Electronic Supporting Information for

Bicyclic enol cyclocarbamates inhibit Penicillin-binding proteins

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Supporting Experimental information

NMR spectra of the compound series

MIC assay, optical density curves and microscopy pictures

Determination of MIC: *B. subtilis* was cultured with the indicated concentration of enol cyclocarbamate derivative for 20 hours at 35 °C in a 96-well plate in cation adjusted Mueller-Hinton broth. The OD_{600} was determined and then resazurin was added. The bacterial suspension was incubated for 20 minutes at 37 °C, after which the fluorescence intensity was measured at 581 nm. The MIC value was considered the lowest value at which the OD600 did not reveal growth and at which the resazurin was not converted into resorufin. The assay was realized following the CLSI protocol (Clinical and Laboratory Standards Institute).

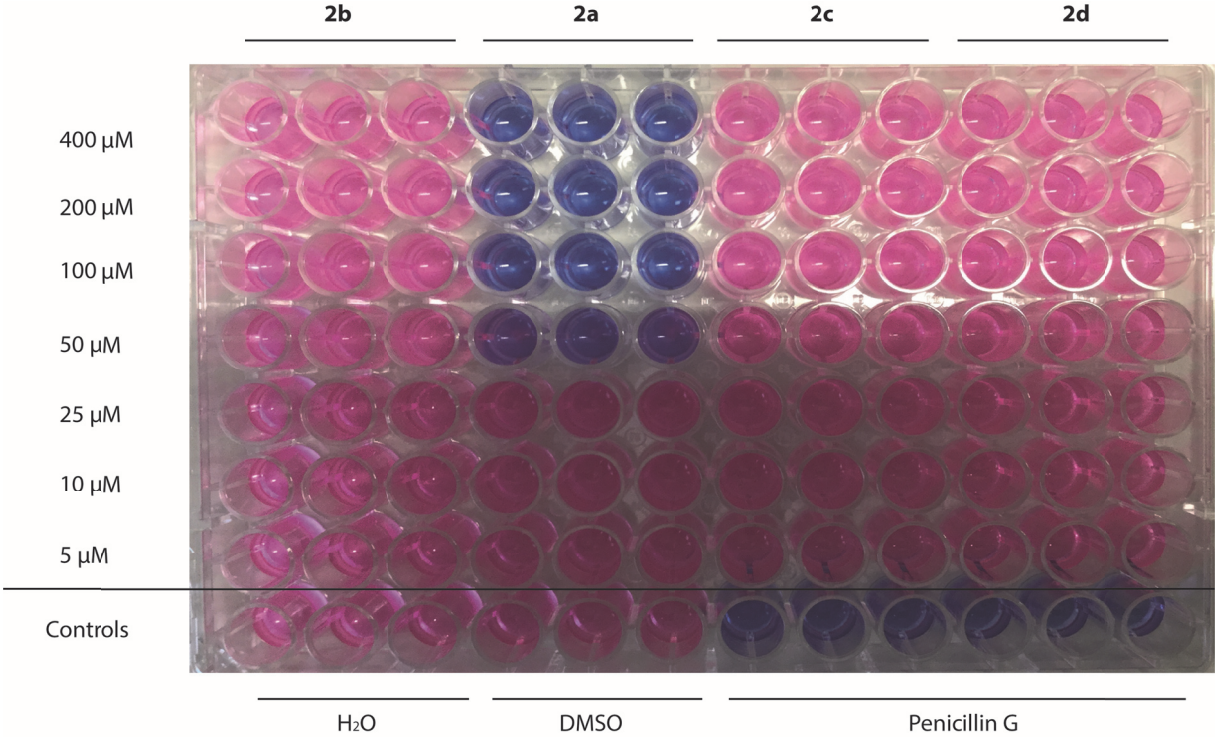


Figure S1. Example of 96 well-plate for the MIC determination of **2a**, **2b**, **2c**, **2d** against *B. subtilis* 168. Resazurin (blue) is reduced to resorufin (pink) when the cells are viable.

Having established which compounds display antibacterial activity, the mode of action was studied with growth curve assays. *B. subtilis* and *S. pneumoniae* were cultured in the presence of a serial dilution of the lipocyclocarbamate in LB broth and the absorbance at 600 nm or 595 nm was measured over time and plotted. Cells were cultured in the presence of 0.05% DMSO and kanamycin as a controls. The *Z* isomer of **4b** delayed growth of *B. subtilis* (Figure S2) (The *E*-isomer of **4b** also inhibited bacterial growth in a similar fashion). Inactive compounds **3c** and **4a** had a marginal effect on *B. subtilis* growth (Figure S3).

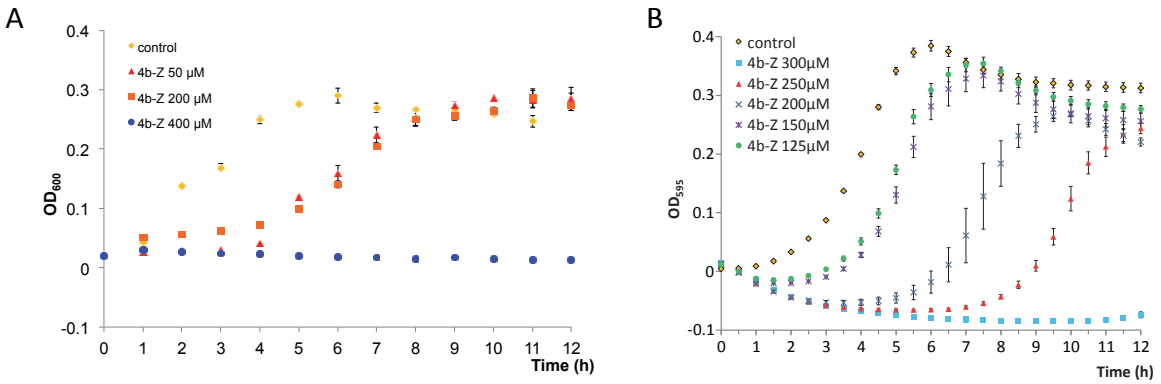


Figure S2. Growth curves of *B. subtilis* 168 (A) and *S. pneumoniae* (B) in the presence of derivatives **4b-Z**. *Remark: some curves have been omitted for sake of clarity (example: for the case of non-active concentrations where all the curves are on top of each other)*

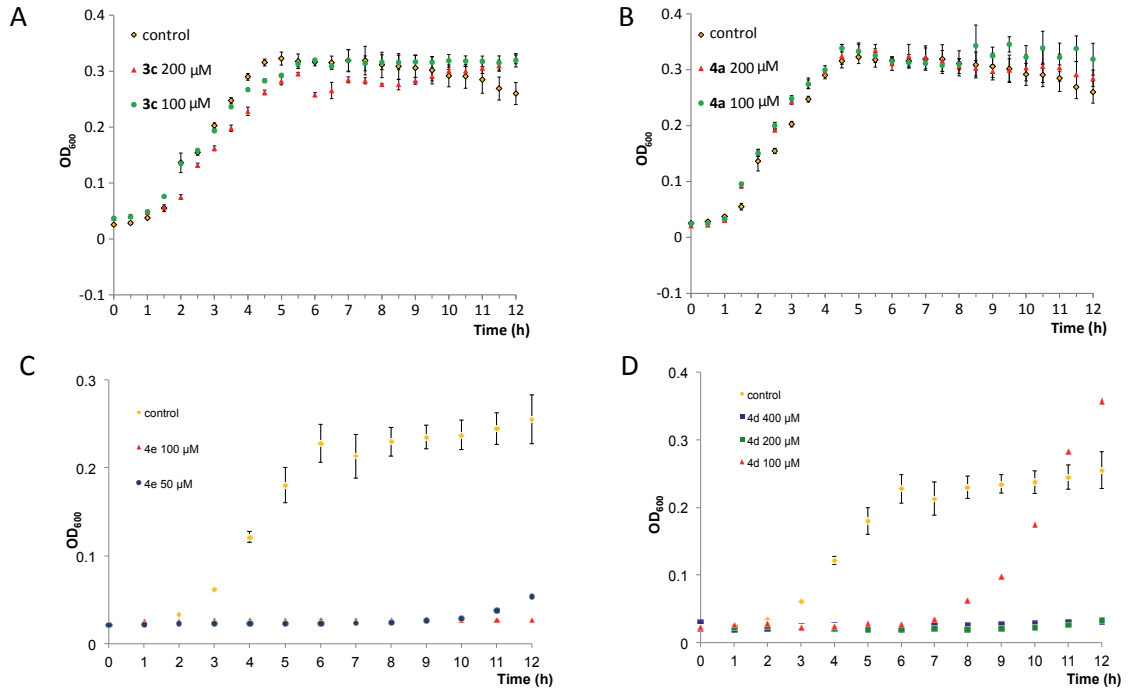


Figure S3. Growth curves of *B. subtilis* 168 in the presence of derivatives **3c** (A), **4a** (B), **4e** (C), **4d** (D). *Remark: some curves have been omitted for sake of clarity.*

The observed delay in bacterial growth for **2a**, **4b**, **4d** and **4e** is prototypical for bacteriolytic compounds and prompted us to perform time-lapse microscopy on *S. pneumoniae* in order to confirm that these enol cyclocarbamate derivatives are bacteriolytic. For this purpose, we grew *S. pneumoniae* on semi-solid media containing surfaces that contained different concentrations (50-100 µM) of **2a** and monitored the growth of seven different microcolonies per condition using time-lapse microscopy. Compound **2a** rapidly lysed the cells at 75 µM, while at 50 µM normal growth was observed (Figure S4).

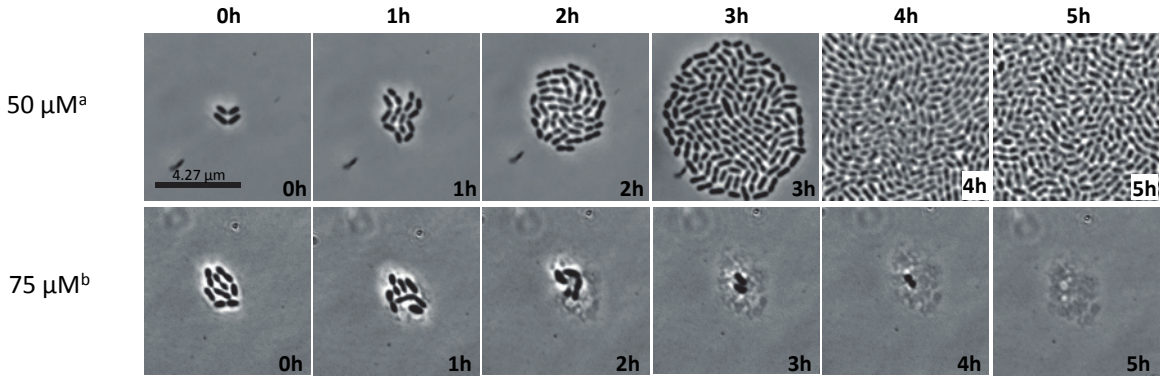


Figure S4. Time-lapse microscopy to study the bacteriolytic effect of the enol cyclocarbamate compounds. 7 different cells were tracked for each concentration of compound. [a] A normal growth was observed in presence of 50 µM and 60 µM (data not shown) of compound. [b] Bacteriolytic effect on pneumococcal cells in presence of 75 µM of compound.

The effect of the enol cyclocarbamates on peptidoglycan synthesis was determined using HADA labeling. *B. subtilis* (uncropped image, Figure S5) and *S. pneumoniae* (uncropped images, Figure S8) were for this purpose cultured with the enol cyclocarbamates, penicillin G and DMSO for 20 minutes and subsequently incubated with HADA for 5 minutes. The labeling of peptidoglycan was subsequently visualized with fluorescence microscopy. Fluorescent labeling of the septum was blocked by active compounds but not by inactive compounds. The fluorescence intensity of individual cells was quantified to determine the inhibitory effect of the enol cyclocarbamates on the incorporation of the fluorescent amino-acid analog HADA into peptidoglycan in live *B. subtilis* and the fluorescence intensity was plotted in a box-and-whisker plot. Addition of compound **2a**, **4d** or penicillin G led to a significant reduction in fluorescent labeling (Figure S6)

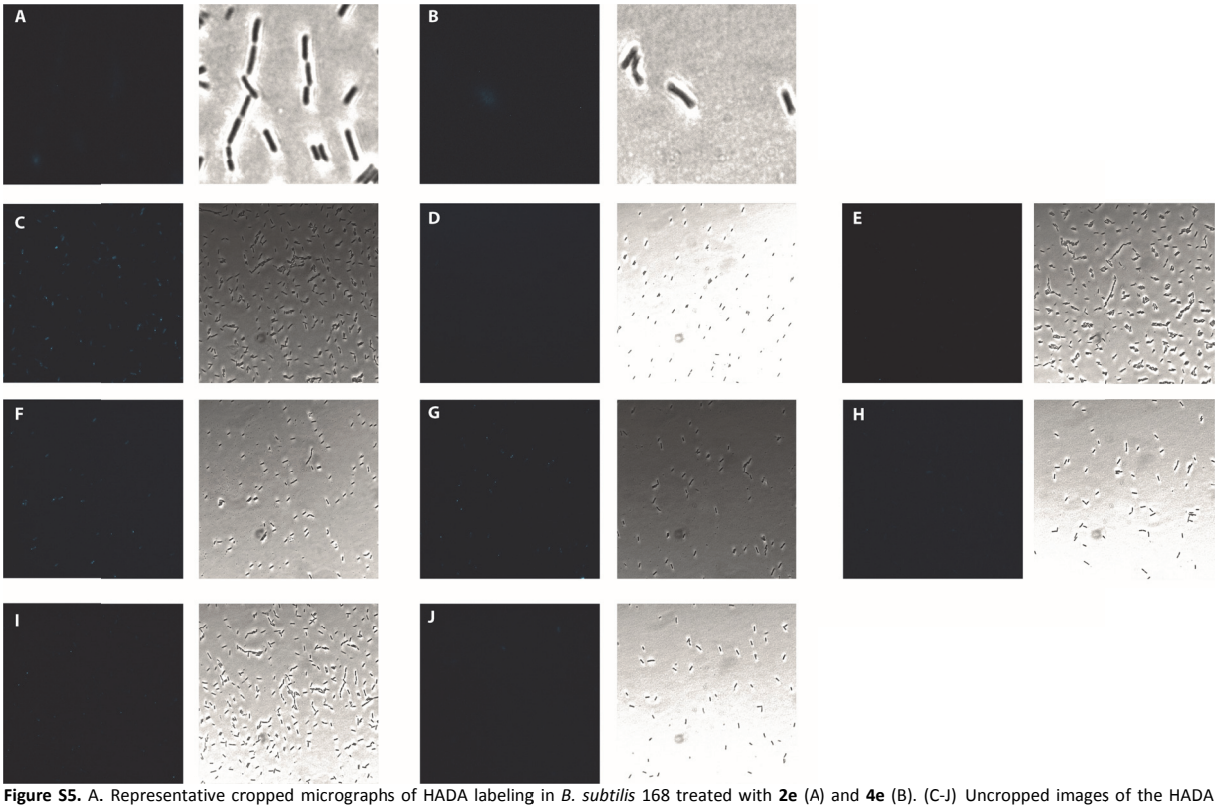


Figure S5. A. Representative cropped micrographs of HADA labeling in *B. subtilis* 168 treated with **2e** (A) and **4e** (B). (C-J) Uncropped images of the HADA labeling experiments depicted in Figure 3, Figure S5A and Figure S5B. Cells were incubated with 100 μM of enol carbamate for 20 minutes and then with HADA (0.5 mM) for 5 minutes. 0.05% DMSO/H_2O and 100 μM Penicillin G were used as controls. Representative fluorescence and bright-field micrographs of *B. subtilis* cells treated with (C) 0.05% DMSO/H_2O, (D) Penicillin G, (E) **2a,** (F) **2d**, (G) **4b**, (H) **4d**, (I) **2e** and (J) **4e**.

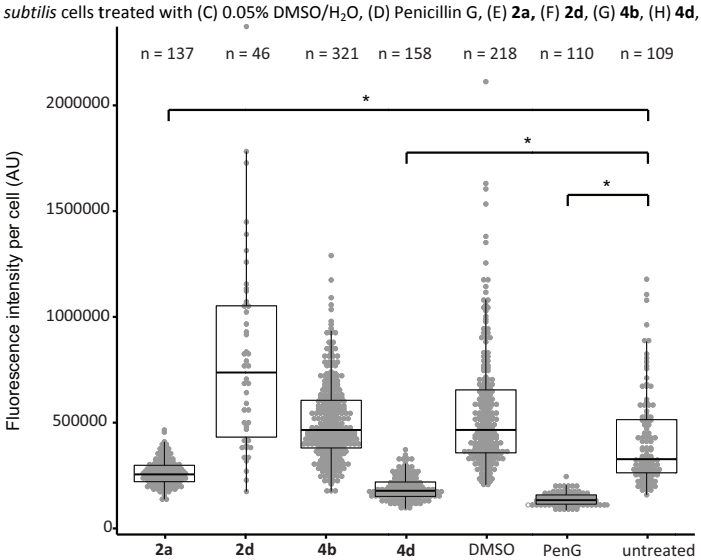


Figure S6. Inhibition incorporation of the fluorescent amino-acid analog HADA into peptidoglycan in live *B. subtilis* was determined using a wide-field fluorescence microscope. Cells were incubated for 20 min with compound, followed by 5-min labeling with HADA, after which cells were fixed with 70% ethanol. Fluorescence intensity per cell of n number of cells was extracted (grey dots, bin-size 20000). The box-and-whiskers plot shows the median of the sample (thick bar), the first and third quartiles (thin bars), and the values within 1.5 times the length of the box (whiskers). A * denotes a p-value <0.001 according the Wilcoxon rank sum test with continuity correction. The compounds **2d**, **2a**, **4b**, **4d** and Penicillin G (Pen G) were dissolved in 4% DMSO.

To determine if the synthesized enol cyclocarbamates derivatives inhibit the incorporation of HADA by permeabilizing the membrane, we stained the cells with a membrane impermeable DNA stain. *B. subtilis* was cultured in the presence or absence of **4b** or **4d** (250 μM) for 3 hours, after which the cells were collected by centrifugation and stained with propidium iodide (red, membrane impermeable) and SYTO 9 (green, membrane permeable). Bacterial cells that have an intact cell membrane will be stained with SYTO 9 exclusively, while permeabilized cells will be stained with both fluorescent dyes. *B. subtilis* treated with DMSO showed exclusive staining with SYTO 9 (Figure S7A). Nisin or Penicillin G were used as positive controls for membrane permeabilization and peptidoglycan synthesis inhibition, and cells that were incubated with these compounds were indeed stained by both fluorescent dyes (Figure S7C and S7D). Incubating *B. subtilis* with propargyl ether **4b**,

which delayed growth, resulted in a comparable image to DMSO-treated cells (Figure S7B). Although an increased number of cells were stained with both dyes when they were treated with PEGylated derivative **4d** (Figure S7E), the majority of the cells is still exclusively labeled with SYTO 9, indicating that the membrane is not disrupted in these cells even after incubating the cells for 3h with 250 μM of **4d** suggesting that lipocyclocarbamate derivative **4d** most probably does not inhibit bacterial growth via the same mechanism as Nisin or Penicillin G.

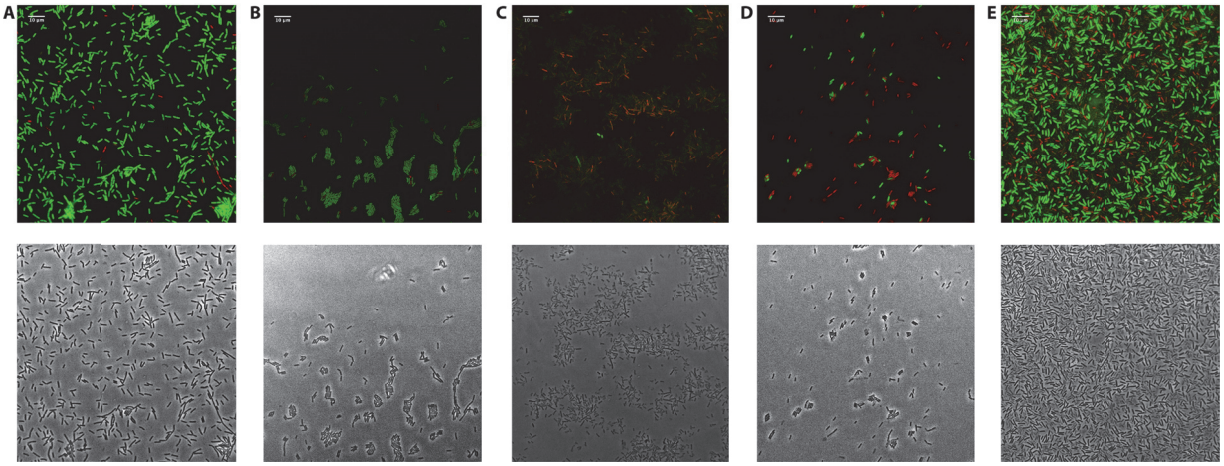


Figure S7. Uncropped fluorescent microscopy images of the live/death cell-staining assay. *B. subtilis* was cultured with DMSO (4% DMSO in H_2O) (A), lipocyclocarbamate **4b** (250 μM, 4% DMSO in H_2O) (B), Nisin (3 μg/mL) (C) or Penicillin G (200 μg/mL) (D), lipocyclocarbamate **4d** (250 μM 4% DMSO in H_2O) (E) for 3 h at 37 °C. Top panels: fluorescence image of Syto9 (green signal) and propidium iodide (red signal) staining. Bottom panels: Bright-field image of the same cells. Scale bar = 10 μm. Propidium iodide labels cells only when the membrane has been compromised, while Syto 9 labels any cell. The pore forming antimicrobial peptide Nisin shows a very characteristic profile that we did not observe for our compounds **4b** and **4d**.

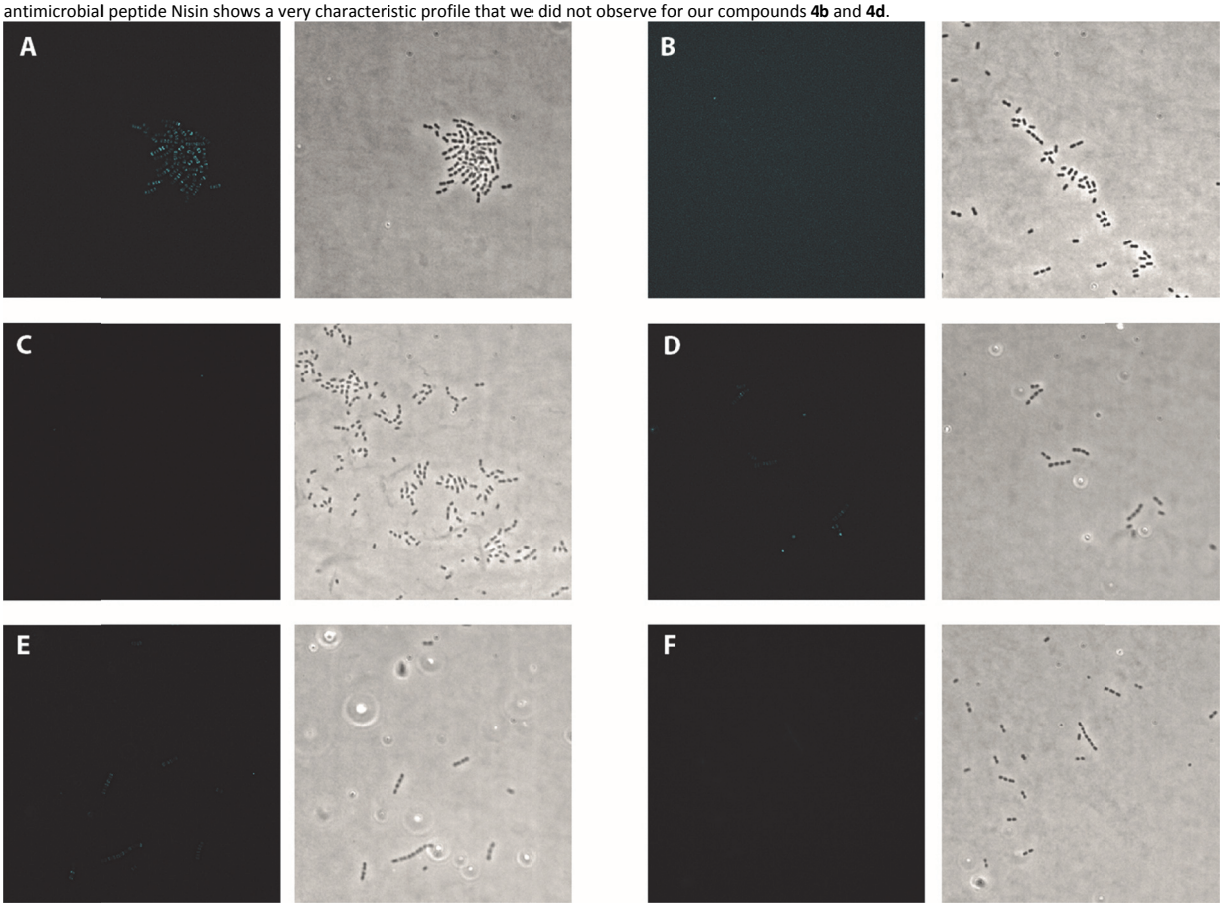


Figure S8. Uncropped images of the HADA labeling in *Streptococcus pneumoniae* as depicted in Figure 3G-L. *Streptococcus pneumoniae* cells were incubated with 100 μM of enol carbamate for 20 minutes and then with HADA (0.5 mM) for 5 minutes. 0.05% DMSO/H_2O and 100 μM Penicillin G were used as controls. Representative fluorescence and bright-field micrographs of cells treated with (A) 0.05% DMSO/H_2O, (B) Penicillin G, (C) **2a**, (D) **2d**, (E) **4b**, (F) **4d**.

The effect of the panel of enol cyclocarbamates on penicillin-binding protein (PBPs) activity was determined using Bocillin FL. 100 μM of compounds **2-4** were added to *B. subtilis* and the cells were incubated for 60 minutes. Next, the non-reacted

PBPs were visualized by lysing the cells and labeling the PBPs with Bocillin FL (45 nM). By comparing the labeling profile of DMSO treated cells with that of enol cyclocarbamate treated cells, the inhibited PBPs could be identified. A single PBP was inhibited by compounds **2a**, **2e**, **4d** and **4e** (Figure S9**,** S10). The other compounds did not seem to affect the PBP labeling profile (Figure S10).

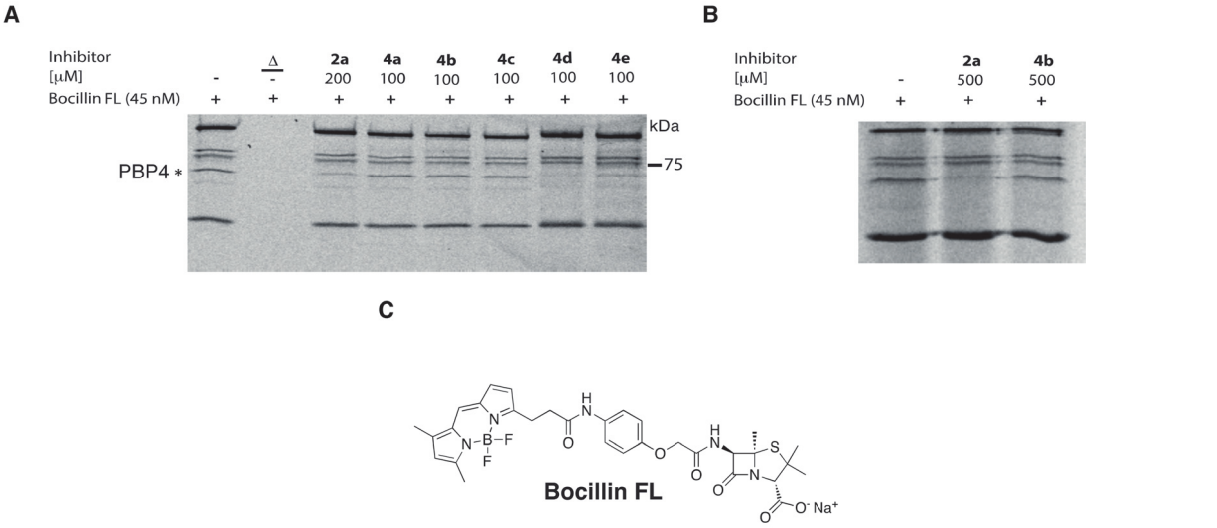


Figure S9. *Bacillus subtilis.* Competition assay with enol cyclocarbamate **2a** at 200 μM or **4a**, **4b**, **4c**, **4d** and **4e** at 100 μM. (A) Competition assay of **2a** and **4b** 500 μM on lysates (B). Structure of Bocillin-FL.

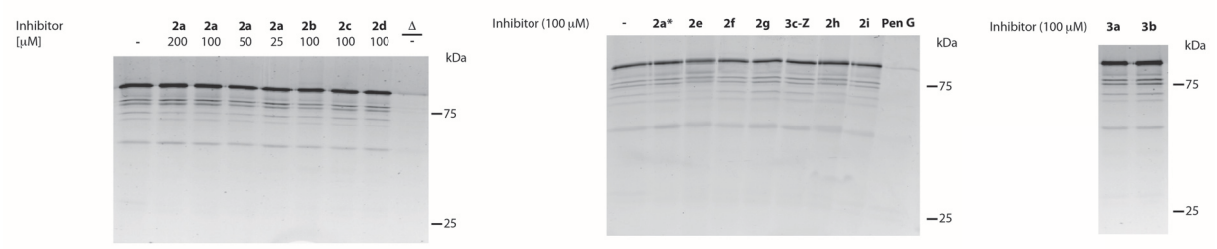


Figure S10. *Bacillus subtilis.* Competition assay with enol cyclocarbamate **2a**, **2b**, **2c**, **2d**, **2e**, **2f**, **2g**, **2h**, **2i**, **3a**, **3b**, **3c** and Penicillin G (PenG). PBPs were visualized with 45 nM Bocillin-FL.

Finally, the inhibitory effect of the enol cyclocarbamates in *S. pneumoniae* was determined with Bocillin FL. Bacteria were cultured with the compounds, lysed and the active PBPs were labeled with Bocillin FL. Separation of the protein by gel electrophoresis and visualization of the labeled proteins by fluorescence scanning revealed that both PBP1a/1b and PBP3 were inhibited by **2a**. Treating the cells with 200 μM of **2a** led to an approximately 40-60% reduction in the labeling of PBP1a/1b and the PBP3 band, as judged by quantification of the fluorescence intensity (Figure S11B). A new fluorescently labeled protein appeared in cell lysates treated with **2a**, but not **2d** (Figure S11A). Addition of PMSF (300 μM) during the cell lysis did not inhibit the formation of this band, but did improve the inhibition (Figure S11C and S11D). However, it did not affect Bocillin FL labeling profile in cells treated with **2d** or **3c** (Figure S11C and S11D), which were inactive in the viability assay.

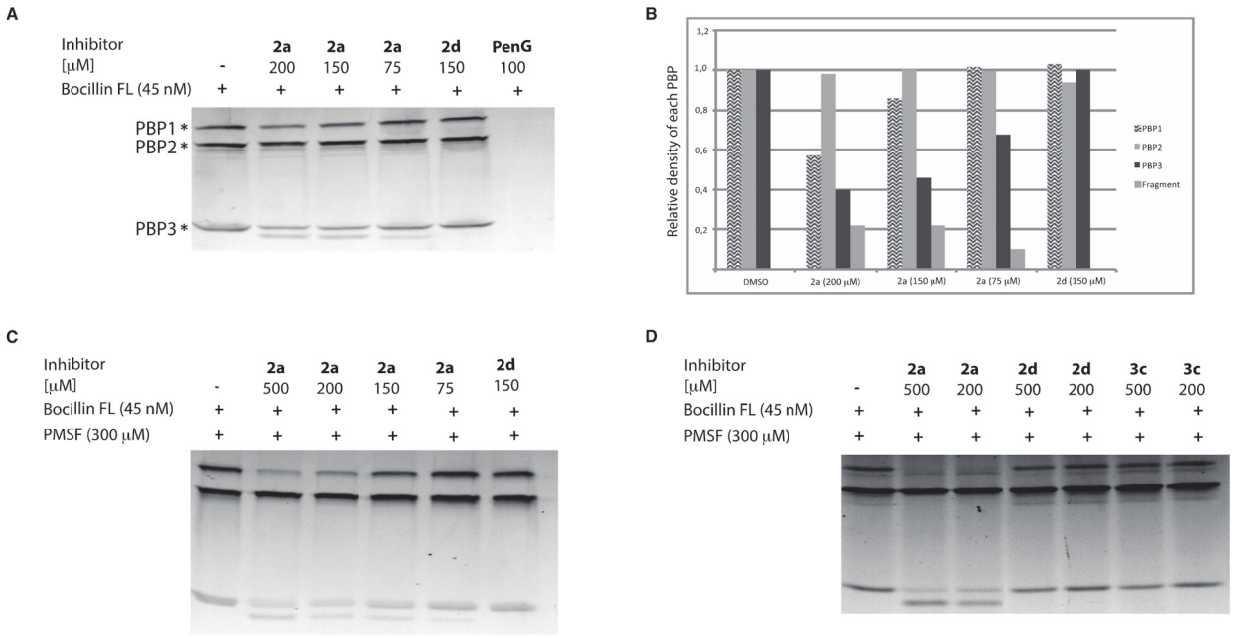


Figure S11. *Streptococcus pneumoniae.* Competition assay with different concentrations enol cyclocarbamate **2a**, **2d** and **3c-Z** and Bocillin-FL (A, C and D) without PMSF (A) or in presence of PMSF at 300 μM (C and D). (B) Quantification of the fluorescence intensity of labeled PBPs in (A) using ImageJ. Relative density of each PBP is calculated by dividing the intensity of the cells treated with inhibitor with the non-treated cells.

We labeled the PBP4 null strain with HADA in the presence or absence of enol cyclocarbamate to verify if the observed decrease in HADA incorporation is caused by PBP4 inhibition. In the absence of compound, strong fluorescent labeling of the septum was observed (Figure S12A). Incubating the cells with 100 μM of **2a** for 20 minutes prior to the addition of HADA completely blocked labeling of the septum (Figure S12B). As expected, compound **2d**, which did not block HADA labeling in the *B. subtilis* 168 strain, also did not affect HADA labeling in the PBP4 null strain (Figure S12C). Strong septal labeling was observed in this case.

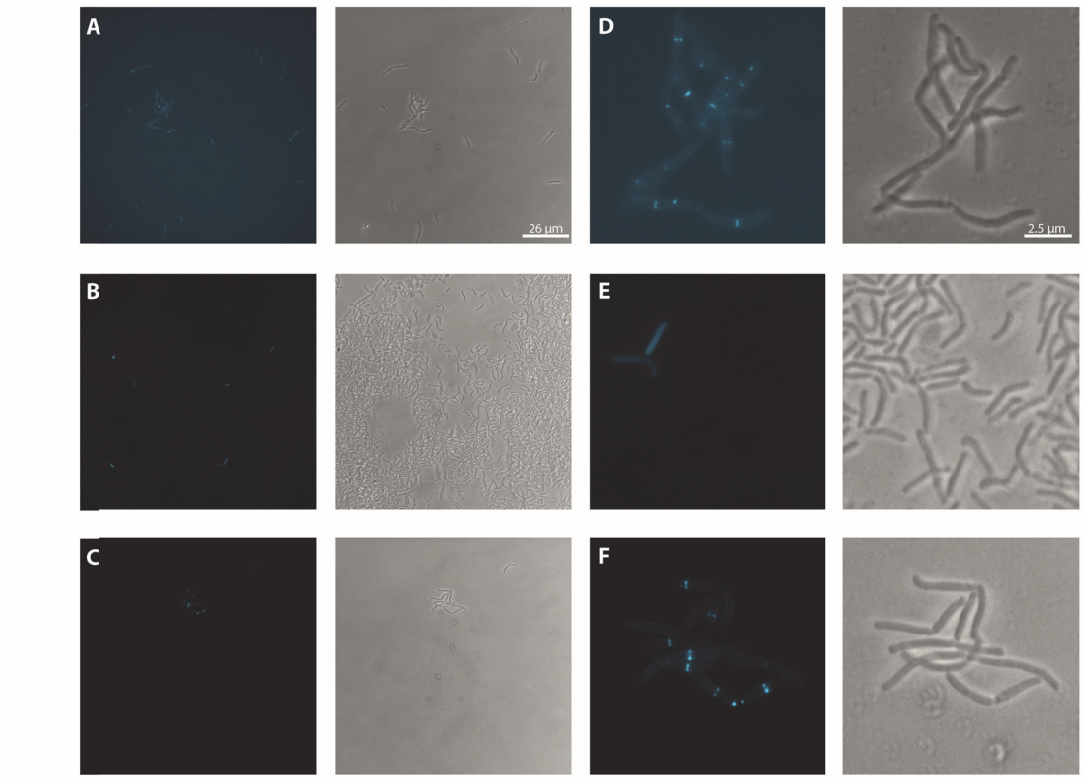


Figure S12. HADA labeling of *Bacillus subtilis PBP4-null strain*. Cells were incubated with 4% DMSO/H_2O (A), **2a** (100 μM) (B) and **2d** (100 μM) (C) during 20 minutes and then with HADA (0.5 mM) during 5 minutes. Uncropped pictures on the left, cropped pictures on the right.

Supplemental experimental

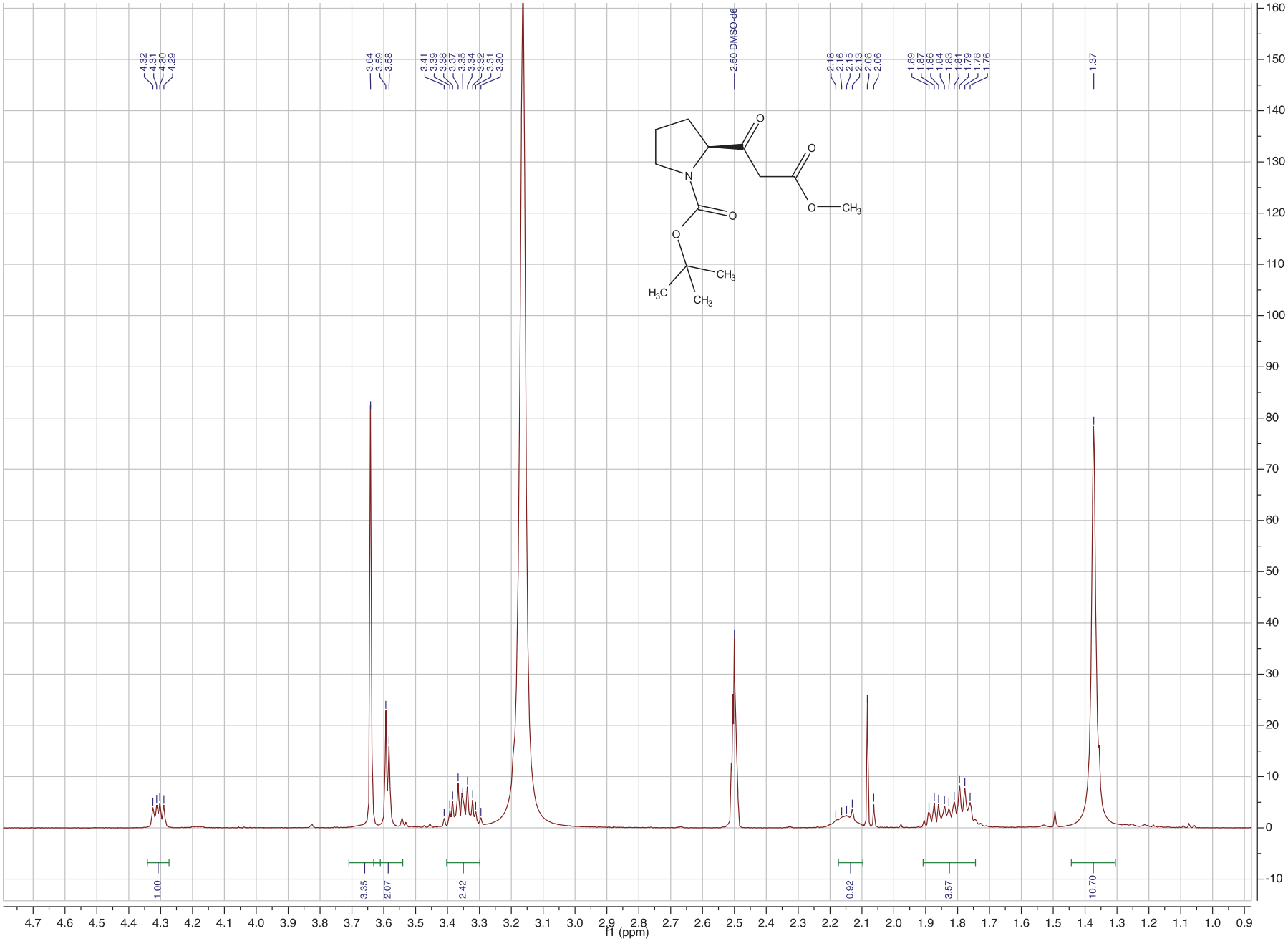
Fluorescence microscopy –

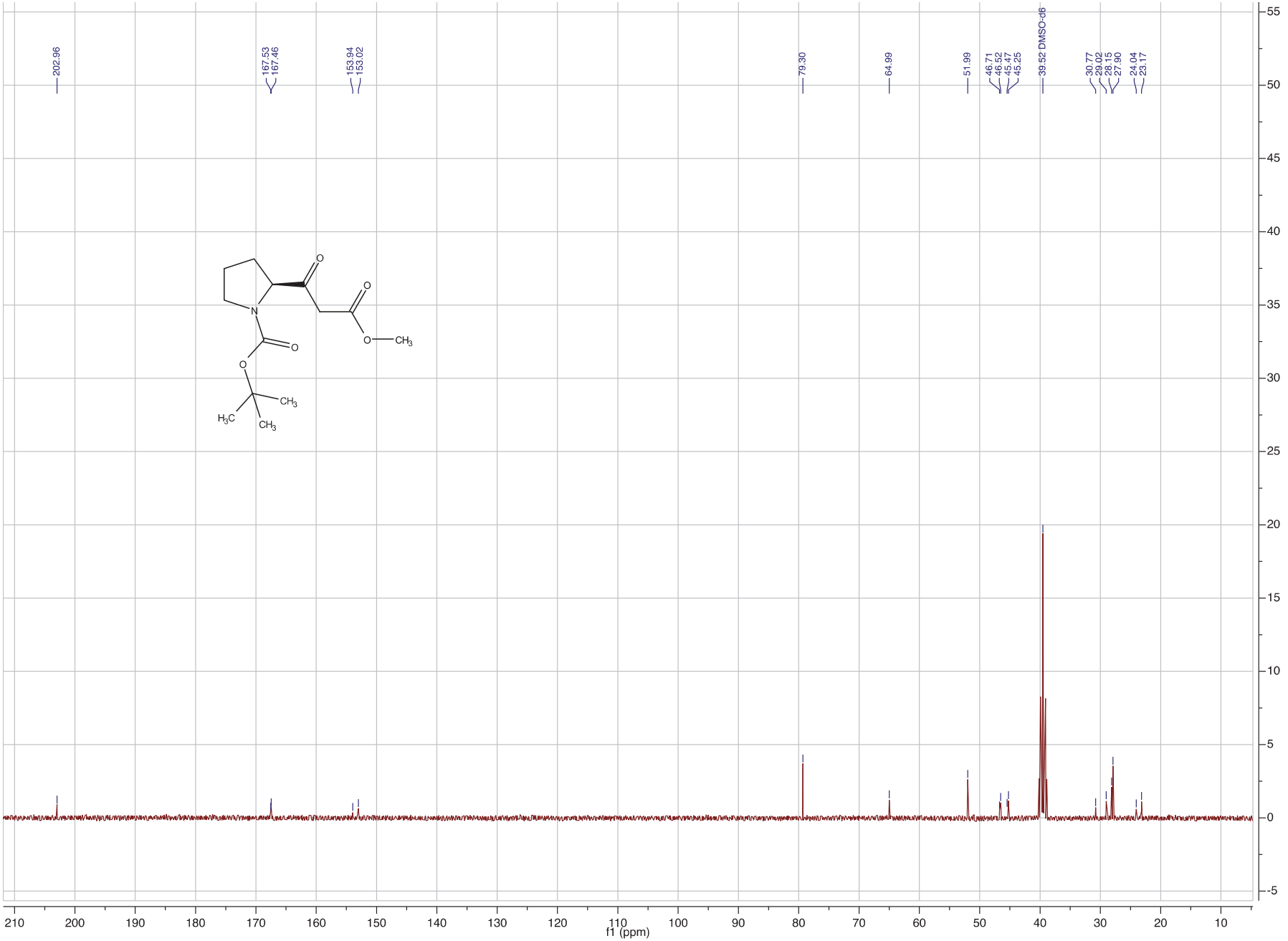
B. subtilis 168 was diluted from an overnight culture to an OD_{600} of 0.02 in casein hydrolysate medium[34] and grown at 37 °C until an OD_{600} of 0.2, at which point the compounds were added to the medium. At different time intervals (1 h and 3 h) cells were collected by centrifugation (2 minutes, 20000g) and resuspended in PBS ($1/10^{th}$ of the original culture volume). Depending on the experiment, fluorescent dyes were added to this suspension to visualize cell integrity (Syto9/Propidium iodide) (final concentrations propidium iodide, 20 μM; Syto9, 3.34 μM). After incubation for up to 15 minutes at 37 °C, bacteria were immobilized by putting 3-μL drops of the suspension on agarose pads (1% in PBS). Bacteria were imaged under a Nikon Ti-E inverted microscope equipped with a CFI Plan Apochromat DM 100× oil objective, using appropriate filter sets for the dyes used. Digital images were recorded using a Hamamatsu Orca Flash 4.0 (V2) camera and prepared using Adobe Photoshop.

