Supplementary Information

Synthesis and biological evaluation of santacruzamate A and analogs as potential anticancer agents

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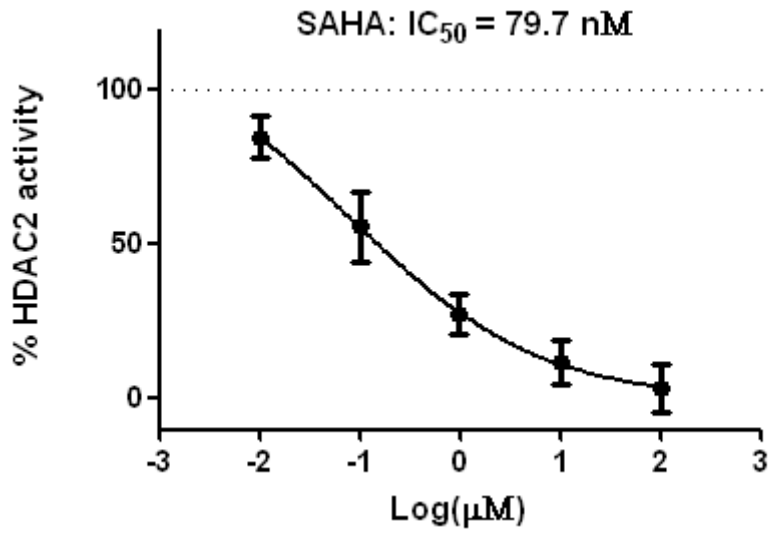

Fig. S1 Effect of HDAC2 activity by SAHA at different concentrations

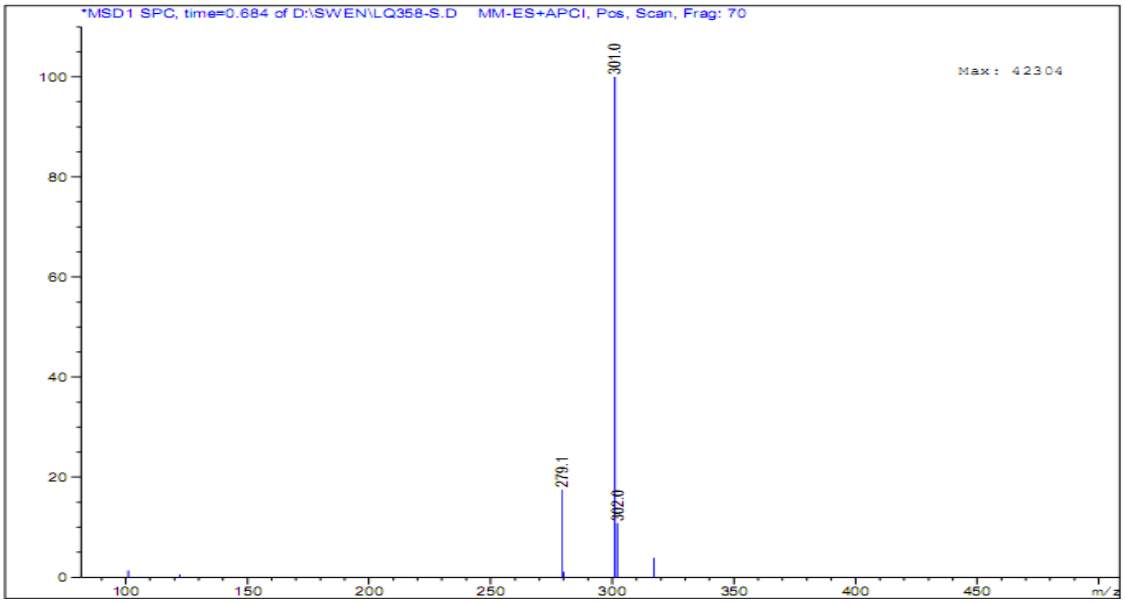

Fig. S2 Mass spectroscopy detection of **1a** in the diluted buffer to test its stability.

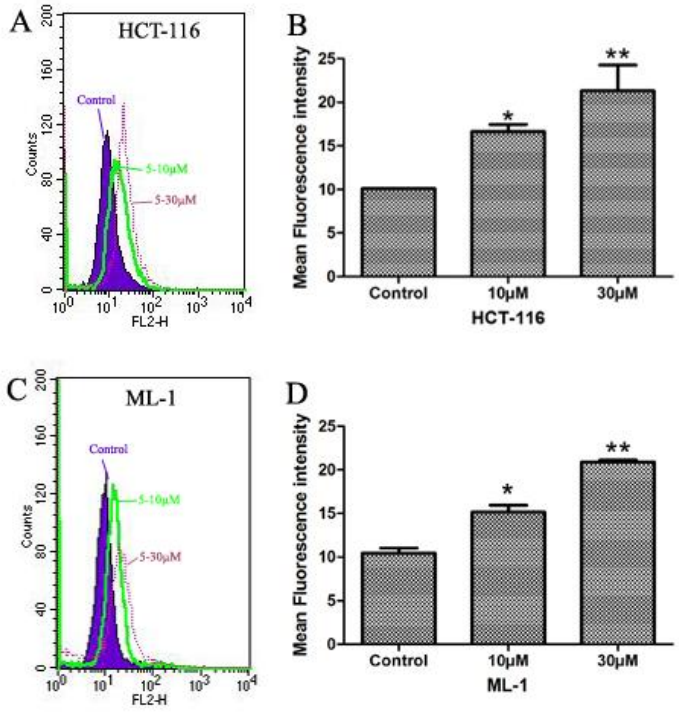

Fig. S3 Effect of **5** on reactive oxygen species generation

General information

All solvents were commercially available and used without a further purification unless stated. The chemicals used were either purchased from commercial sources or prepared according to literature procedures. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance spectrometer 400 at 400 MHz and 100 MHz respectively. Chemical shifts are given in ppm (δ) referenced to $CDCl_3$ with 7.26 for ^{1}H and 77.10 for ^{13}C, and to d_6-DMSO with 2.50 for ^{1}H and 39.50 for ^{13}C. In the case of multiplet, the signals are reported as intervals. Signals are abbreviated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Coupling constants are expressed in hertz. The progress of the reactions was monitored by thin-layer chromatography on a glass plate coated with silica gel with fluorescent indicator (GF254). Column chromatography was performed on silica gel (200-300 mesh). High-resolution mass spectrometric data (HRMS) were collected on a Shimadzu LCMS-IT-TOF mass spectrometer. Purity of all final compounds was

determined by Agilent 1260 HPLC system using an Eclipse XDB-C18 column. In HPLC conditions, flow rate was set at 1 mL/min, and gradient elution was uesd 70% MeOH/H_2O run for 12 mins. All biological assays were run using established protocols or following kit directions. Brief experimental details are provided below.

