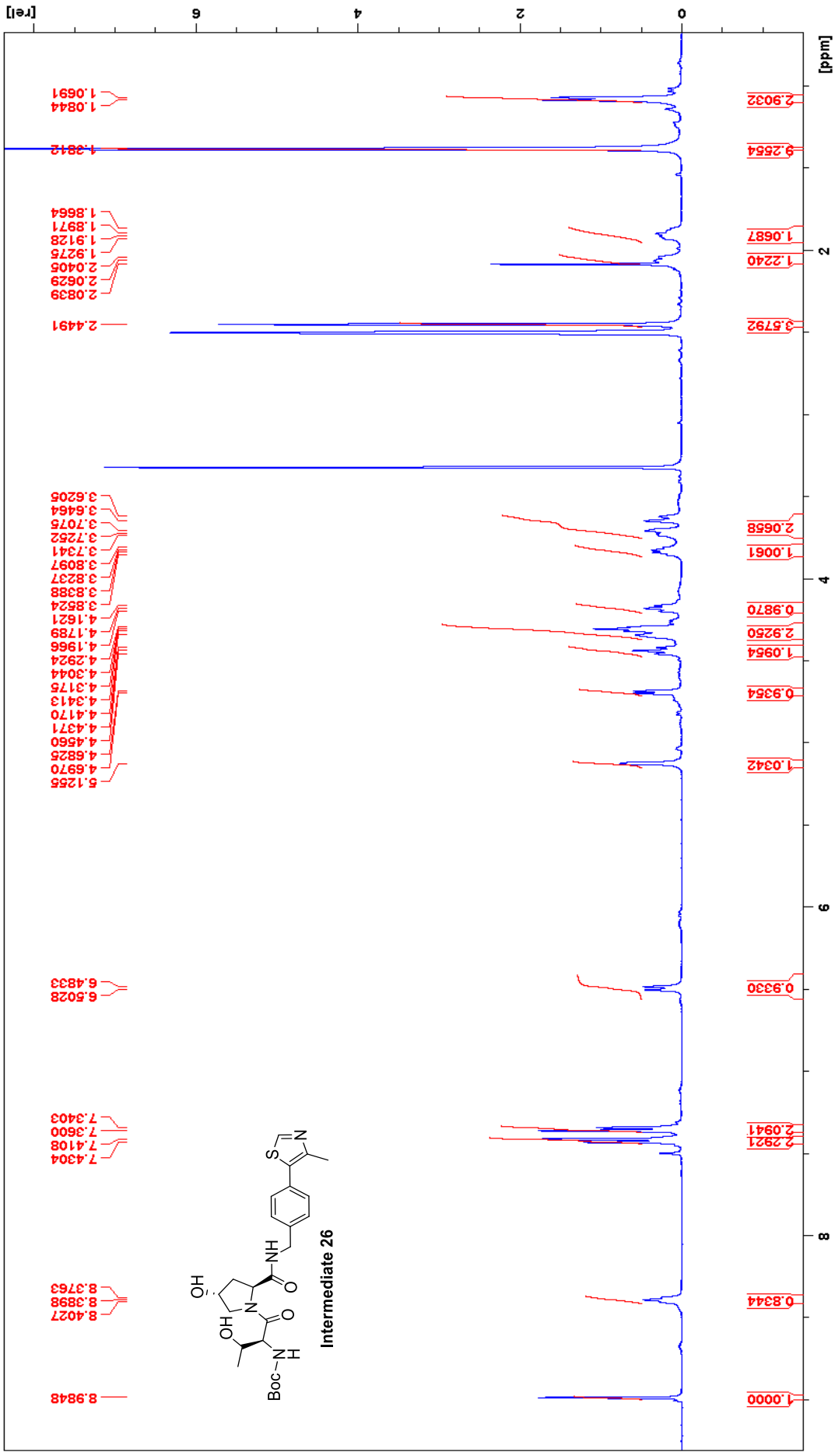
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