Time lapse microscopy - S. pneumoniae

Time-lapse microscopy assays were performed as described previously.[29] Briefly, the effect of compound **2a** on single pneumococcal cells was followed in time by microscopy (Nikon Eclipse Ti) using strain D39. The cells were pre-grown in C+Y acid medium until OD_{595} = 0.2. Then, 100-fold dilution of culture was spotted on slides with acrylamide-C+Y with or without different concentrations of the compound. Images were acquired every 10 minutes during 12h.

2. NMR spectrum

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30.77
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CH3
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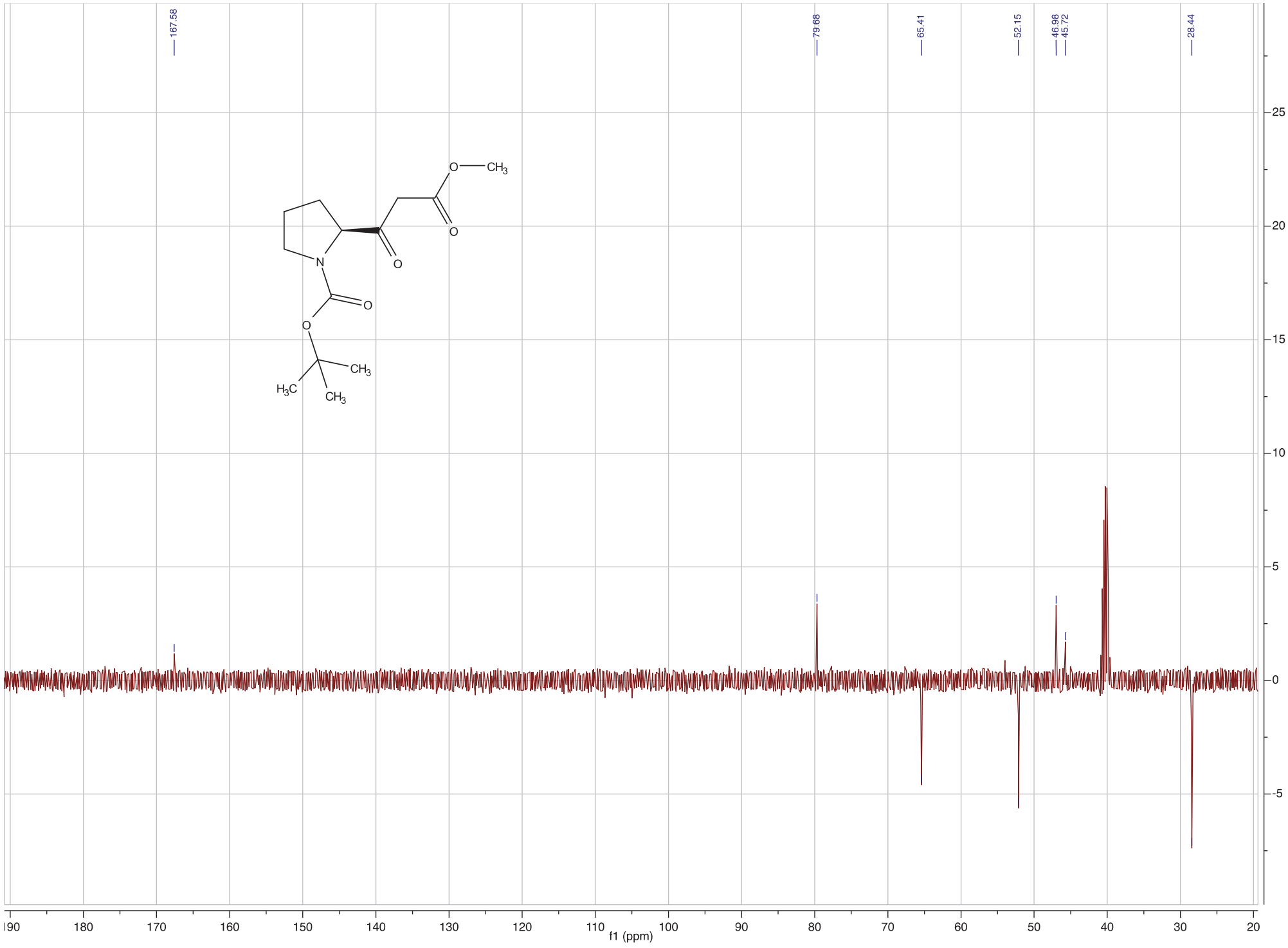
CH3
f1 (ppm)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CH3
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20
15
10
5
0
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30
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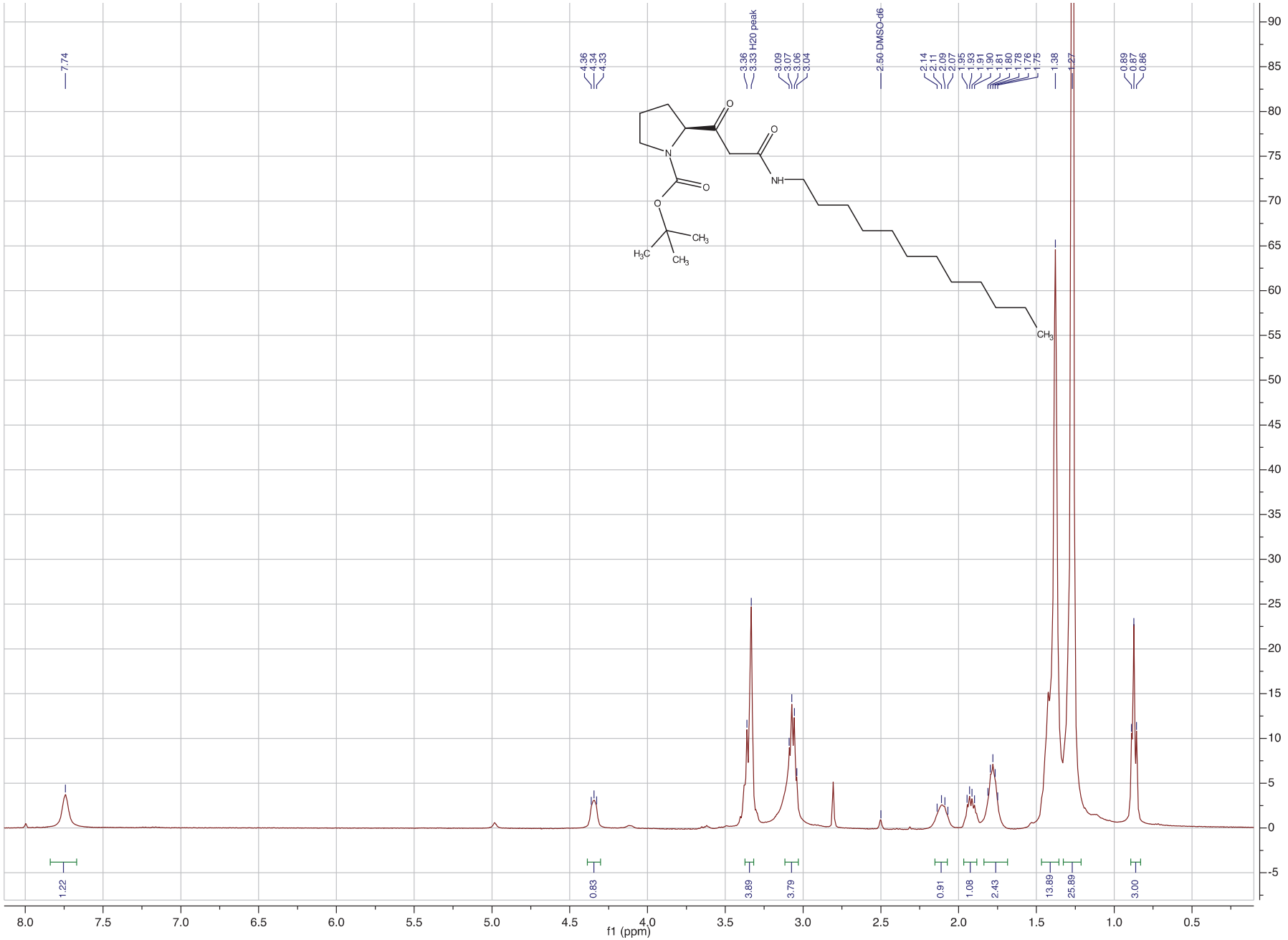
f1 (ppm)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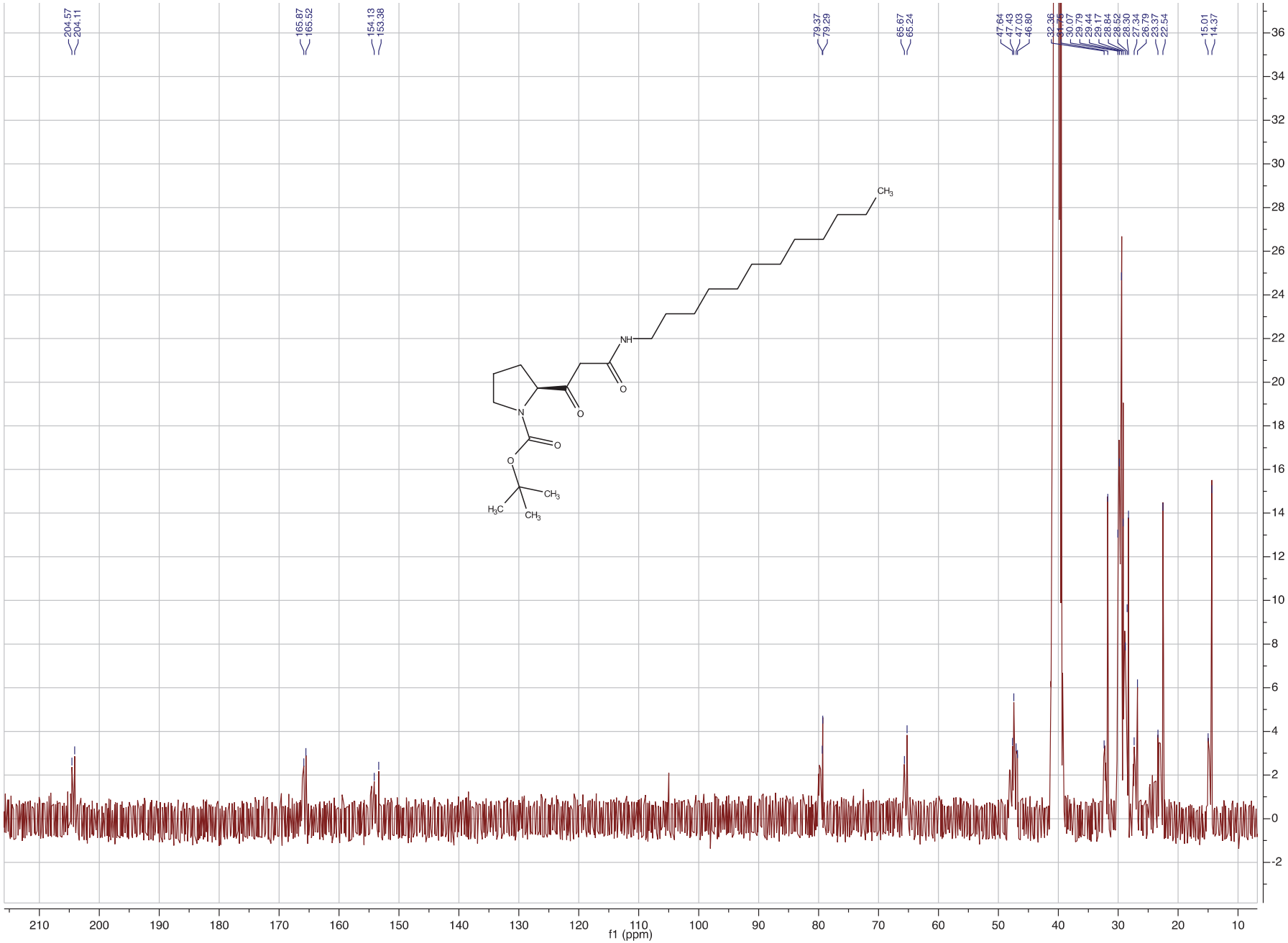
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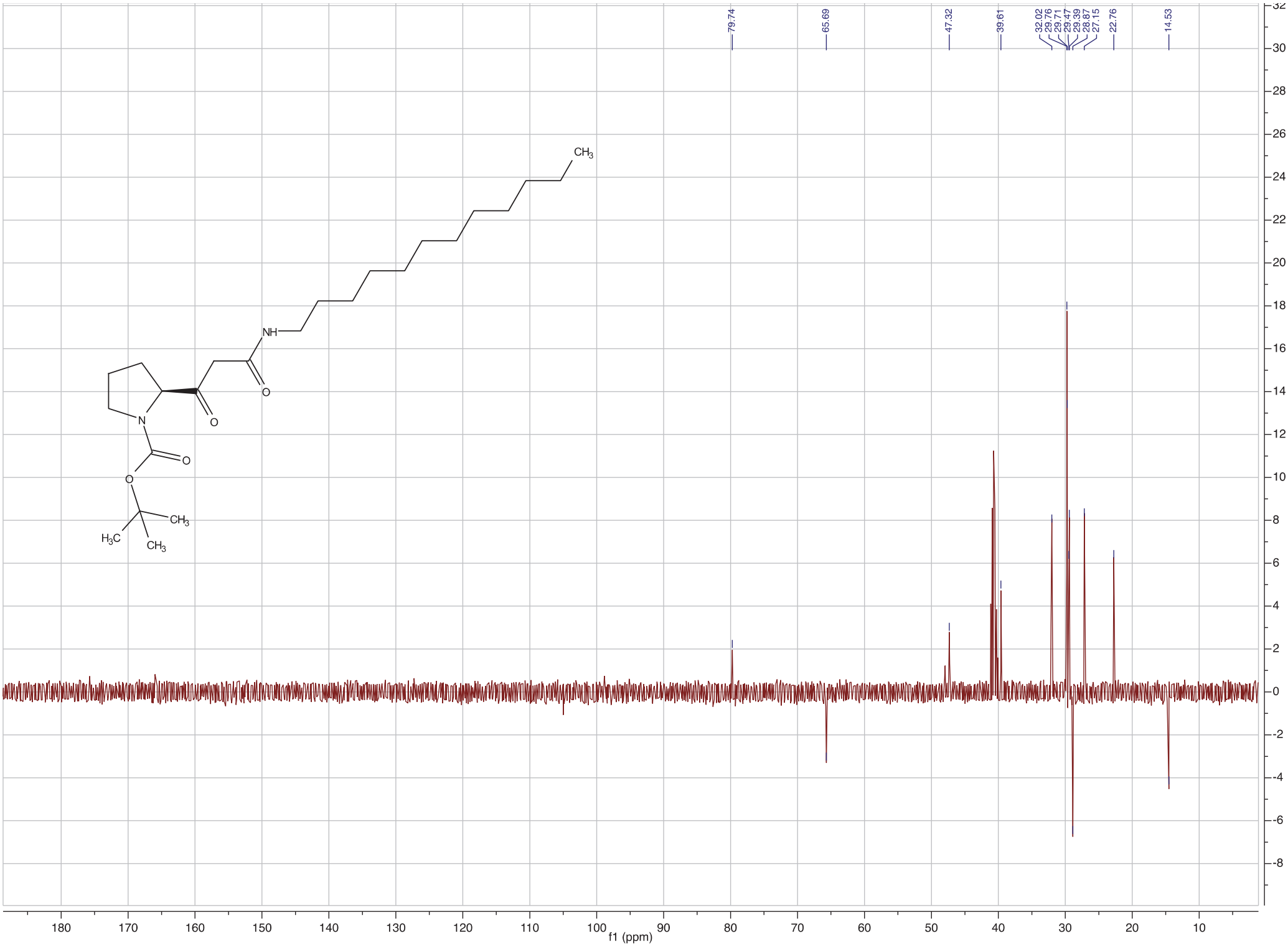
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H3C
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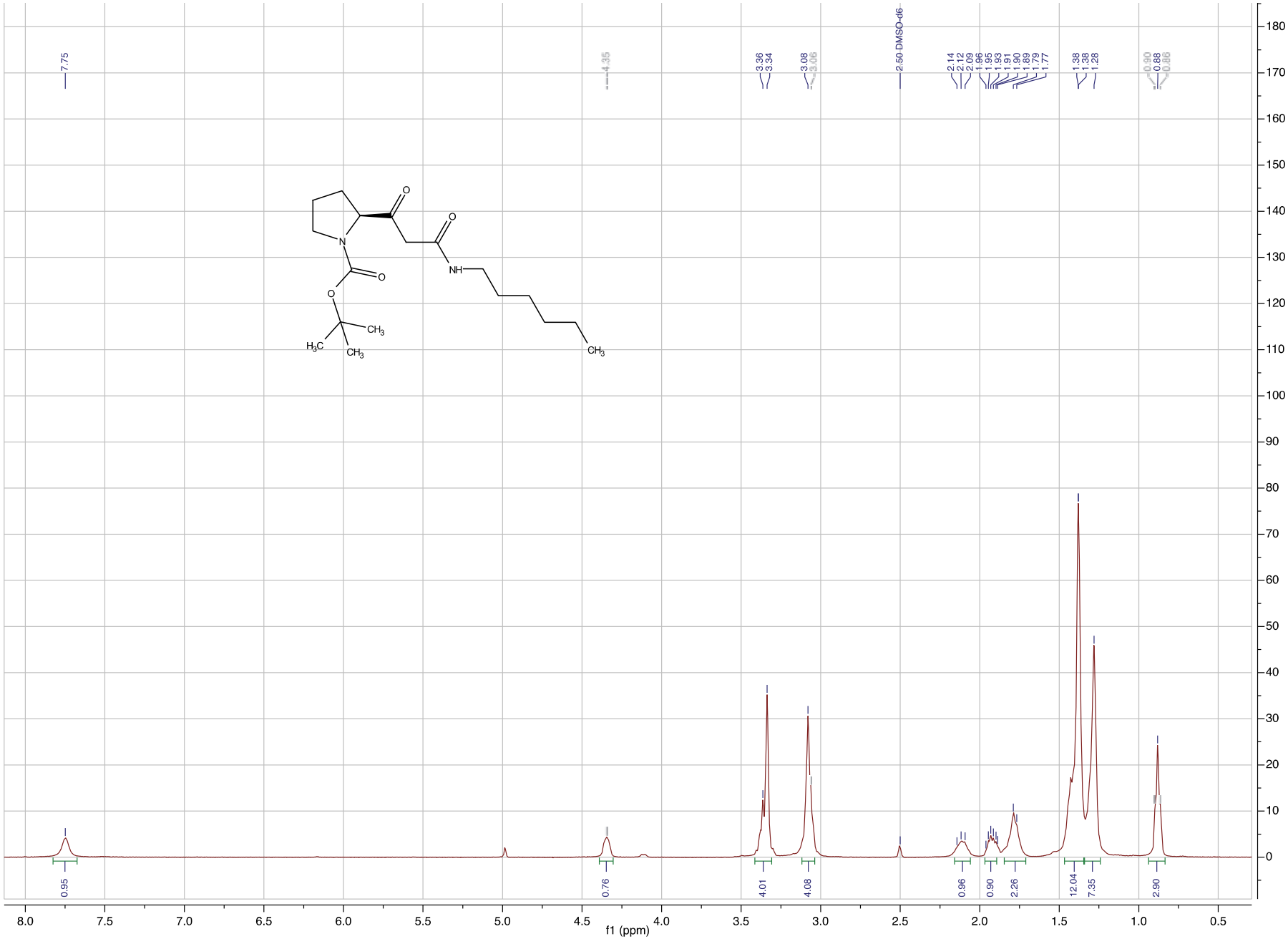
f1 (ppm)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H3C
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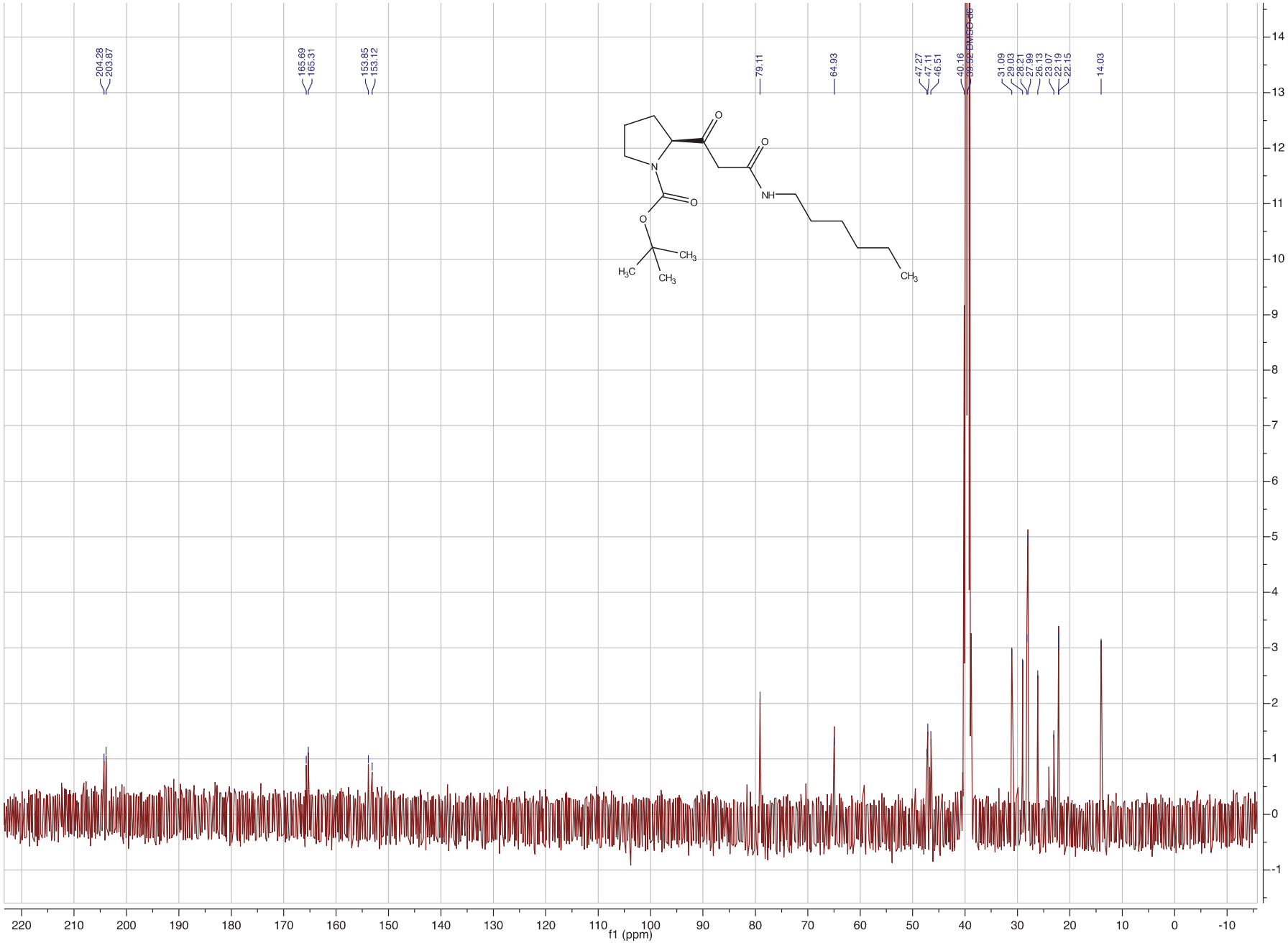
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H3C
CH3
CH3
f1 (ppm)


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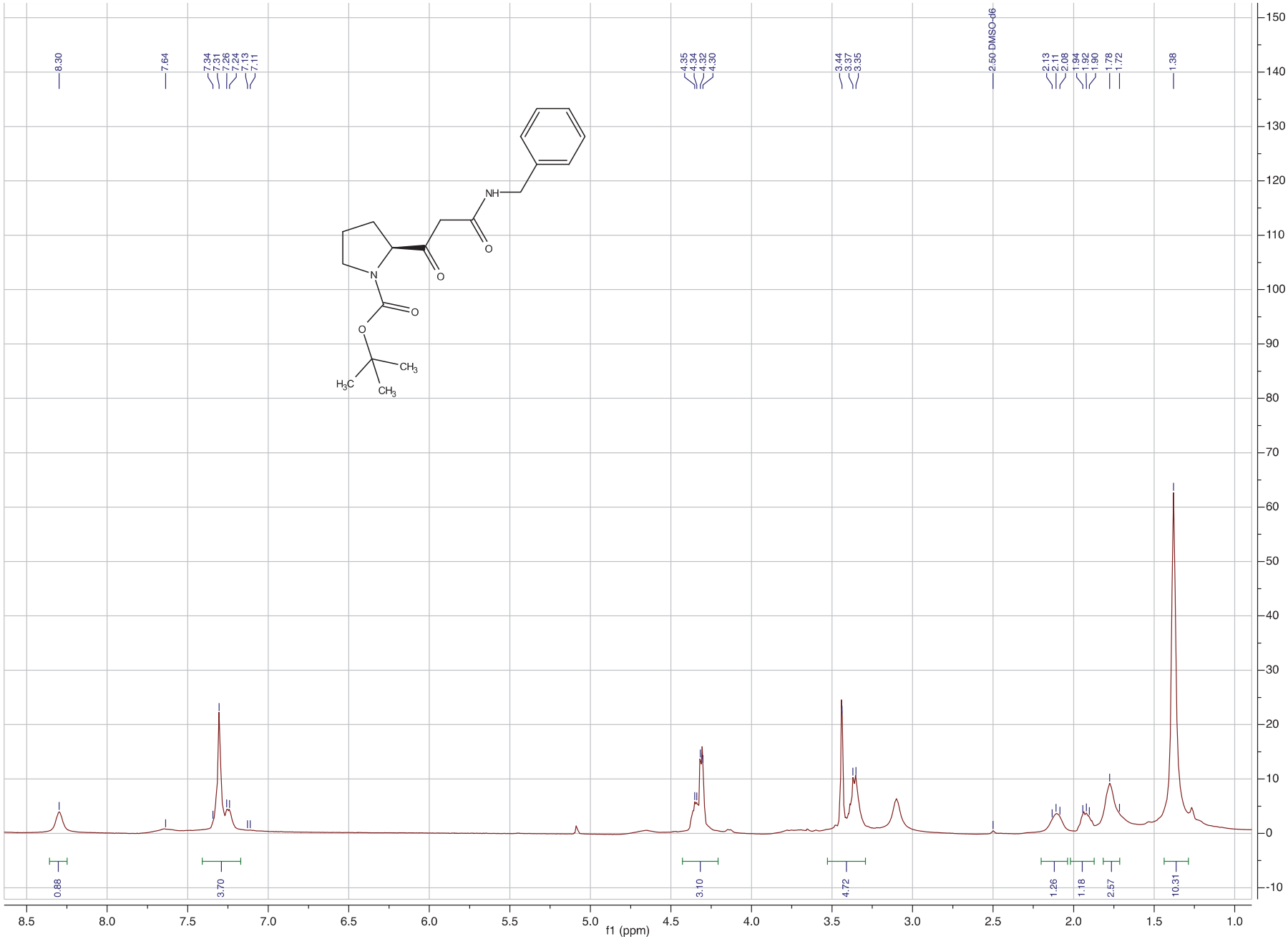
f1 (ppm)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1.92
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1.38
NH
O
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O
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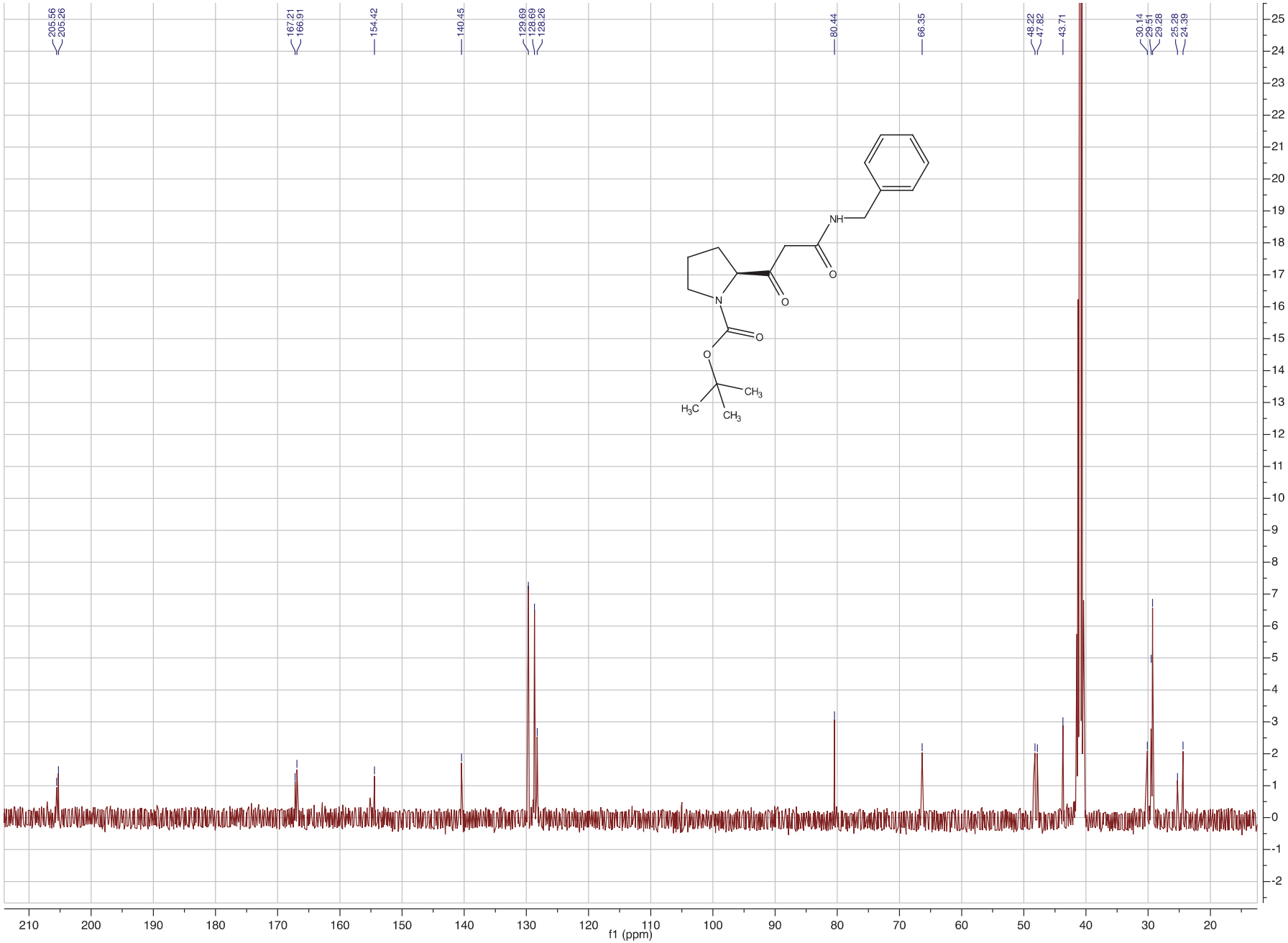
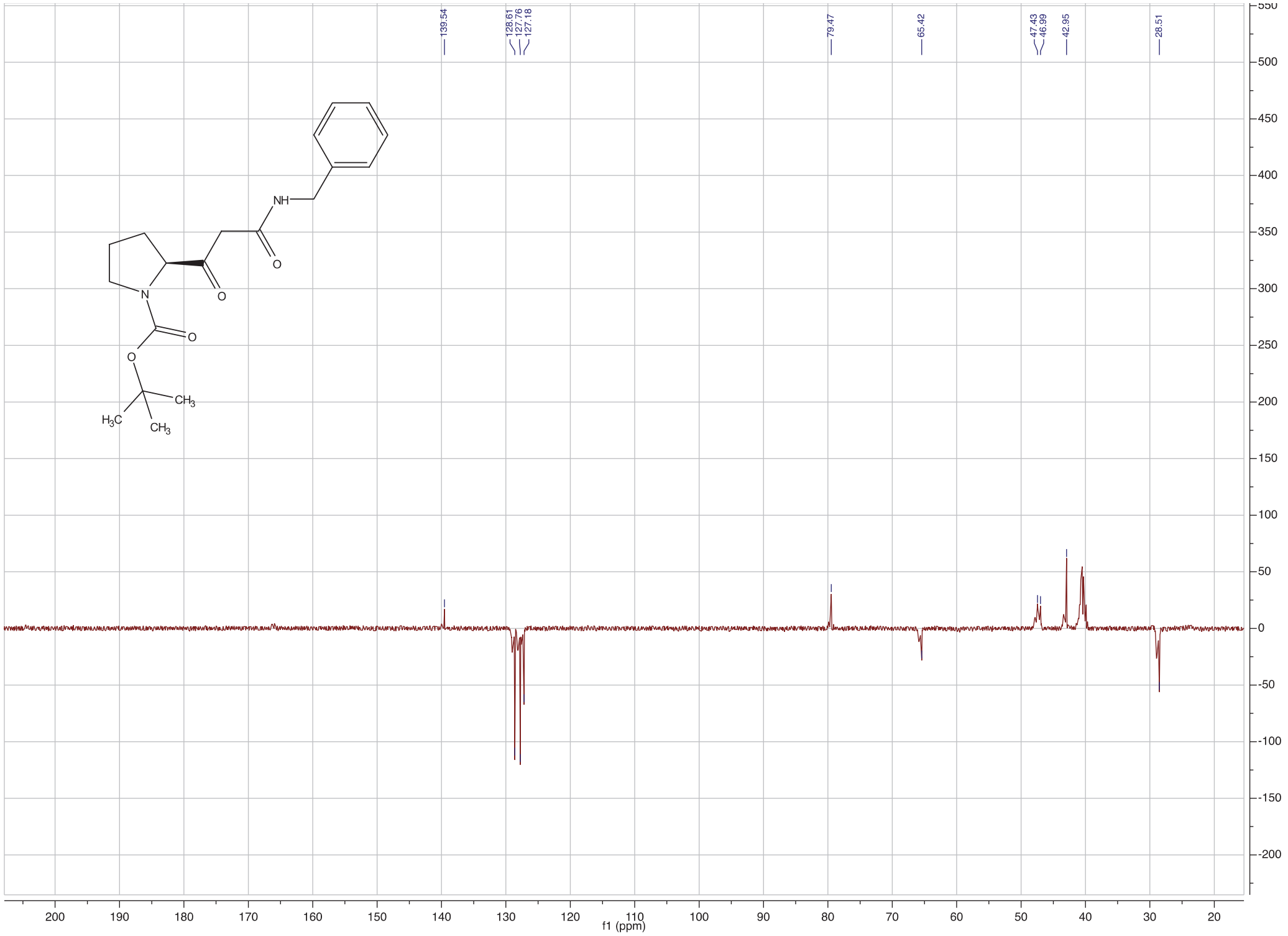
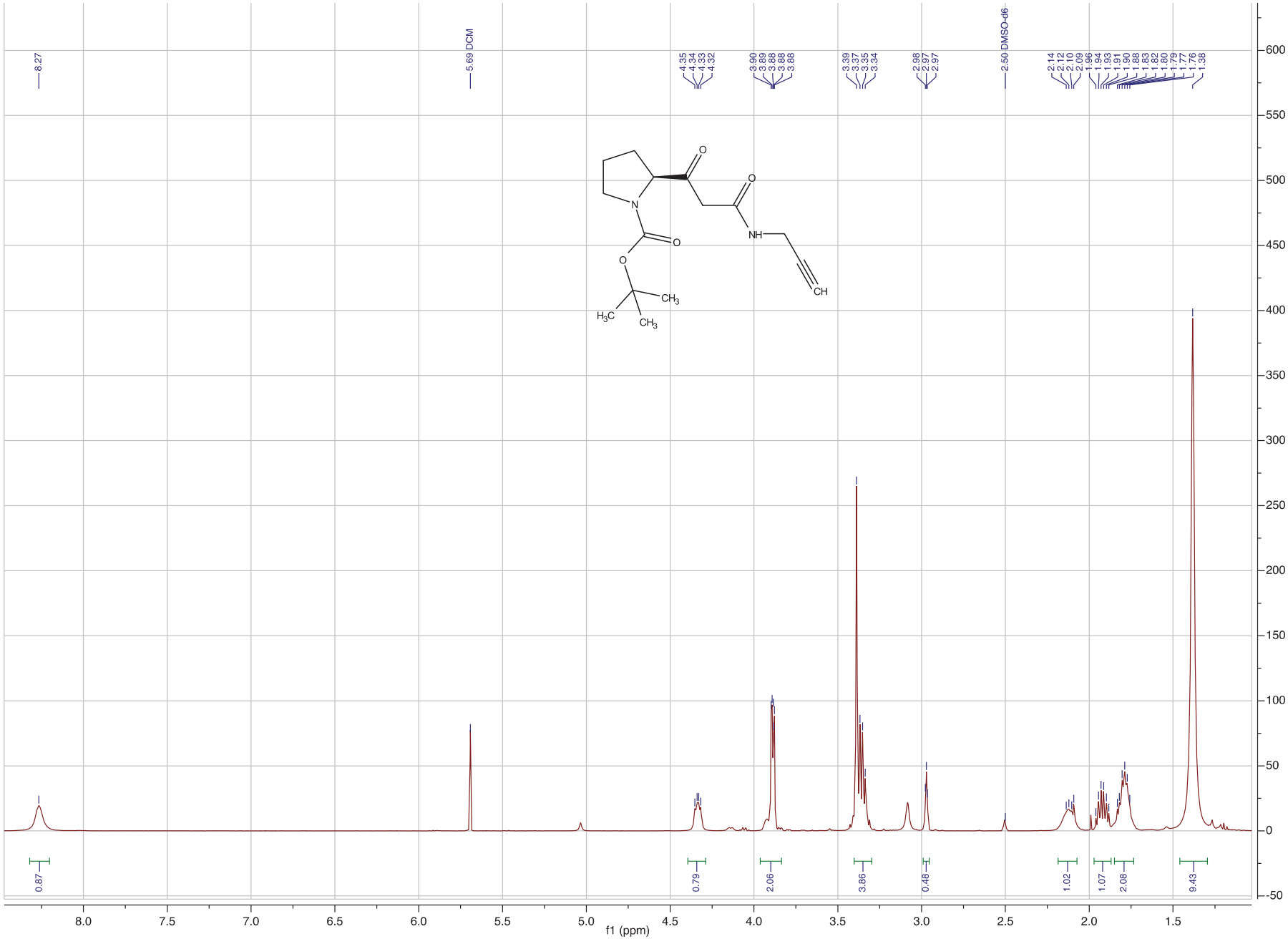
CH3
H3C
CH3
0.88
3.70
3.10
4.72
1.26
1.18
2.57
10.31
f1 (ppm)


205.56
205.26
167.21
166.91
154.42
140.45
129.69
128.69
128.26
80.44
66.35
48.22
47.82
43.71
30.14
29.51
29.28
25.28
24.39
NH
O
O
N
O
O
O
CH3
H3C
CH3
f1 (ppm)


139.54
128.61
127.76
127.18
79.47
65.42
47.43
46.99
42.95
28.51
NH
O
O
N
O
O
O
CH3
H3C
CH3
f1 (ppm)