The Human colorectal carcinoma cell line HCT-116 was maintained in McCoy's 5a Medium, the myeloblastic leukemia cells ML-1 in RPMI 1640 Medium, and the normal colon epithelial cell line CCD841 in Eagle's Minimum Essential Medium. All cell line medium were supplemented with 10% fetal bovine serum at 37^{o}C and 5% CO_2. The biological data are presented as the means and SD of at least three independent measurements. Statistical analysis (one way ANOVA or unpaired t-test) was used to determine differences between means of different experimental groups. Significance level for all statistical comparisons was set at P<0.05. Graphpad Prism 5.0 software (US) was used to draw pictures.

Synthesis and characterization

General procedure to synthesize 7a-d: The synthesis of 4-((ethoxycarbonyl)amino)butanoic acid (**7a**) was exemplified here. To a stirred mixture of γ-aminobutyric acid (**6a**) (2.0 g, 19.39 mmol) in H_2O (15 mL) and THF (15 mL) was added K_2CO_3 (6.7 g, 48.49 mmol), and then added dropwise a solution of ethyl chloroformate (2.23 mL, 23.27 mmol) in THF (5 mL) within 30 mins. The reaction mixture was stirred overnight at rt before it was diluted with H_2O (20 mL) and washed with petroleum ether (3 × 10 mL). The aqueous phase was acidified until to pH 2 with 4 M HCl in an ice bath and extracted with EtOAc (3 × 20 mL). The EtOAC layers were dried over Na_2SO_4 and concentrated under a vacuum to give **7a** as a white solid (3.21 g, 95%).

Synthesis of 4-((*tert*-butoxycarbonyl)amino)butanoic acid (8): To a stirred mixture of γ-aminobutyric acid (**6a**) (2.0 g, 19.39 mmol) in H_2O (20 mL) and THF (20 mL),

was added NaOH (1.55 g, 38.79 mmol), and then added dropwise a solution of $(Boc)_2O$ (5.08 g, 23.27 mmol) in THF (10 mL) within 30 mins. The mixture was stirred for 5 hours before it was diluted with H_2O (20 mL) and washed with petroleum ether (3 × 10 mL). The aqueous phase was acidified until to pH 3 with 4 M HCl in an ice bath and then extracted with EtOAc (3 × 20 mL). The EtOAc layers were dried over Na_2SO_4 and concentrated to give **8** as a transparent liquid (4.19 g, 100% yield).

General procedure to synthesize 1a-d, 2 and 3: The synthesis of ethyl (4-oxo-4-(phenethylamino) butyl) carbamate (**1a**) was exemplified here. To a solution of **7a** (346.95 mg, 1.98 mmol) in anhydrous CH_2Cl_2 (8 mL) cooled at 0 ℃ was added 2-phenylethanamine (207.9 μL, 1.65 mmol), Et_3N (344.1 μL, 2.48 mmol), EDCI (0.411 g, 2.15 mmol), and DMAP (20.2 mg, 0.16 mmol). The reaction solution was stirred at 0 ℃ for 1 h, and then overnight at rt before it was diluted with additional CH_2Cl_2 (20 mL). The solution was then washed sequentially with 1.0 M HCl (10 mL), saturated $NaHCO_3$ (10 mL), H_2O (10 mL), and brine (10 mL). The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by silica column chromatography to give **1a**.

Synthesis of 4-amino-*N*-phenethylbutanamide (9): To a solution of **3** (0.2 g, 652.74 μmol) in anhydrous CH_2Cl_2 (5 mL) was injected TFA (0.5 mL) at rt, and then the mixture was stirred at rt for 2 hours. The solution was concentrated in a vacuo and basified to pH 8 with saturated aq. $NaHCO_3$, and then extracted with EtOAc (3 × 20 mL). The organic layers were dried over Na_2SO_4 and concentrated to give **9** as a yellow liquid (195 mg, 100%).

Synthesis of 4-(3-ethylureido)-*N*-phenethylbutanamide (4): To a solution of **9** (139 mg, 673.83 μmol) in THF (3 mL) was injected isocyanatoethane (160 μL, 2.02 mmol), and the mixture was stirred at rt overnight. After the reaction was complete, the reaction solution was extracted with CH_2Cl_2 (30 mL), washed with H_2O (5 mL) and brine (5 mL), dried over Na_2SO_4 and concentrated in a vacuo. The residue was

purified through silica column chromatography to provide **4** as a white solid (83 mg, 44.1%).

Ethyl (4-oxo-4-(phenethylamino)butyl)carbamate (1a). A white solid, 75% yield. HPLC Retention time (Rt) = 2.615 min, 97.81%. mp: 111-112 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 – 7.29 (m, 2H), 7.24 – 7.19 (m, 3H), 5.90 (s, 1H), 4.90 (s, 1H), 4.09 (q, *J* = 7.1 Hz, 2H), 3.53 (q, *J* = 6.9 Hz, 2H), 3.17 (q, *J* = 6.3 Hz, 2H), 2.83 (t, *J* = 7.0 Hz, 2H), 2.17 (t, *J* = 7.0 Hz, 2H), 1.79 (dt, *J* = 13.4, 6.8 Hz, 2H), 1.23 (t, *J* = 7.1 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.7, 157.2, 139.0, 128.8, 128.7, 126.5, 60.8, 40.7, 40.2, 35.7, 33.7, 26.1, 14.7 ppm. IR υ 3348, 3287, 1655, 1541, 1298, 1230 cm^{-1}. ESI-HRMS for $[C_{15}H_{22}N_2O_3Na]^+$, calcd: 301.1523; found: 301.1530.