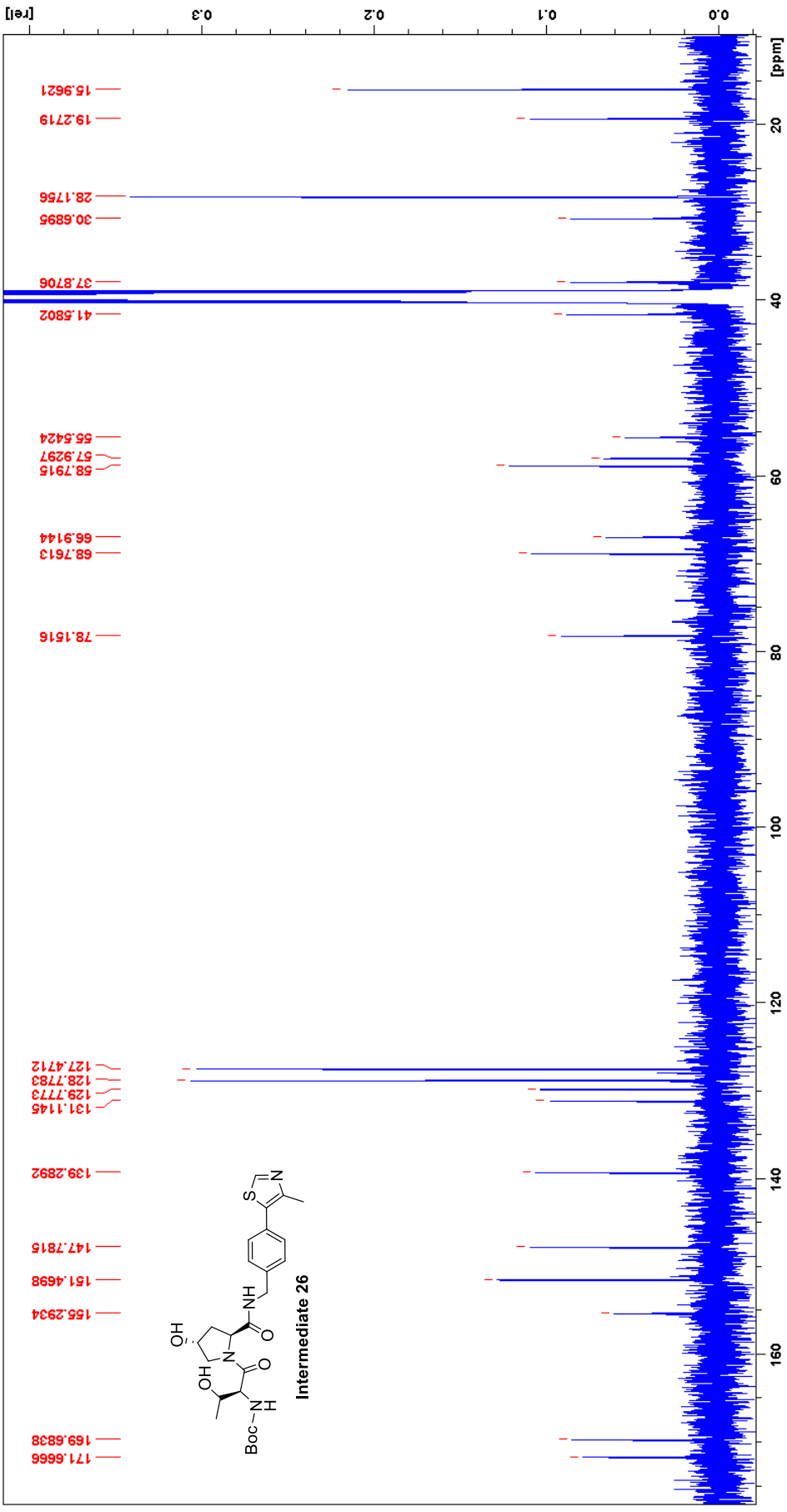
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Intermediate 26