8.27
5.69 DCM
4.35
4.34
4.33
4.32
3.90
3.89
3.88
3.88
3.88
3.39
3.37
3.35
3.34
2.98
2.97
2.97
2.50 DMSO-d6
2.14
2.12
2.10
2.09
1.96
1.94
1.93
1.91
1.90
1.88
1.83
1.82
1.80
1.79
1.77
1.76
1.38
O
O
N
O
NH
O
CH
CH3
H3C
CH3
600
550
500
450
400
350
300
250
200
150
100
50
0
-50
0.87
0.79
2.06
3.86
0.48
1.02
1.07
2.08
9.43
8.0
7.5
7.0
6.5
6.0
5.5
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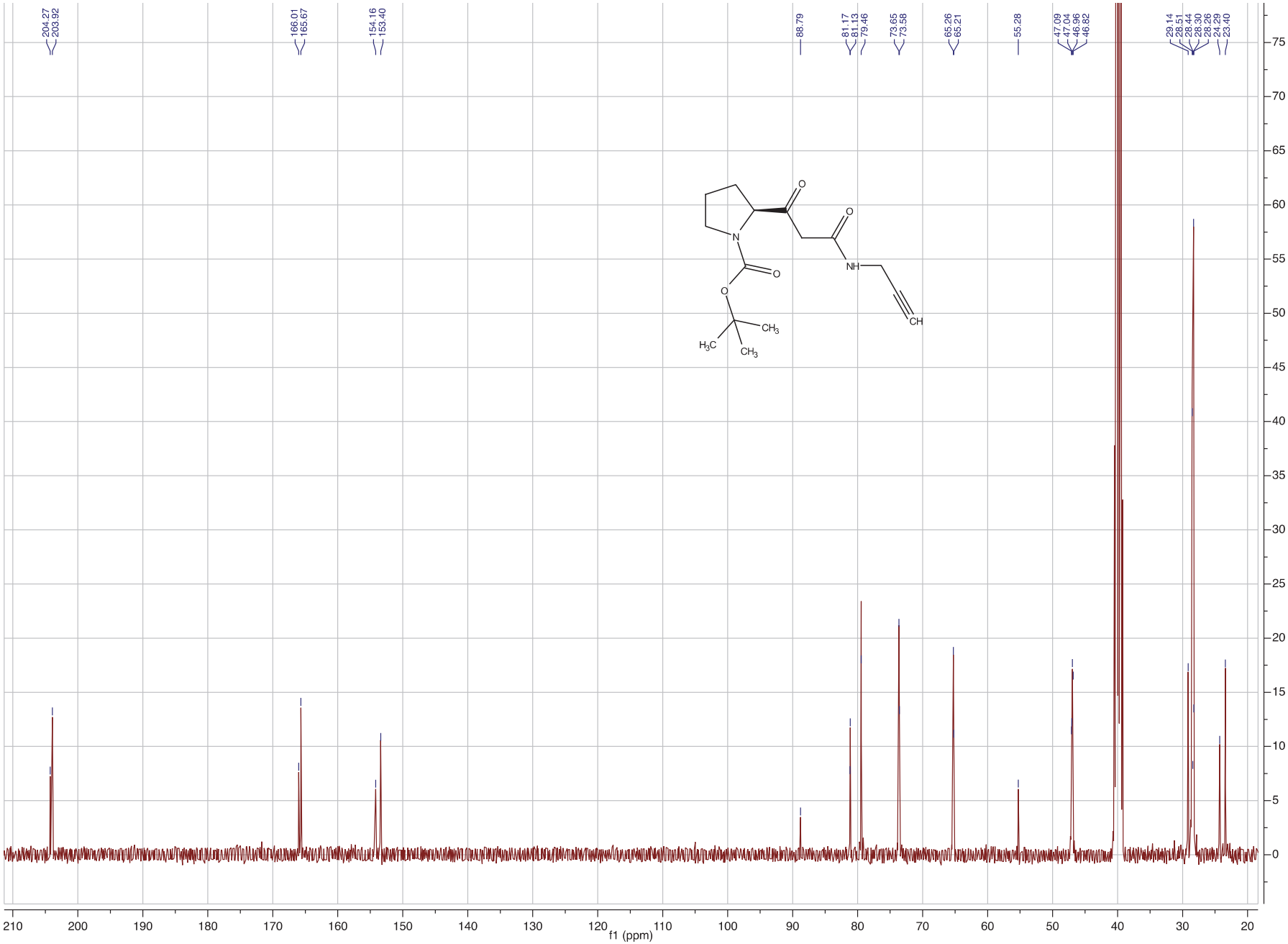
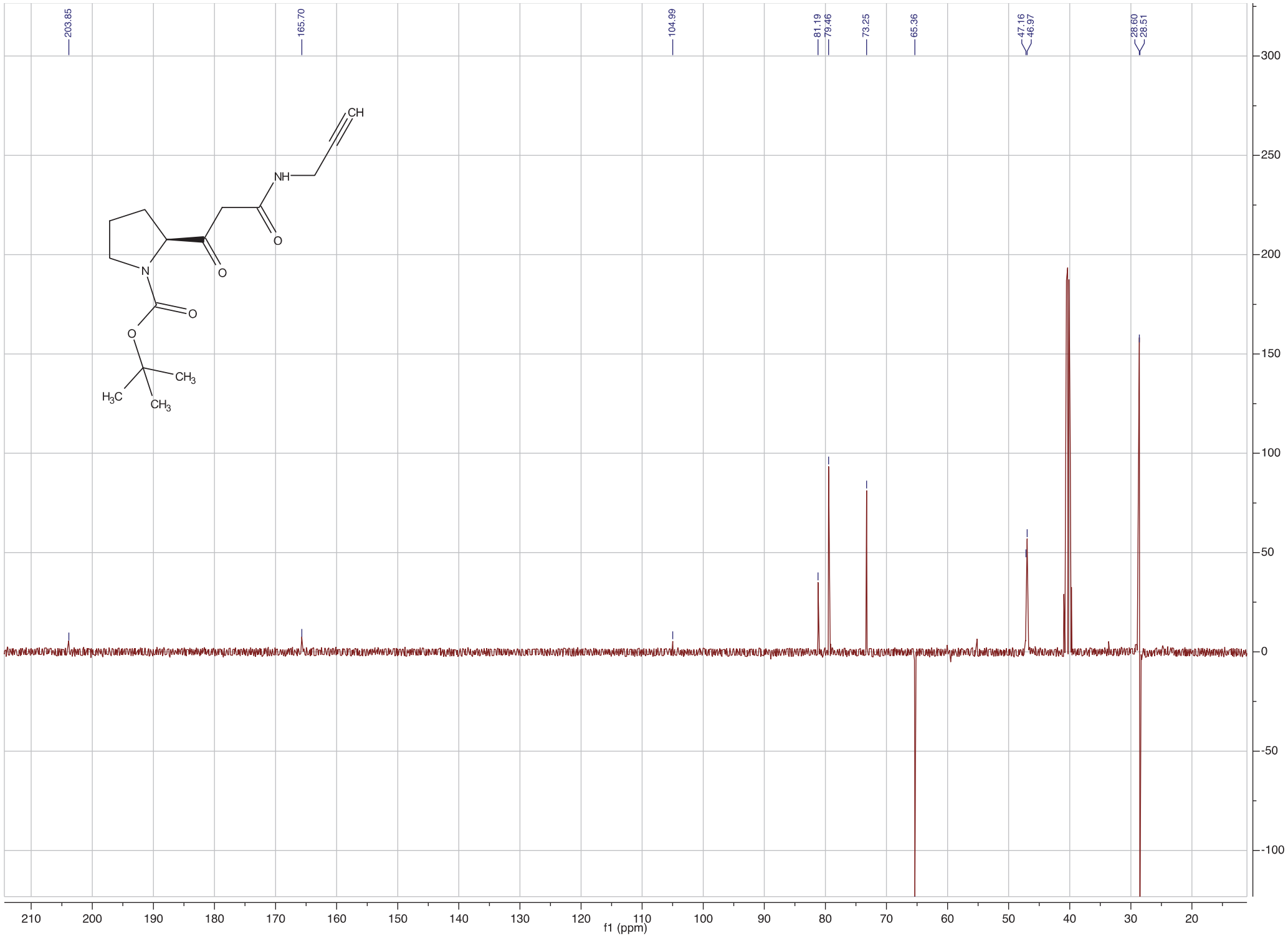
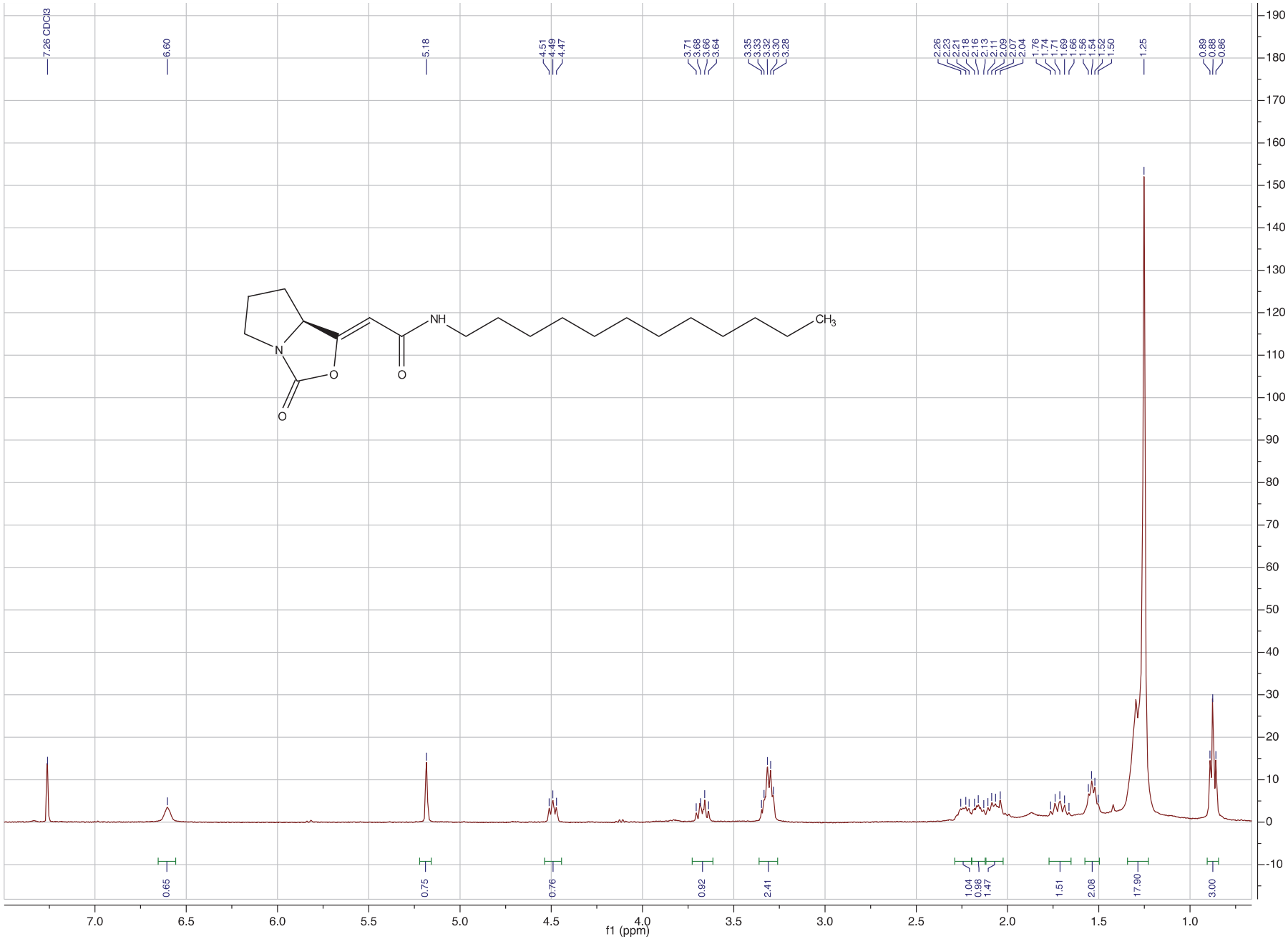
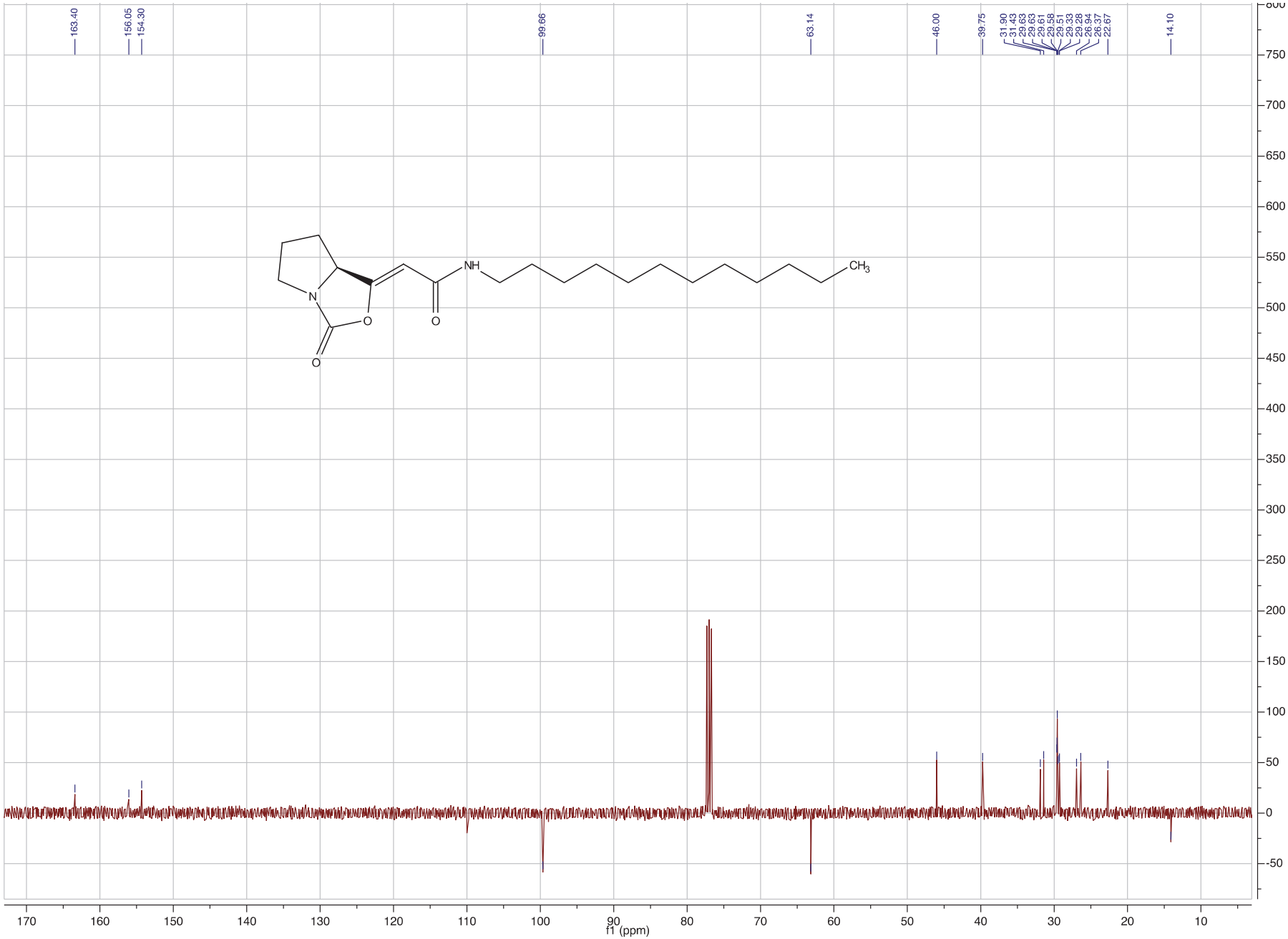
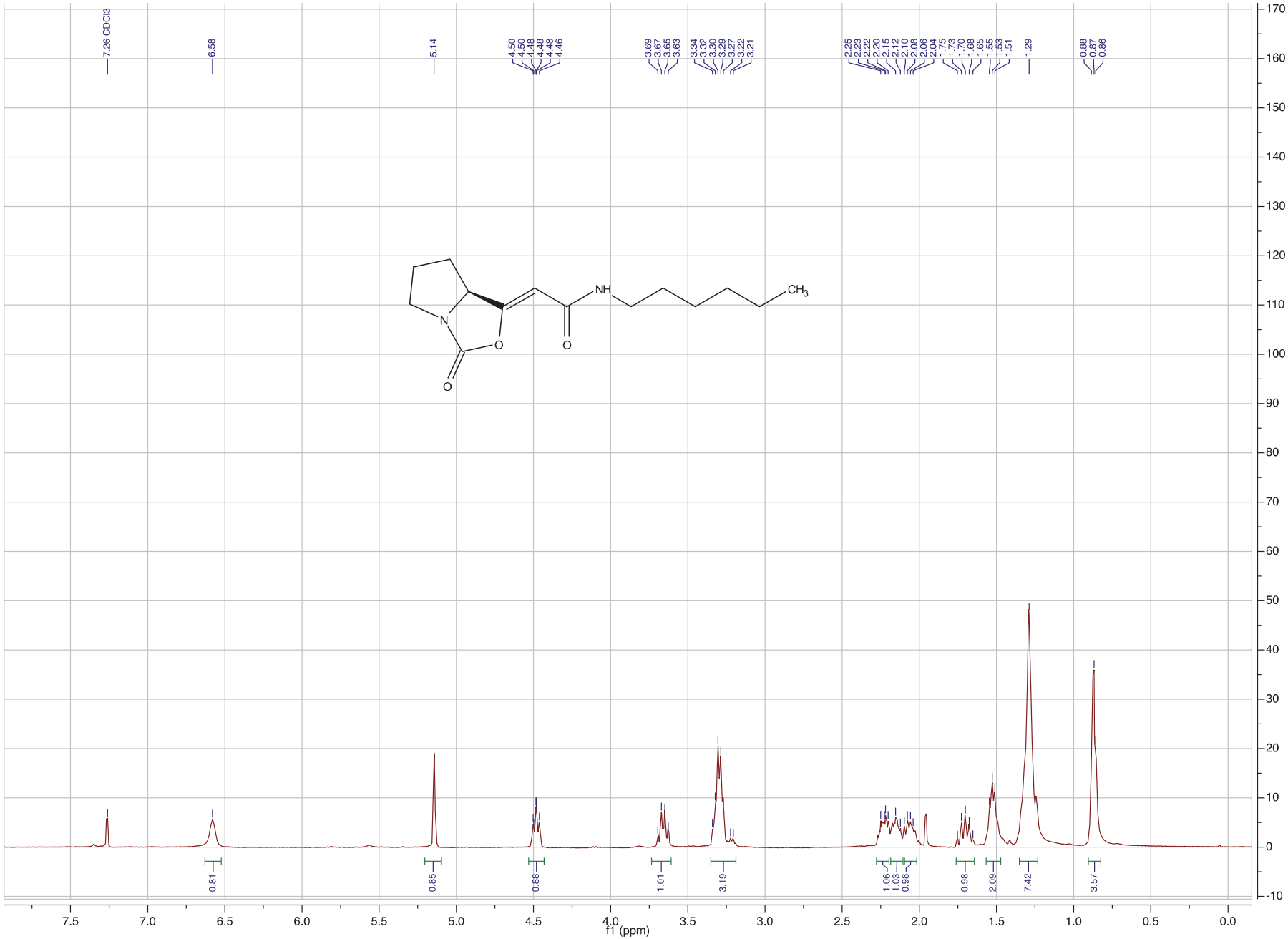
3.0
2.5
2.0
1.5
f1 (ppm)

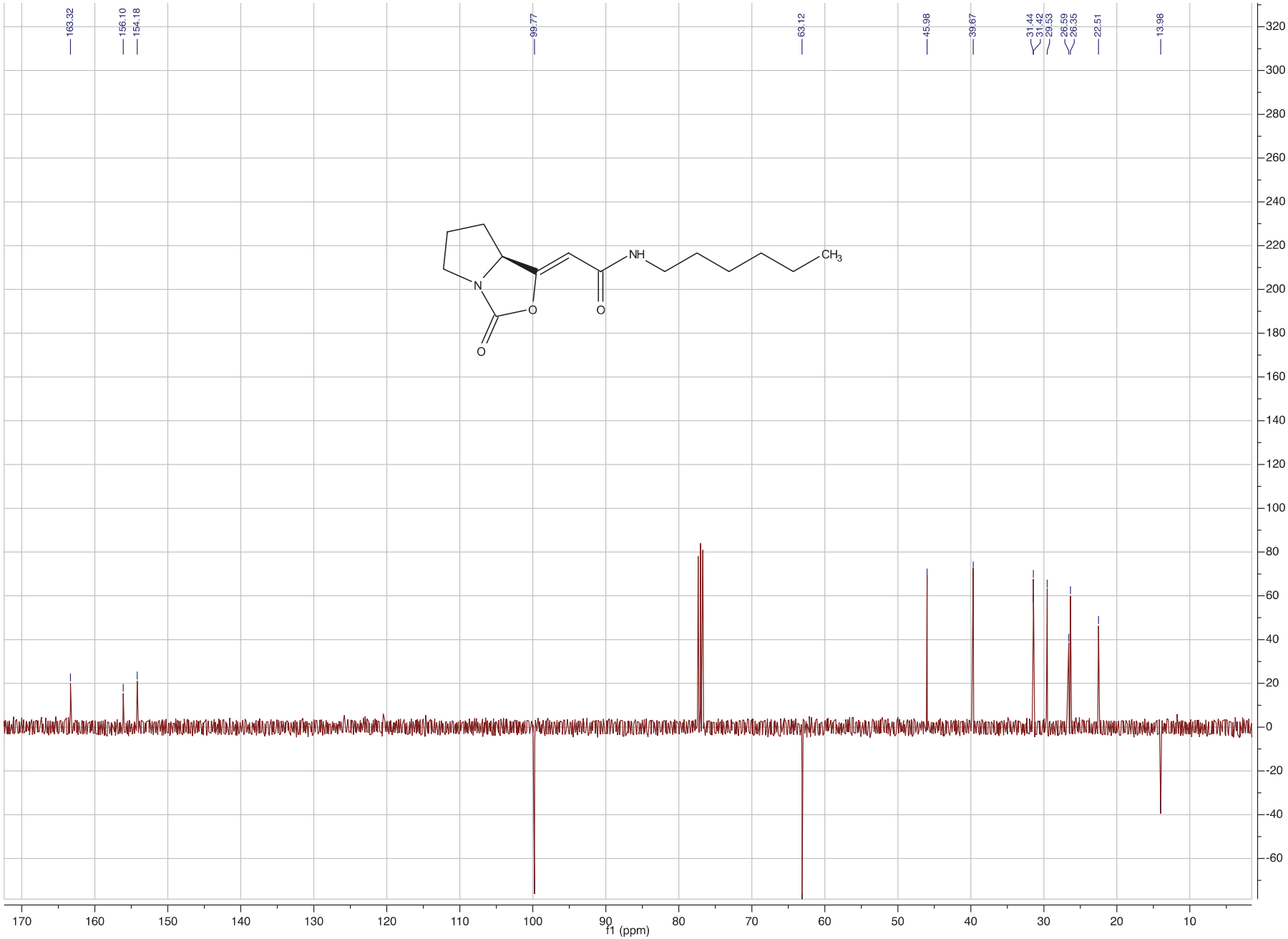

204.27
203.92
166.01
165.67
154.16
153.40
88.79
81.17
81.13
79.46
73.65
73.58
65.26
65.21
55.28
47.09
47.04
46.96
46.82
29.14
28.51
28.44
28.30
28.26
24.29
23.40
O
O
N
NH
O
O
CH
CH3
H3C
CH3
f1 (ppm)

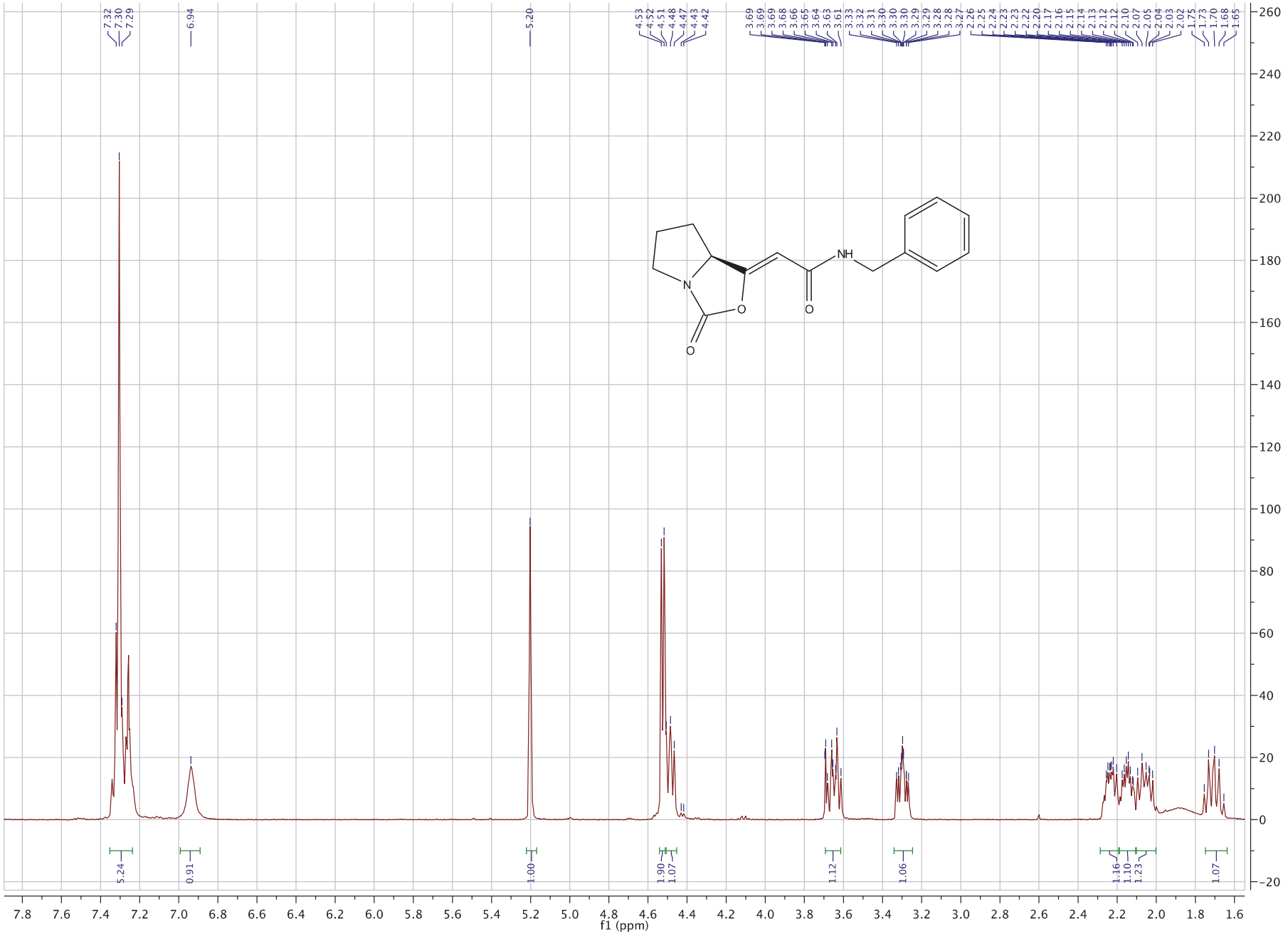

203.85
165.70
104.99
81.19
79.46
73.25
65.36
47.16
46.97
28.60
28.51
CH
NH
O
O
N
O
O
CH3
H3C
CH3
300
250
200
150
100
50
0
-50
-100
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
f1 (ppm)


7.26 CDCl3
6.60
5.18
4.51
4.49
4.47
3.71
3.68
3.66
3.64
3.35
3.33
3.32
3.30
3.28
2.26
2.23
2.21
2.18
2.16
2.13
2.11
2.09
2.07
2.04
1.76
1.74
1.71
1.69
1.66
1.56
1.54
1.52
1.50
1.25
0.89
0.88
0.86
NH
CH3
N
O
O
O
0.65
0.75
0.76
0.92
2.41
1.04
0.98
1.47
1.51
2.08
17.90
3.00
f1 (ppm)

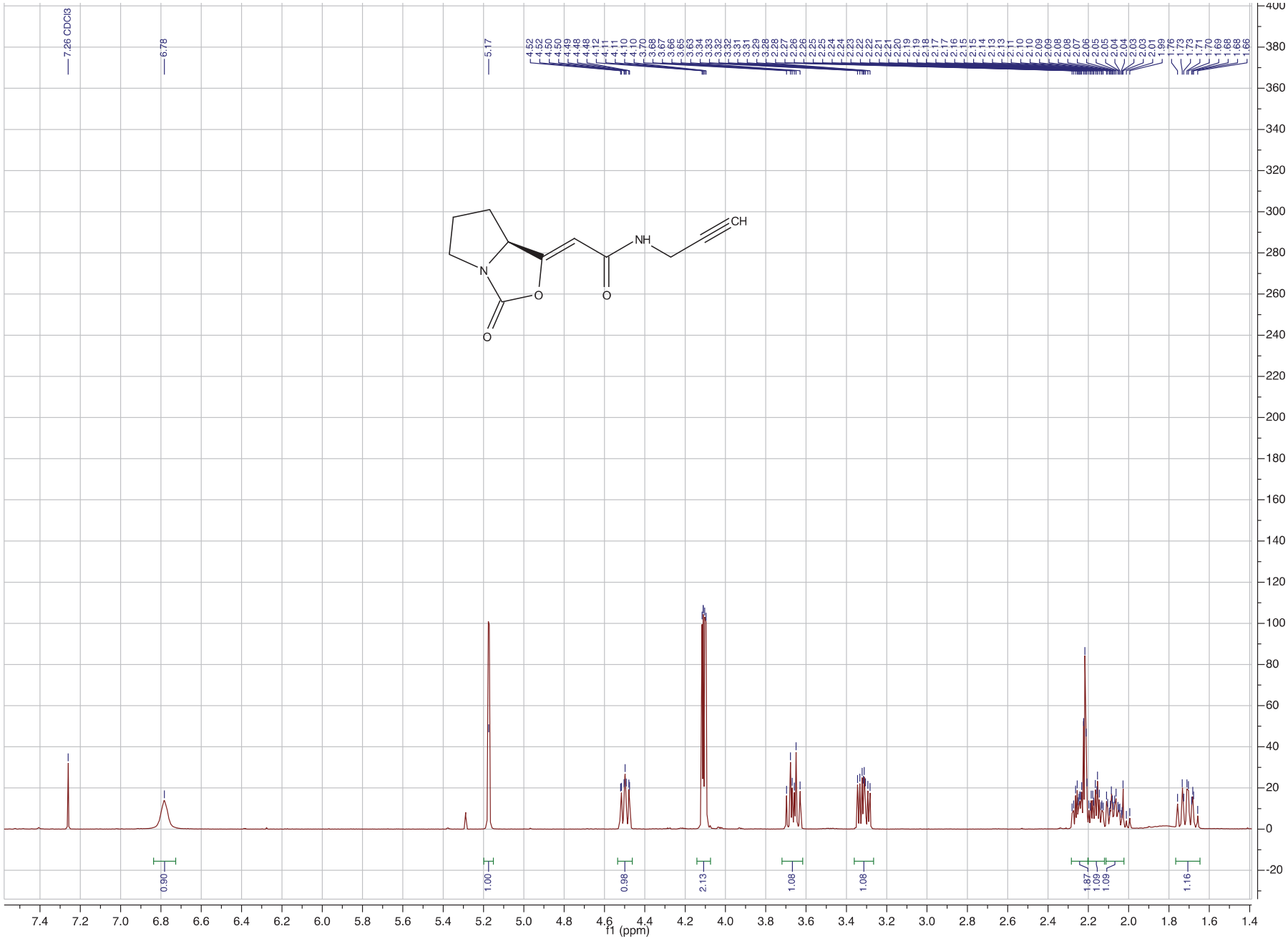

163.40
156.05
154.30
99.66
63.14
46.00
39.75
31.90
31.43
29.63
29.63
29.61
29.58
29.51
29.33
29.28
26.94
26.37
22.67
14.10
NH
CH3
N
O
O
O
f1 (ppm)

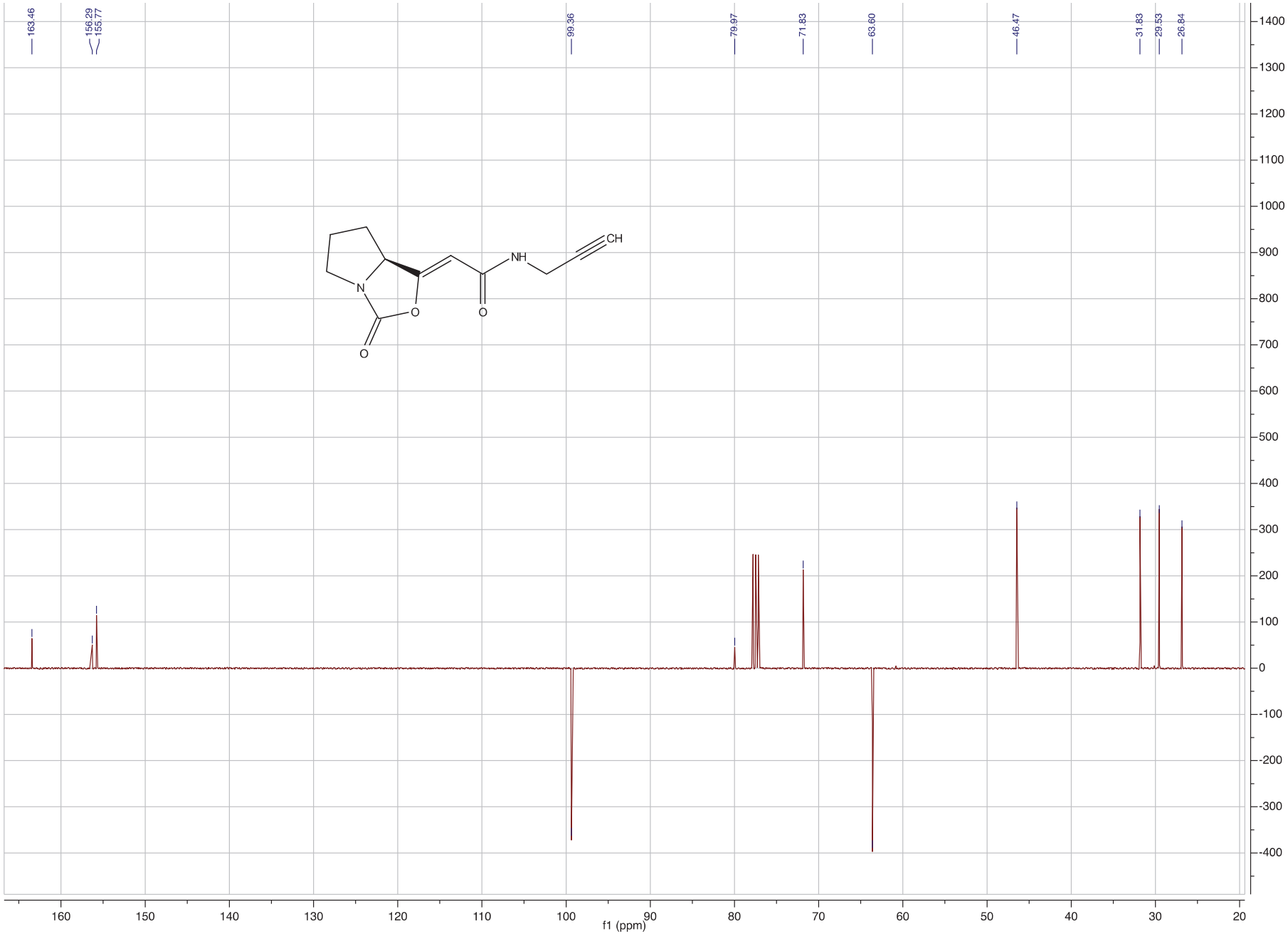

7.26 CDCl3
6.58
5.14
4.50
4.50
4.48
4.48
4.48
4.48
4.46
3.69
3.67
3.65
3.63
3.34
3.32
3.30
3.29
3.27
3.22
3.21
2.25
2.23
2.22
2.20
2.15
2.12
2.10
2.08
2.06
2.04
1.75
1.73
1.70
1.68
1.65
1.55
1.53
1.51
1.29
0.88
0.87
0.86
NH
CH3
N
O
O
O
0.81
0.85
0.88
1.01
3.19
1.06
1.03
0.98
0.98
2.09
7.42
3.57
f1 (ppm)

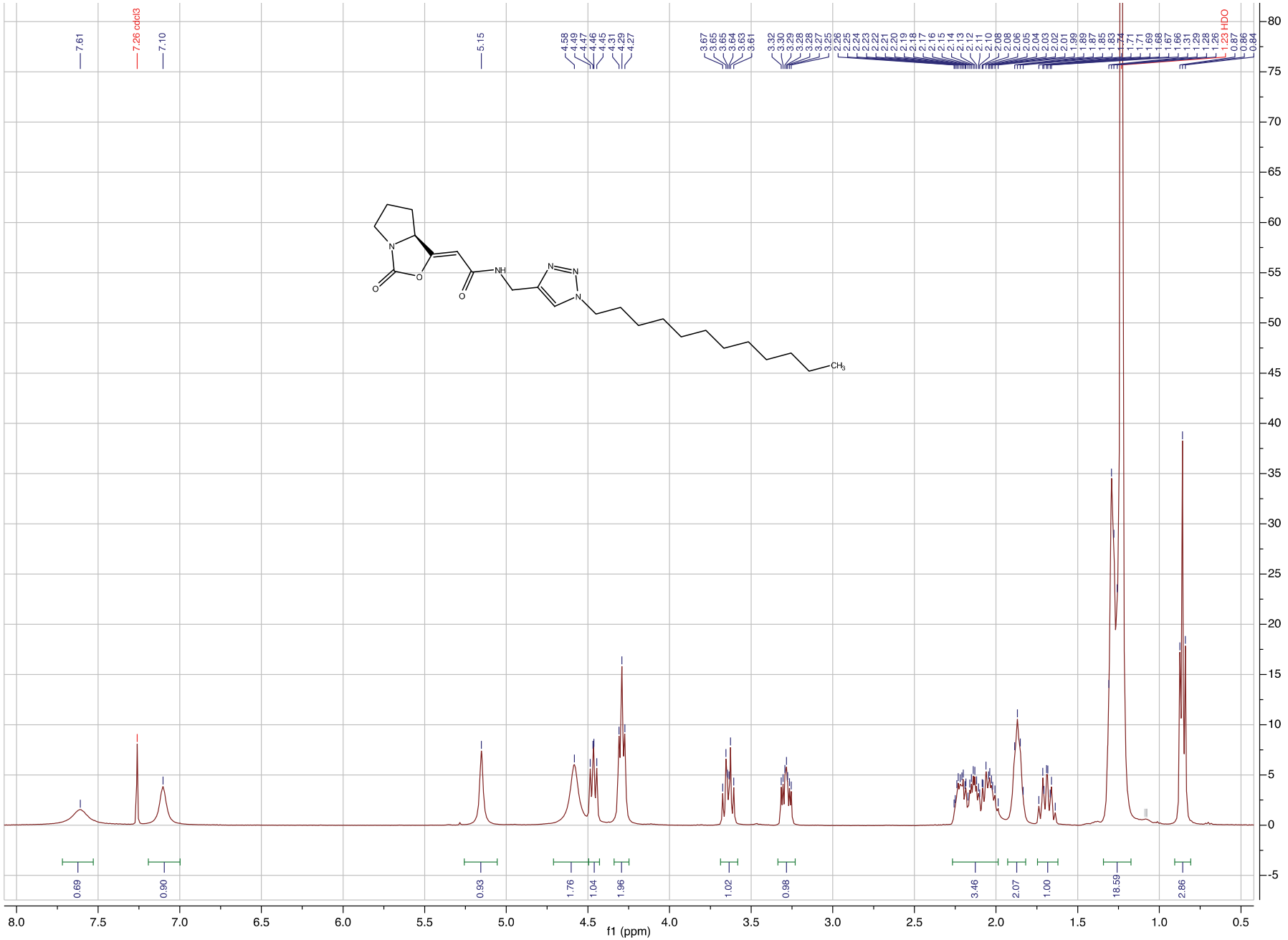

163.32
156.10
154.18
99.77
63.12
45.98
39.67
31.44
31.42
29.53
26.59
26.35
22.51
13.98
NH
CH3
N
O
O
O
f1 (ppm)

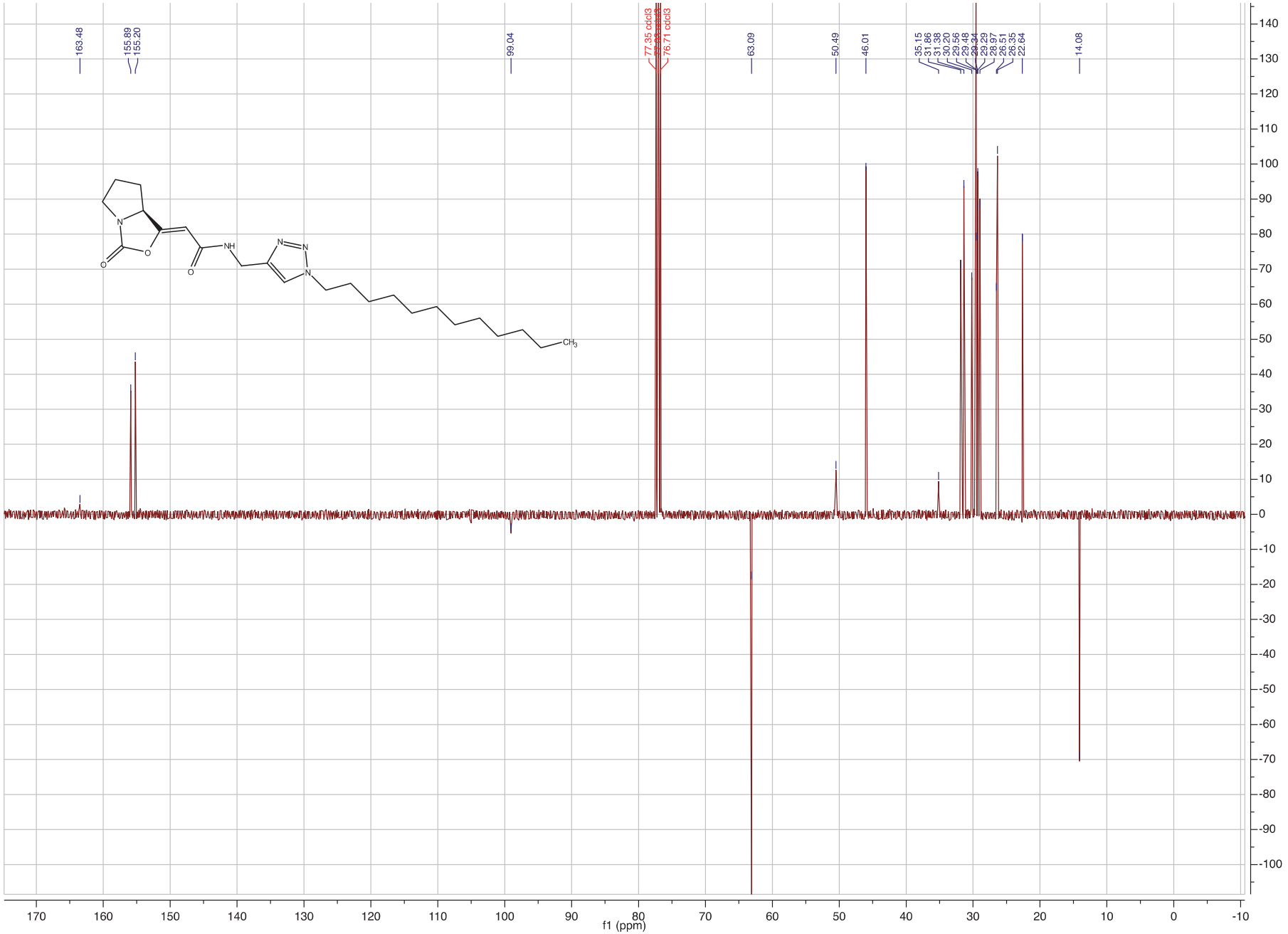

7.32
7.30
7.29
6.94
5.20
4.53
4.52
4.51
4.48
4.47
4.43
4.42
3.69
3.69
3.69
3.68
3.66
3.65
3.64
3.63
3.61
3.33
3.32
3.31
3.30
3.30
3.30
3.29
3.29
3.28
3.28
3.27
2.26
2.25
2.24
2.23
2.23
2.22
2.20
2.17
2.16
2.15
2.14
2.13
2.12
2.12
2.10
2.07
2.05
2.04
2.03
2.02
1.75
1.73
1.70
1.68
1.65
NH
N
O
O
O
5.24
0.91
1.00
1.90
1.07
1.12
1.06
1.16
1.10
1.23
1.07
f1 (ppm)

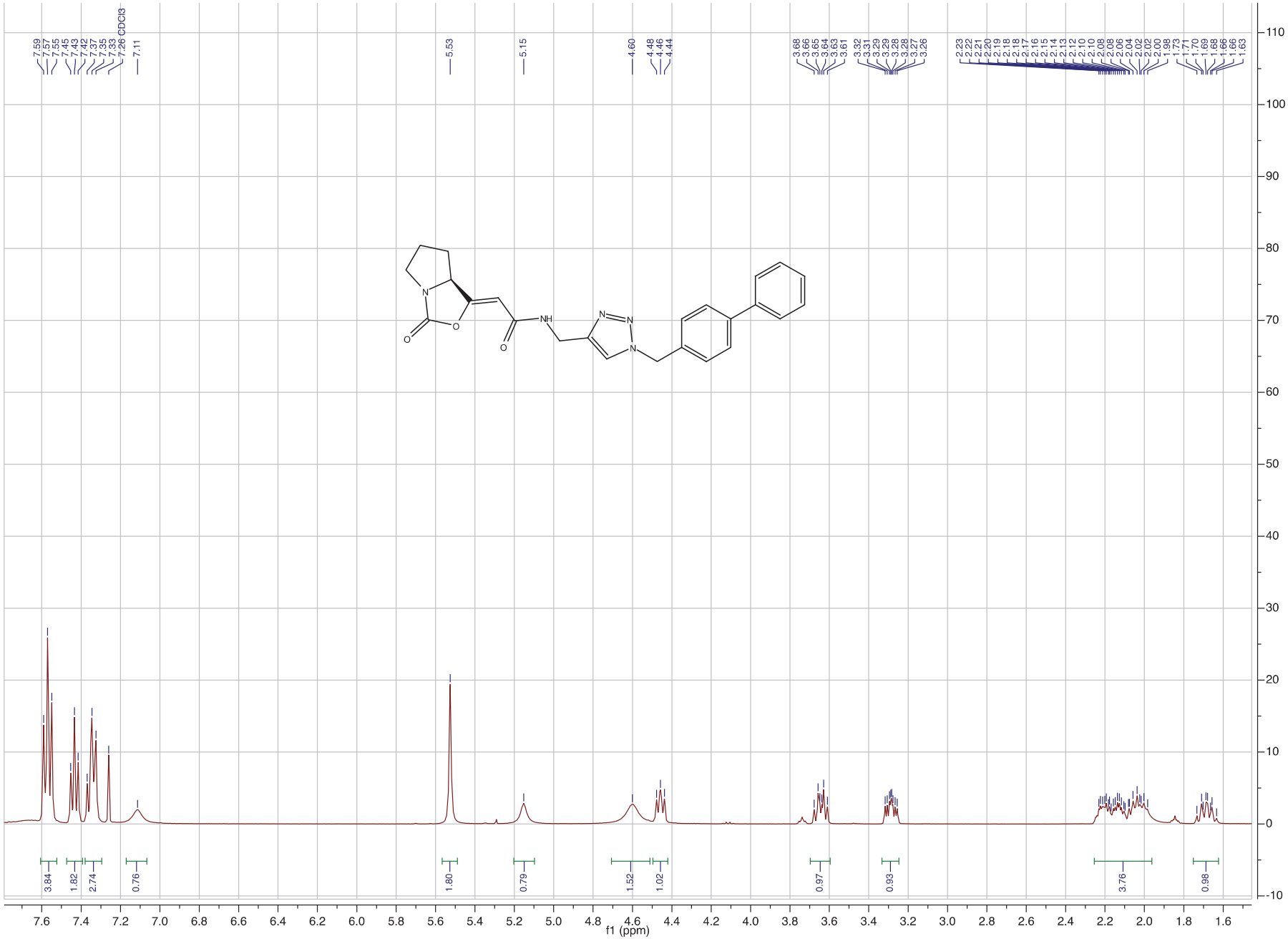

163.48
156.11
154.85
138.42
128.76
127.75
127.48
99.57
63.28
46.14
43.56
31.54
26.50
NH
N
O
O
O
240
220
200
180
160
140
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
-120
-140
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
f1 (ppm)