Ethyl (6-oxo-6-(phenylamino)hexyl)carbamate (1b). A white solid, 77% yield. HPLC Rt = 2.886 min, 100%. mp: 111-112 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52 (d, *J* = 7.9 Hz, 2H), 7.31 (t, *J* = 7.9 Hz, 2H), 7.10 (t, *J* = 7.4 Hz, 1H), 4.68 (brs, 1H), 4.10 (dd, *J* = 13.9, 6.9 Hz, 2H), 3.17 (m, 2H), 2.36 (t, *J* = 7.4 Hz, 2H), 1.76 (dt, *J* = 14.9, 7.4 Hz, 2H), 1.55 (dt, *J* = 14.2, 7.2 Hz, 2H), 1.46 – 1.37 (m, 2H), 1.23 (t, *J* = 7.1 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.6, 157.0, 138.3, 129.0, 124.1, 120.0, 60.8, 40.7, 37.4, 29.8, 26.3, 25.2, 14.7 ppm. IR υ 3316, 2931, 2858, 1681, 1540, 1442, 1272, 1146 cm^{-1}. ESI-HRMS for $[C_{15}H_{23}N_2O_3]^+$, calcd: 279.1630; found: 279.1703.

Ethyl (5-(benzylamino)-5-oxopentyl)carbamate (1c). A white solid. 83.5% yield. HPLC Rt = 2.573 min, 100%. mp: 111-112 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 – 7.31 (m, 2H), 7.29 – 7.27 (m, 3H), 5.92 (s, 1H), 4.77 (s, 1H), 4.43 (d, *J* = 5.7 Hz, 2H), 4.05 (q, *J* = 6.8 Hz, 2H), 3.18 (dd, *J* = 12.6, 6.2 Hz, 2H), 2.24 (t, *J* = 7.4 Hz, 2H), 1.74 – 1.66 (m, 3H), 1.60 – 1.50 (m, 2H), 1.21 (t, *J* = 7.0 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.8, 157.0, 138.5, 128.7, 127.8, 127.4, 60.7, 43.6, 40.2, 35.9, 29.5, 22.7, 14.7 ppm. IR υ 3308, 2950, 1687, 1539, 1275, 1049, 721 cm^{-1}. ESI-HRMS for $[C_{15}H_{23}N_2O_3]^+$, calcd: 279.1630; found: 279.1699.

Ethyl (3-oxo-3-((3-phenylpropyl)amino)propyl)carbamate (1d). A white solid, 78% yield. HPLC Rt = 3.212 min, 99.16%. mp: 98-100 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 – 7.27 (m, 2H), 7.23 – 7.14 (m, 3H), 5.60 (s, 1H), 5.28 (s, 1H), 4.09 (q, *J* = 6.8 Hz, 2H), 3.44 (m, 2H), 3.29 (dd, *J* = 13.0, 7.0 Hz, 2H), 2.65 (t, *J* = 7.6 Hz, 2H), 2.35 (t, *J* = 5.7 Hz, 2H), 1.88 – 1.80 (m, 2H), 1.22 (t, *J* = 7.1 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.3, 157.0, 141.4, 128.5, 128.4, 126.1, 60.9, 39.3, 37.1, 36.2, 33.4, 31.2, 14.7 ppm. IR υ 3310, 2949, 1658, 1551, 1256, 1048, 695 cm^{-1}. ESI-HRMS for $[C_{15}H_{23}N_2O_3]^+$, calcd: 279.1630; found: 279.1693.

Ethyl (4-((2-(1H-indol-3-yl)ethyl)amino)-4-oxobutyl)carbamate (2). A white solid, 73% yield. HPLC Rt = 2.525 min, 96.73%. mp: 81-84 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 (s, 1H), 7.61 (d, *J* = 7.9 Hz, 1H), 7.38 (d, *J* = 8.1 Hz, 1H), 7.23 – 7.19 (m, 1H), 7.15 – 7.11 (m, 1H), 7.05 (s, 1H), 5.97 (brs, 1H), 4.12 – 4.06 (m, 2H), 3.61 (dd, *J* = 12.5, 6.6 Hz, 2H), 3.16 (t, *J* = 6.4 Hz, 2H), 2.99 (t, *J* = 6.8 Hz, 2H), 2.16 (t, *J* = 6.9 Hz, 2H), 1.81 – 1.77 (m, 2H), 1.23 (t, *J* = 7.1 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.9, 157.2, 136.5, 127.4, 122.3, 122.0, 119.2, 118.6, 112.6, 111.4, 60.8, 40.3, 39.9, 33.7, 26.0, 25.3, 14.7 ppm. IR υ 3389, 3252, 3082, 2957, 1693, 1638, 1550, 1449, 1273, 746 cm^{-1}. ESI-HRMS for $[C_{17}H_{24}N_3O_3]^+$, calcd: 318.1739, found: 318.1803.

***Tert*-butyl (4-oxo-4-(phenethylamino)butyl)carbamate (3)**. A yellow solid, 81.2% yield. HPLC Rt = 4.431 min, 96.75%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 – 7.29 (m, 2H), 7.24 – 7.19 (m, 3H), 6.04 (s, 1H), 4.72 (s, 1H), 3.53 (dd, *J* = 12.9, 7.0 Hz, 2H), 3.12 (m, 2H), 2.83 (t, *J* = 7.0 Hz, 2H), 2.16 (t, *J* = 7.0 Hz, 2H), 1.80 – 1.74 (m, 2H), 1.43 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.7, 156.5, 139.0, 128.8, 128.6, 126.5, 79.3, 40.7, 39.8, 35.7, 33.7, 28.5, 26.4 ppm.

4-(3-Ethylureido)-N-phenethylbutanamide (4). A white solid. 34.6% yield. HPLC t_R = 2.464 min, 100%. mp: 165-166 ℃.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 – 7.29 (m, 2H), 7.24 – 7.19 (m, 3H), 6.19 (s, 1H), 4.82 (s, 1H), 4.55 (s, 1H), 3.51 (dd, *J* = 13.2, 6.8 Hz, 2H), 3.20 (m, 4H), 2.83 (t, *J* = 7.1 Hz, 2H), 2.19 (t, *J* = 6.6 Hz, 2H), 1.78 (m,

2H), 1.13 (t, J = 7.2 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.6, 161.2, 140.5, 129.8, 129.5, 127.3, 42.0, 40.4, 36.5, 35.8, 34.4, 27.8, 15.8 ppm. IR υ 3356, 3274, 2956, 2484, 2409, 1624, 1472, 752 cm^{-1}. ESI-HRMS for $[C_{15}H_{24}N_3O_2]^+$, calcd: 278.1790; found: 278.1859.