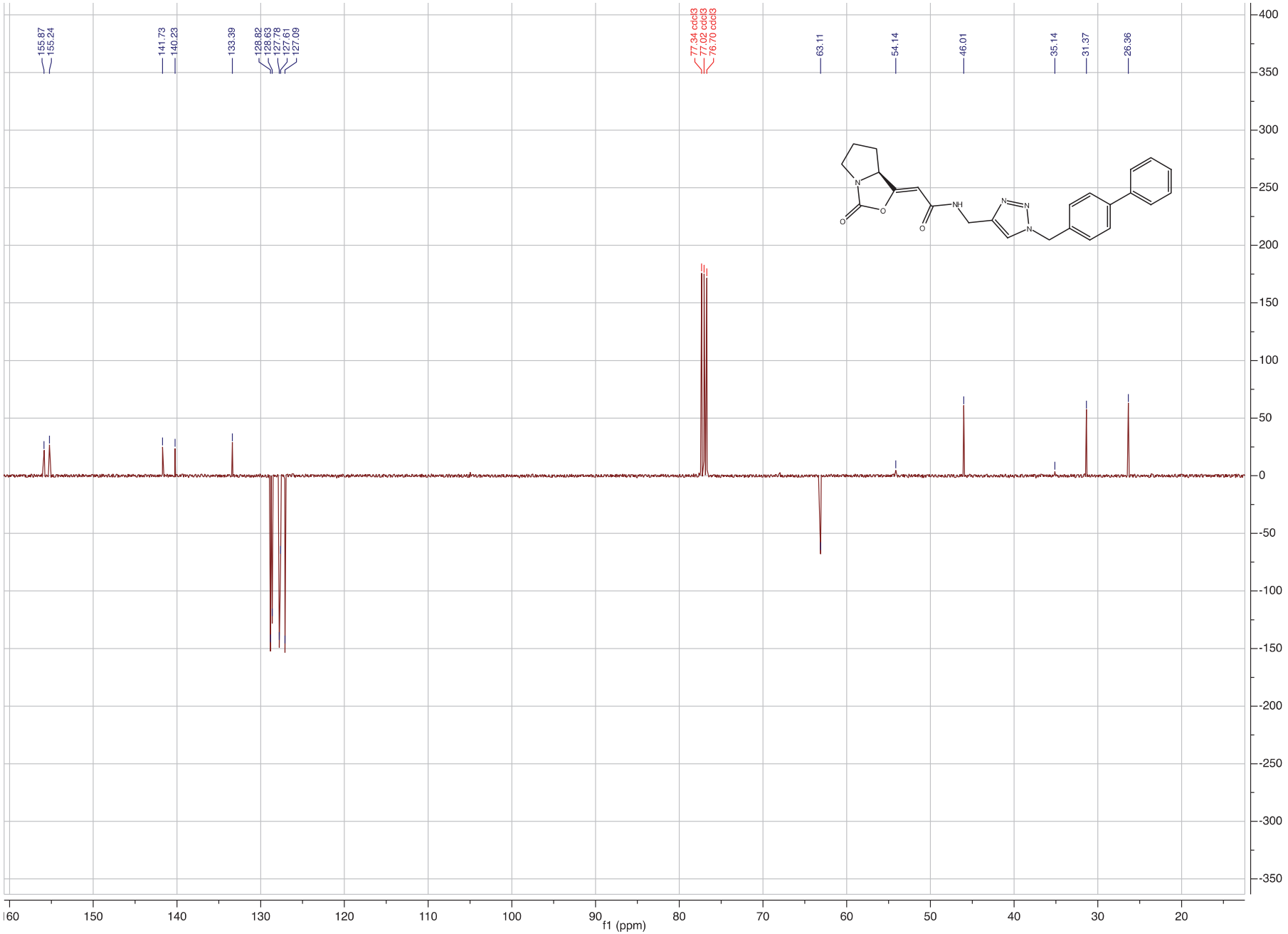

7.26 CDCl3
6.78
5.17
NH
CH
N
O
O
O
f1 (ppm)

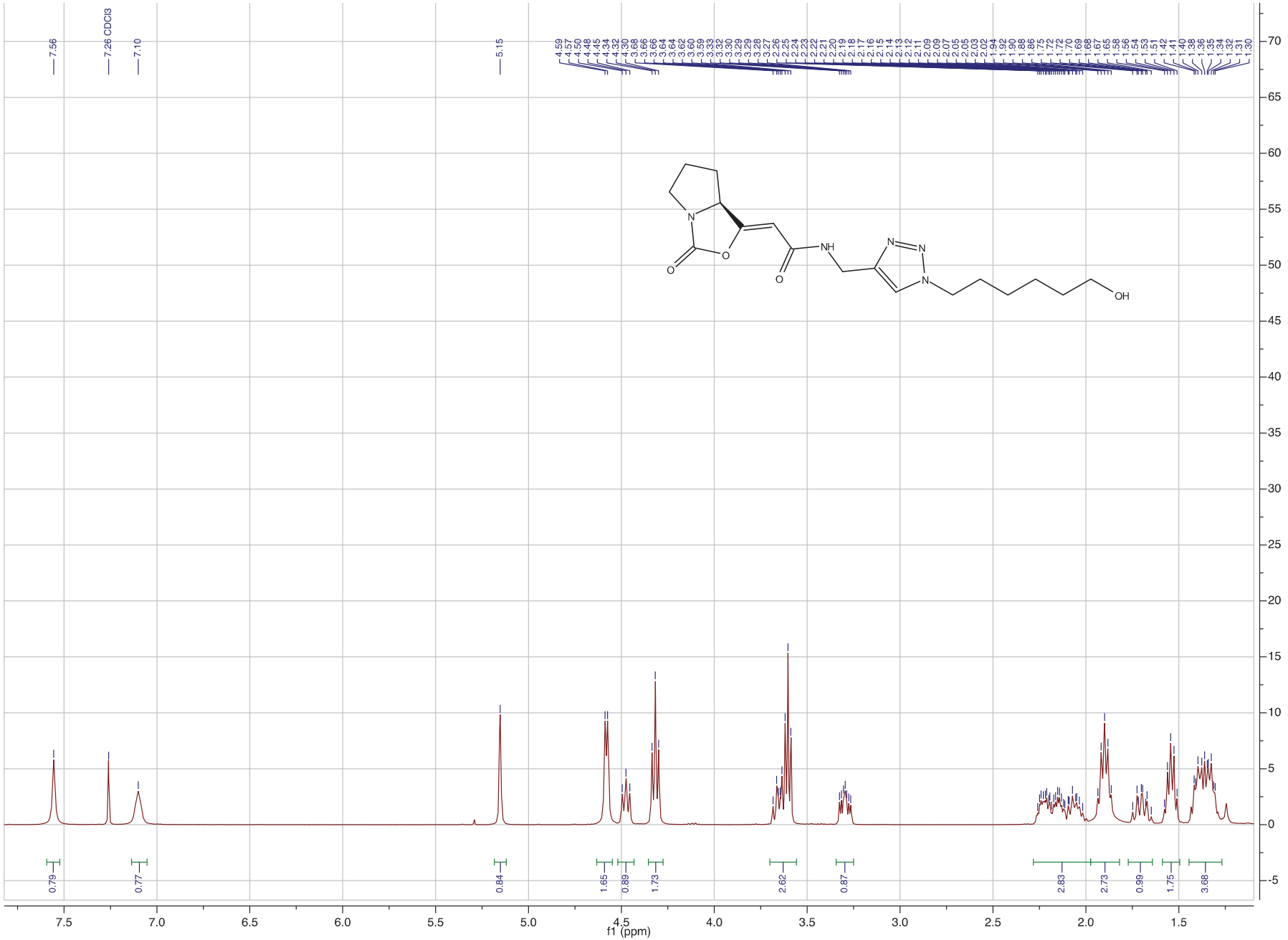

163.46
156.29
155.77
99.36
79.97
71.83
63.60
46.47
31.83
29.53
26.84
NH
CH
N
O
O
O
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
-200
-300
-400
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
f1 (ppm)

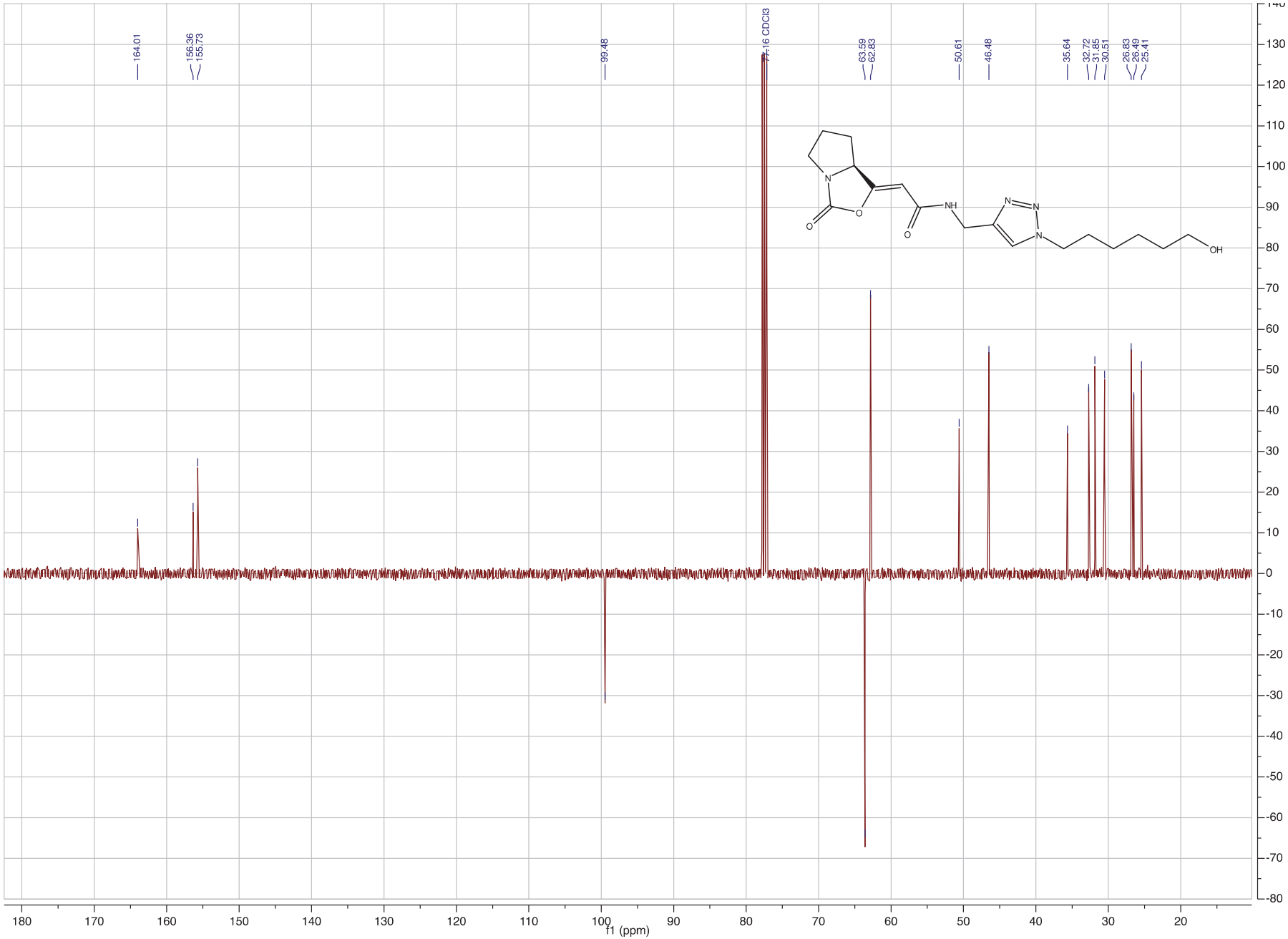

7.61
7.26 cdcl3
7.10
5.15
4.58
4.49
4.47
4.46
4.45
4.31
4.29
4.27
3.67
3.65
3.65
3.64
3.63
3.61
3.32
3.30
3.29
3.28
3.28
3.27
3.25
2.26
2.25
2.24
2.23
2.22
2.21
2.20
2.19
2.18
2.17
2.16
2.15
2.14
2.13
2.12
2.11
2.10
2.08
2.08
2.06
2.05
2.04
2.03
2.02
2.01
1.99
1.89
1.87
1.85
1.83
1.74
1.71
1.71
1.69
1.68
1.67
1.66
1.31
1.29
1.28
1.26
1.23 HDO
0.87
0.86
0.84
NH
N
N
N
O
O
O
CH3
0.69
0.90
0.93
1.76
1.04
1.96
1.02
0.98
3.46
2.07
1.00
18.59
2.86
f1 (ppm)

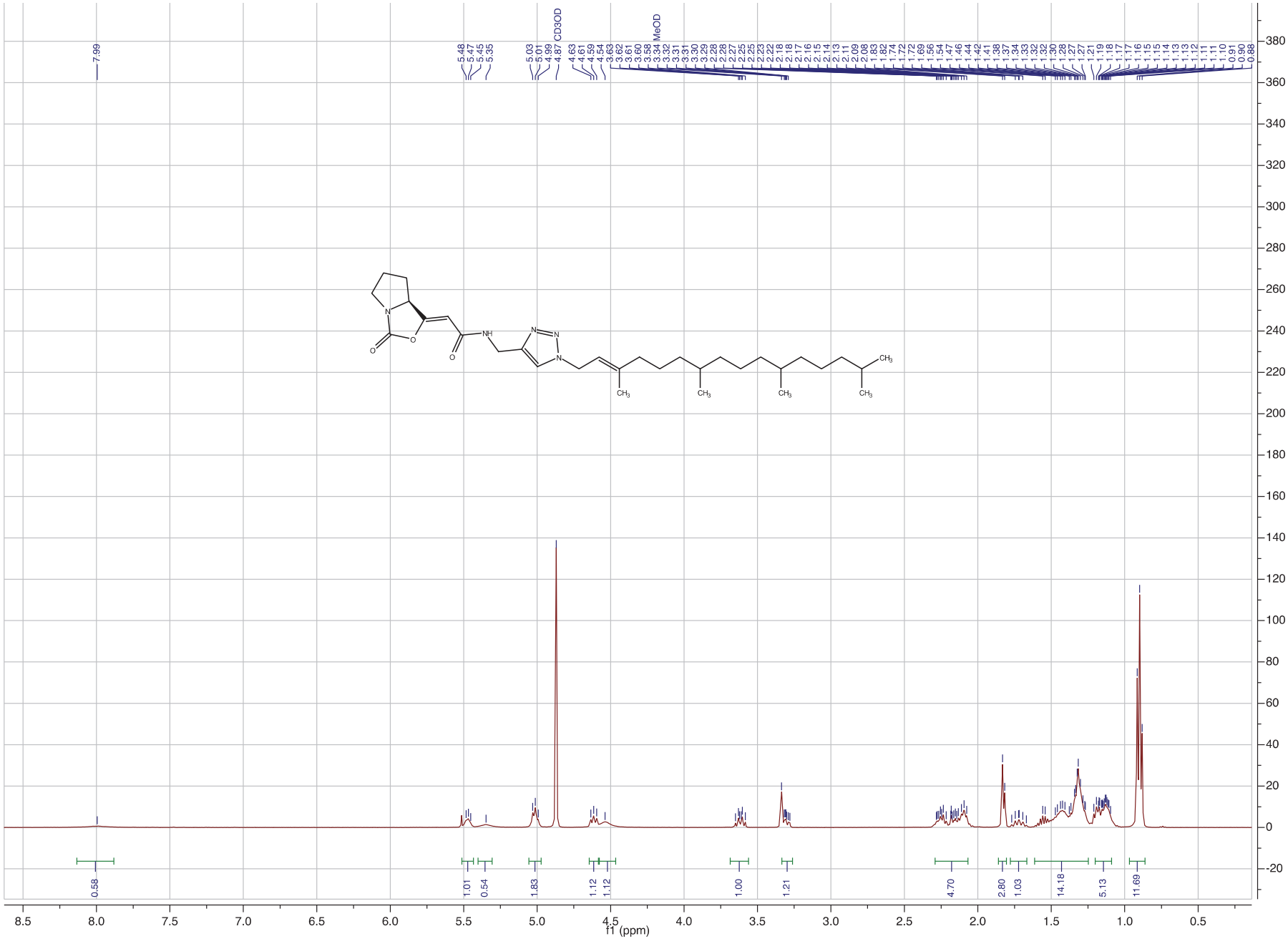

163.48
155.89
155.20
99.04
77.35 cdcl3
77.03 cdcl3
76.71 cdcl3
63.09
50.49
46.01
35.15
31.86
31.38
30.20
29.56
29.48
29.24
28.97
26.51
26.35
22.64
14.08
f1 (ppm)

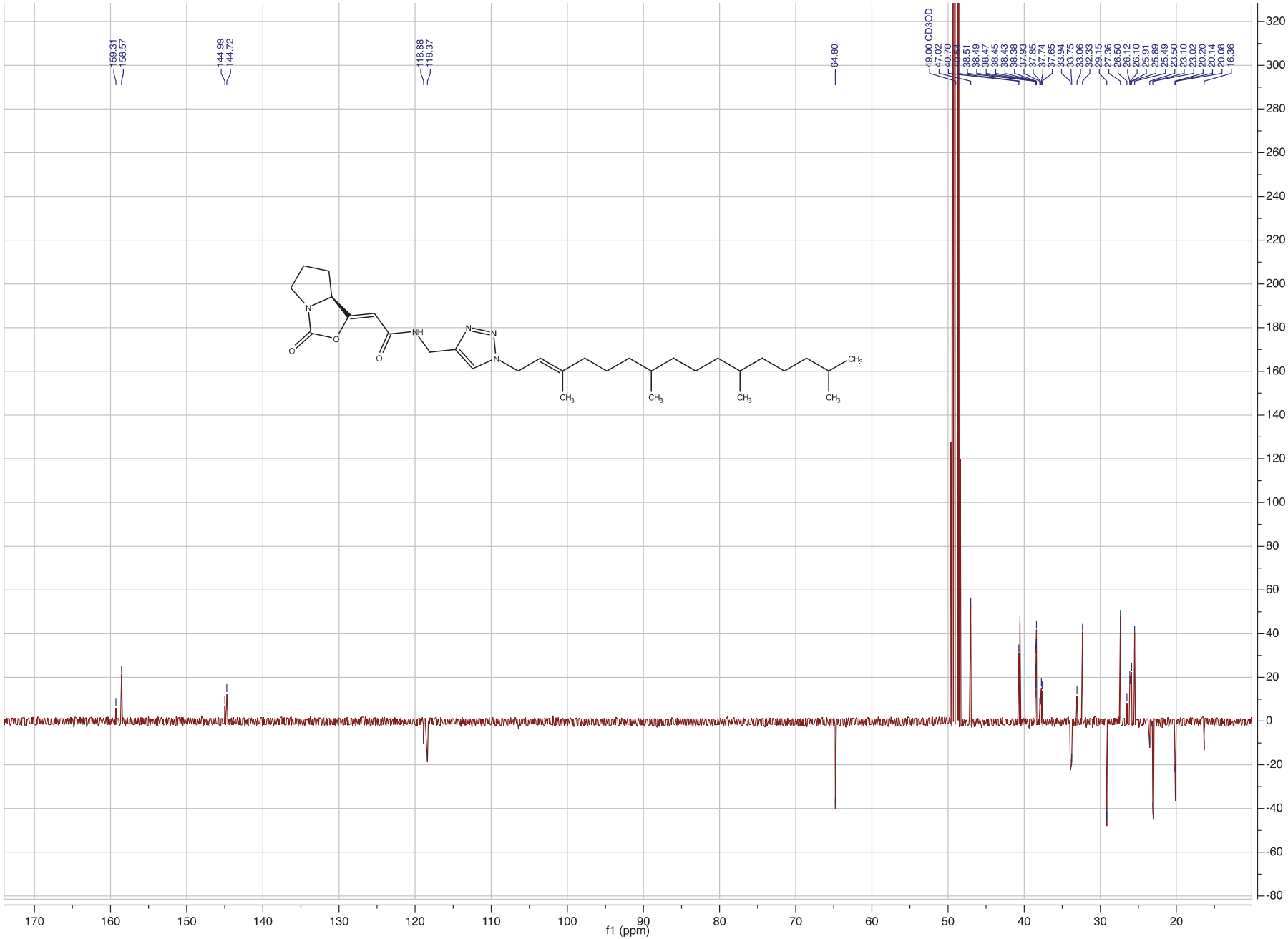

7.59
7.57
7.55
7.45
7.43
7.42
7.37
7.35
7.33
7.26 CDCl3
7.11
5.53
5.15
4.60
4.48
4.46
4.44
3.68
3.66
3.65
3.64
3.63
3.61
3.32
3.31
3.29
3.29
3.28
3.28
3.27
3.26
2.23
2.22
2.21
2.20
2.19
2.18
2.18
2.17
2.16
2.15
2.14
2.13
2.12
2.10
2.10
2.08
2.08
2.06
2.04
2.02
2.02
2.00
1.98
1.73
1.71
1.70
1.69
1.68
1.66
1.66
1.63
N
O
O
NH
O
N
N
N
3.84
1.82
2.74
0.76
1.80
0.79
1.52
1.02
0.97
0.93
3.76
0.98
f1 (ppm)

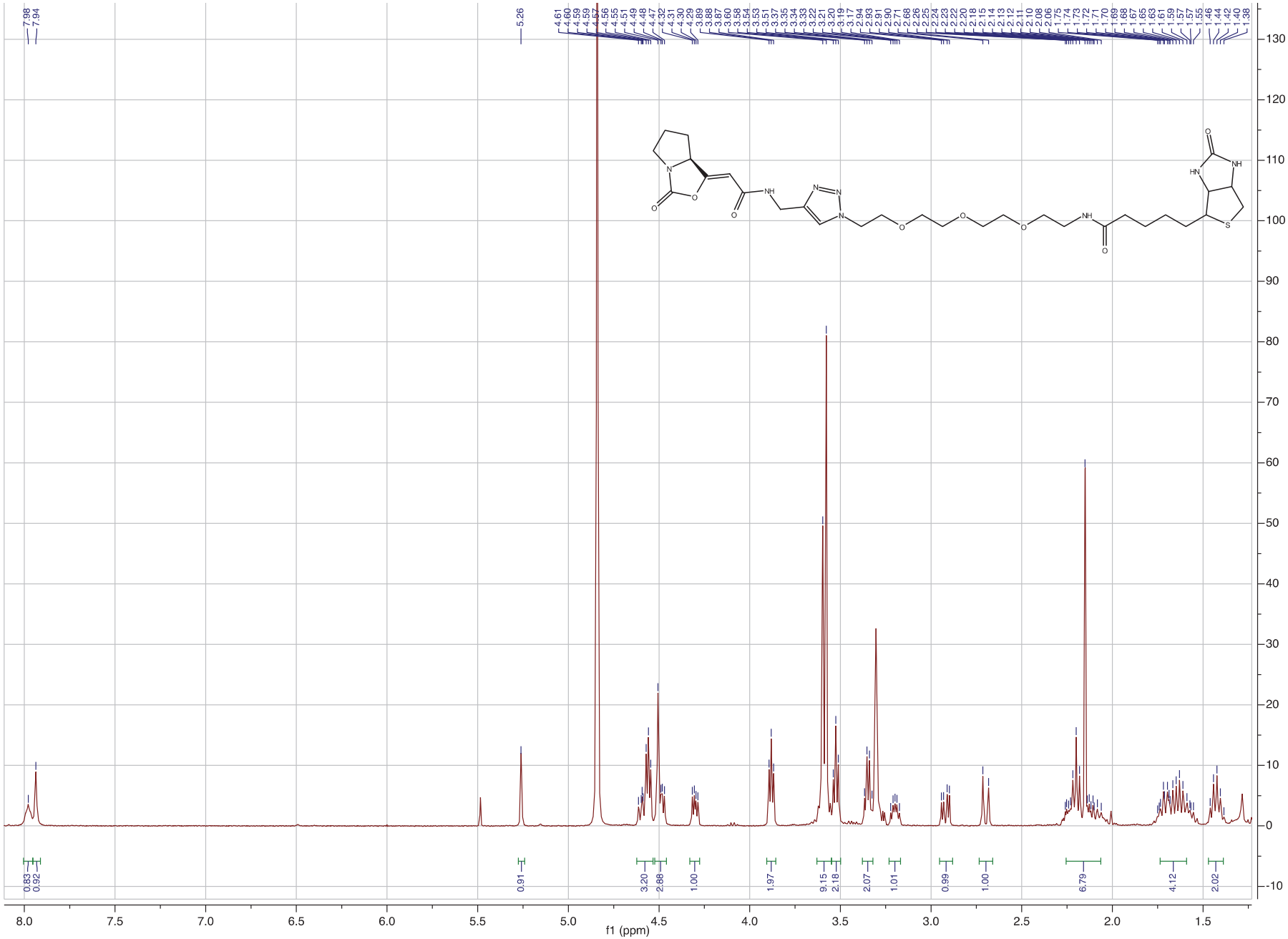

155.87
155.24
141.73
140.23
133.39
128.82
128.53
127.78
127.61
127.09
77.34 cdcl3
77.02 cdcl3
76.70 cdcl3
63.11
54.14
46.01
35.14
31.37
26.36
400
350
300
250
200
150
100
50
0
-50
-100
-150
-200
-250
-300
-350
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
f1 (ppm)

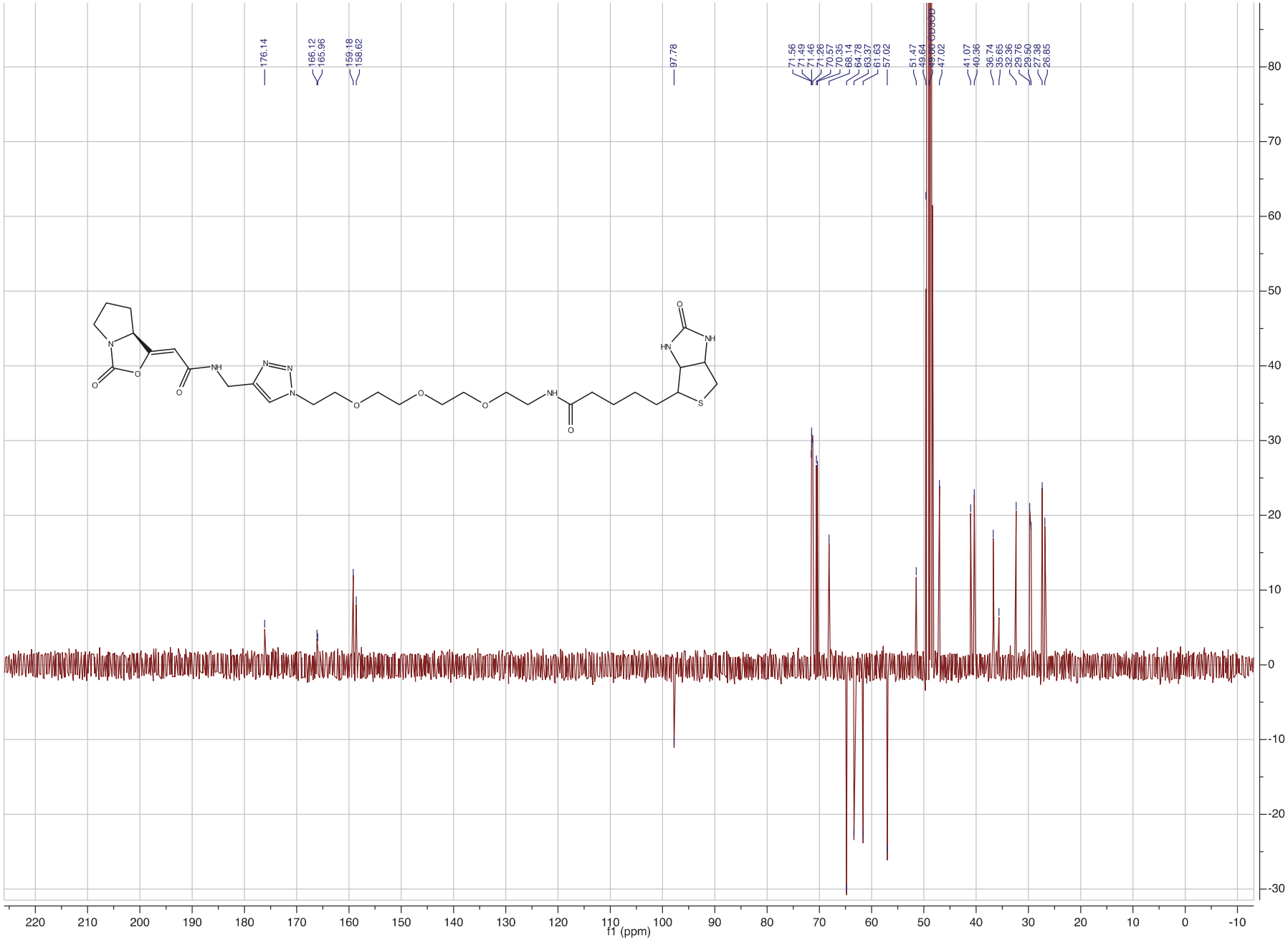

7.56
7.26 CDCl3
7.10
5.15
4.59 4.57 4.50 4.48 4.45 4.34 4.32 4.30 3.68 3.66 3.64 3.62 3.60 3.59 3.33 3.32 3.30 3.29 3.29 3.28 3.27 2.26 2.25 2.24 2.23 2.22 2.21 2.20 2.19 2.18 2.17 2.16 2.15 2.14 2.13 2.12 2.11 2.09 2.09 2.07 2.05 2.05 2.03 2.02 1.94 1.92 1.90 1.88 1.86 1.75 1.72 1.72 1.70 1.69 1.68 1.67 1.65 1.58 1.56 1.54 1.53 1.51 1.42 1.41 1.40 1.38 1.36 1.35 1.34 1.32 1.31 1.30
N
O
O
O
NH
N
N
N
OH
0.79
0.77
0.84
1.65
0.89
1.73
2.62
0.87
2.83
2.73
0.99
1.75
3.68
f1 (ppm)


164.01
156.36
155.73
99.48
77.16 CDCl3
63.59
62.83
50.61
46.48
35.64
32.72
31.85
30.51
26.83
26.49
25.41
O
N
O
O
NH
N
N
N
OH
f1 (ppm)

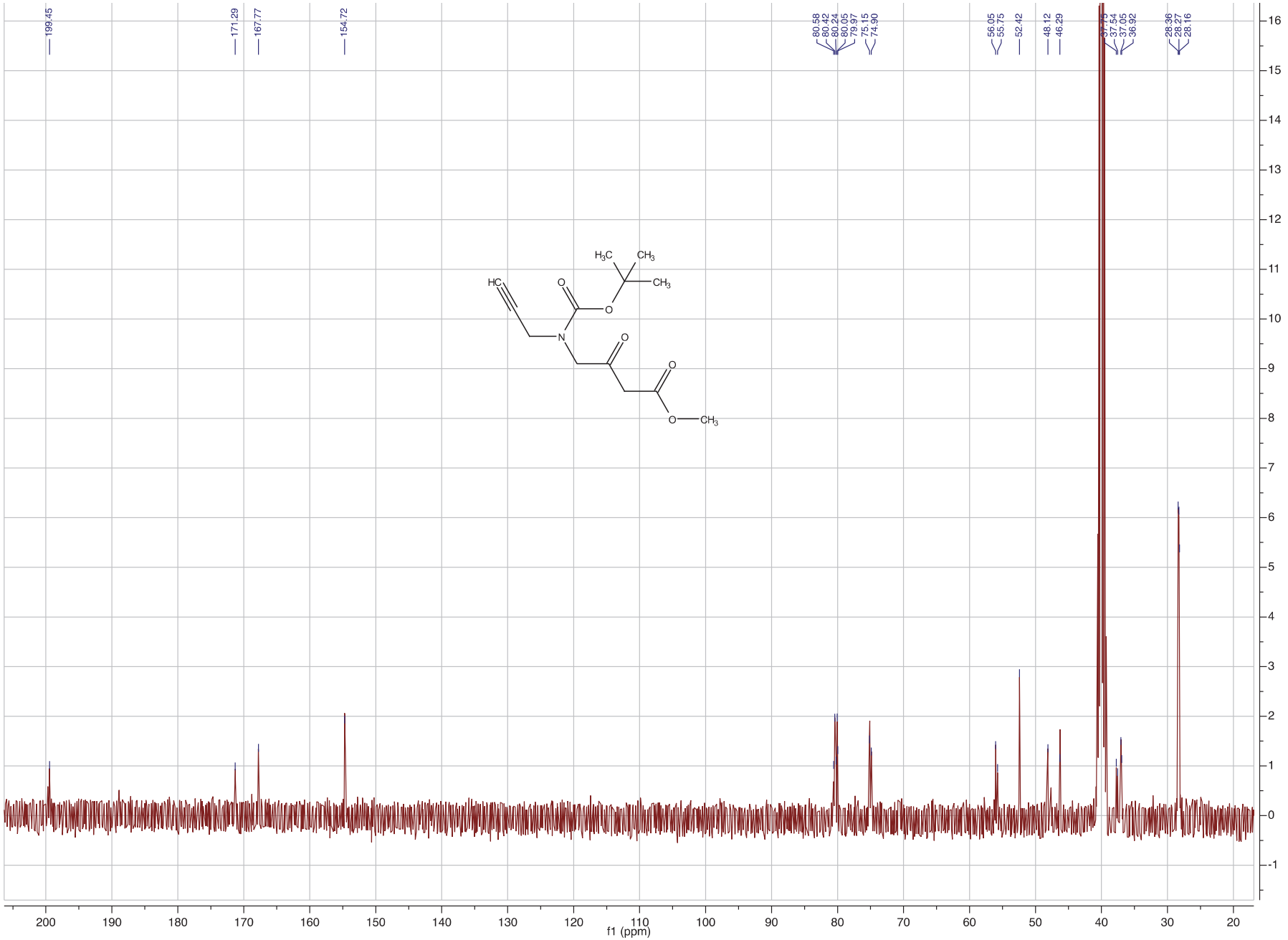

7.99
5.48
5.47
5.45
5.35
5.03
5.01
4.99
4.87 CD3OD
4.63
4.61
4.59
4.54
3.63
3.62
3.61
3.60
3.58
3.34 MeOD
3.32
3.31
3.31
3.30
3.29
2.28
2.28
2.27
2.25
2.23
2.22
2.18
2.17
2.16
2.15
2.14
2.13
2.11
2.09
2.08
1.83
1.82
1.74
1.72
1.69
1.56
1.54
1.47
1.46
1.44
1.42
1.41
1.38
1.37
1.34
1.33
1.32
1.32
1.30
1.28
1.27
1.27
1.21
1.19
1.18
1.17
1.17
1.16
1.15
1.15
1.14
1.13
1.13
1.12
1.11
1.11
1.10
0.91
0.90
0.88
0.58
1.01
0.54
1.83
1.12
1.12
1.00
1.21
4.70
2.80
1.03
14.18
5.13
11.69
f1 (ppm)


159.31
158.57
144.99
144.72
118.88
118.37
64.80
49.00 CD3OD
47.02
40.70
40.63
38.51
38.49
38.47
38.45
38.43
38.38
37.93
37.85
37.74
37.65
33.94
33.75
33.06
32.33
29.15
27.36
26.50
26.12
26.10
25.91
25.89
25.49
23.50
23.10
23.02
20.20
20.14
20.08
16.36
f1 (ppm)

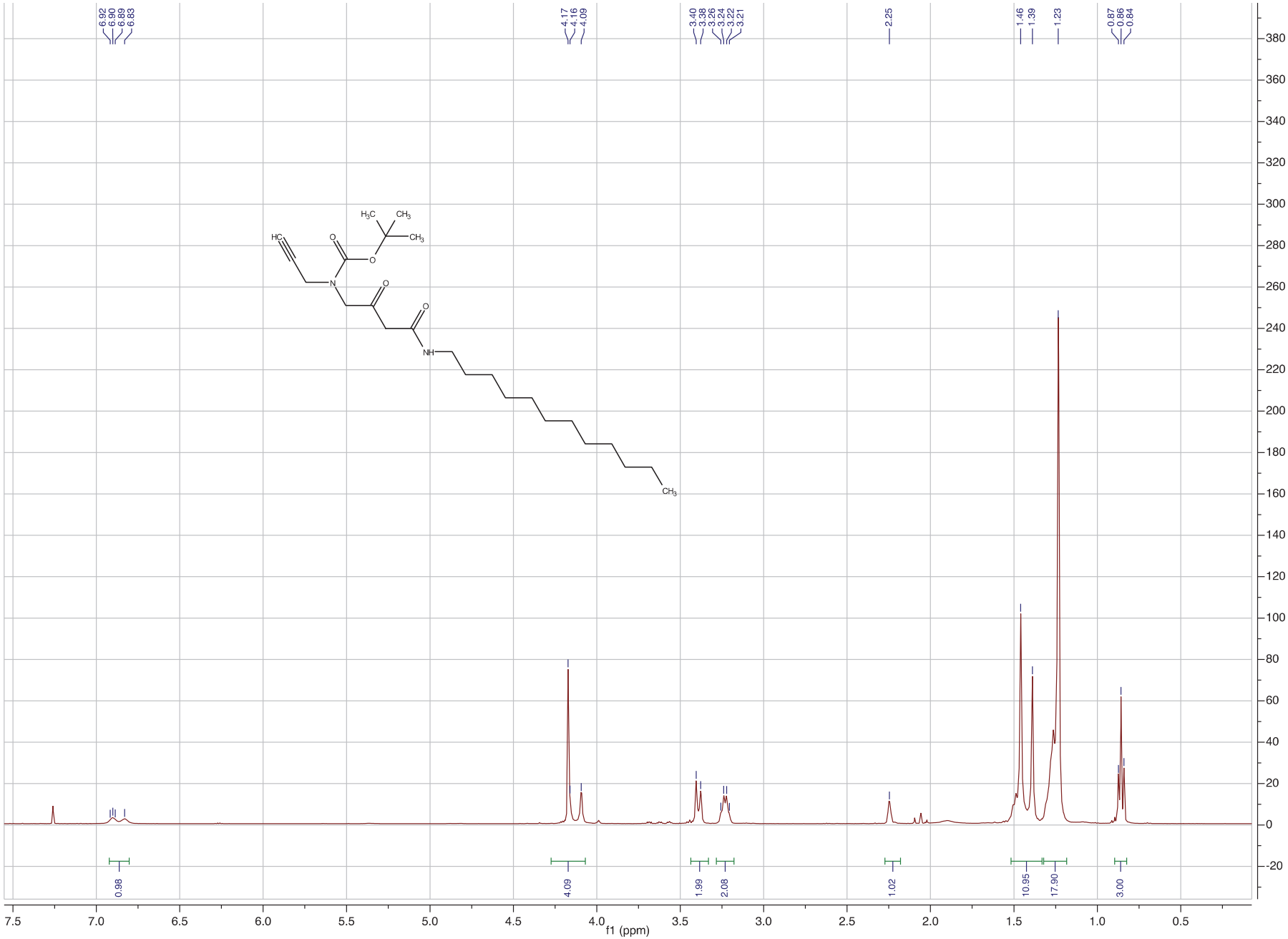

7.98
7.94
5.26
4.61
4.60
4.59
4.59
4.57
4.56
4.55
4.51
4.49
4.48
4.47
4.32
4.31
4.30
4.29
3.89
3.88
3.87
3.60
3.58
3.54
3.53
3.51
3.37
3.35
3.34
3.33
3.22
3.21
3.20
3.19
3.17
2.94
2.93
2.91
2.90
2.71
2.68
2.26
2.25
2.24
2.23
2.22
2.20
2.18
2.15
2.14
2.13
2.12
2.11
2.10
2.08
2.06
1.75
1.74
1.73
1.72
1.71
1.70
1.69
1.68
1.67
1.65
1.63
1.61
1.59
1.57
1.55
1.46
1.44
1.42
1.40
1.38
0.83
0.92
0.91
3.20
2.88
1.00
1.97
9.15
2.18
2.07
1.01
0.99
1.00
6.79
4.12
2.02
f1 (ppm)

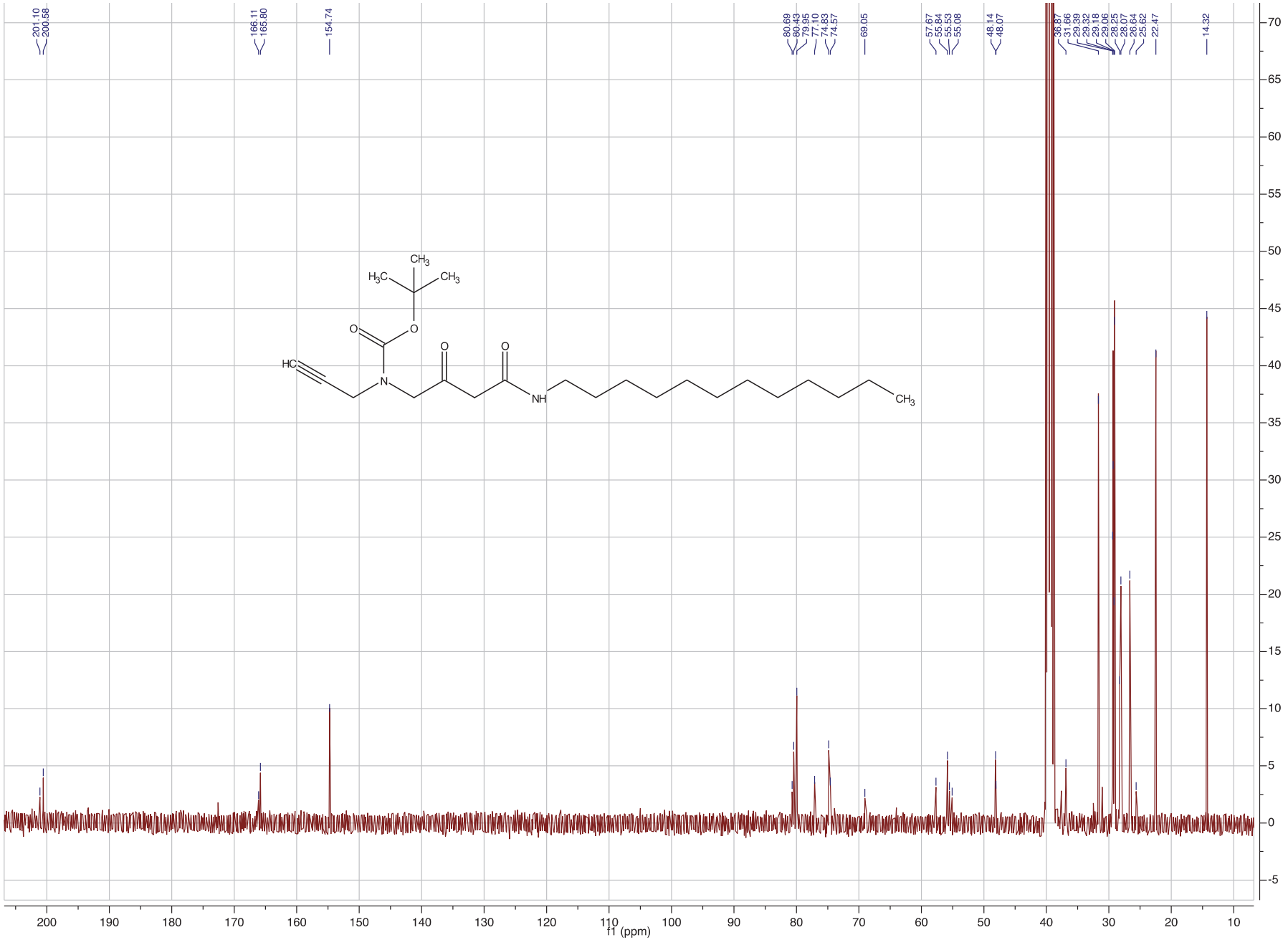

176.14
166.12
165.96
159.18
158.62
97.78
71.56
71.49
71.46
71.26
70.57
70.35
68.14
64.78
63.37
61.63
57.02
51.47
49.64
47.02
41.07
40.36
36.74
35.65
32.36
29.76
29.50
27.38
26.85
f1 (ppm)

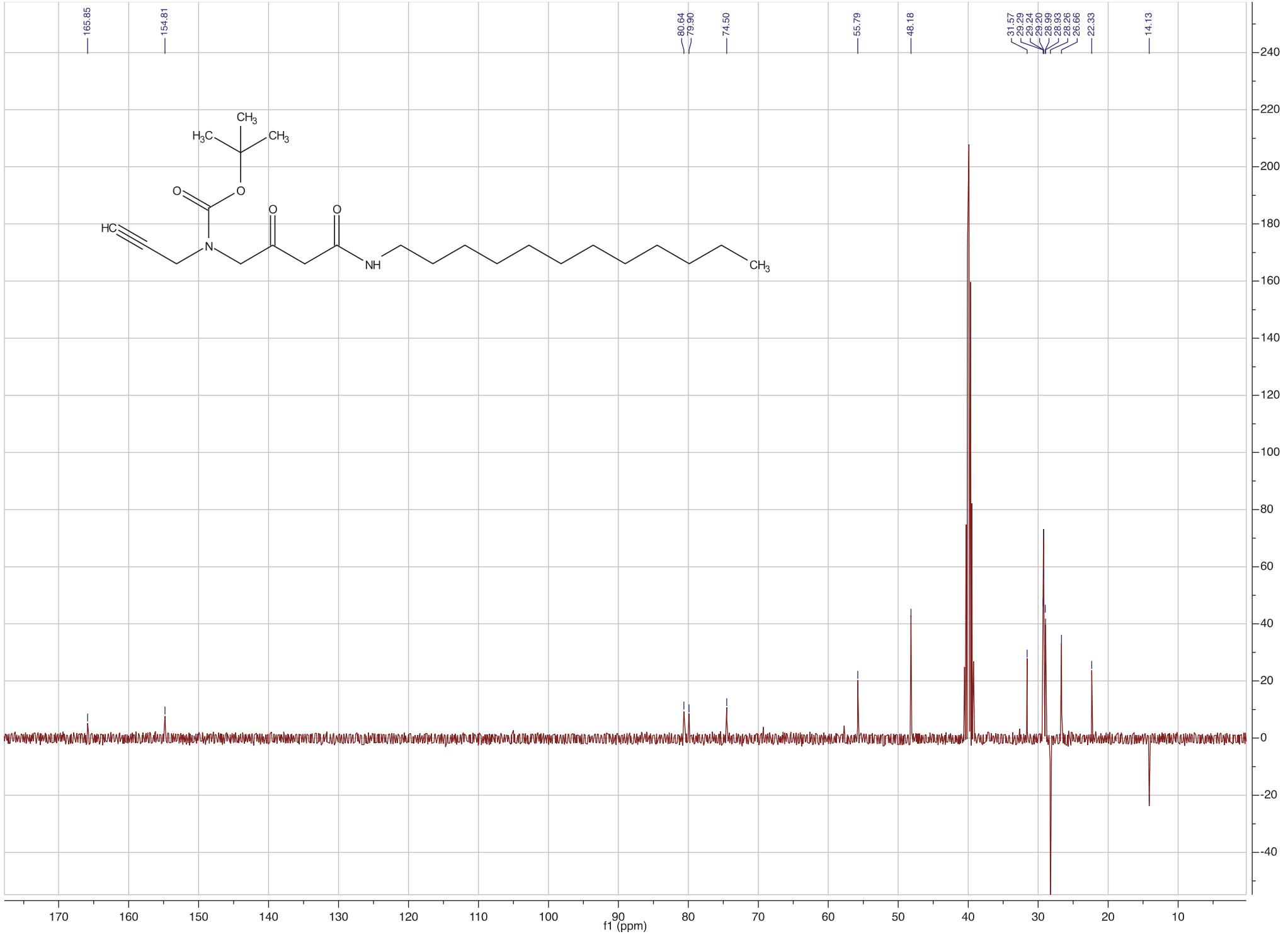

7.26 CDCl3
4.25
4.18
4.13
4.11
4.10
4.10
4.09
3.75
3.50
3.50
2.26
2.26
2.25
1.48
1.43
HC
O
O
H3C
O
N
CH3
H3C
O
CH3
0.80
1.87
1.06
2.84
1.94
1.00
9.38
f1 (ppm)