4-(3-Ethylthioureido)-N-phenethylbutanamide (5). Following the procedure to synthesize compound **4**, **5** was obtained from **9** and isothiocyanate as a white solid at 43.5% yield. HPLC Rt = 2.683 min, 98.72%. mp: 98-102 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 – 7.30 (m, 2H), 7.24 – 7.17 (m, 3H), 5.64 (s, 1H), 3.56 – 3.46 (m, 6H), 2.82 (t, J = 6.9 Hz, 2H), 2.26 – 2.23 (m, 2H), 1.90 – 1.86 (m, 2H), 1.24 (t, J = 7.2 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 181.3, 173.2, 138.6, 128.6, 128.6, 126.5, 43.9, 40.8, 39.2, 35.5, 33.3, 24.7, 14.3 ppm. IR υ 3320, 3072, 2873, 1642, 1550, 1356, 707 cm^{-1}. ESI-HRMS for $[C_{15}H_{24}N_3OS]^+$, calcd: 294.1562; found: 294.1634.

The HDAC2 activity assay

HDAC2 Fluorimetric Drug Discovery Kit from Enzo Life Sciences, a complete system for screening and characterizing modulators of HDAC2 activity, was utilized to determine percent inhibition of compounds **1a-d**, and **2-5.** SAHA (Vorinostat; Sigma Aldrich, St. Louis, MO, USA) served as a control for the enzyme inhibition assay. Enzyme inhibition assays were performed with varying concentrations of **1a-d**, and **2-5**. Briefly, components were added sequentially to a black, flat-bottom 96-well microtiter plate (included in the assay kit) as described by the manufacturer's protocol, and the reaction mixture was incubated for 30 min at 37 ℃. The potent HDAC inhibitor trichostatin A (included in the assay kit) was added to the HDAC assay developer at a final reaction concentration of 1 μM to stop deacetylation and initiate the release of the fluorophore. Fluorescence was measured on a microplate reader (SpectraMax M5 Molecular Devices, Sunnyvale, CA, USA) using an excitation wavelength of 480 nm and a detection wavelength of 530 nm.

Stability test of synthetic santacruzuamt A (1a)

Due to our disparity with the previous reported results, the stability test of compound **1a** was performed. A solution of **1a** in DMSO was diluted in the buffer employed for the enzymatic assay and incubated for 1 hour, and finally taken for mass spectroscopy detection. The results showed that the major peak of species corresponded to compound **1a**, suggesting compound **1a** was stabile during under the enzymatic assay conditions.

MTS assay

HCT-116 and CCD841 cells in logarithmic growing phase were seeded in 96-well plates with 3,000 cells in each well, and ML-1 was seeded in 96-well plates with 30,000 cells in each well. Various concentrations of compound **5** were added to the wells and the plates were placed in a cell culture incubator for 72 h. The samples were then incubated with MTS reagent (20 μl/well) and incubated for another 4 h. The absorbance in each well at 490 nm was measured using a Multiskan plate reader (Thermo Scientific, USA).[1]

Colony formation assay

Cells in logarithmic phase were seeded in 6-well plates with 500 cells in each well. Various concentrations of compound **5** were added to the wells. The cells were cultured for two weeks before fixation and staining with crystal violet. The samples were photographed and the colonies were counted as describe previously.[2]

Cell cycle analysis

Cells in logarithmic phase were seeded in 6-well plates with a density of $2x10^5$ cells per well. Different concentrations of compound **5** were added and the plates were incubated for 24 h. The cells were harvested and fixed with 75% alcohol at 4˚C for 4 h and then incubated with RNase at 37˚C for 30 min followed by addition of PI.

Finally, cell cycle distribution were detected by using the BD FACSCalibur flow cytometer.[3]

Annexin V/PI assay

Apoptosis was detected using Annexin V/PI double staining as described previously.[1] After a drug treatment, the cells were harvested, washed and counted. The cells were then stained with Annexin V and PI in binding buffer for 15 min and cell viability was analyzed using the FACSCalibur flow cytometer (BD Co., USA).

Mitochondria reactive oxygen species (ROS) detection

Cells were stained for 30 min with Mito-SOX for ROS detection. The samples were harvested and analyzed using a BD Calibur flow cytometry as described previously.[4]