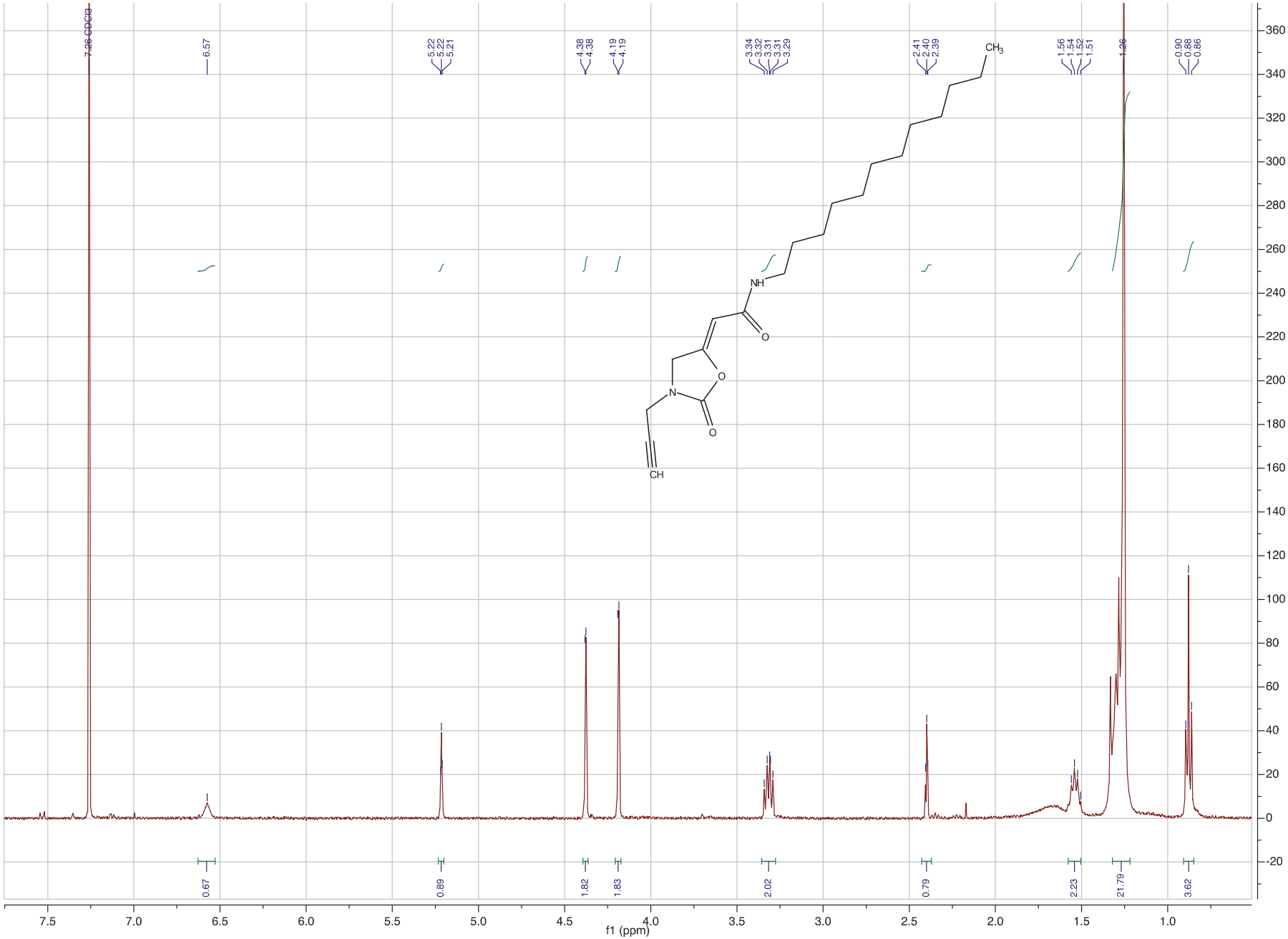

199.45
171.29
167.77
154.72
80.58
80.42
80.24
80.05
79.97
75.15
74.90
56.05
55.75
52.42
48.12
46.29
37.54
37.05
36.92
28.36
28.27
28.16
HC
O
O
N
O
O
O
CH3
H3C
CH3
CH3
f1 (ppm)

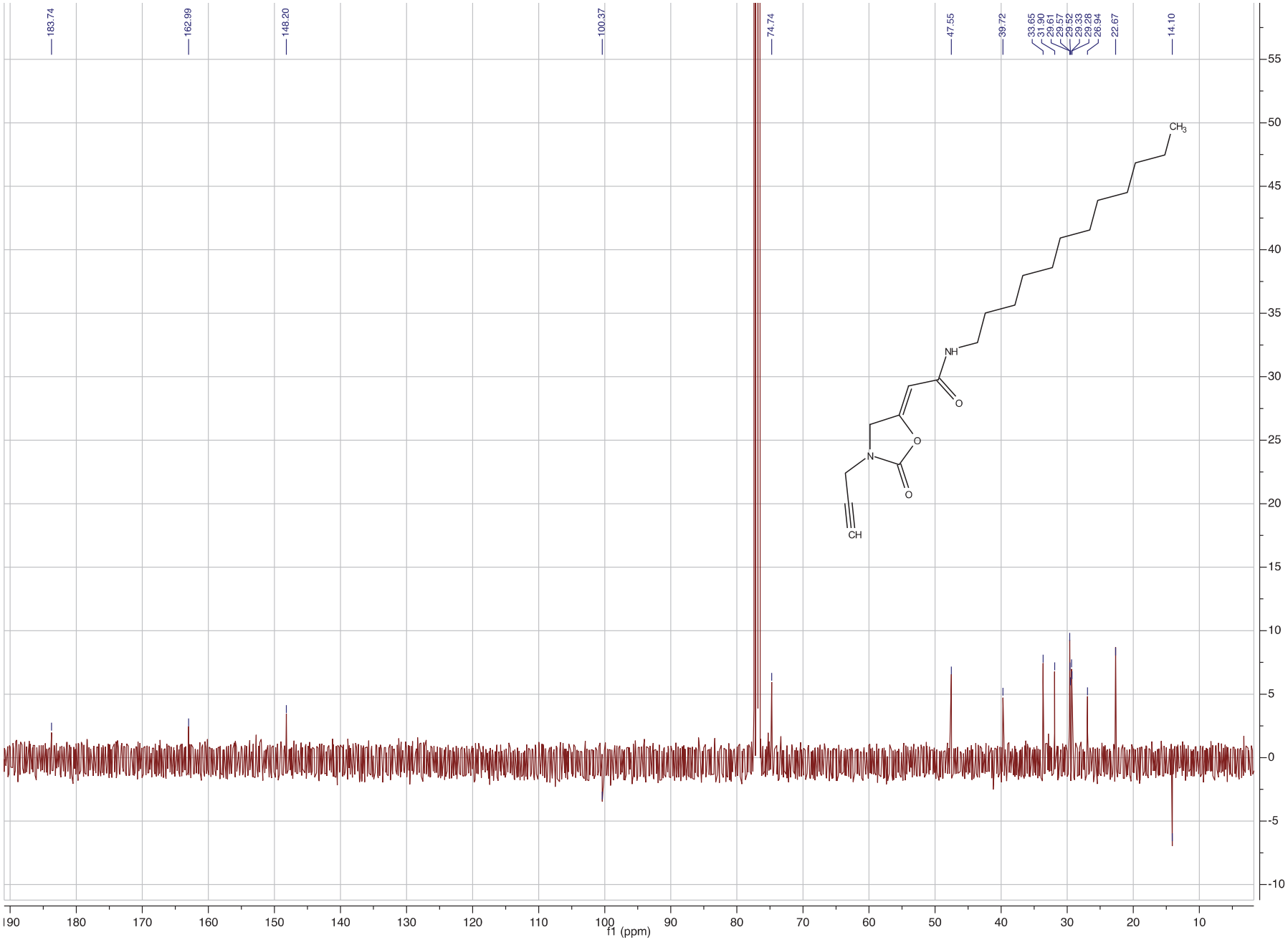

199.13
170.81
167.48
154.70
154.64
80.57
80.35
79.90
79.84
74.74
55.97
52.21
47.92
46.28
28.29
CH3
H3C
CH3
O
O
O
O
HC
N
O
CH3
450
400
350
300
250
200
150
100
50
0
-50
-100
-150
-200
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
f1 (ppm)

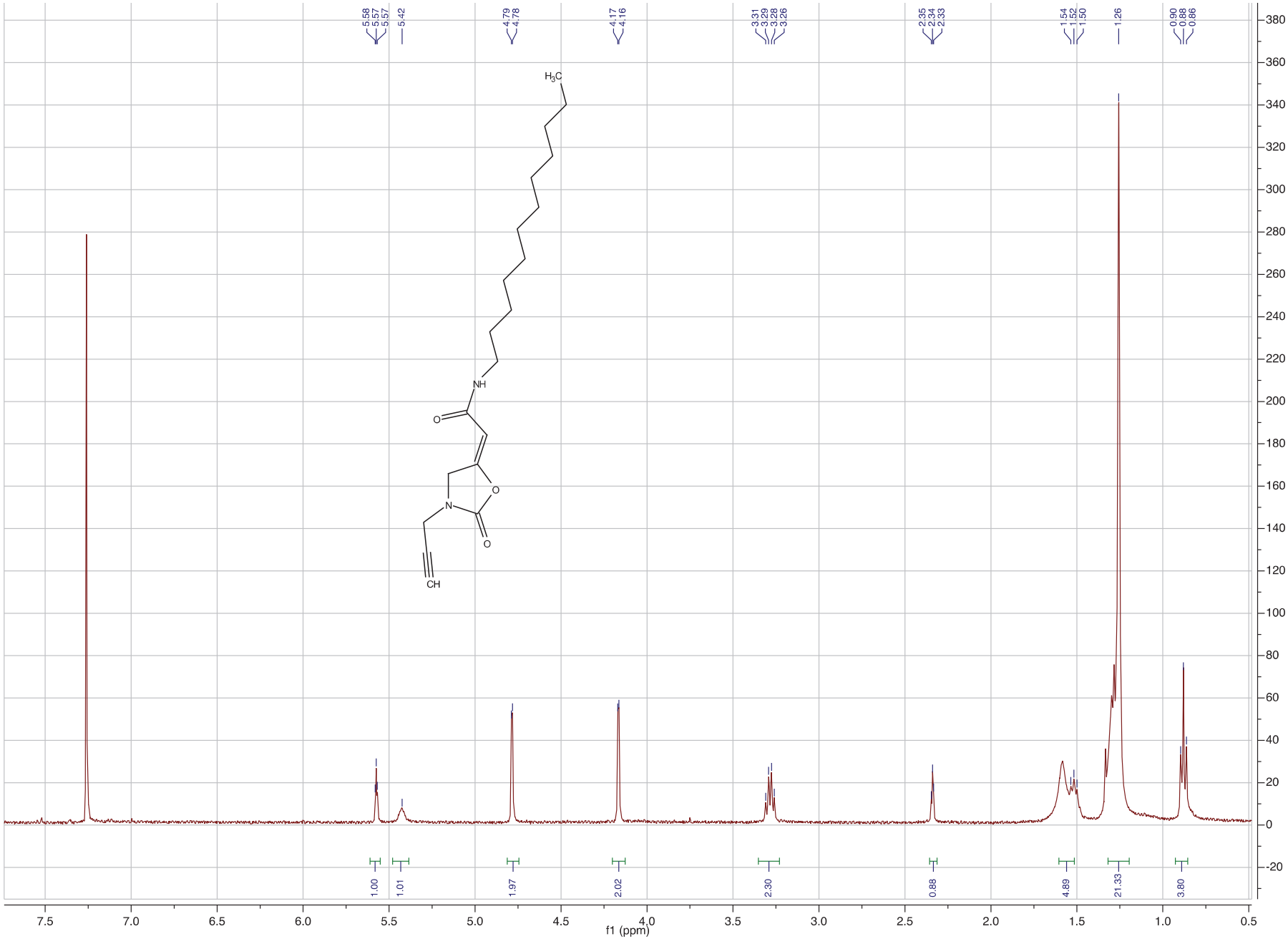

6.92
6.90
6.89
6.83
4.17
4.16
4.09
3.40
3.38
3.26
3.24
3.22
3.21
2.25
1.46
1.39
1.23
0.87
0.86
0.84
HC
O
O
N
O
O
NH
H3C
CH3
CH3
CH3
0.98
4.09
1.99
2.08
1.02
10.95
17.90
3.00
f1 (ppm)

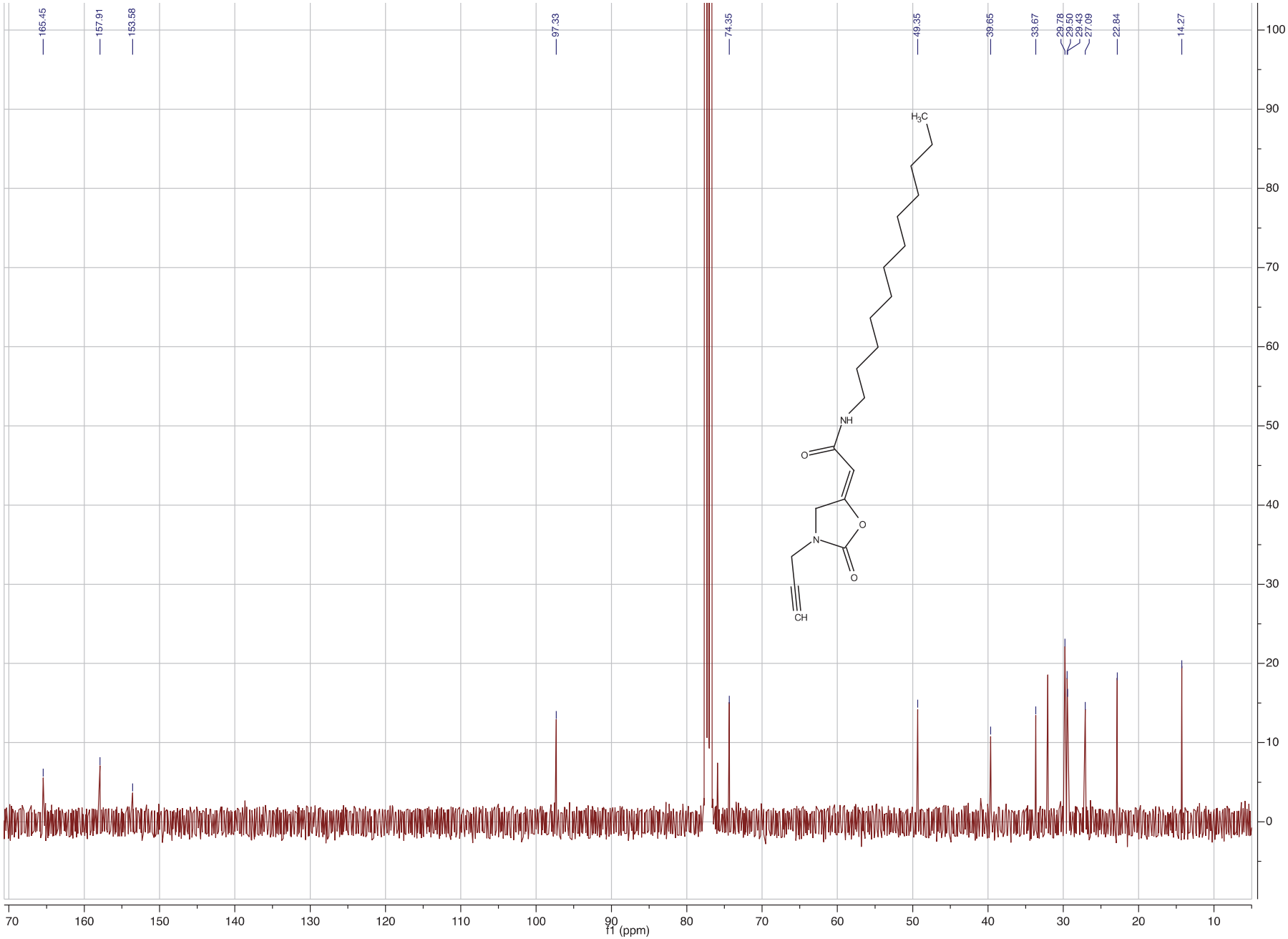

201.10
200.58
166.11
165.80
154.74
80.69
80.43
79.95
77.10
74.83
74.57
69.05
57.67
55.84
55.53
55.08
48.14
48.07
38.87
31.66
29.39
29.32
29.18
29.06
28.25
28.07
26.64
25.62
22.47
14.32
CH3
H3C
CH3
O
O
O
O
HC
N
NH
CH3
f1 (ppm)

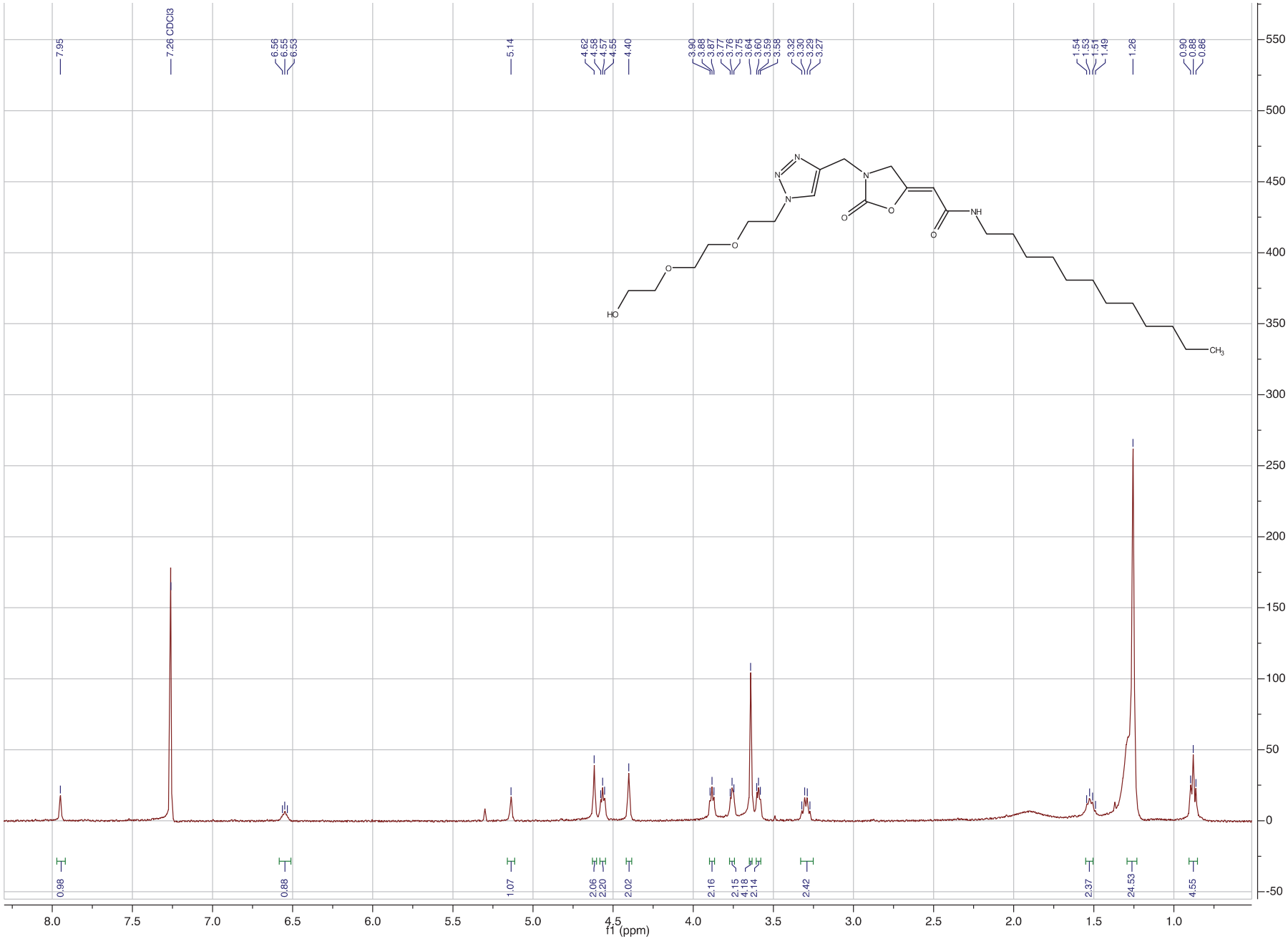

165.85
154.81
80.64
79.90
74.50
55.79
48.18
31.57
29.29
29.24
29.20
28.99
28.93
28.26
26.66
22.33
14.13
CH3
H3C
CH3
O
O
O
O
HC
N
NH
CH3
240
220
200
180
160
140
120
100
80
60
40
20
0
-20
-40
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

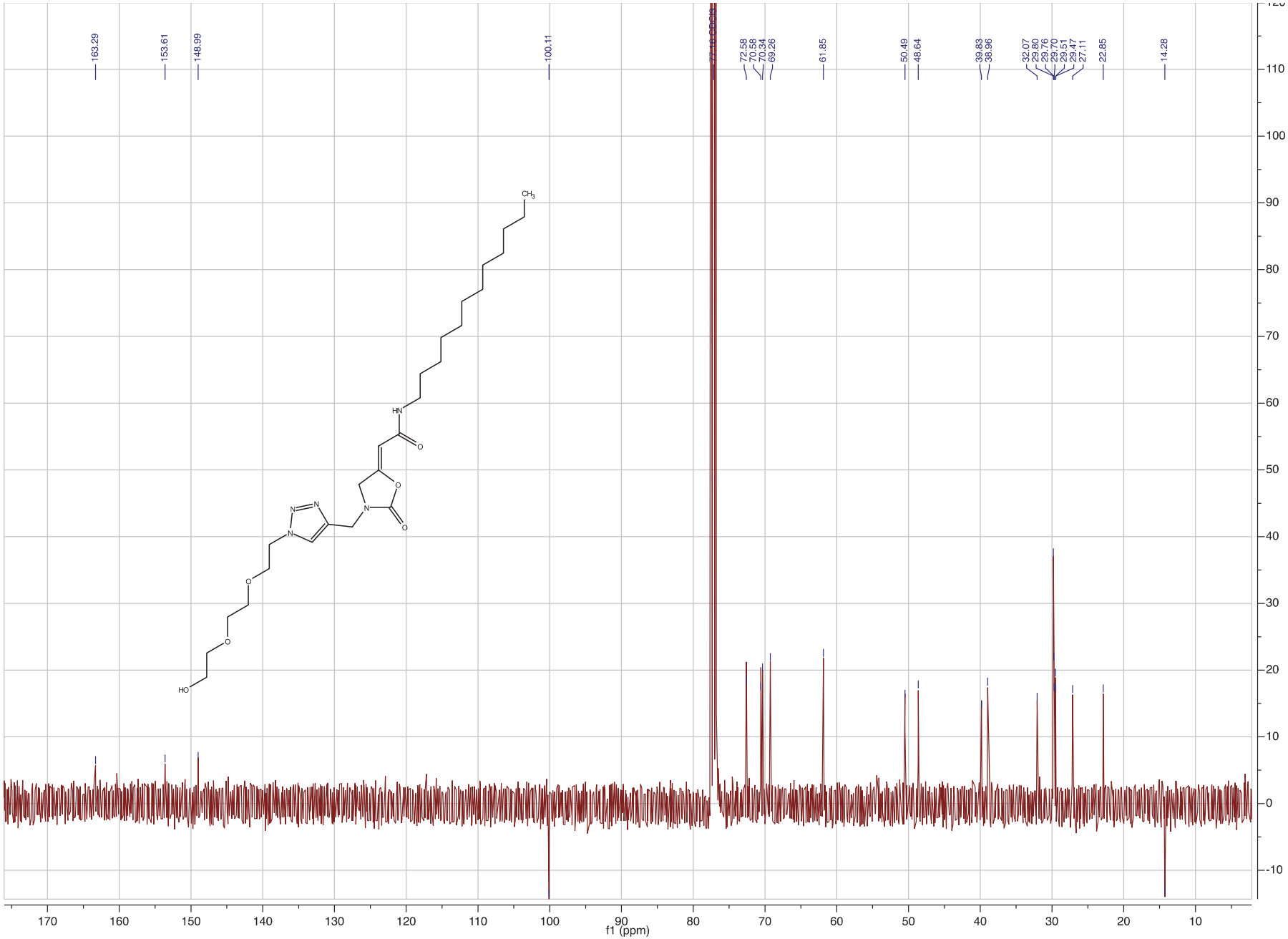

7.26 CDCl3
6.57
5.22
5.22
5.21
4.38
4.38
4.19
4.19
3.34
3.32
3.31
3.31
3.29
2.41
2.40
2.39
1.56
1.54
1.52
1.51
1.26
0.90
0.88
0.86
CH3
NH
O
O
N
O
O
CH
0.67
0.89
1.82
1.83
2.02
0.79
2.23
21.79
3.62
f1 (ppm)

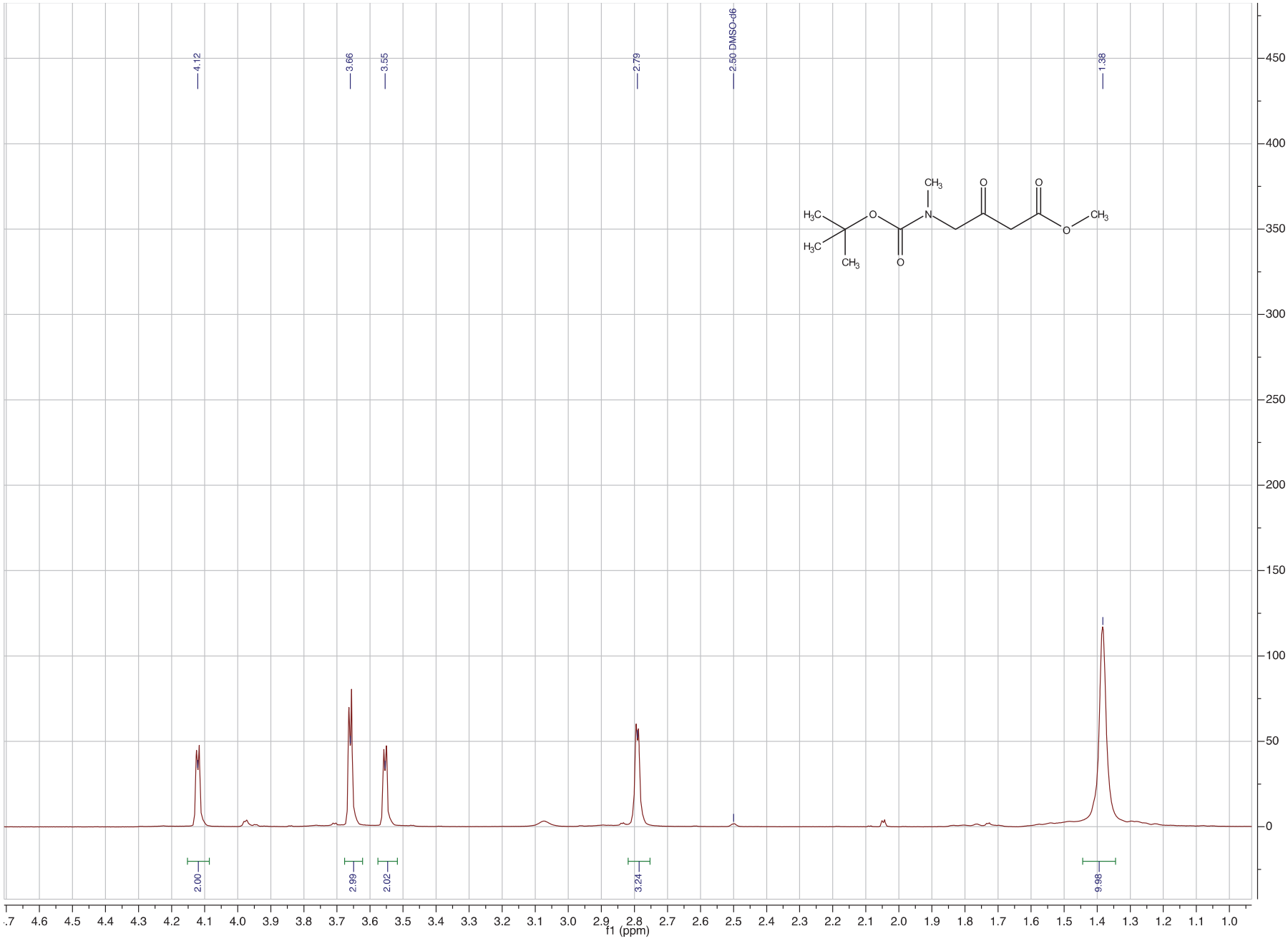

183.74
162.99
148.20
100.37
74.74
47.55
39.72
33.65
31.90
29.61
29.57
29.52
29.33
29.28
26.94
22.67
14.10
CH3
NH
O
O
N
O
O
CH
f1 (ppm)

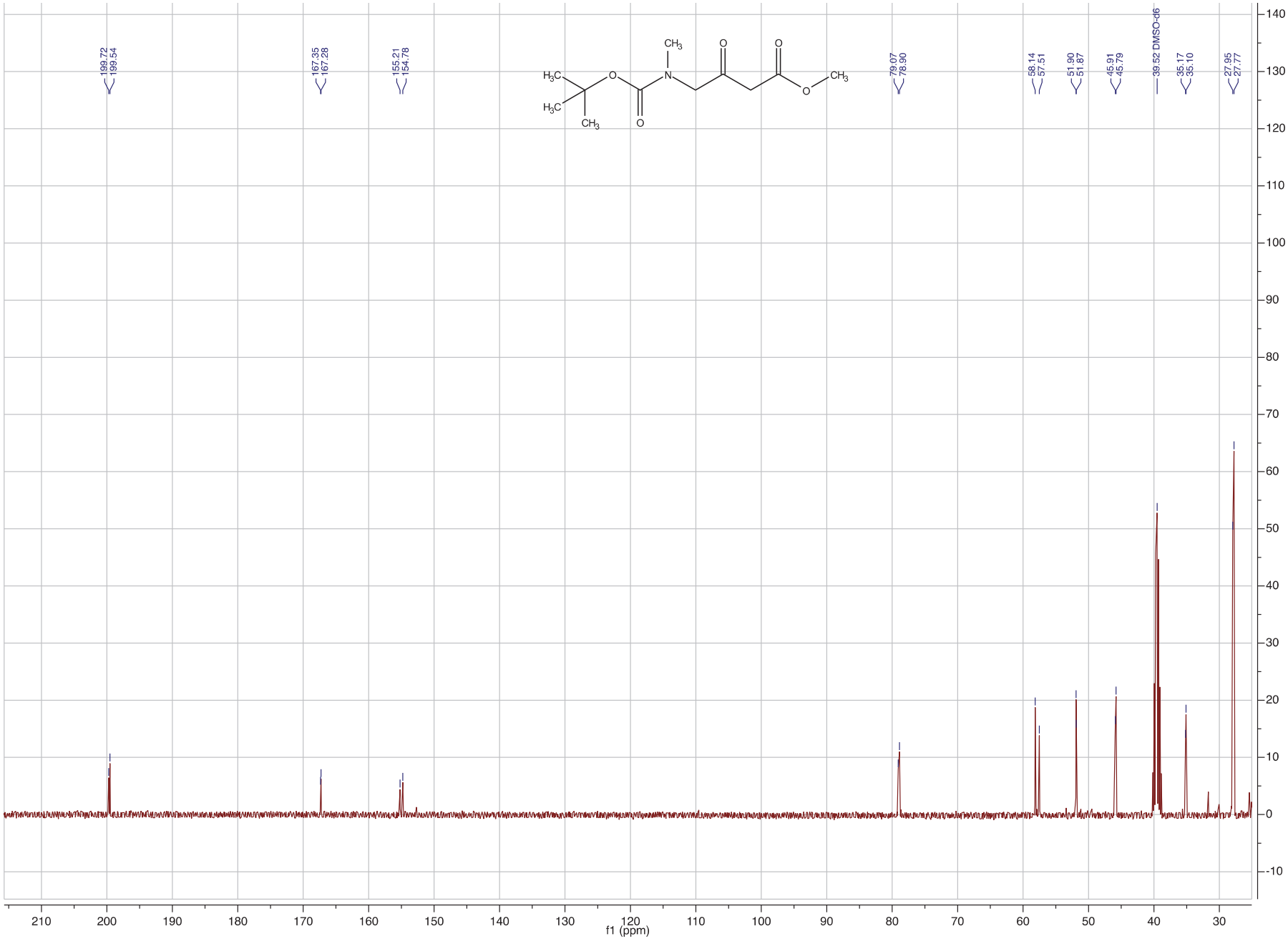

5.58
5.57
5.57
5.42
4.79
4.78
4.17
4.16
3.31
3.29
3.28
3.26
2.35
2.34
2.33
1.54
1.52
1.50
1.26
0.90
0.88
0.86
H3C
NH
O
O
N
O
O
CH
1.00
1.01
1.97
2.02
2.30
0.88
4.89
21.33
3.80
f1 (ppm)

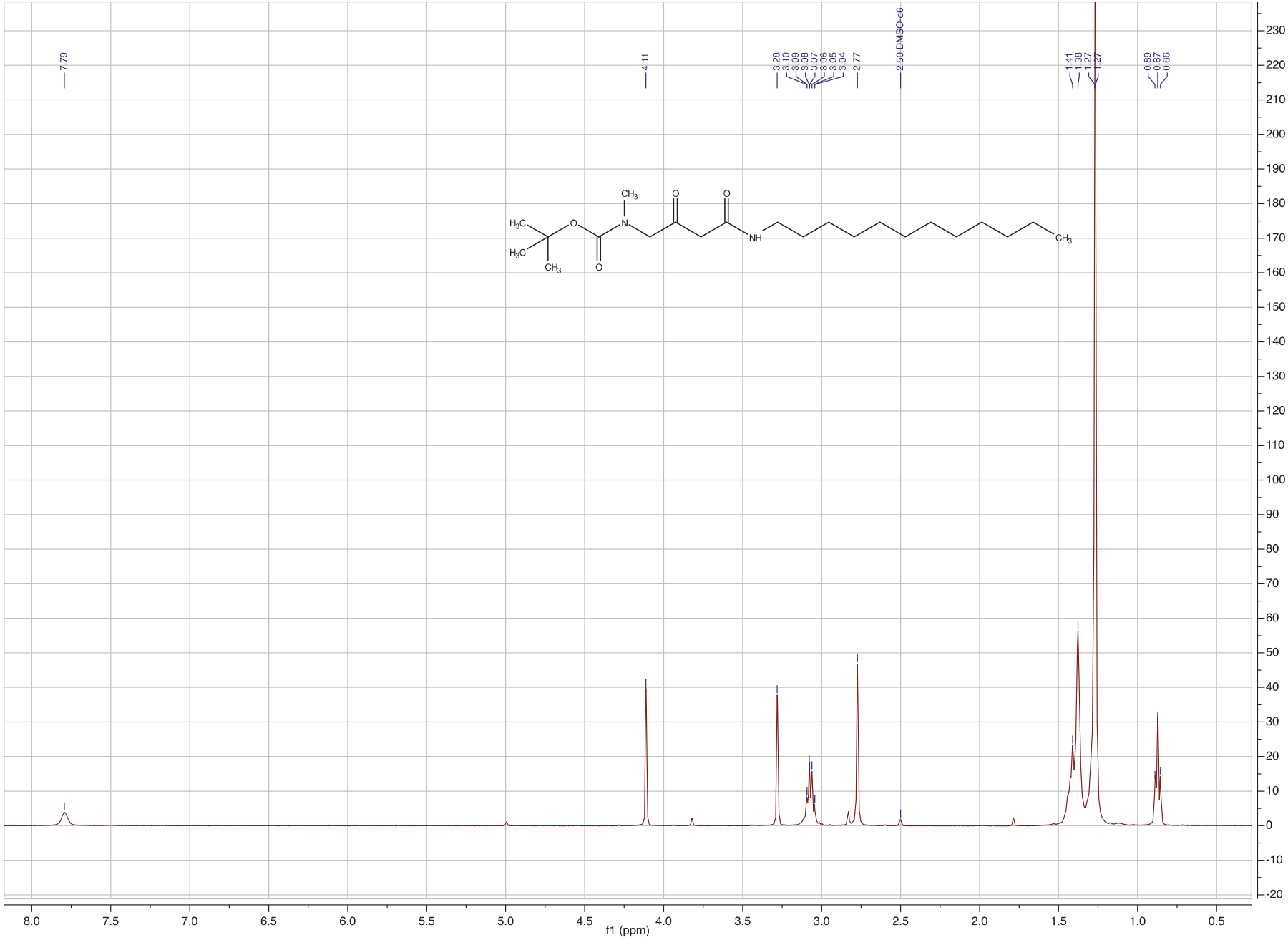

165.45
157.91
153.58
97.33
74.35
49.35
39.65
33.67
29.78
29.50
29.43
27.09
22.84
14.27
H3C
NH
O
O
N
O
O
CH
100
90
80
70
60
50
40
30
20
10
0
70
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

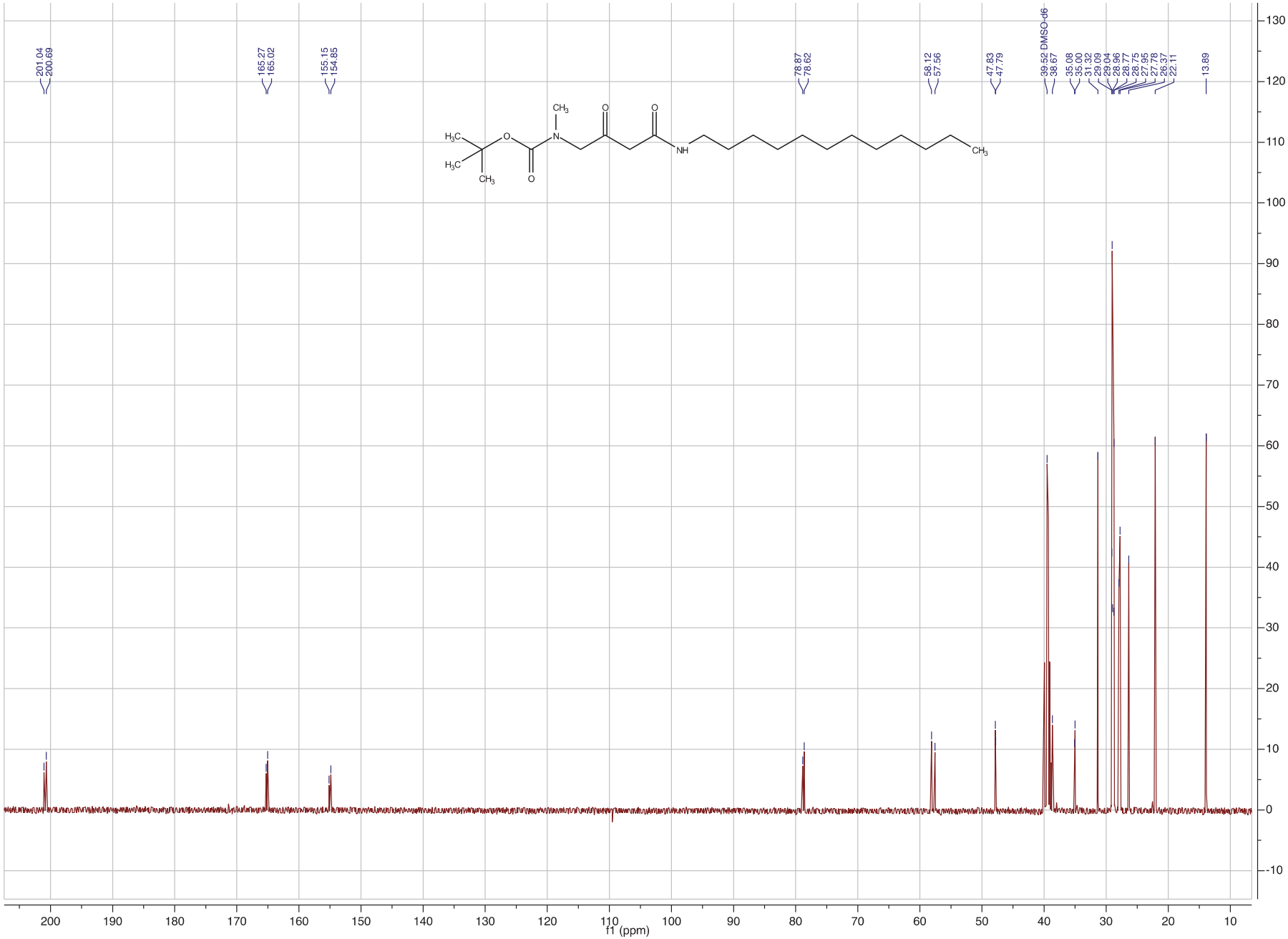

7.95
7.26 CDCl3
6.56
6.55
6.53
5.14
4.62
4.58
4.57
4.55
4.40
3.90
3.88
3.87
3.77
3.76
3.75
3.64
3.60
3.59
3.58
3.32
3.30
3.29
3.27
1.54
1.53
1.51
1.49
1.26
0.90
0.88
0.86
HO
O
O
O
N
N
N
O
O
O
NH
CH3
0.98
0.88
1.07
2.06
2.20
2.02
2.16
2.15
4.18
2.14
2.42
2.37
24.53
4.55
f1 (ppm)

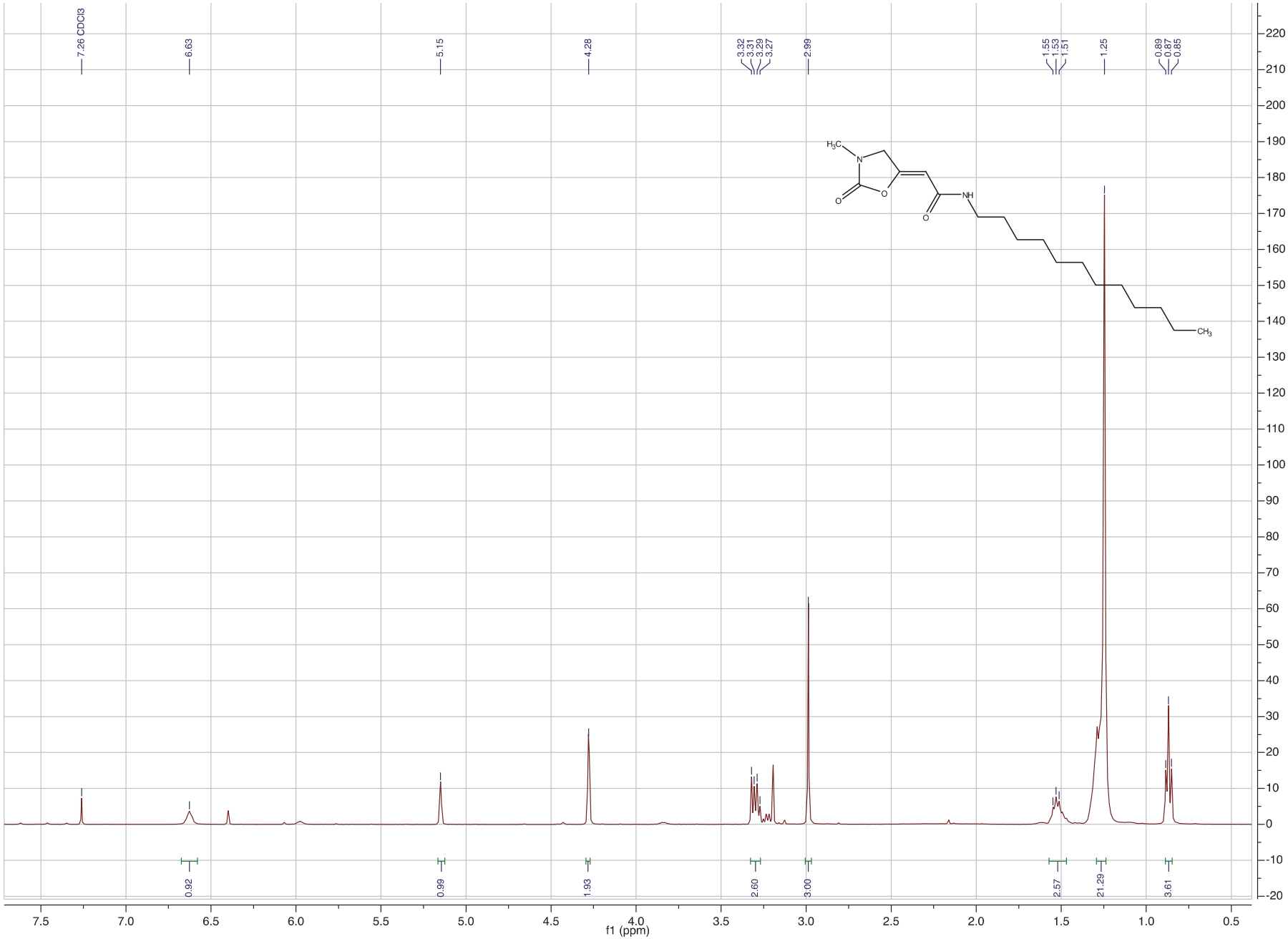

163.29
153.61
148.99
100.11
72.58
70.58
70.34
69.26
61.85
50.49
48.64
39.83
38.96
32.07
29.80
29.76
29.70
29.51
29.47
27.11
22.85
14.28
CH3
HN
O
N
N
N
O
O
O
O
HO
f1 (ppm)

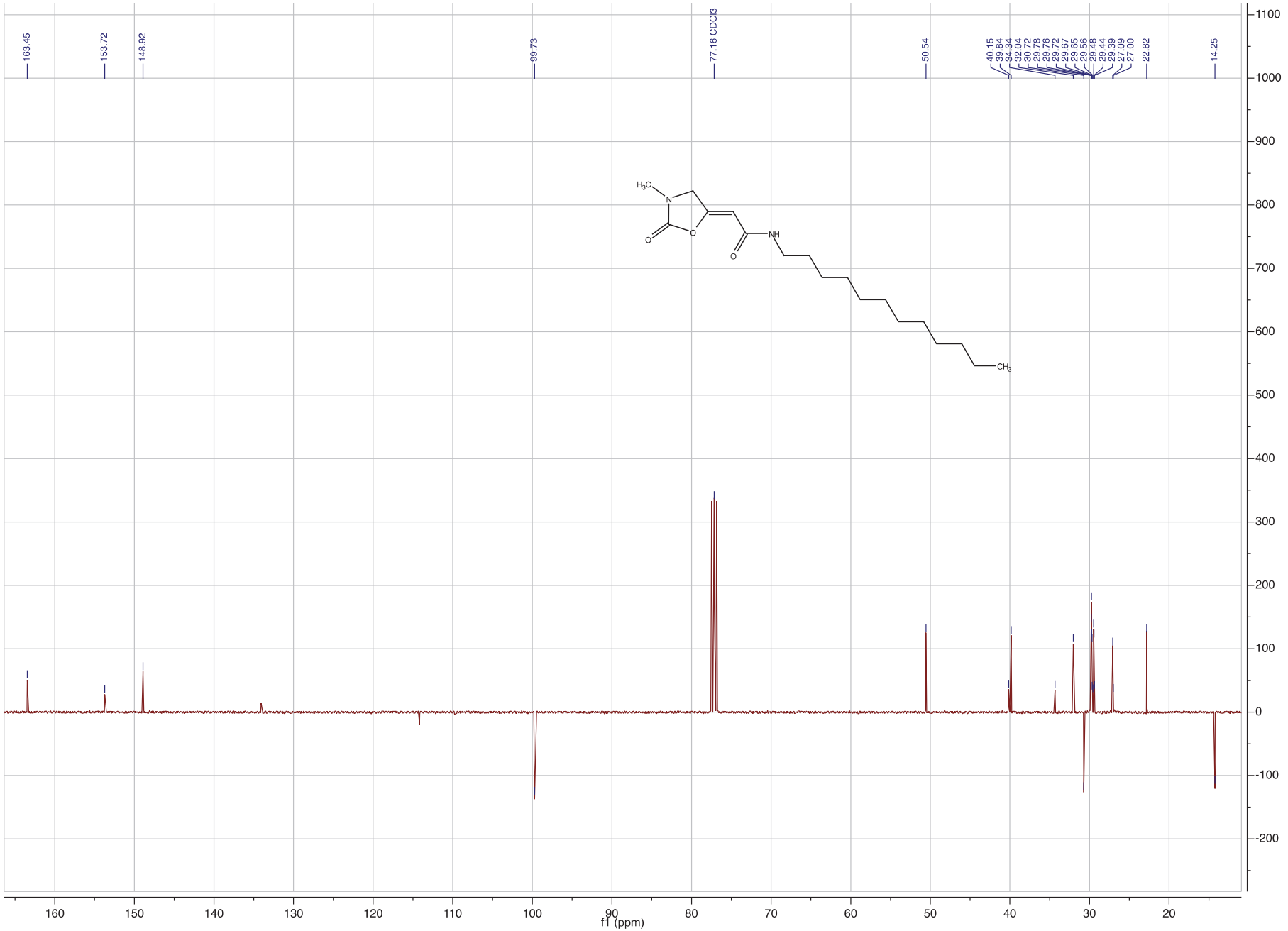

4.12
3.66
3.55
2.79
2.50 DMSO-d6
1.38
2.00
2.99
2.02
3.24
9.98
f1 (ppm)