References

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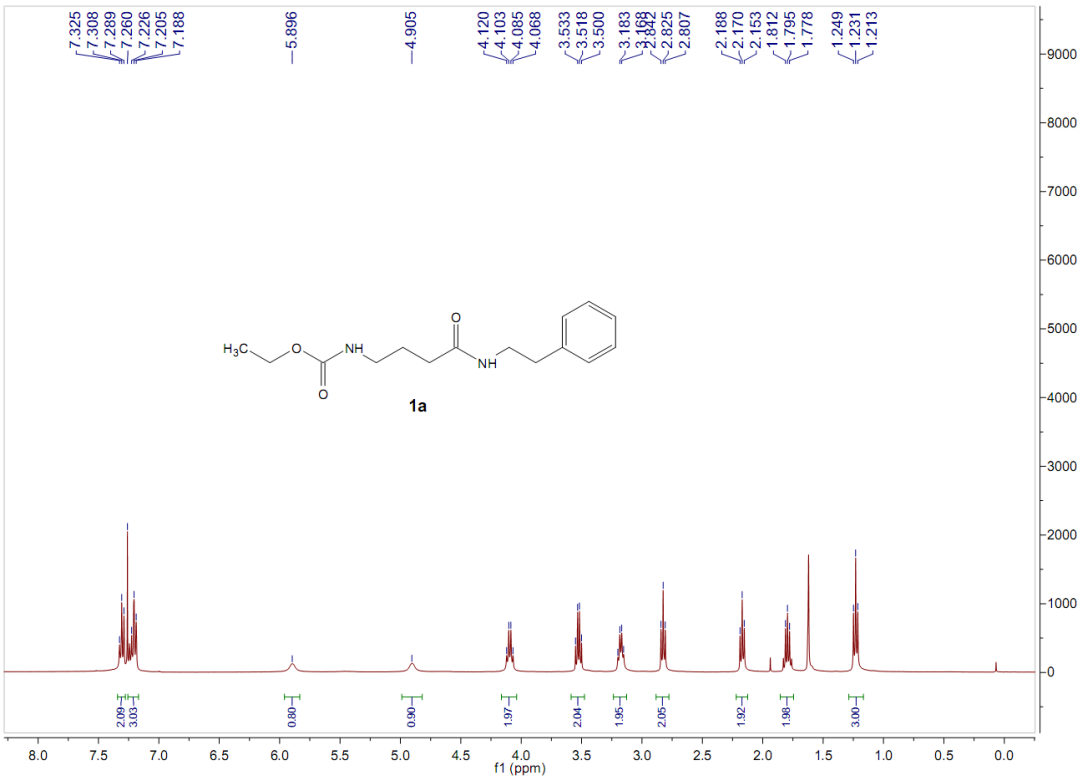
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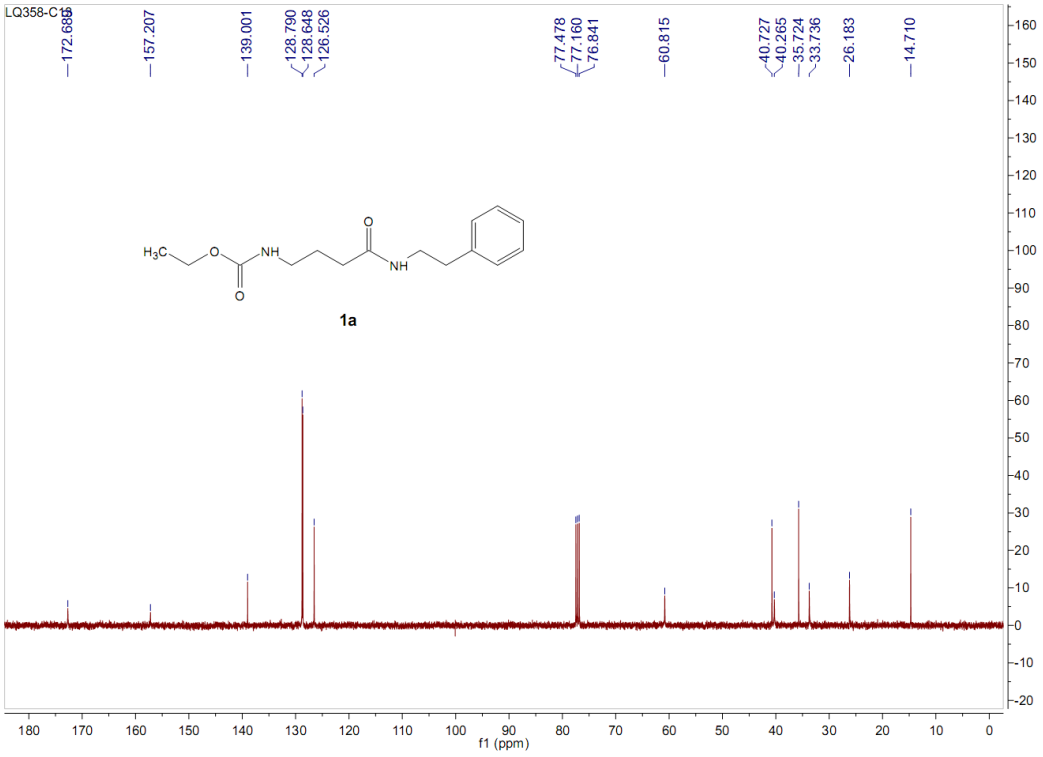