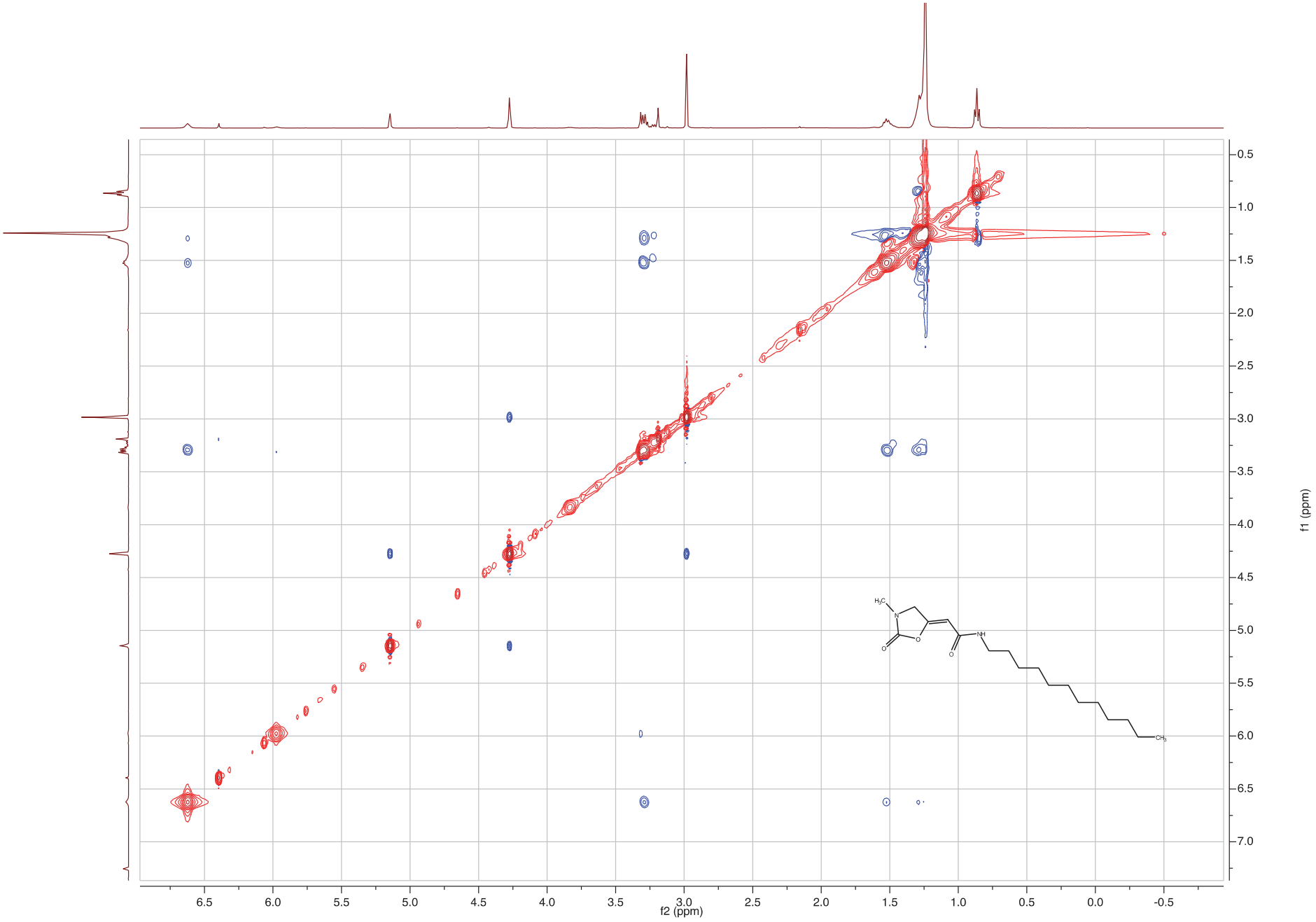

199.72
199.54
167.35
167.28
155.21
154.78
79.07
78.90
58.14
57.51
51.90
51.87
45.91
45.79
39.52 DMSO-d6
35.17
35.10
27.95
27.77
f1 (ppm)

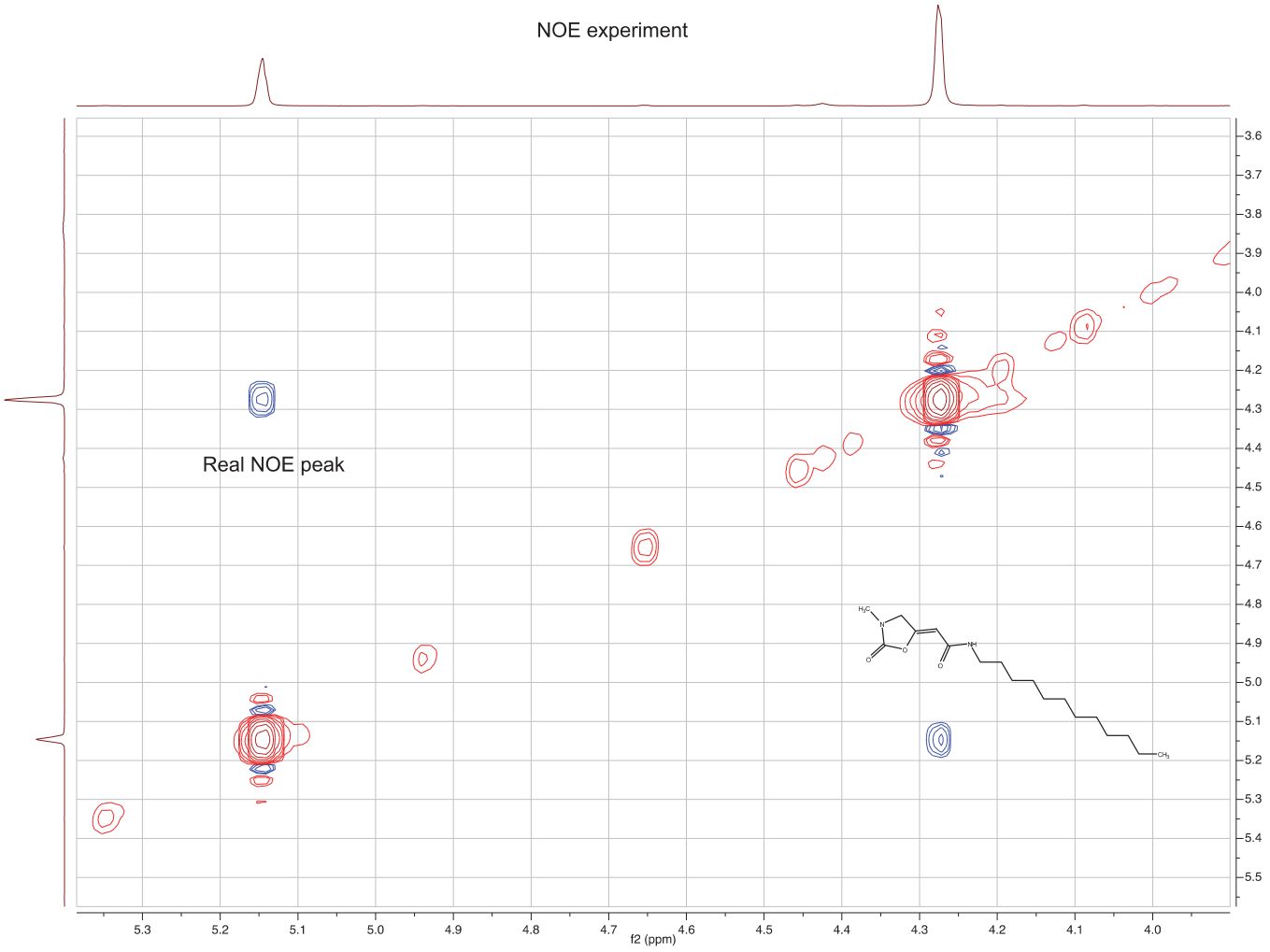

7.79
4.11
3.28
3.10
3.09
3.08
3.07
3.06
3.05
3.04
2.77
2.50 DMSO-d6
1.41
1.38
1.27
1.27
0.89
0.87
0.86
CH3
O
O
N
O
O
H3C
H3C
CH3
NH
CH3
f1 (ppm)

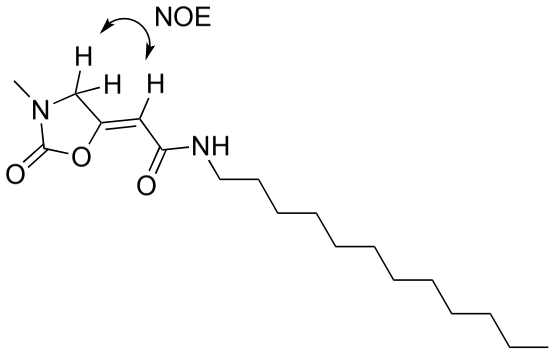

201.04
200.69
165.27
165.02
155.15
154.85
78.87
78.62
58.12
57.56
47.83
47.79
39.52 DMSO-d6
38.67
35.08
35.00
31.32
29.09
29.04
28.96
28.77
28.75
27.95
27.78
26.37
22.11
13.89
CH3
O
O
N
O
H3C
H3C
CH3
O
NH
CH3
f1 (ppm)


7.26 CDCl3
6.63
5.15
4.28
3.32
3.31
3.29
3.27
2.99
1.55
1.53
1.51
1.25
0.89
0.87
0.85
0.92
0.99
1.93
2.60
3.00
2.57
21.29
3.61
f1 (ppm)

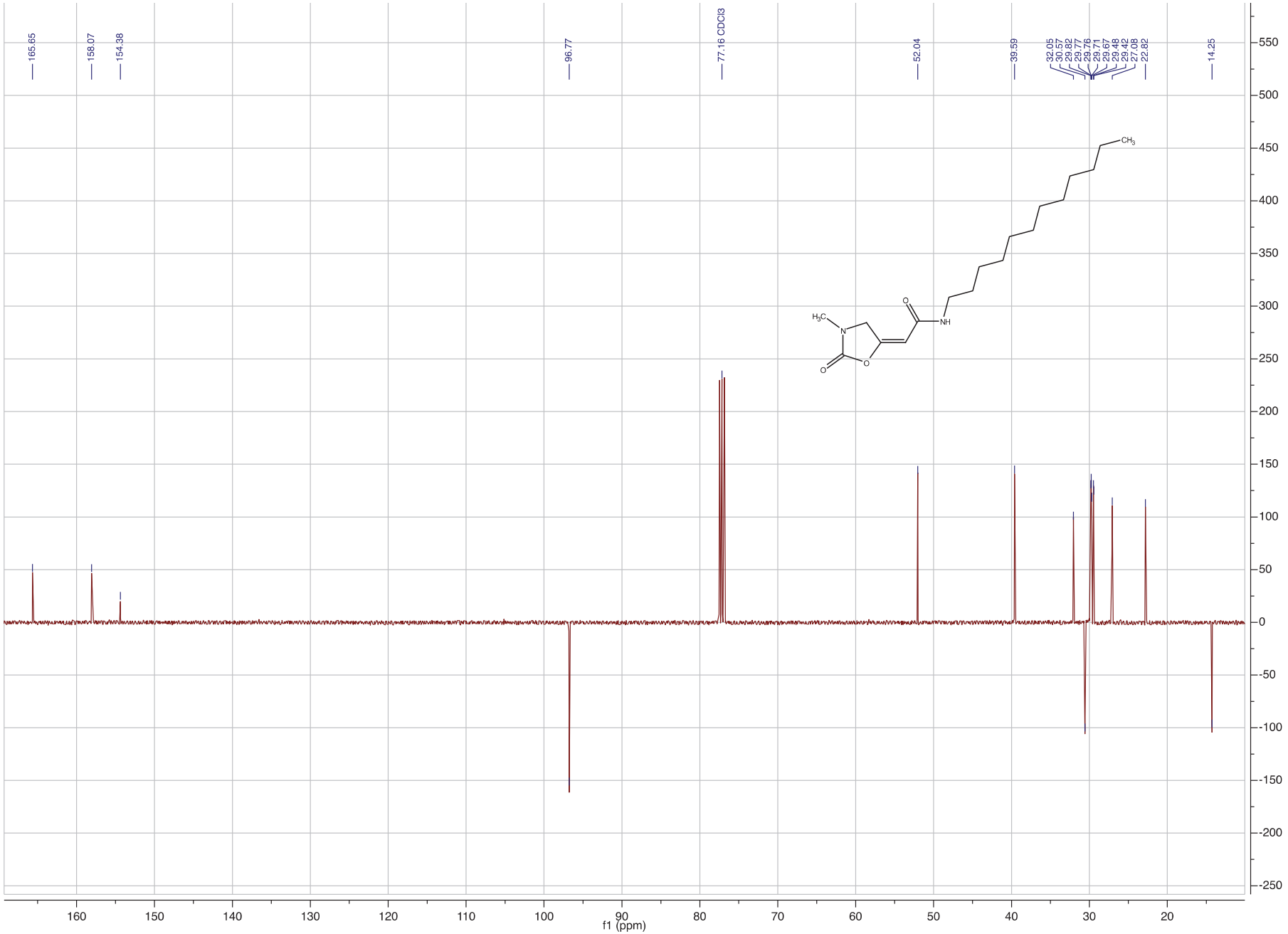

163.45
153.72
148.92
99.73
77.16 CDCl3
50.54
40.15
39.84
34.34
32.04
30.72
29.78
29.76
29.72
29.67
29.65
29.56
29.48
29.44
29.39
27.69
27.00
22.82
14.25
H3C
N
O
O
O
NH
CH3
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
-200
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
f1 (ppm)

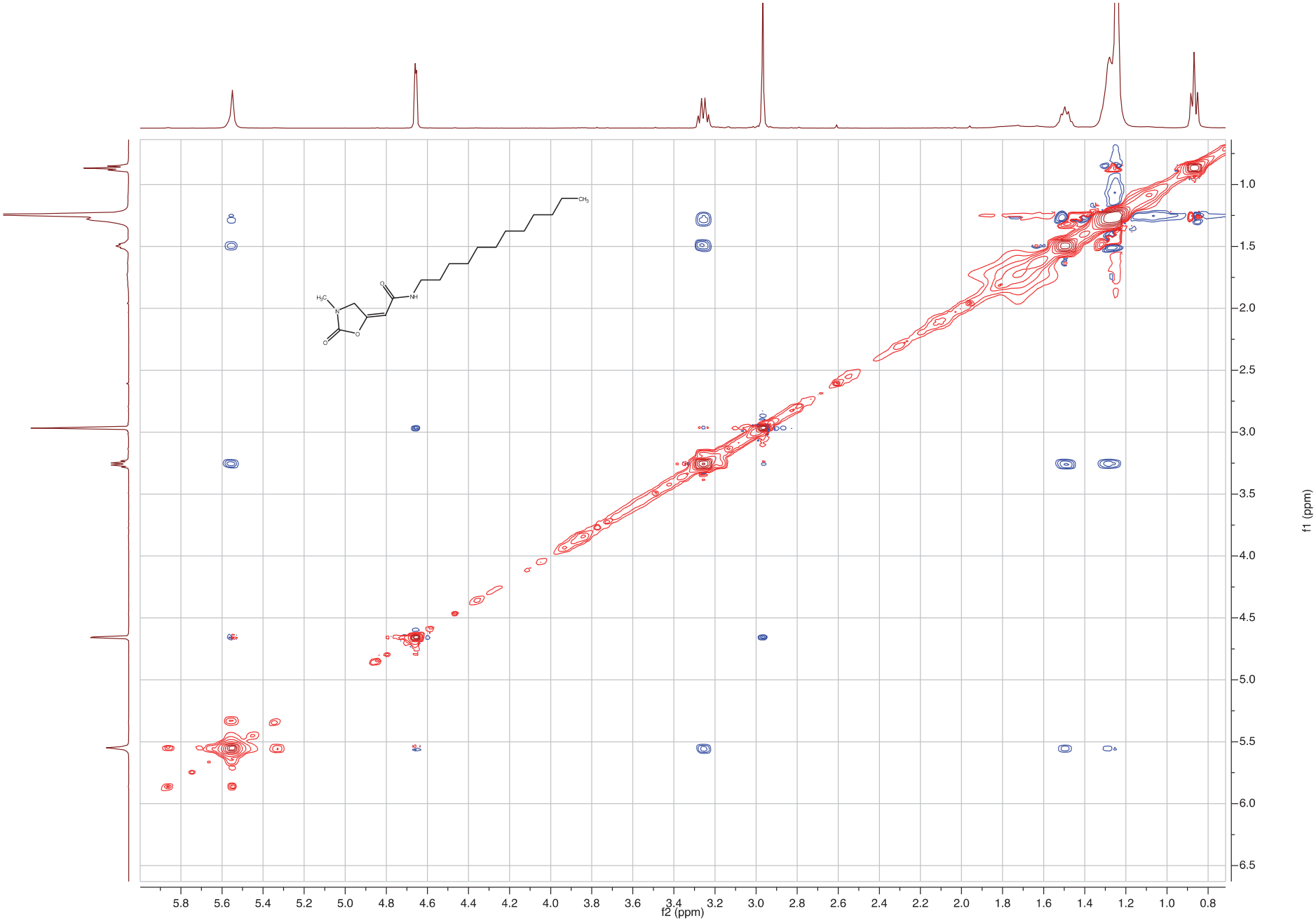

f2 (ppm)
f1 (ppm)
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0

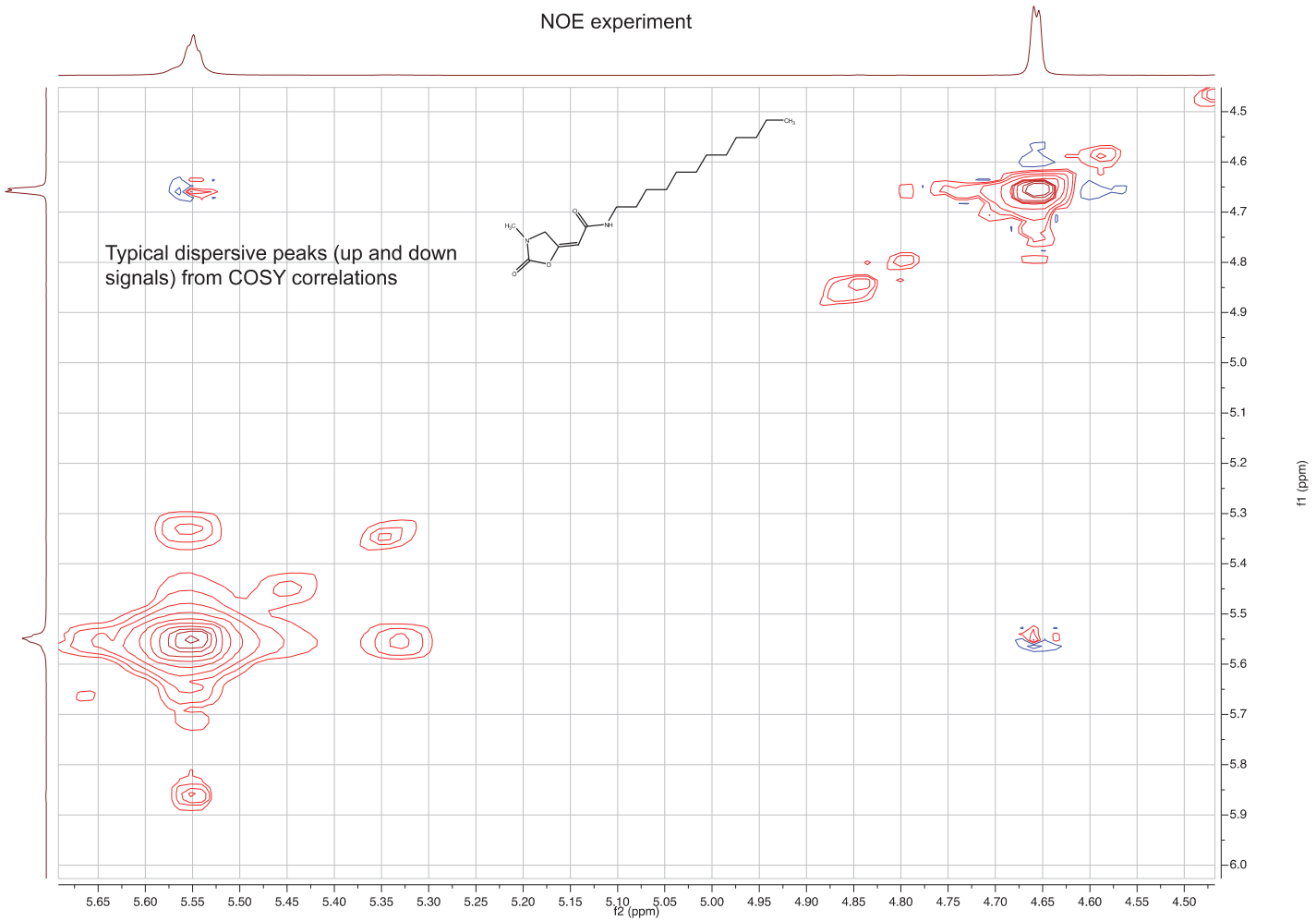

NOE experiment
Real NOE peak
f2 (ppm)
f1 (ppm)

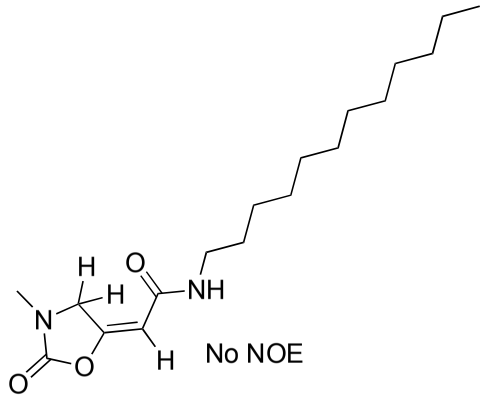

NOE
H
H
H
N
O
O
NH
O

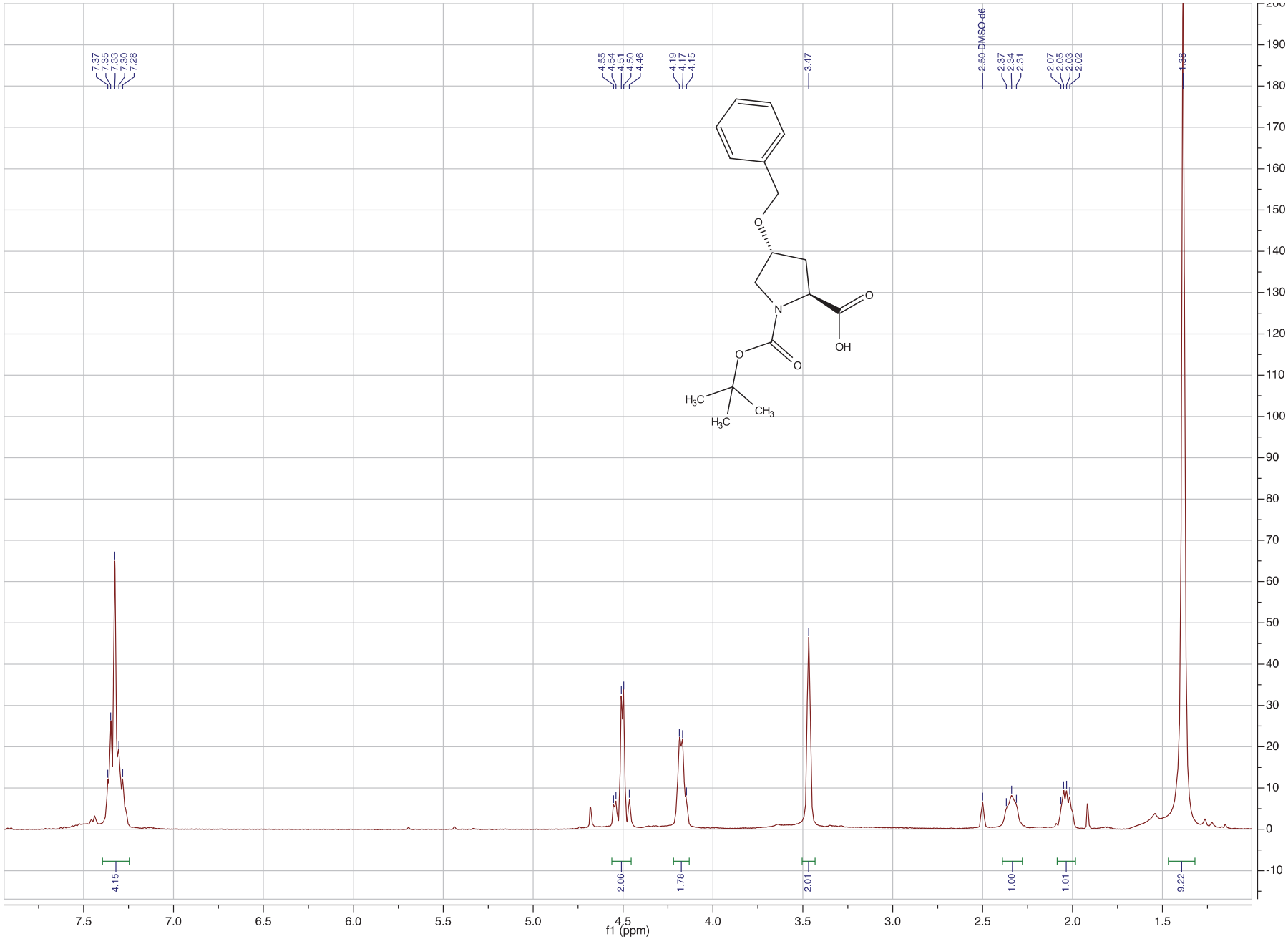

5.55
4.66
4.65
3.28
3.27
3.25
3.23
2.97
1.53
1.51
1.50
1.48
1.46
1.29
1.28
1.24
0.88
0.87
0.85
CH3
H3C
N
O
O
O
NH
1.55
1.72
1.82
2.56
1.89
19.01
3.00
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
f1 (ppm)
400
350
300
250
200
150
100
50
0

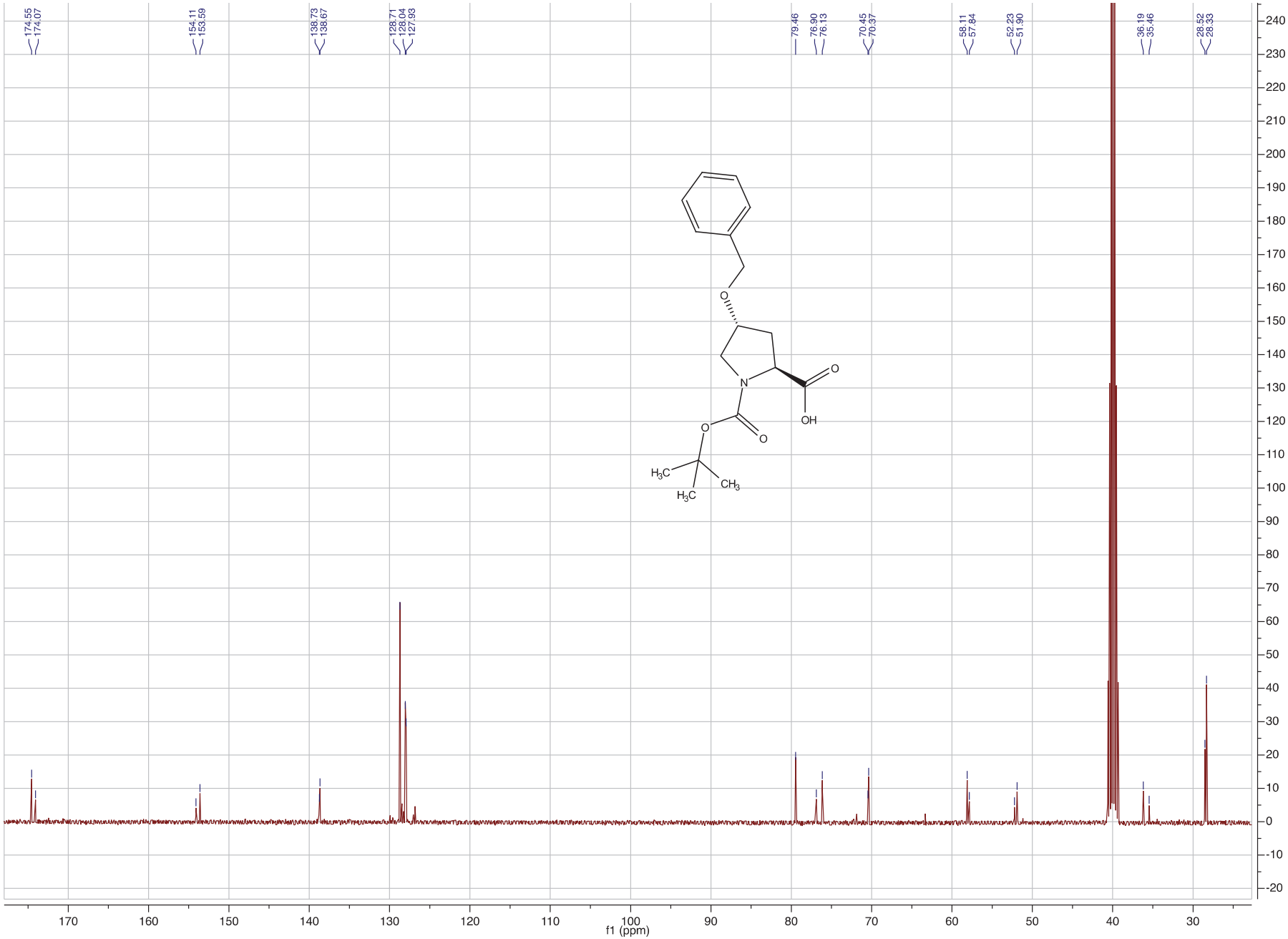

165.65
158.07
154.38
96.77
77.16 CDCl3
52.04
39.59
32.05
30.57
29.82
29.77
29.76
29.71
29.67
29.48
29.42
27.08
22.82
14.25
CH3
H3C
N
O
NH
O
O
550
500
450
400
350
300
250
200
150
100
50
0
-50
-100
-150
-200
-250
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
f1 (ppm)

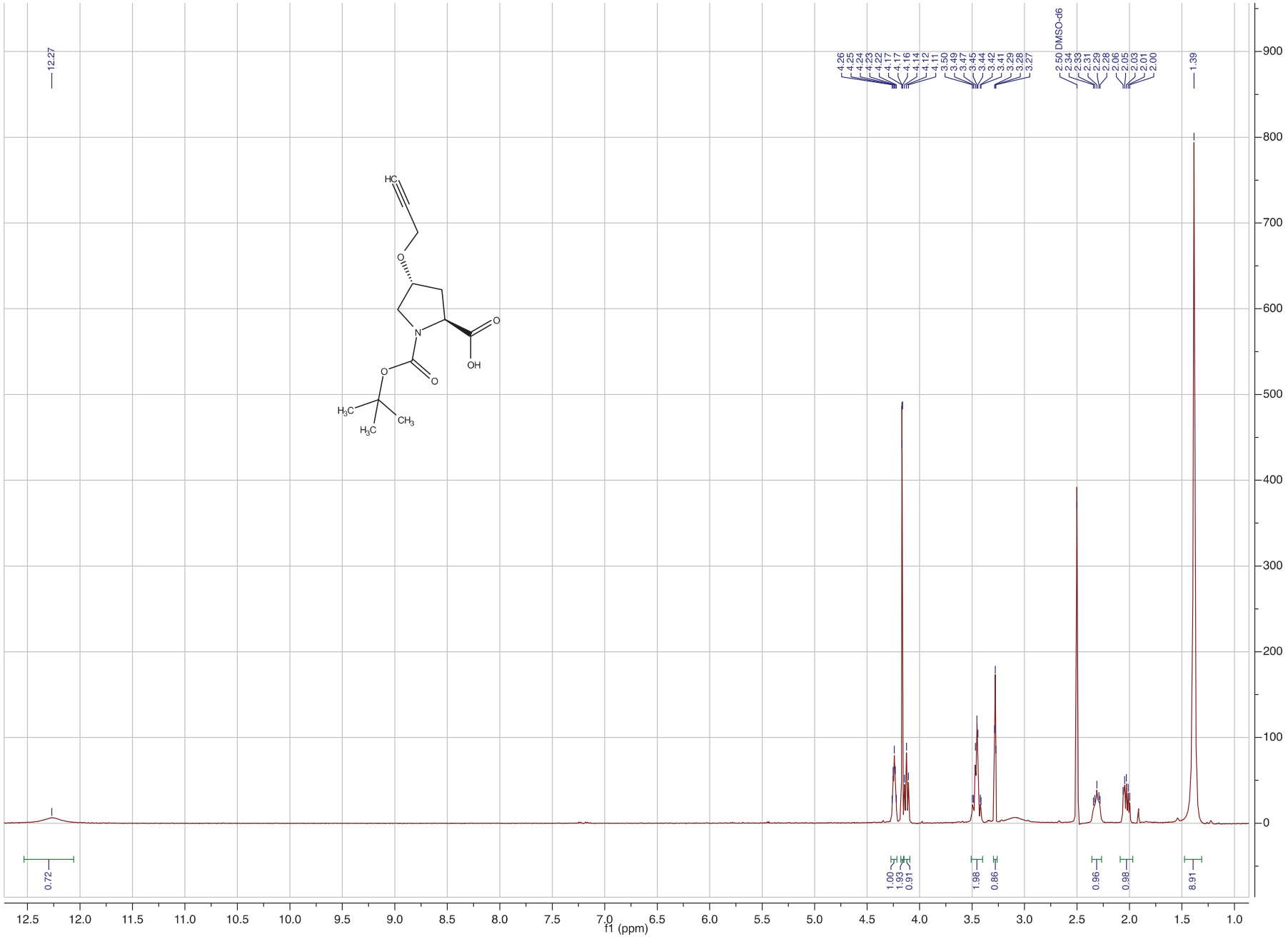

f2 (ppm)
f1 (ppm)


NOE experiment
Typical dispersive peaks (up and down signals) from COSY correlations
f2 (ppm)
f1 (ppm)


H
O
NH
H
N
O
O
H
No NOE


7.37
7.35
7.33
7.30
7.28
4.55
4.54
4.51
4.50
4.46
4.19
4.17
4.15
3.47
2.50 DMSO-d6
2.37
2.34
2.31
2.07
2.05
2.03
2.02
1.38
O
N
O
OH
O
O
H3C
CH3
H3C
4.15
2.06
1.78
2.01
1.00
1.01
9.22
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
f1 (ppm)


174.55
174.07
154.11
153.59
138.73
138.67
128.71
128.04
127.93
79.46
76.90
76.13
70.45
70.37
58.11
57.84
52.23
51.90
36.19
35.46
28.52
28.33
O
N
O
OH
O
O
H3C
CH3
H3C
f1 (ppm)