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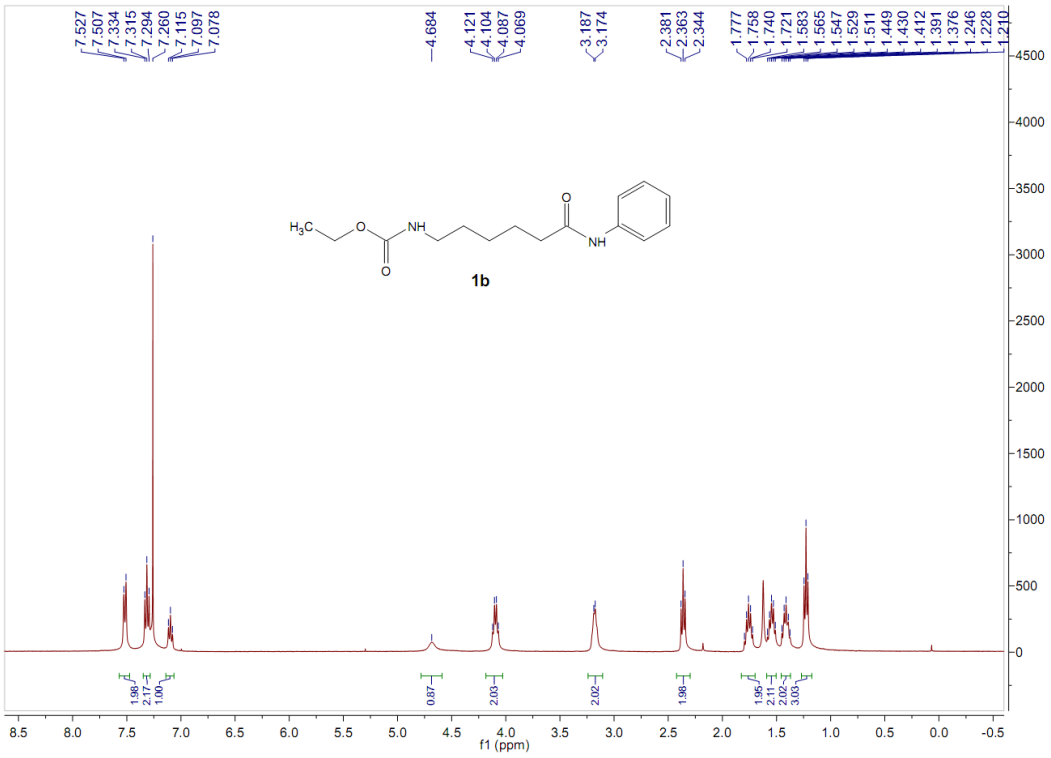
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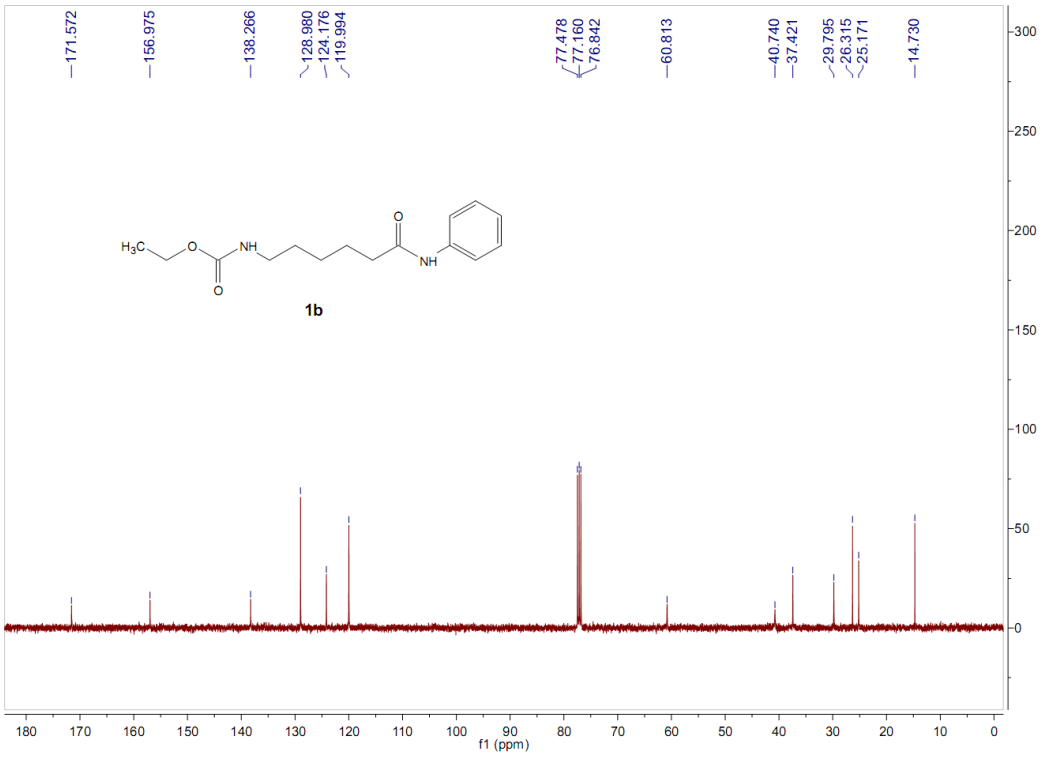
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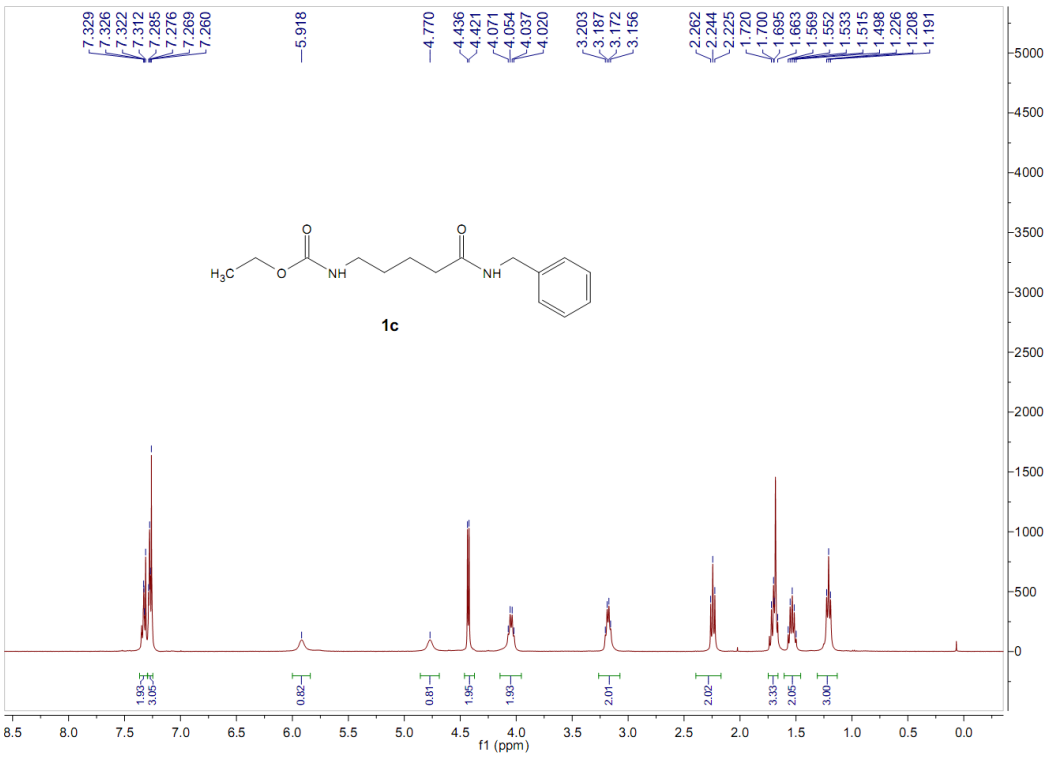
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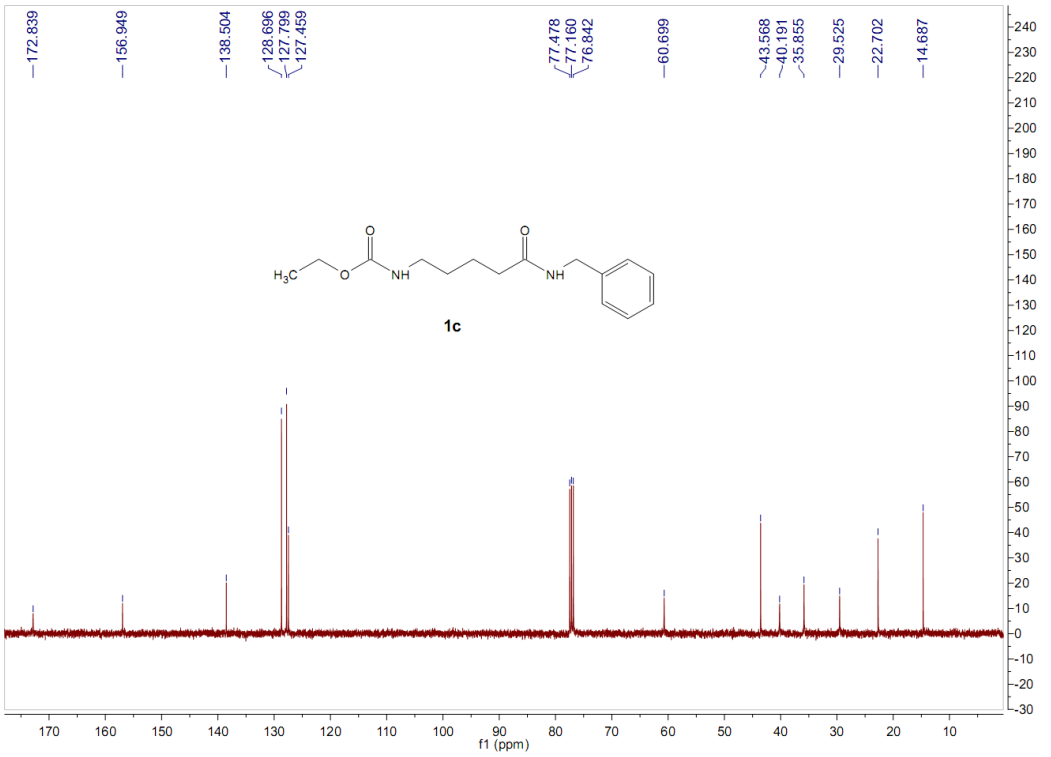
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