12.27
4.26
4.25
4.24
4.23
4.22
4.17
4.16
4.14
4.12
4.11
3.50
3.49
3.47
3.45
3.44
3.42
3.41
3.29
3.28
3.27
2.50 DMSO-d6
2.34
2.33
2.31
2.29
2.28
2.06
2.05
2.03
2.01
2.00
1.39
HC
O
N
O
O
OH
O
H3C
CH3
H3C
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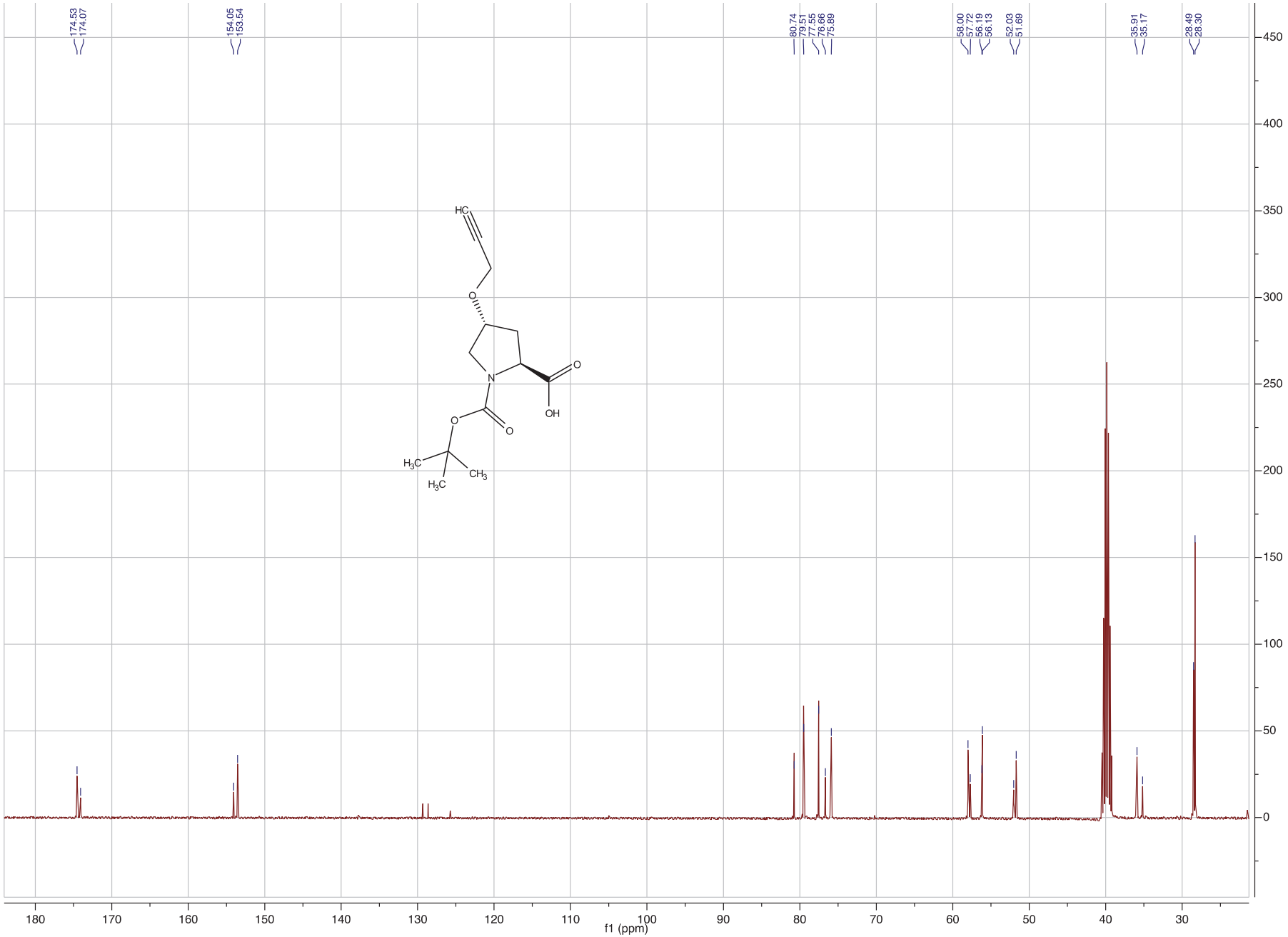
1.00
1.93
0.91
1.98
0.86
0.96
0.98
8.91
f1 (ppm)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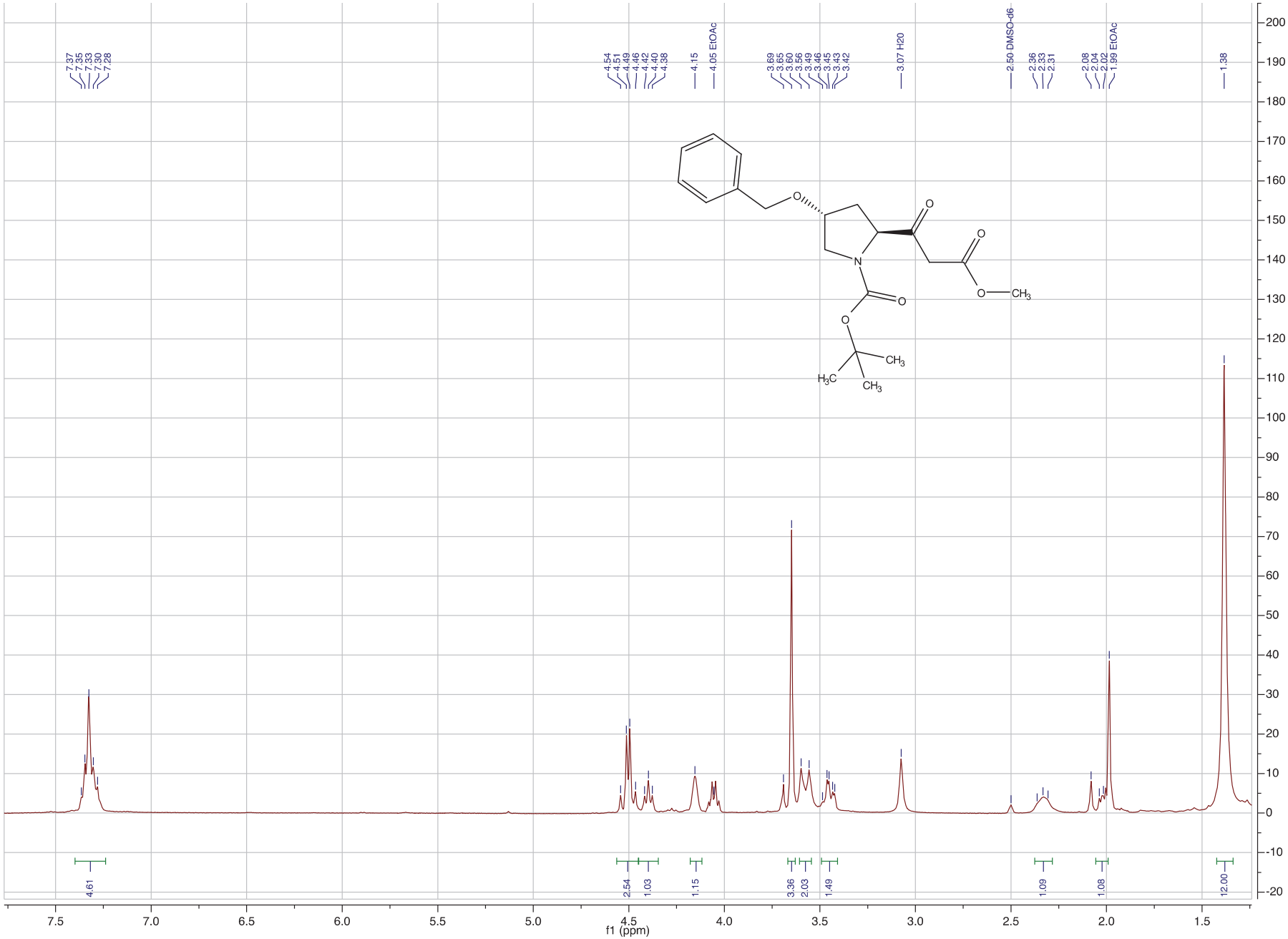
174.07
154.05
153.54
80.74
79.51
77.55
76.66
75.89
58.00
57.72
56.19
56.13
52.03
51.69
35.91
35.17
28.49
28.30
HC
O
N
O
OH
O
O
H3C
CH3
H3C
450
400
350
300
250
200
150
100
50
0
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
f1 (ppm)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7.35
7.33
7.30
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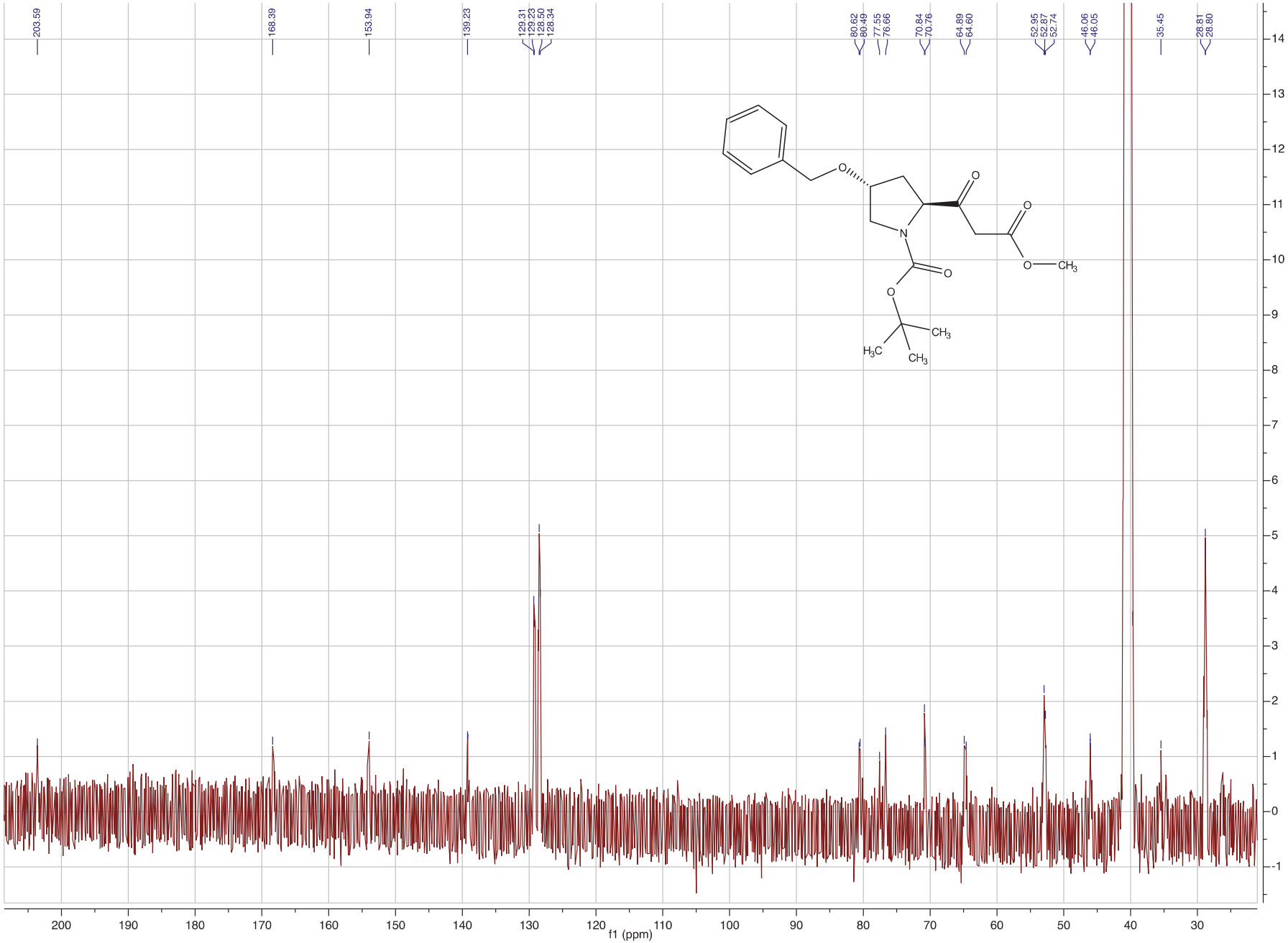
4.51
4.49
4.46
4.42
4.40
4.38
4.15
4.05 EtOAc
3.69
3.65
3.60
3.56
3.49
3.46
3.45
3.43
3.42
3.07 H20
2.50 DMSO-d6
2.36
2.33
2.31
2.08
2.04
2.02
1.99 EtOAc
1.38
4.61
2.54
1.03
1.15
3.36
2.03
1.49
1.09
1.08
12.00
f1 (ppm)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168.39
153.94
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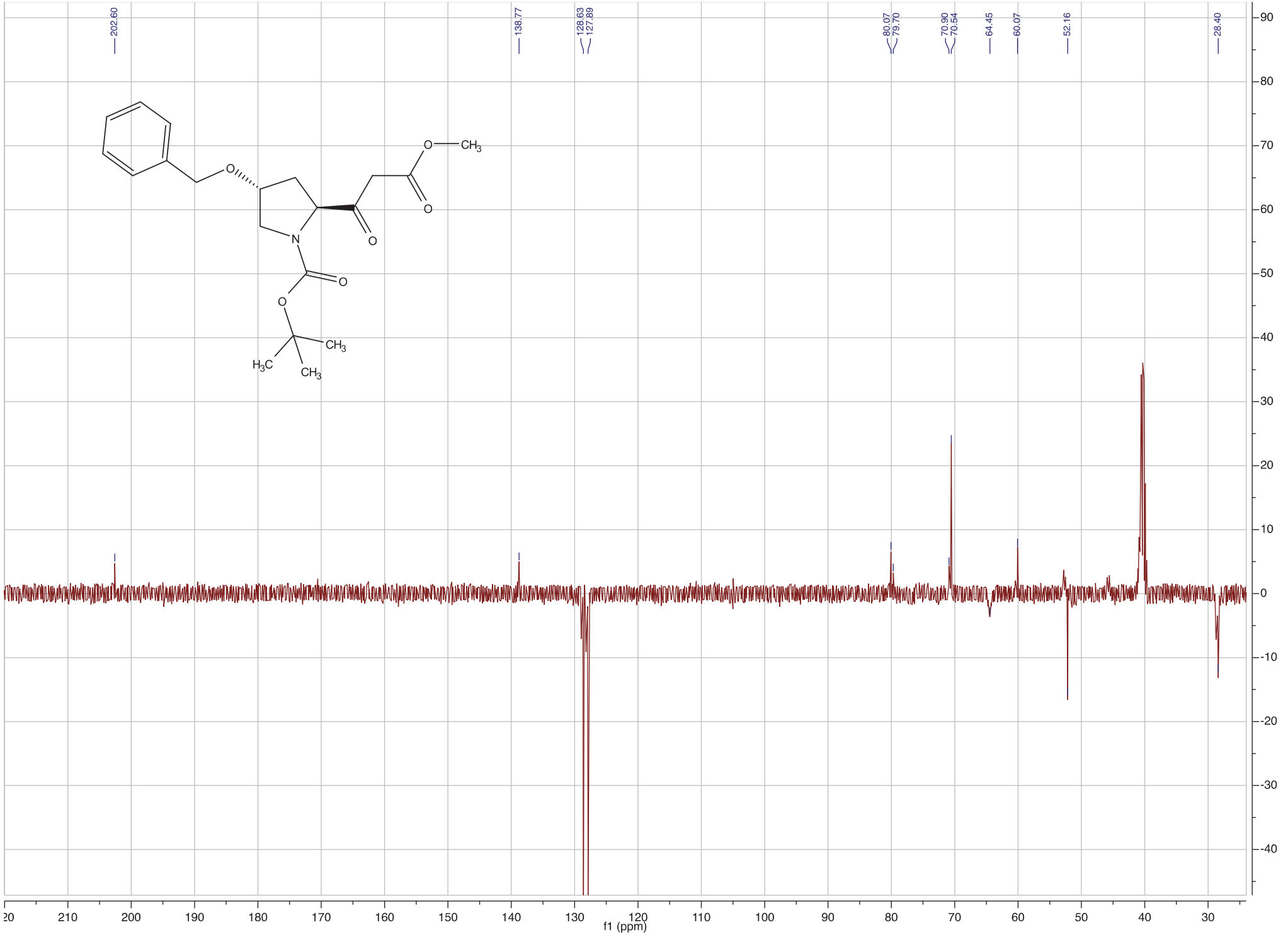
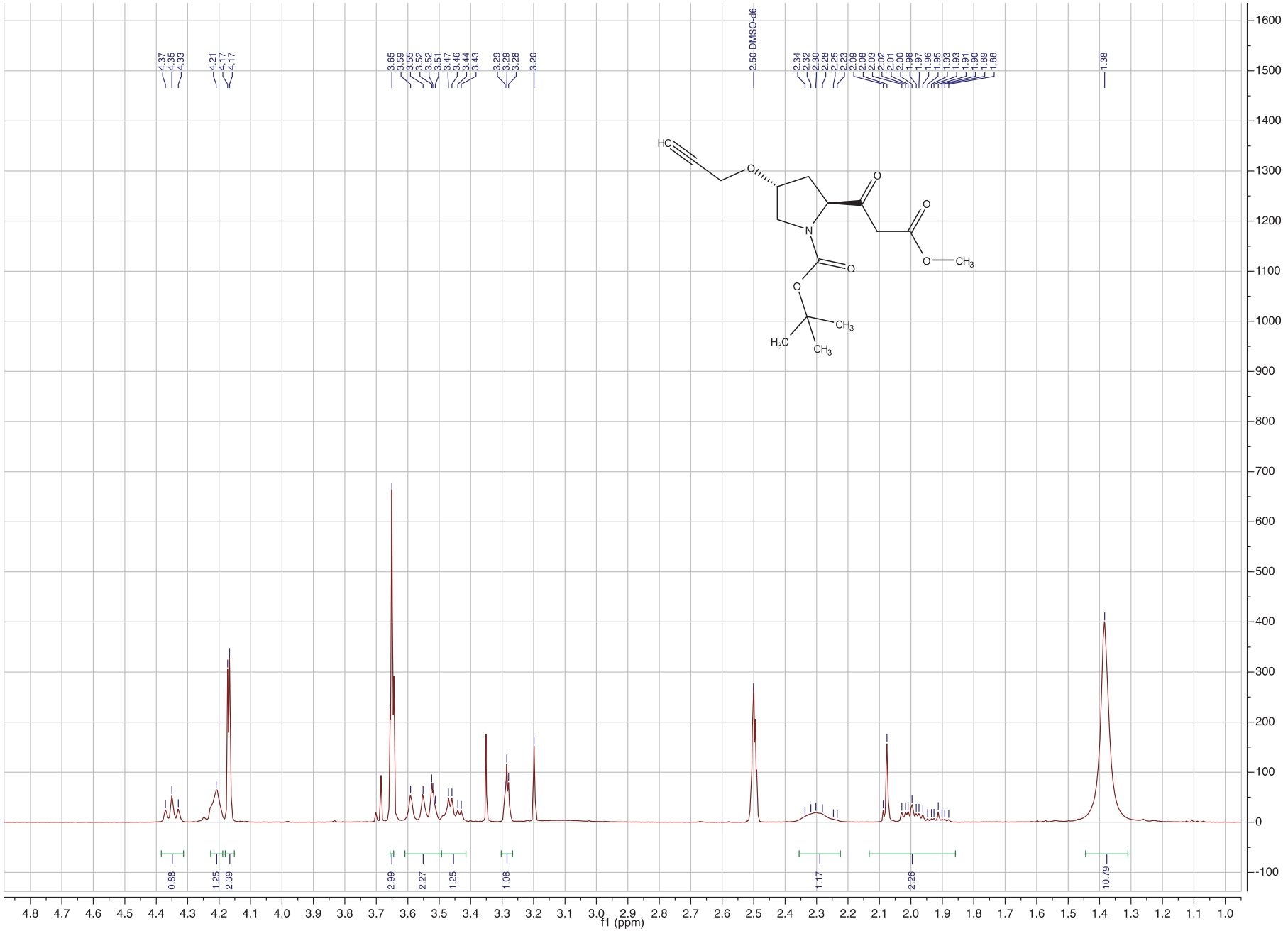
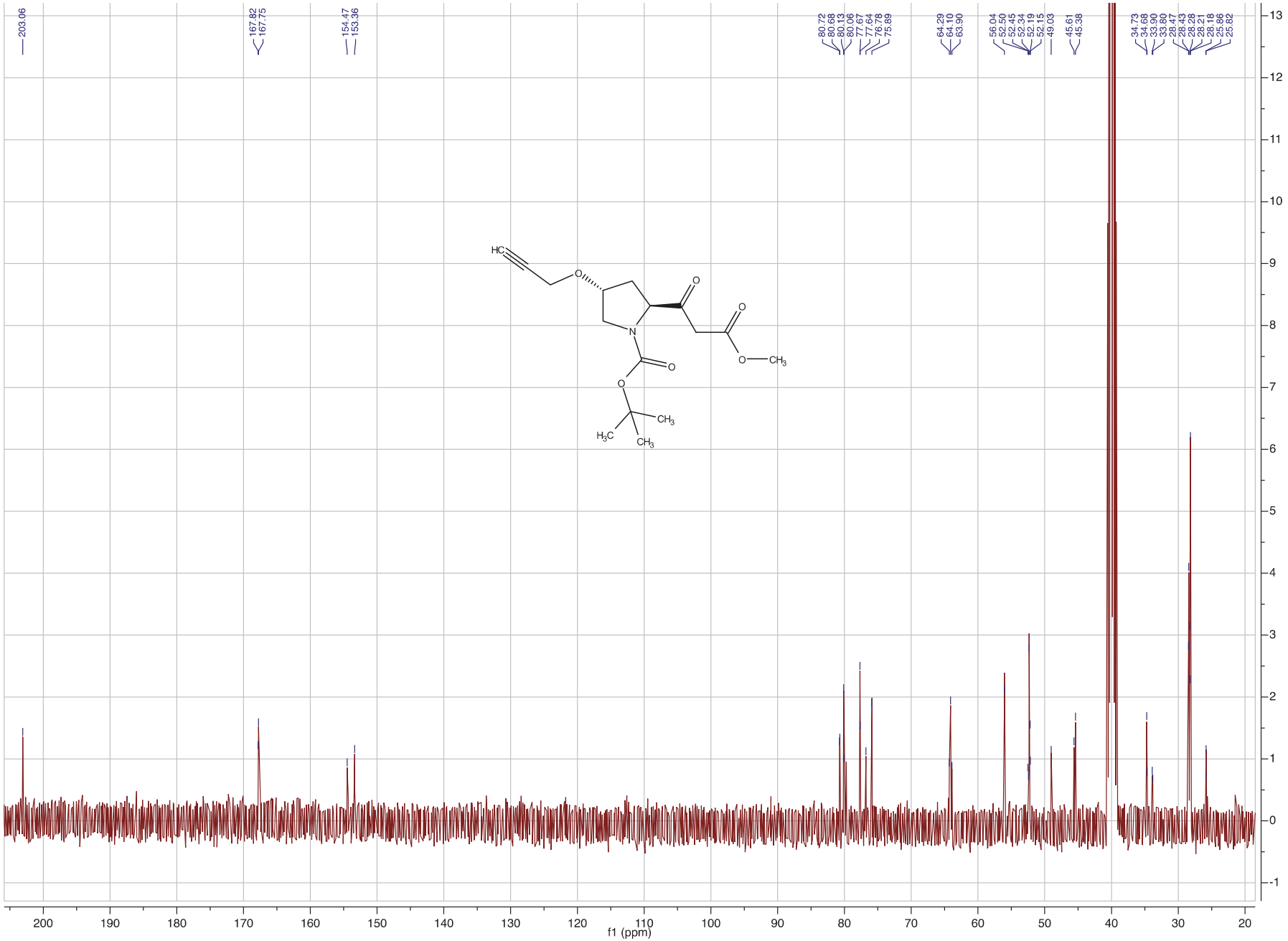
129.31
129.23
128.50
128.34
80.62
80.49
77.55
76.66
70.84
70.76
64.89
64.60
52.95
52.87
52.74
46.06
46.05
35.45
28.81
28.80
O
O
O
O
N
O
O
CH3
H3C
CH3
CH3
f1 (ppm)


202.60
138.77
128.63
127.89
80.07
79.70
70.90
70.54
64.45
60.07
52.16
28.40
O
CH3
O
O
O
N
O
O
CH3
H3C
CH3
f1 (ppm)


4.37
4.35
4.33
4.21
4.17
4.17
3.65
3.59
3.55
3.52
3.52
3.51
3.47
3.46
3.44
3.43
3.29
3.29
3.28
3.20
2.50 DMSO-d6
2.34
2.32
2.30
2.28
2.25
2.23
2.09
2.08
2.03
2.02
2.01
2.00
1.98
1.97
1.96
1.95
1.93
1.93
1.91
1.90
1.89
1.88
1.38
HC
O
O
O
O
O
N
O
O
CH3
H3C
CH3
0.88
1.25
2.39
2.99
2.27
1.25
1.08
1.17
2.26
10.79
f1 (ppm)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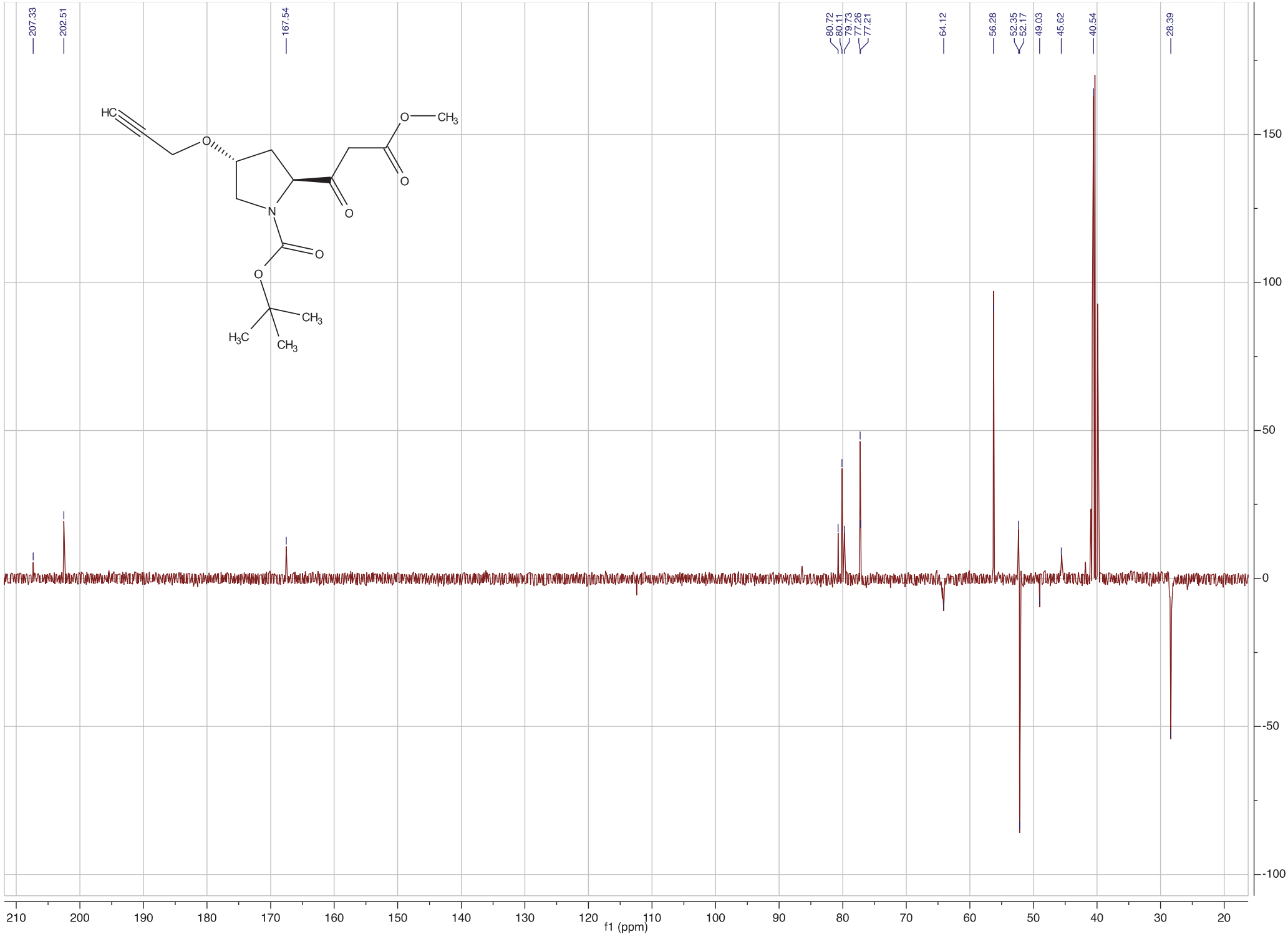
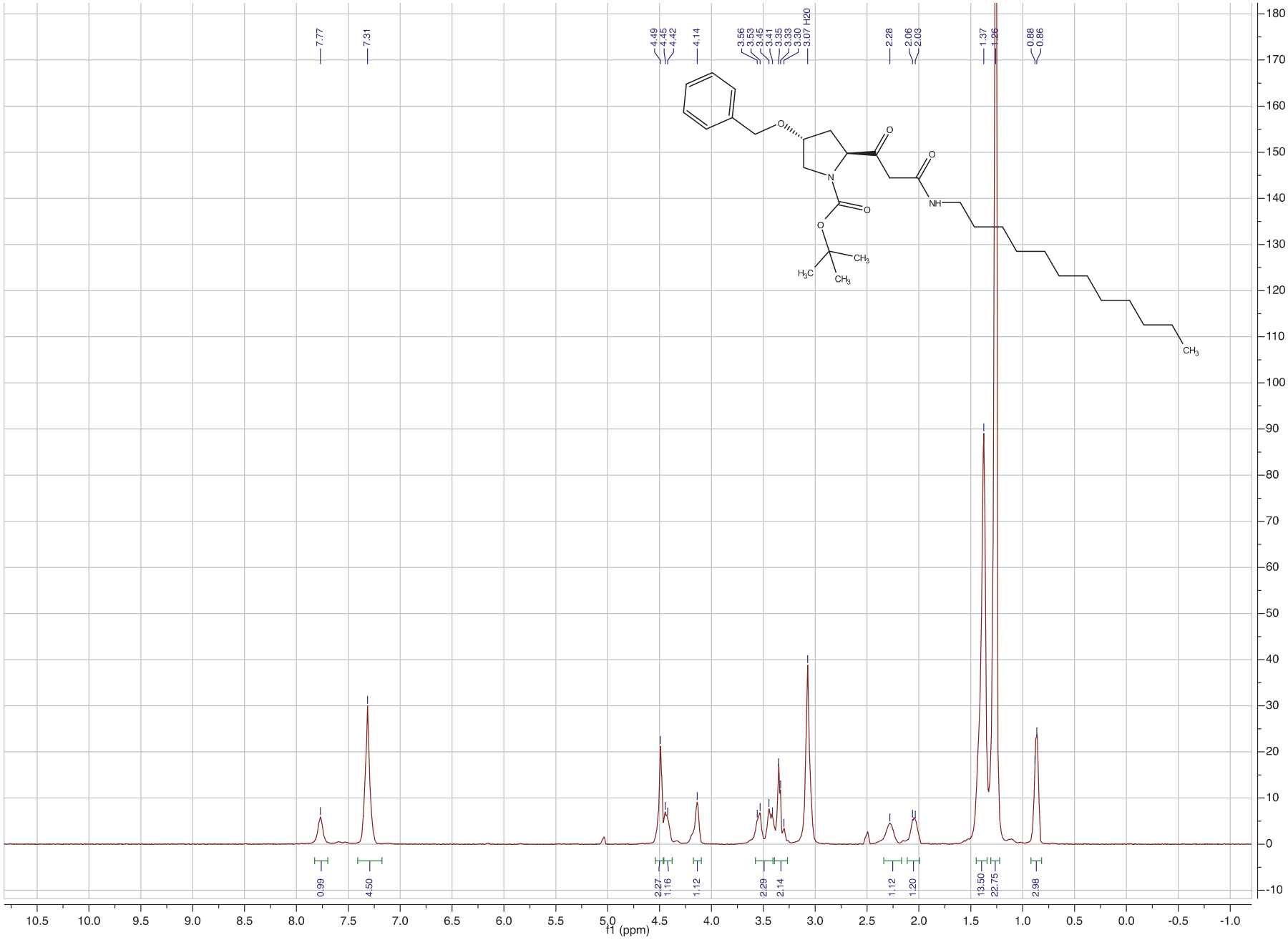
167.82
167.75
154.47
153.36
80.72
80.68
80.13
80.06
77.67
77.64
76.78
75.89
64.29
64.10
63.90
56.04
52.50
52.45
52.34
52.19
52.15
49.03
45.61
45.38
34.73
34.68
33.90
33.80
28.47
28.43
28.28
28.21
28.18
25.86
25.82
HC
O
O
O
N
O
O
O
CH3
H3C
CH3
CH3
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
f1 (ppm)
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-1


207.33
202.51
167.54
80.72
80.11
79.73
77.26
77.21
64.12
56.28
52.35
52.17
49.03
45.62
40.54
28.39
HC
O
O
O
O
O
CH3
N
O
O
CH3
H3C
CH3
150
100
50
0
-50
-100
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
f1 (ppm)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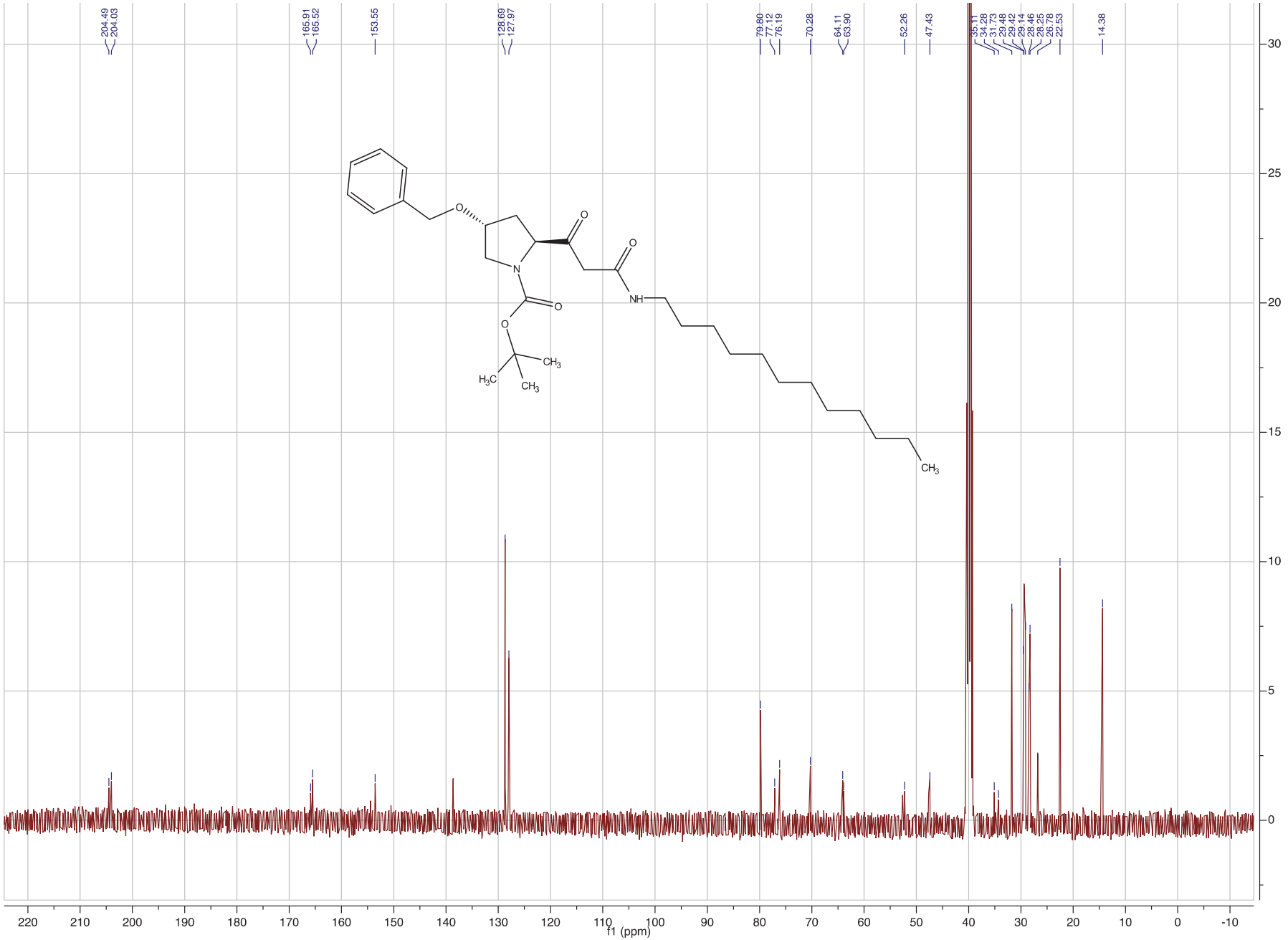
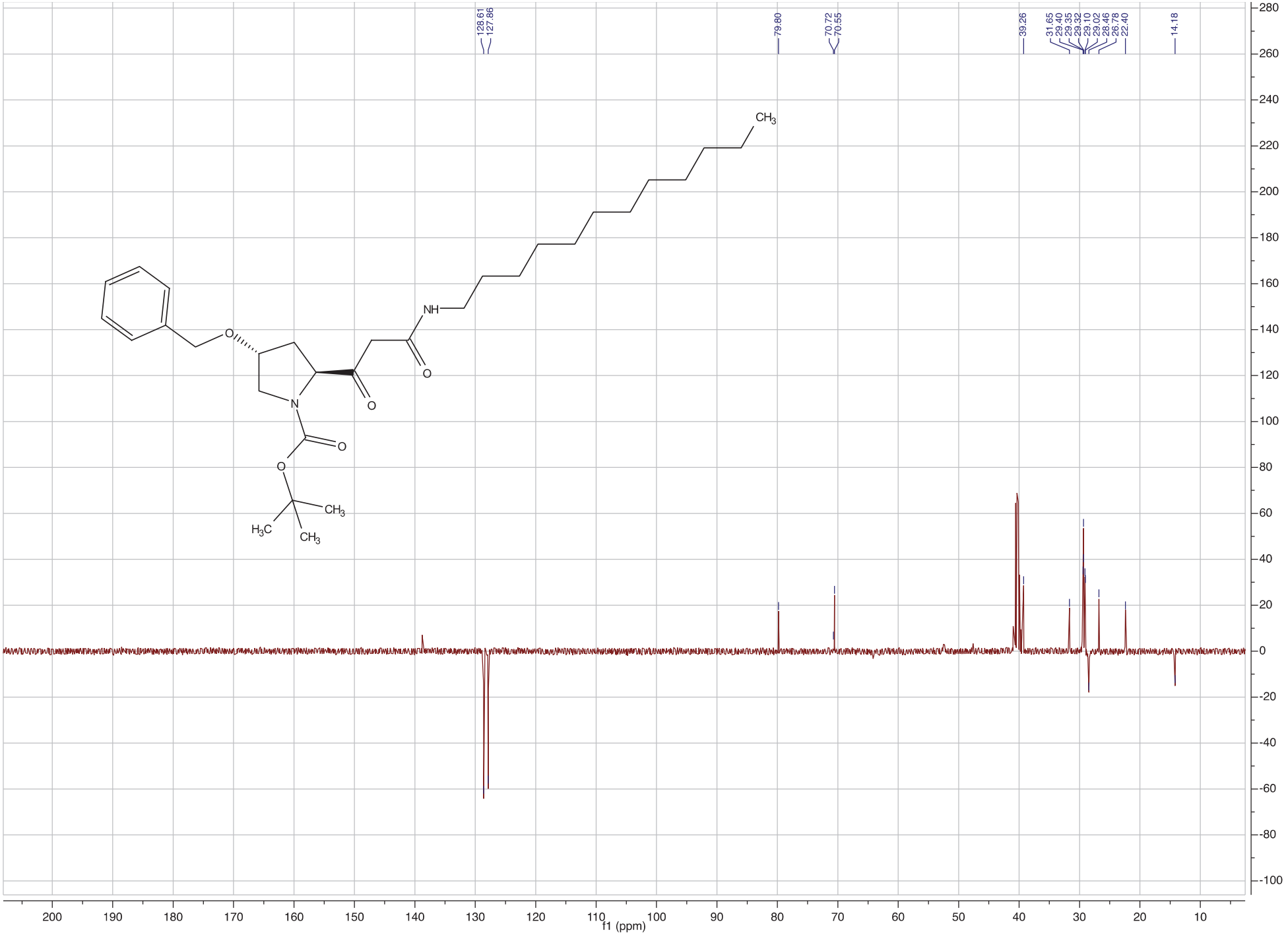
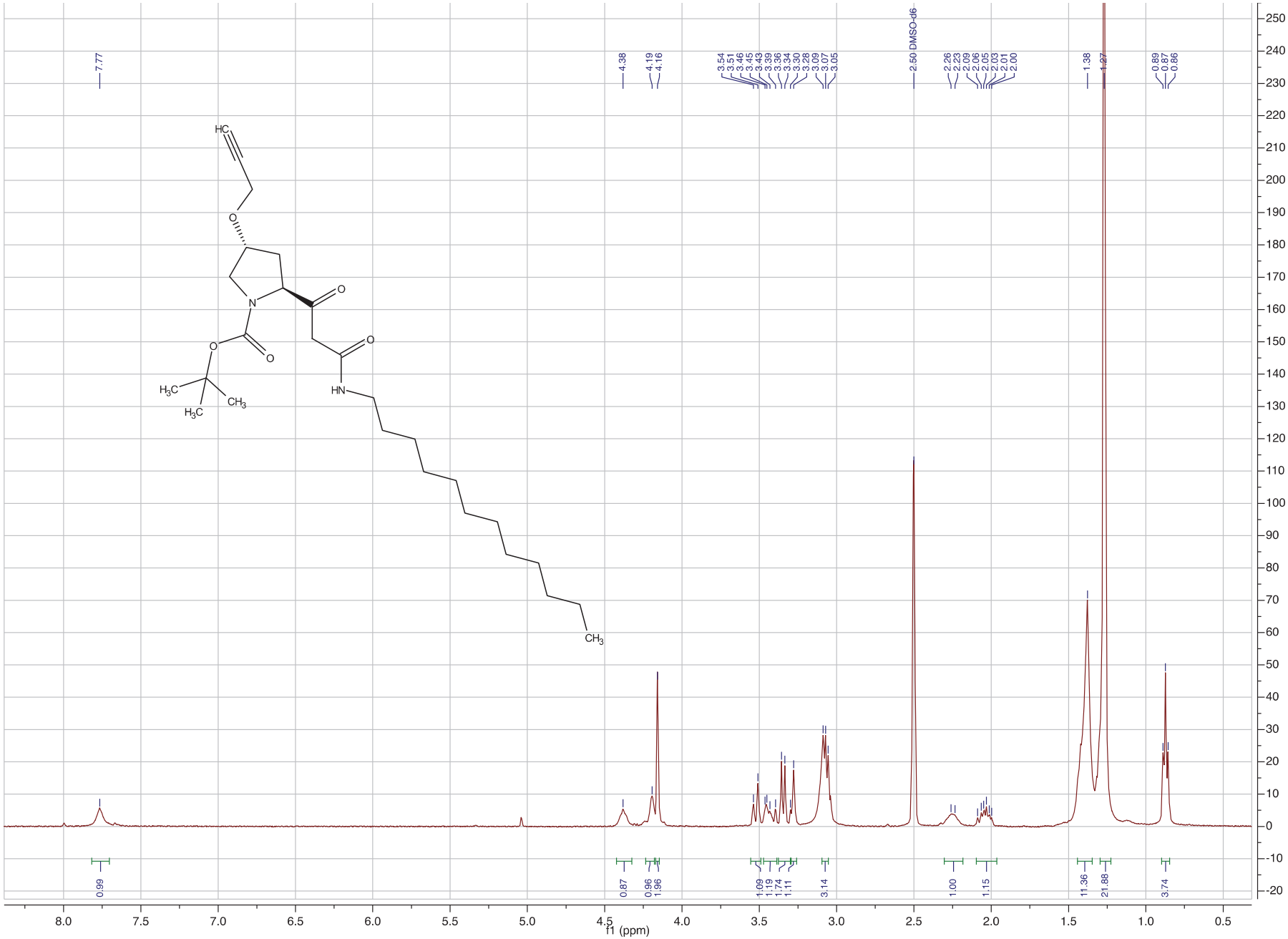
7.31
4.49
4.45
4.42
4.14
3.56
3.53
3.45
3.41
3.35
3.33
3.30
3.07 H20
2.28
2.06
2.03
1.37
1.36
0.88
0.86
0.99
4.50
2.27
1.16
1.12
2.29
2.14
1.12
1.20
13.50
22.75
2.98
f1 (ppm)


204.49
204.03
165.91
165.52
153.55
128.69
127.97
79.80
77.12
76.19
70.28
64.11
63.90
52.26
47.43
35.11
34.28
31.73
29.48
29.42
29.14
28.46
28.25
26.78
22.53
14.38
O
N
O
O
O
O
NH
CH3
H3C
CH3
CH3
30
25
20
15
10
5
0
220
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)


128.61
127.86
79.80
70.72
70.55
39.26
31.65
29.40
29.35
29.32
29.10
29.02
28.46
26.78
22.40
14.18
CH3
NH
O
O
O
N
O
O
CH3
H3C
CH3
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)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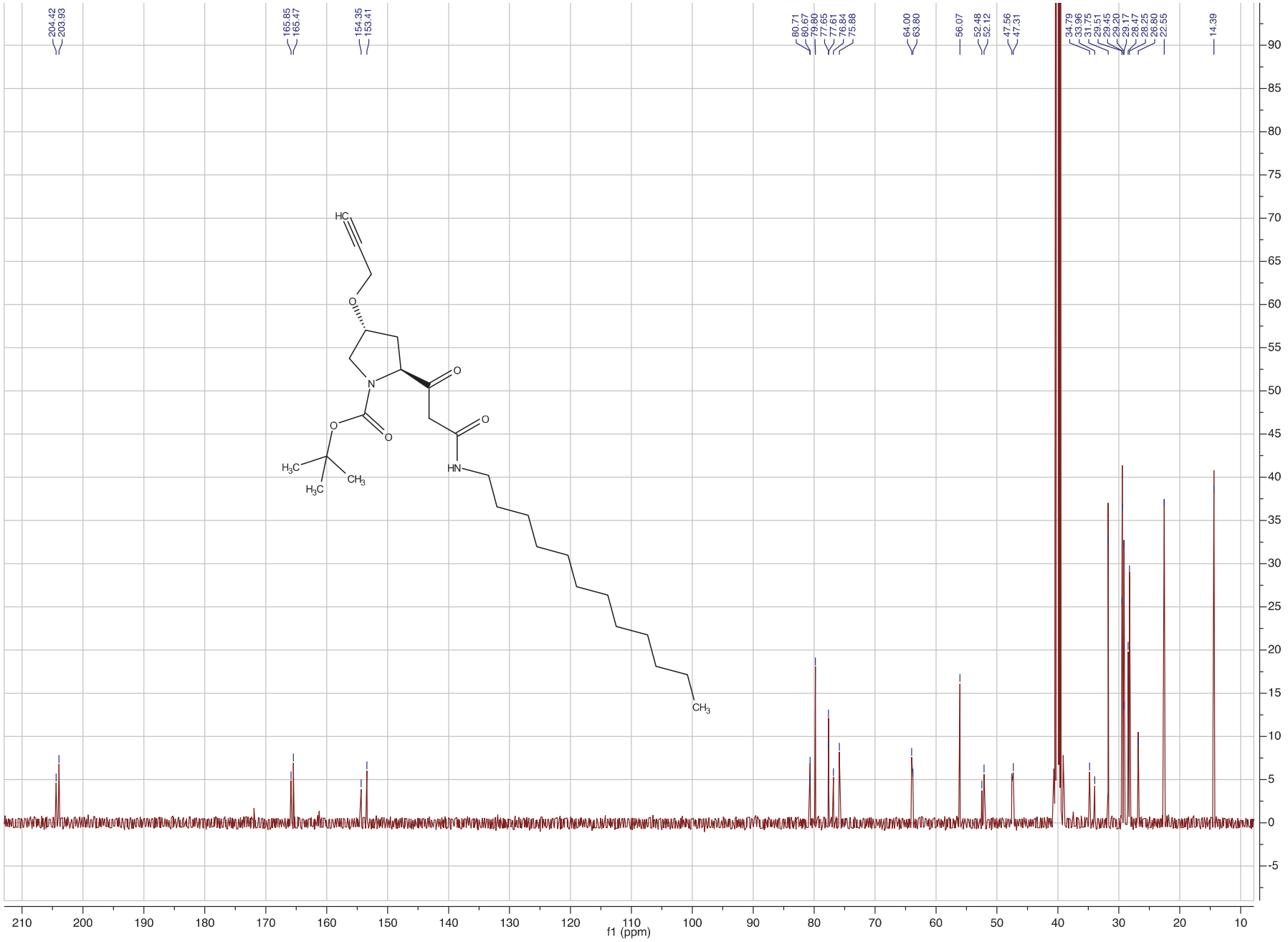
4.38
4.19
4.16
3.54
3.51
3.46
3.43
3.39
3.36
3.34
3.30
3.28
3.09
3.07
3.05
2.50 DMSO-d6
2.26
2.23
2.09
2.06
2.05
2.03
2.01
2.00
1.38
1.27
0.89
0.87
0.86
HC
O
N
O
O
O
O
O
HN
H3C
CH3
H3C
CH3
0.99
0.87
0.96
1.96
1.09
1.19
1.74
1.11
3.14
1.00
1.15
11.36
21.88
3.74
f1 (ppm)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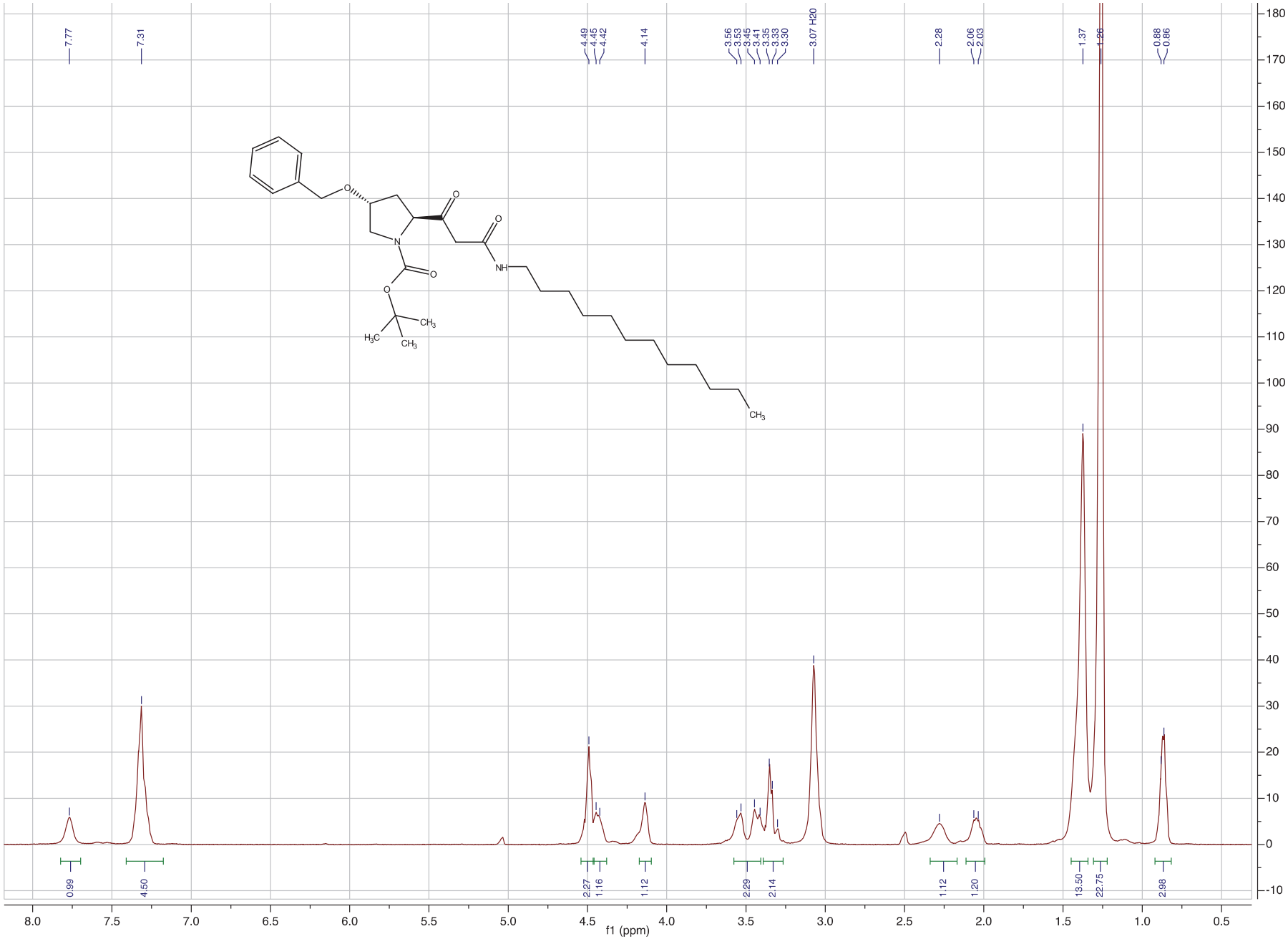
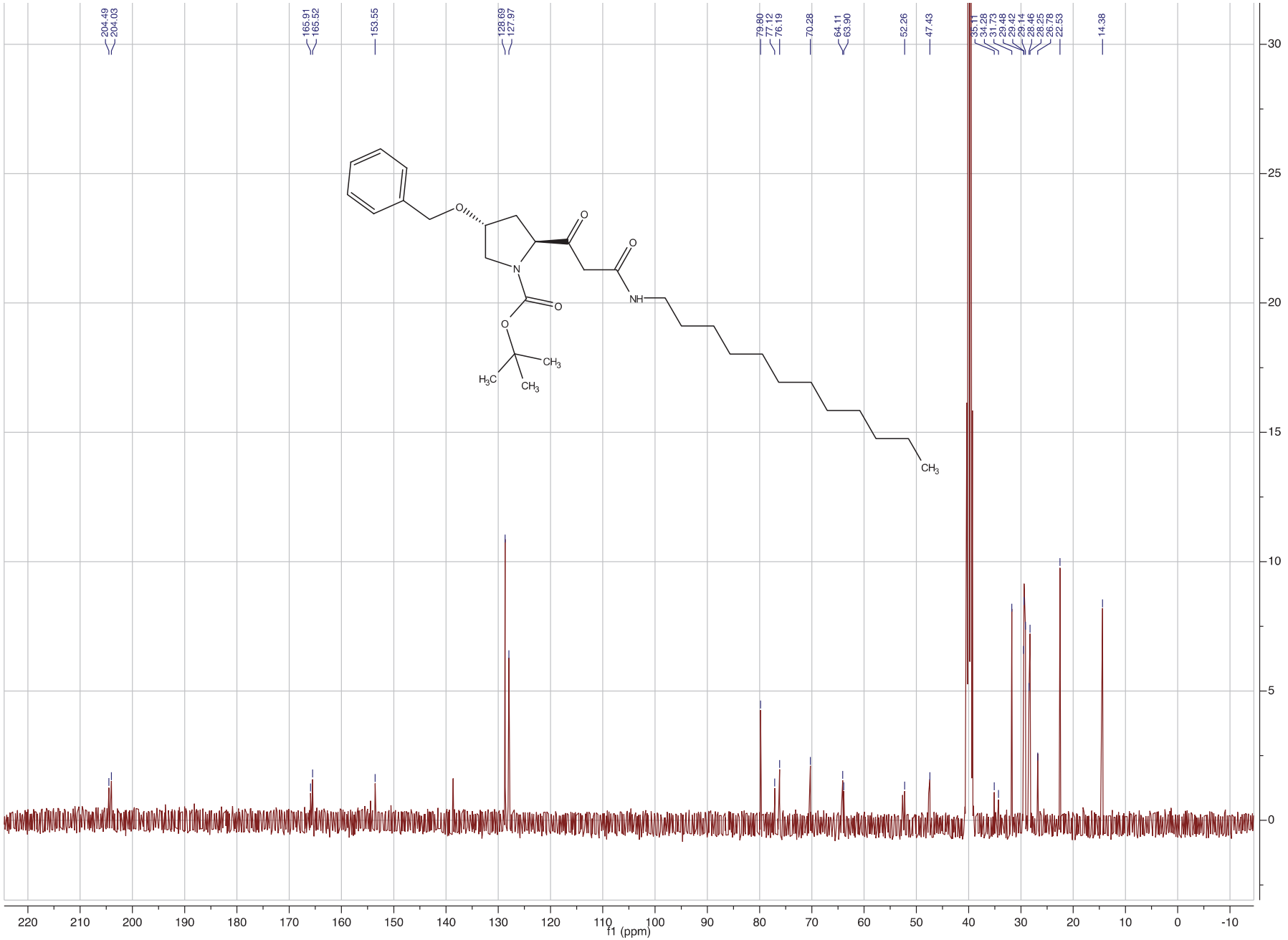
203.93
165.85
165.47
154.35
153.41
80.71
80.67
79.80
77.65
77.61
76.84
75.88
64.00
63.80
56.07
52.48
52.12
47.56
47.31
34.79
33.96
31.75
29.51
29.45
29.20
29.17
28.47
28.25
26.80
22.55
14.39
HC
O
N
O
O
O
O
HN
H3C
CH3
H3C
CH3
f1 (ppm)


7.77
7.31
4.49
4.45
4.42
4.14
3.56
3.53
3.45
3.41
3.35
3.33
3.30
3.07 H20
2.28
2.06
2.03
1.37
1.26
0.88
0.86
O
O
O
N
O
O
NH
CH3
H3C
CH3
CH3
0.99
4.50
2.27
1.16
1.12
2.29
2.14
1.12
1.20
13.50
22.75
2.98
f1 (ppm)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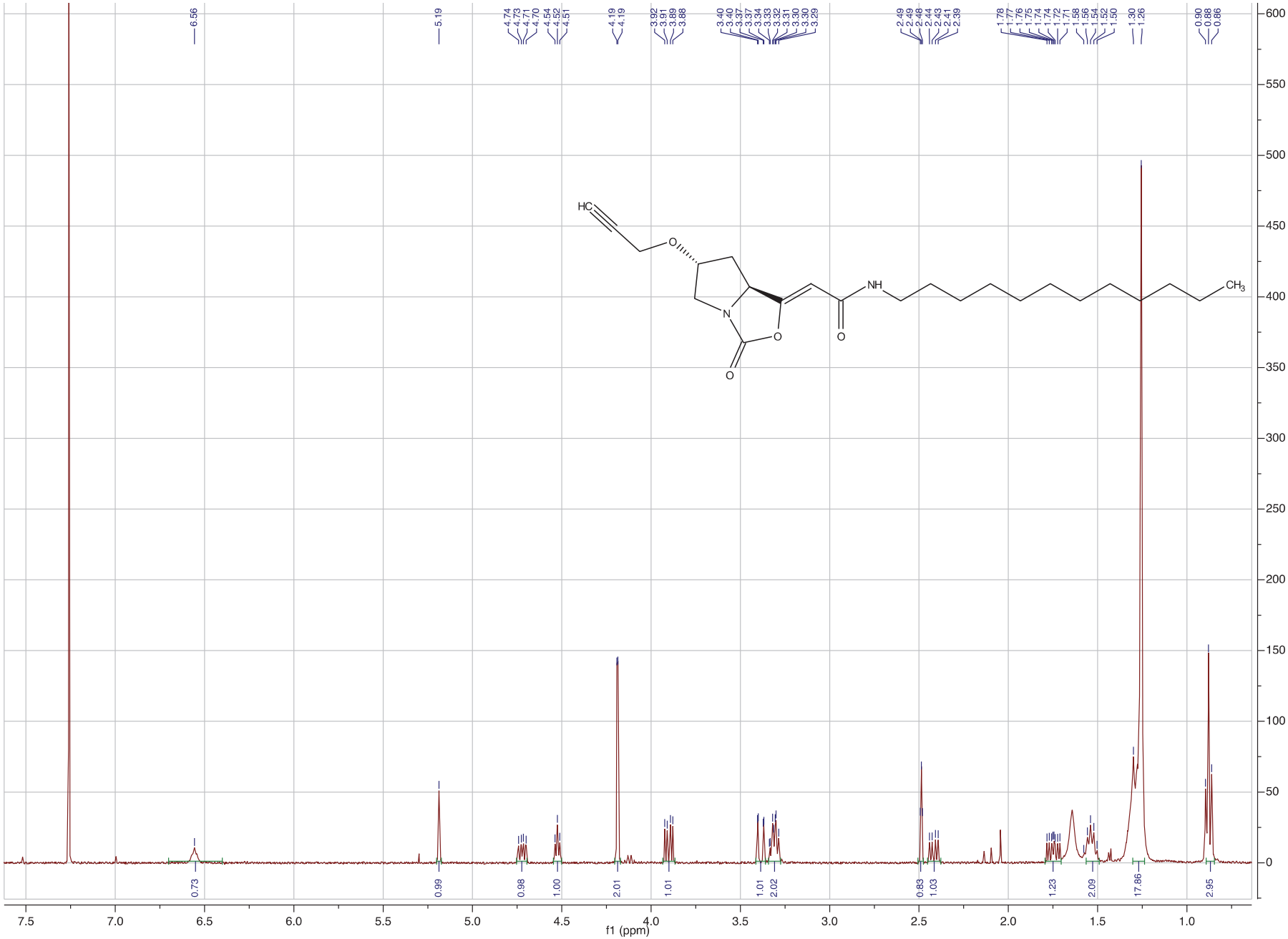
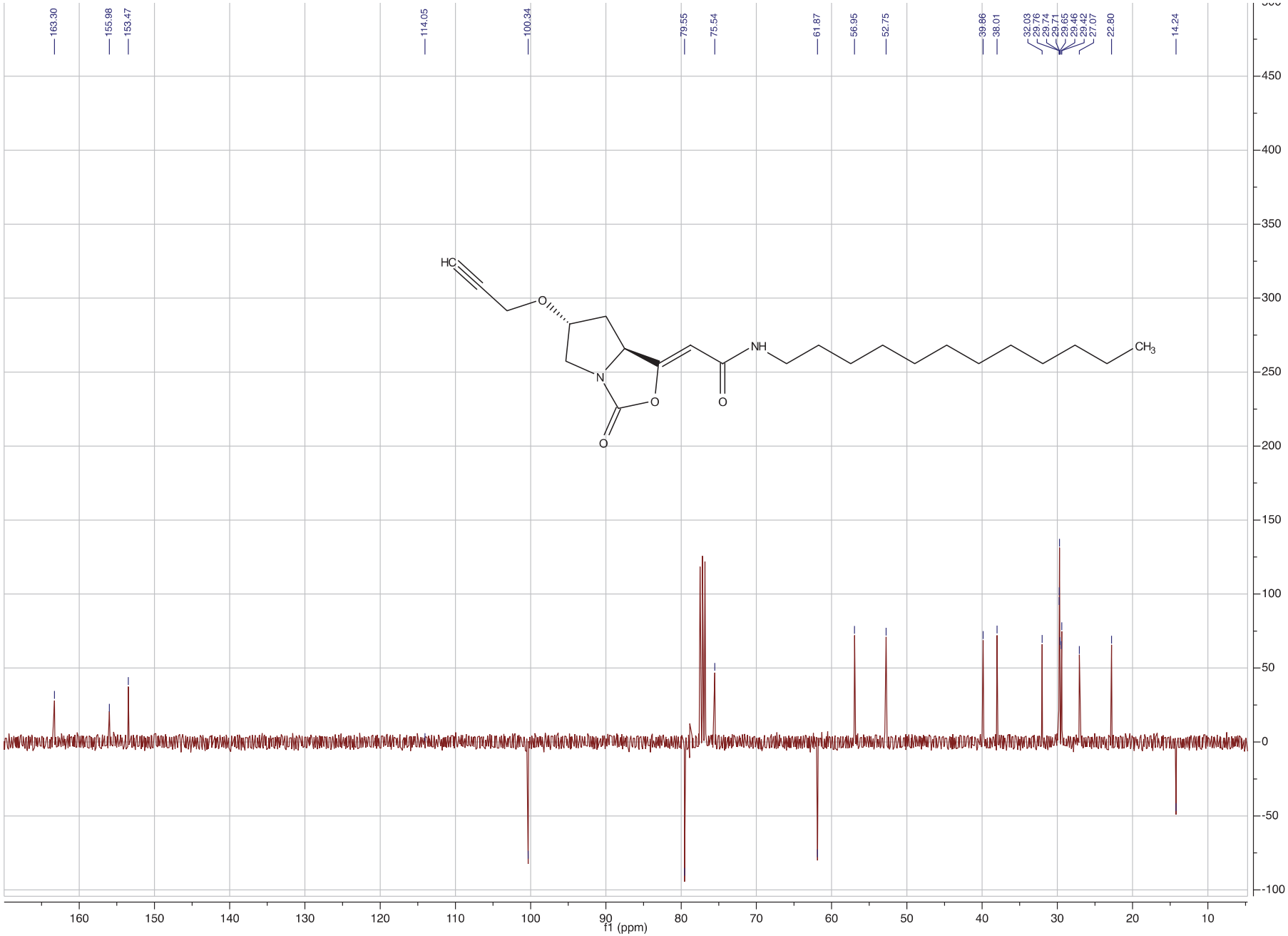
204.03
165.91
165.52
153.55
128.69
127.97
79.80
77.12
76.19
70.28
64.11
63.90
52.26
47.43
35.11
34.28
31.73
29.48
29.42
29.14
28.46
28.25
26.78
22.53
14.38
O
N
O
O
O
O
NH
CH3
H3C
CH3
CH3
30
25
20
15
10
5
0
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)


6.56
5.19
4.74
4.73
4.71
4.70
4.54
4.52
4.51
4.19
4.19
3.92
3.91
3.89
3.88
3.40
3.37
3.37
3.34
3.33
3.32
3.31
3.30
3.30
3.29
2.49
2.49
2.46
2.44
2.43
2.41
2.39
1.78
1.77
1.76
1.75
1.74
1.74
1.72
1.71
1.58
1.56
1.54
1.52
1.50
1.30
1.26
0.90
0.88
0.86
HC
O
N
NH
O
O
O
CH3
0.73
0.99
0.98
1.00
2.01
1.01
1.01
2.02
0.83
1.03
1.23
2.09
17.86
2.95
f1 (ppm)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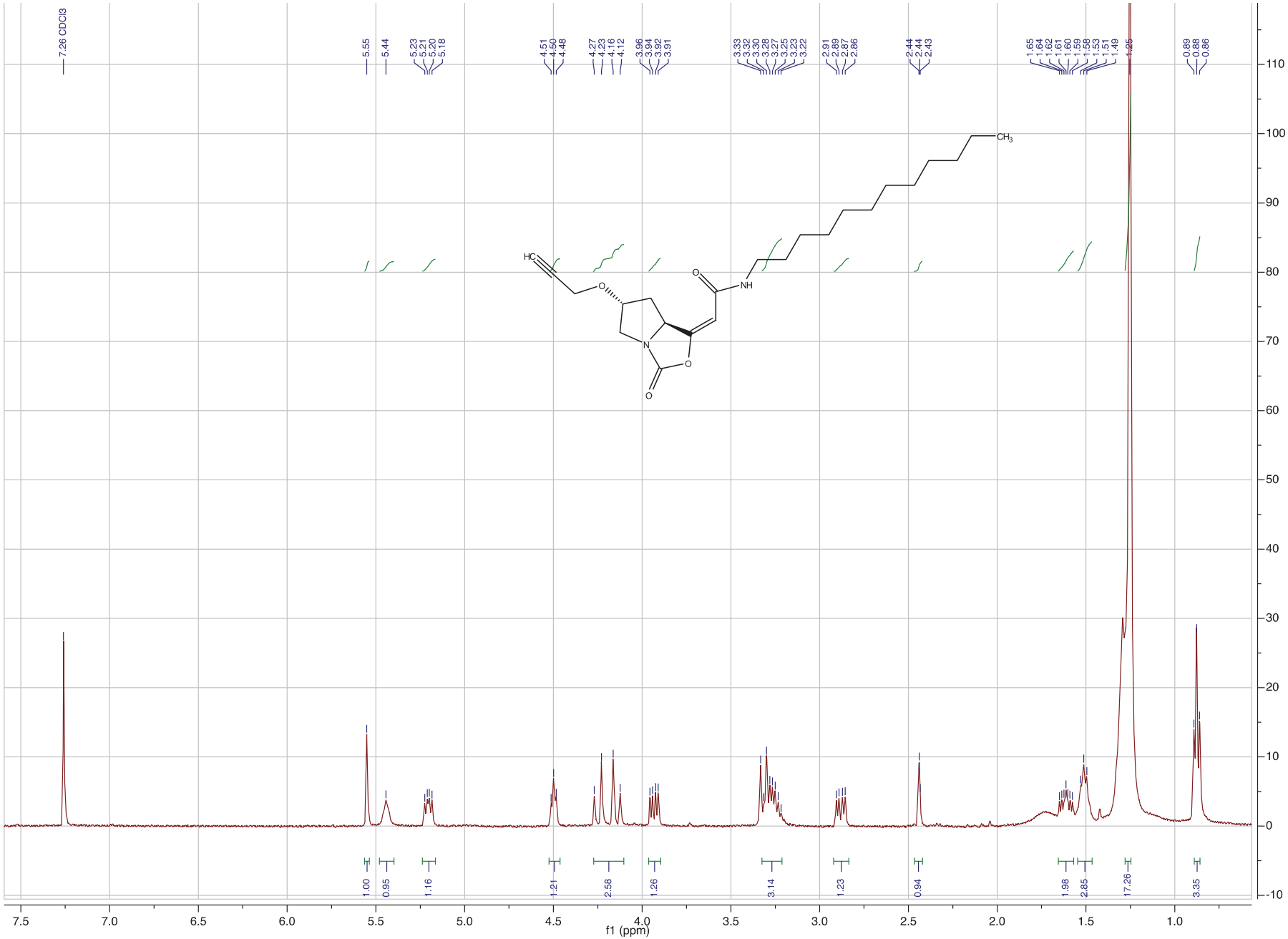
155.98
153.47
114.05
100.34
79.55
75.54
61.87
56.95
52.75
39.86
38.01
32.03
29.76
29.74
29.71
29.65
29.46
29.42
27.07
22.80
14.24
HC
O
N
O
O
O
NH
CH3
450
400
350
300
250
200
150
100
50
0
-50
-100
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)


7.26 CDCl3
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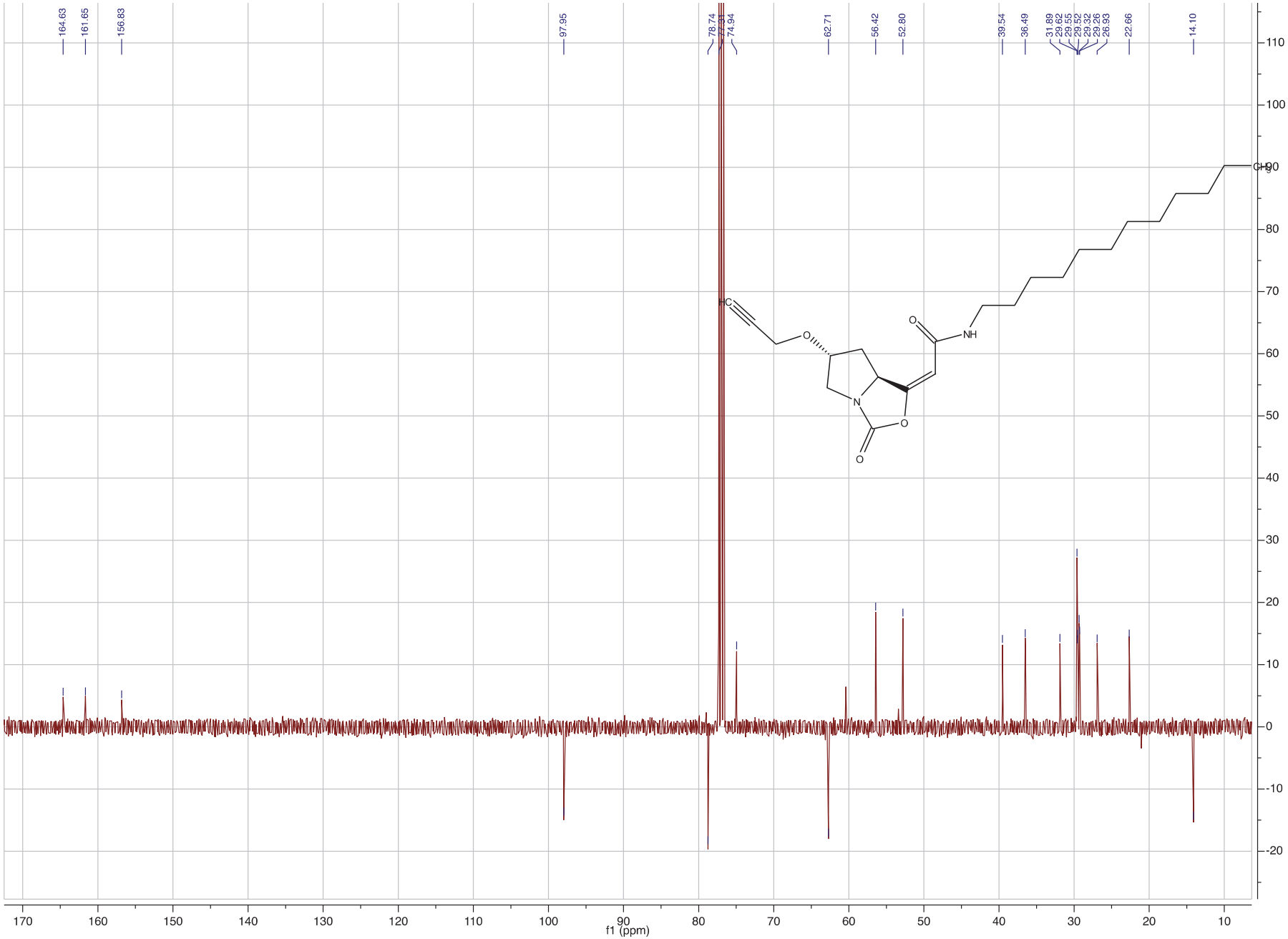
5.44
5.23
5.21
5.20
5.18
4.51
4.50
4.48
4.27
4.23
4.16
4.12
3.96
3.94
3.92
3.91
3.33
3.32
3.30
3.28
3.27
3.25
3.23
3.22
2.91
2.89
2.87
2.86
2.44
2.44
2.43
1.65
1.64
1.62
1.61
1.60
1.59
1.58
1.53
1.51
1.49
1.25
0.89
0.88
0.86
HC
O
O
NH
O
N
O
O
CH3
1.00
0.95
1.16
1.21
2.58
1.26
3.14
1.23
0.94
1.98
2.85
17.26
3.35
f1 (ppm)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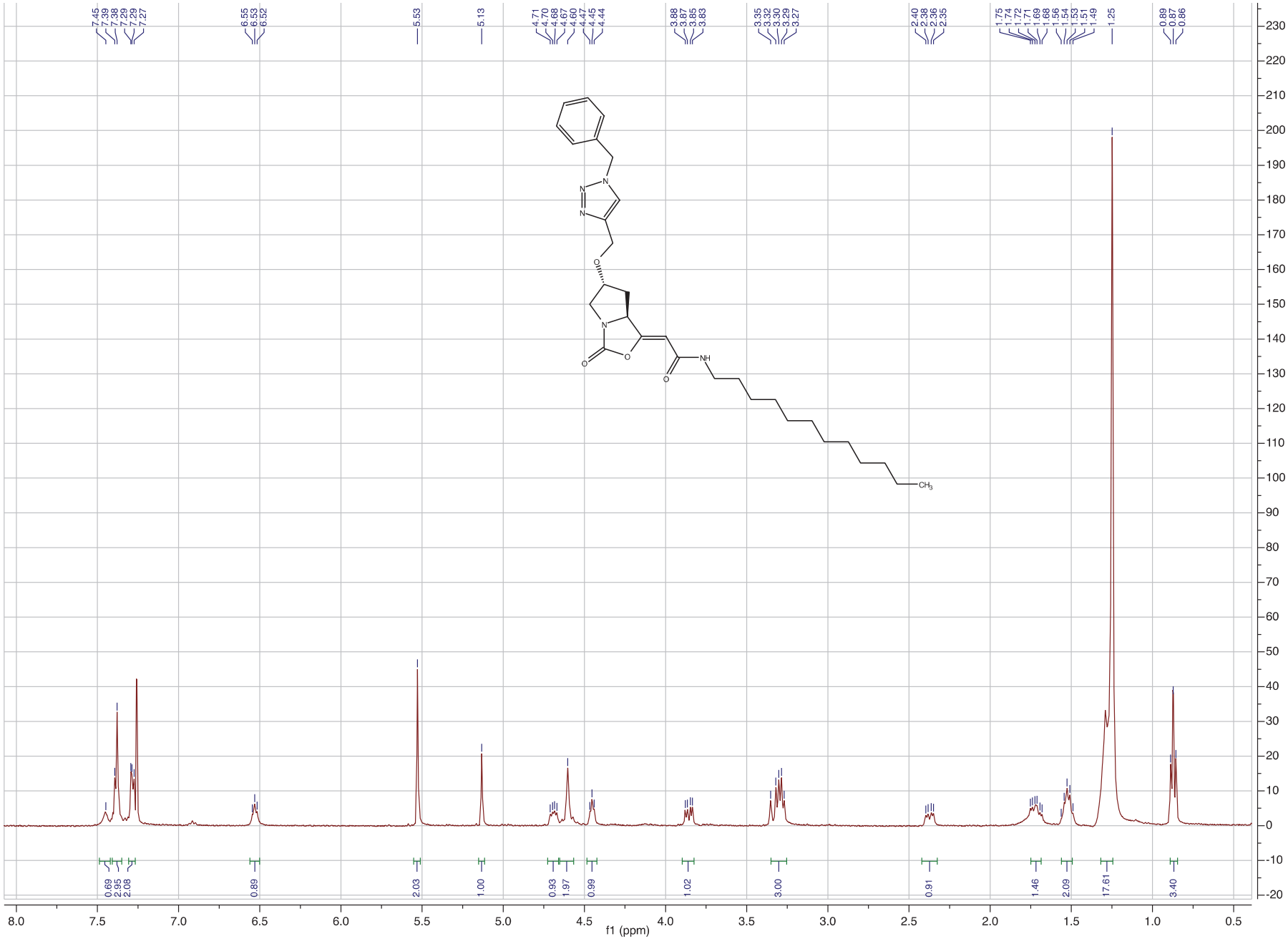
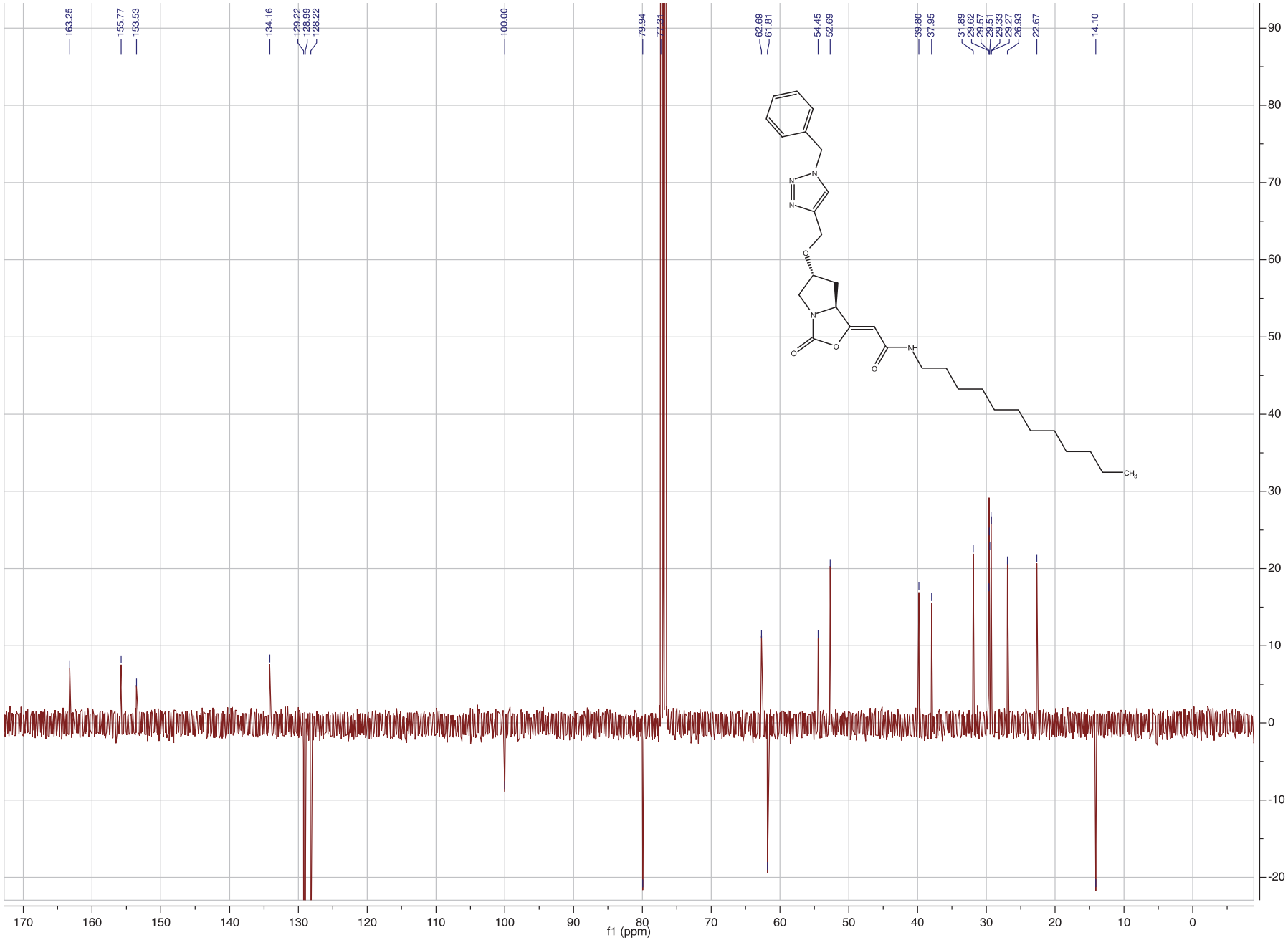
161.65
156.83
97.95
78.74
77.31
74.94
62.71
56.42
52.80
39.54
36.49
31.89
29.62
29.55
29.52
29.32
29.26
26.93
22.66
14.10
HC
O
O
NH
N
O
O
CH3
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)


7.45
7.39
7.38
7.29
7.29
7.27
6.55
6.53
6.52
5.53
5.13
4.71
4.70
4.68
4.67
4.60
4.47
4.45
4.44
3.88
3.87
3.85
3.83
3.35
3.32
3.30
3.29
3.27
2.40
2.38
2.36
2.35
1.75
1.74
1.72
1.71
1.69
1.68
1.56
1.54
1.53
1.51
1.49
1.25
0.89
0.87
0.86
0.69
2.95
2.08
0.89
2.03
1.00
0.93
1.97
0.99
1.02
3.00
0.91
1.46
2.09
17.61
3.40
f1 (ppm)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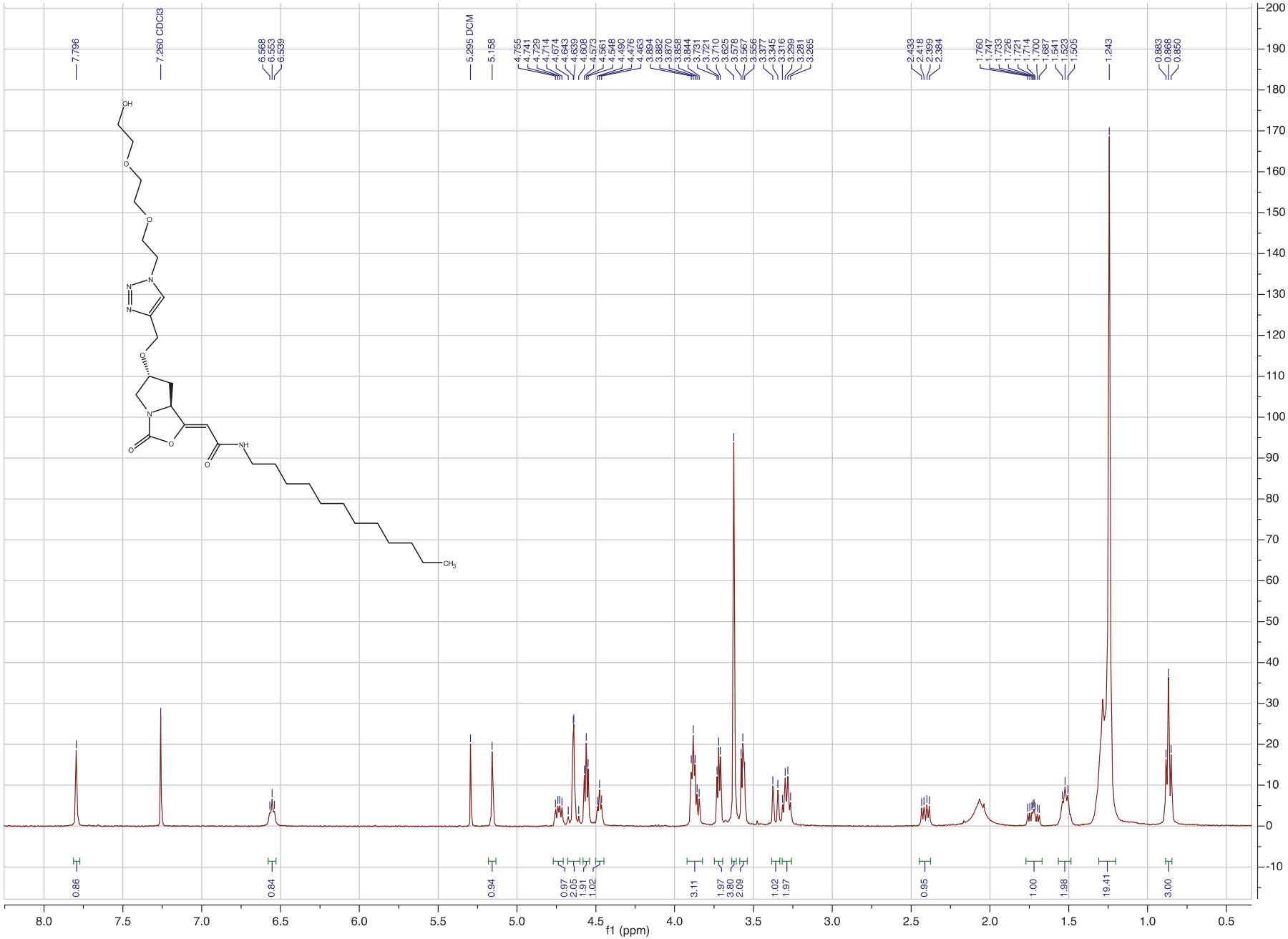
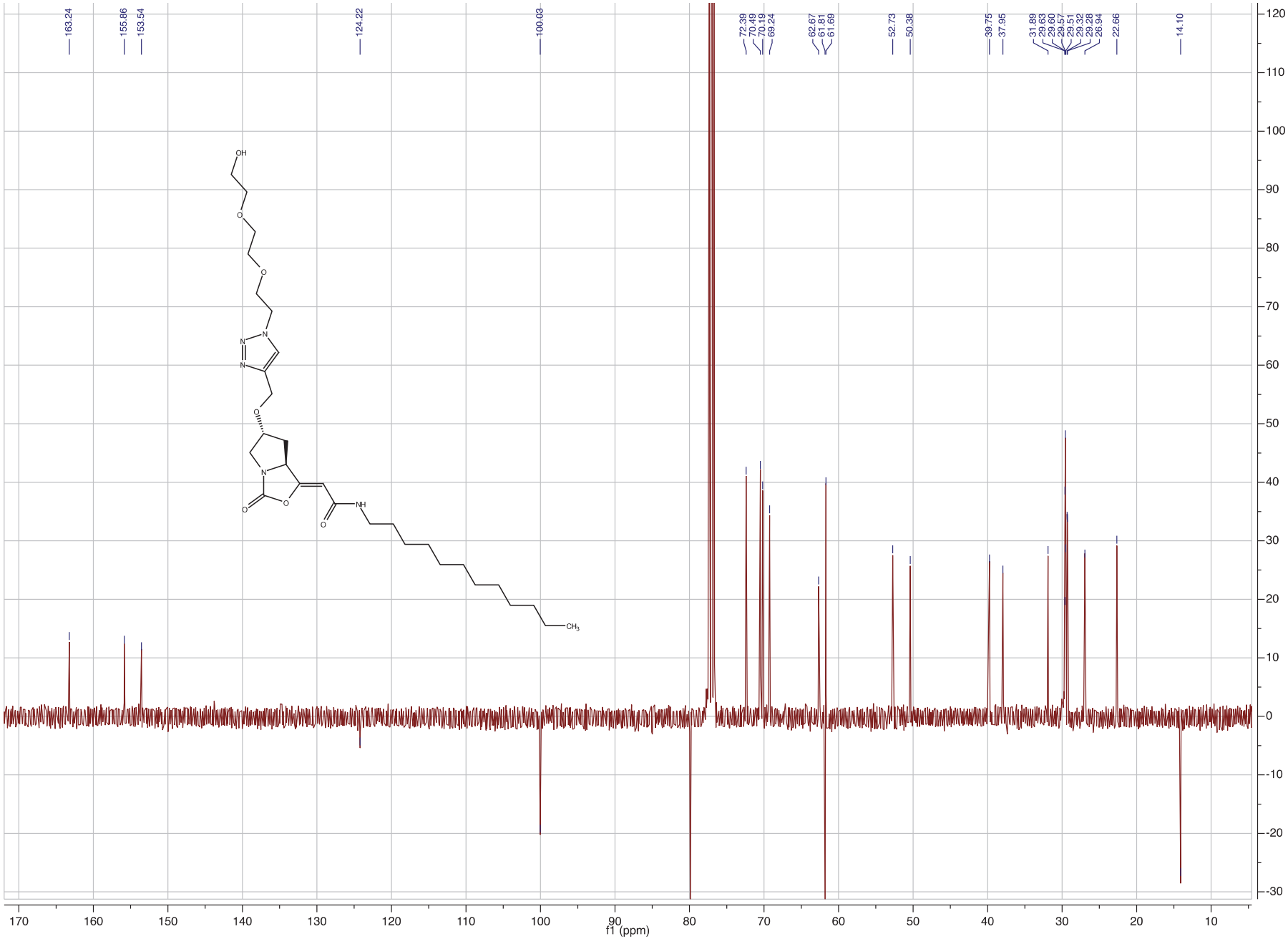
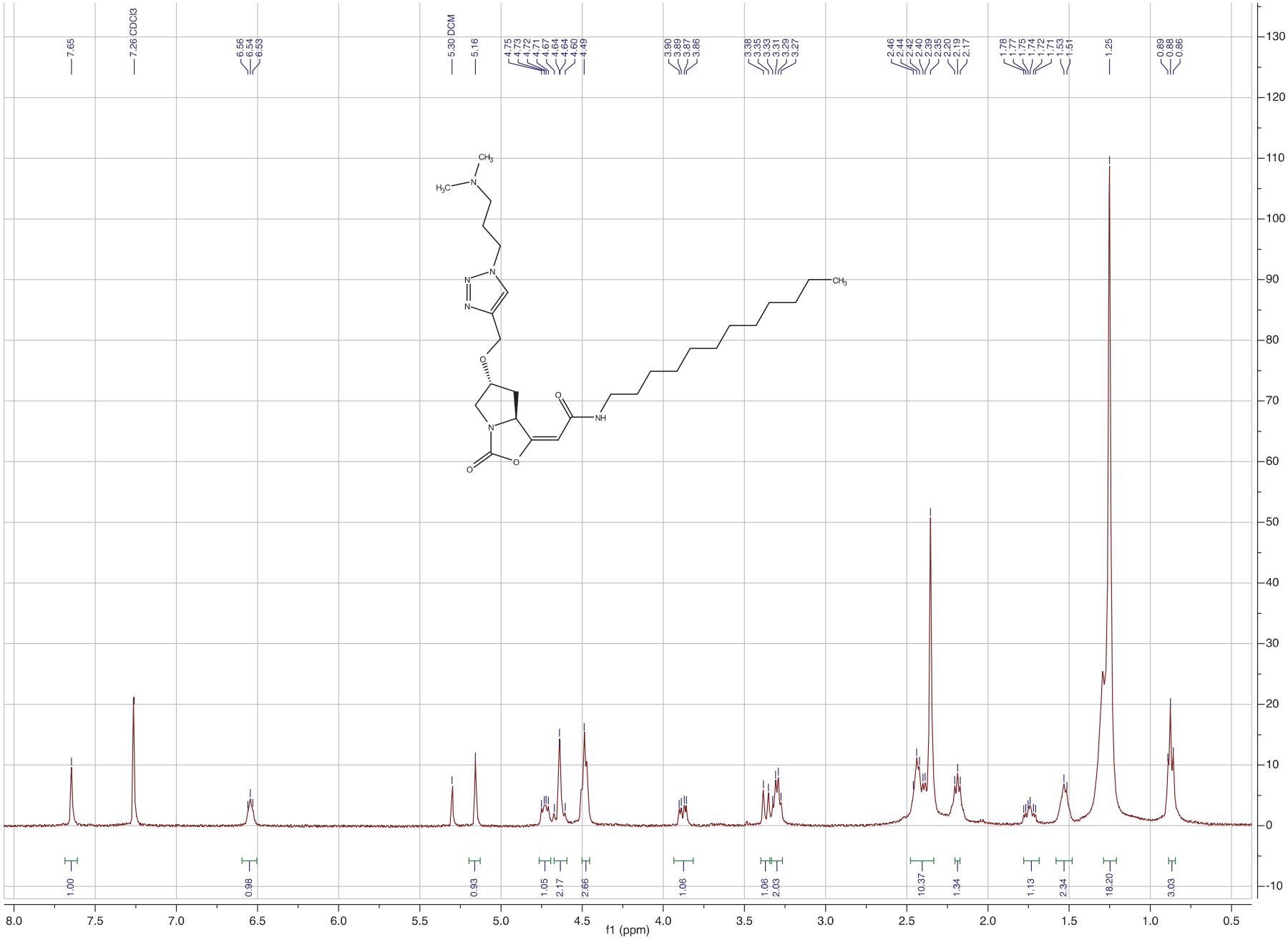
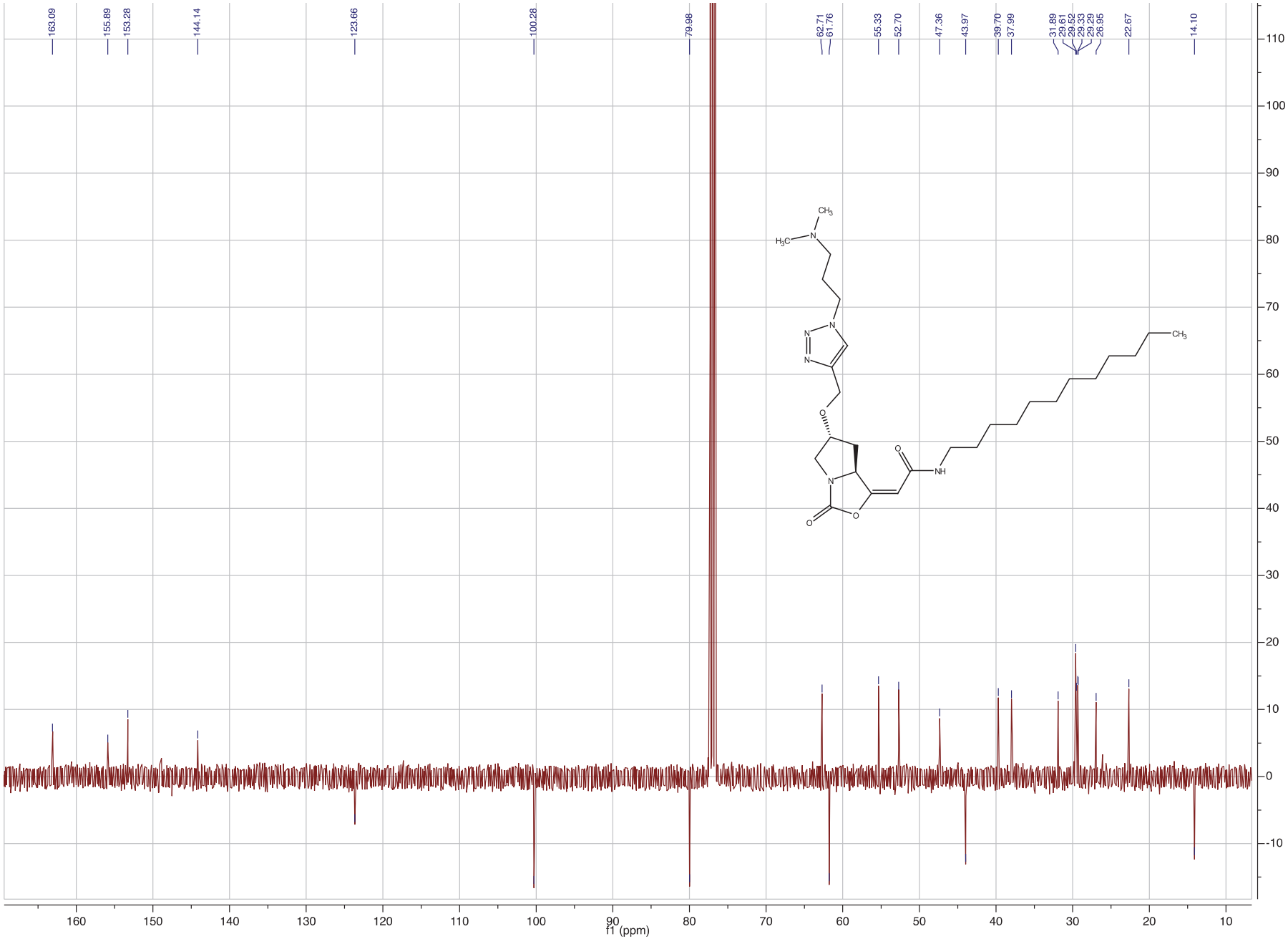
155.77
153.53
134.16
129.22
128.99
128.22
100.00
79.94
77.31
62.69
61.81
54.45
52.69
39.80
37.95
31.89
29.62
29.57
29.51
29.33
29.27
26.93
22.67
14.10
f1 (ppm)


7.796
7.260 CDCl3
6.568
6.553
6.539
5.295 DCM
5.158
4.755
4.741
4.729
4.714
4.674
4.643
4.639
4.608
4.573
4.561
4.548
4.490
4.476
4.463
3.884
3.882
3.870
3.858
3.844
3.731
3.721
3.710
3.625
3.578
3.567
3.556
3.377
3.345
3.316
3.299
3.281
3.265
2.433
2.418
2.399
2.384
1.760
1.747
1.733
1.726
1.721
1.714
1.700
1.687
1.541
1.523
1.505
1.243
0.883
0.868
0.850
OH
O
O
O
N
N
N
O
N
O
O
O
NH
CH3
0.86
0.84
0.94
0.97
2.05
1.91
1.02
3.11
1.97
3.80
2.09
1.02
1.97
0.95
1.00
1.98
19.41
3.00
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
f1 (ppm)
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10


163.24
155.86
153.54
124.22
100.03
72.39
70.49
70.19
69.24
62.67
61.81
61.69
52.73
50.38
39.75
37.95
31.89
29.63
29.60
29.57
29.51
29.32
29.28
26.94
22.66
14.10
OH
O
O
N
N
N
O
N
O
O
O
NH
CH3
f1 (ppm)


7.65
7.26 CDCl3
6.56
6.54
6.53
5.30 DCM
5.16
4.75
4.73
4.72
4.71
4.67
4.64
4.64
4.60
4.49
3.90
3.89
3.87
3.86
3.38
3.35
3.33
3.31
3.29
3.27
2.46
2.44
2.42
2.40
2.39
2.35
2.20
2.19
2.17
1.78
1.77
1.75
1.74
1.72
1.71
1.53
1.51
1.25
0.89
0.88
0.86
1.00
0.98
0.93
1.05
2.17
2.66
1.06
1.06
2.03
10.37
1.34
1.13
2.34
18.20
3.03
f1 (ppm)


163.09
155.89
153.28
144.14
123.66
100.28
79.98
62.71
61.76
55.33
52.70
47.36
43.97
39.70
37.99
31.89
29.61
29.52
29.33
29.29
26.95
22.67
14.10
f1 (ppm)