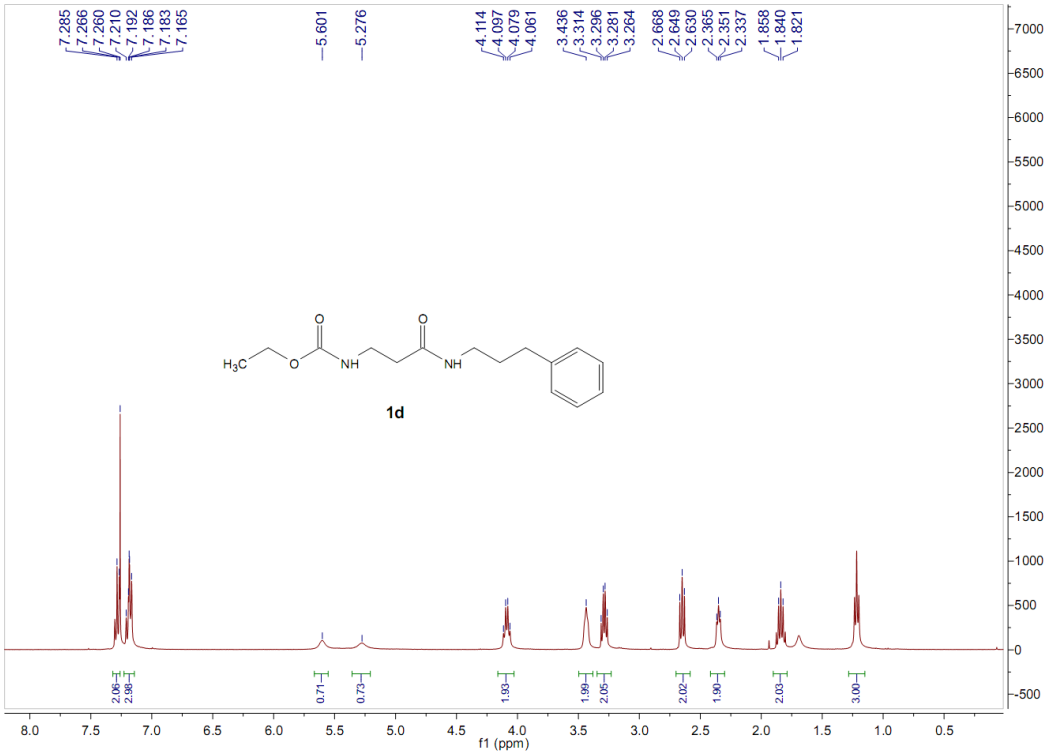
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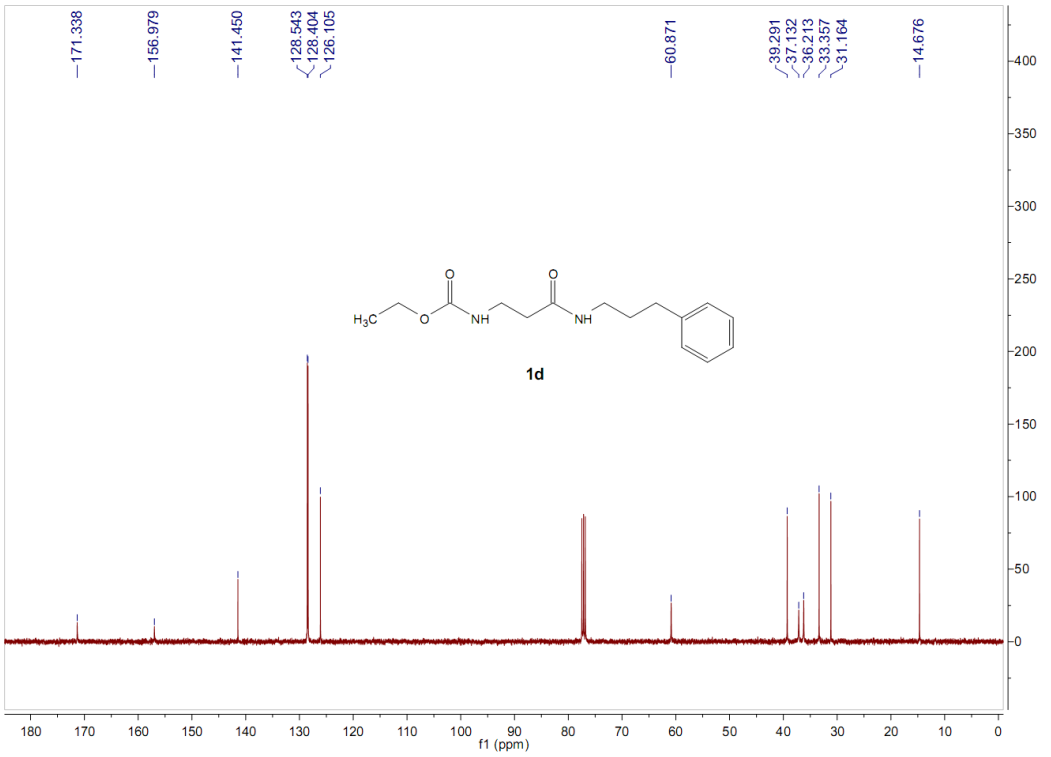
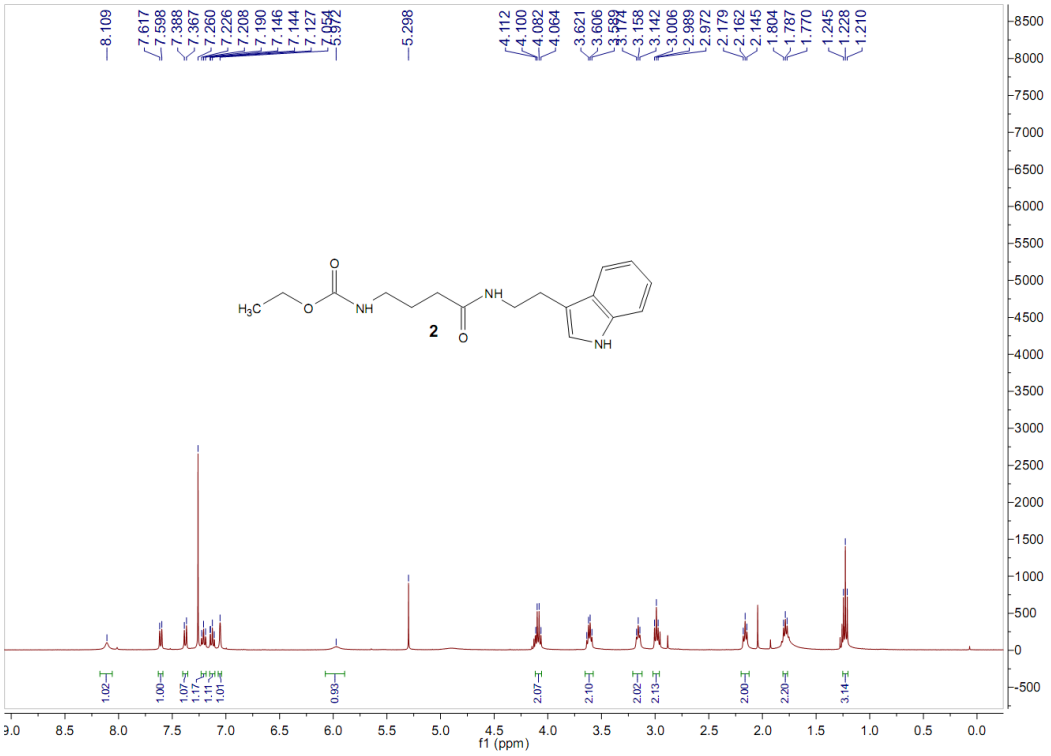
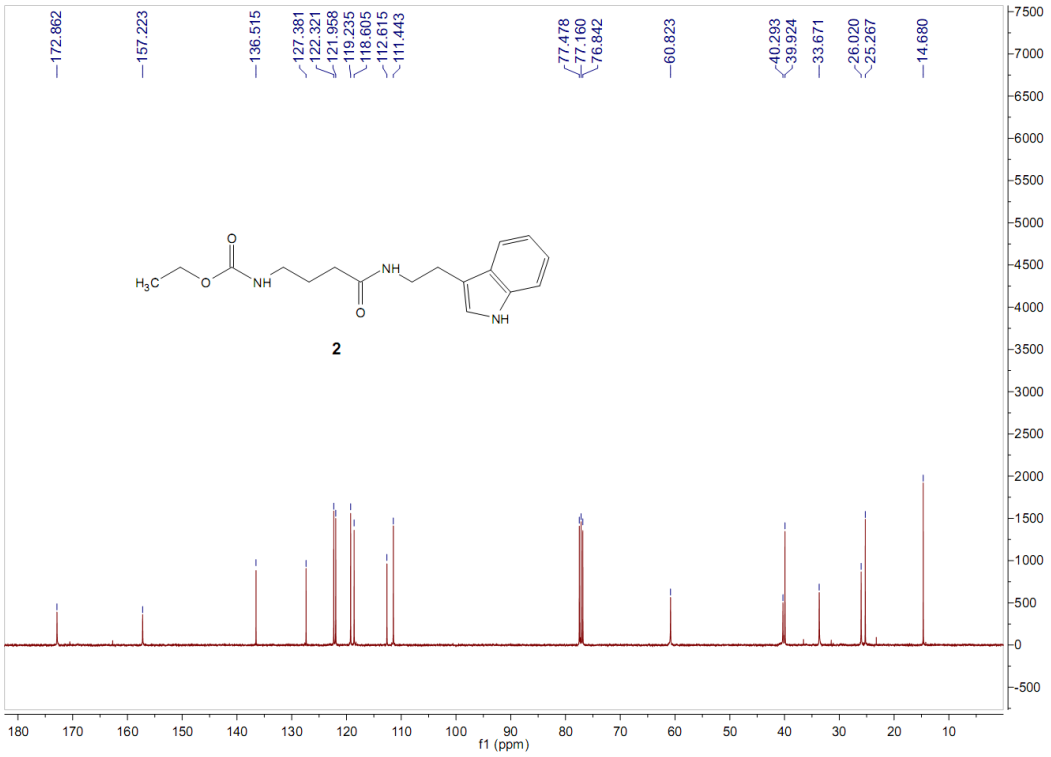
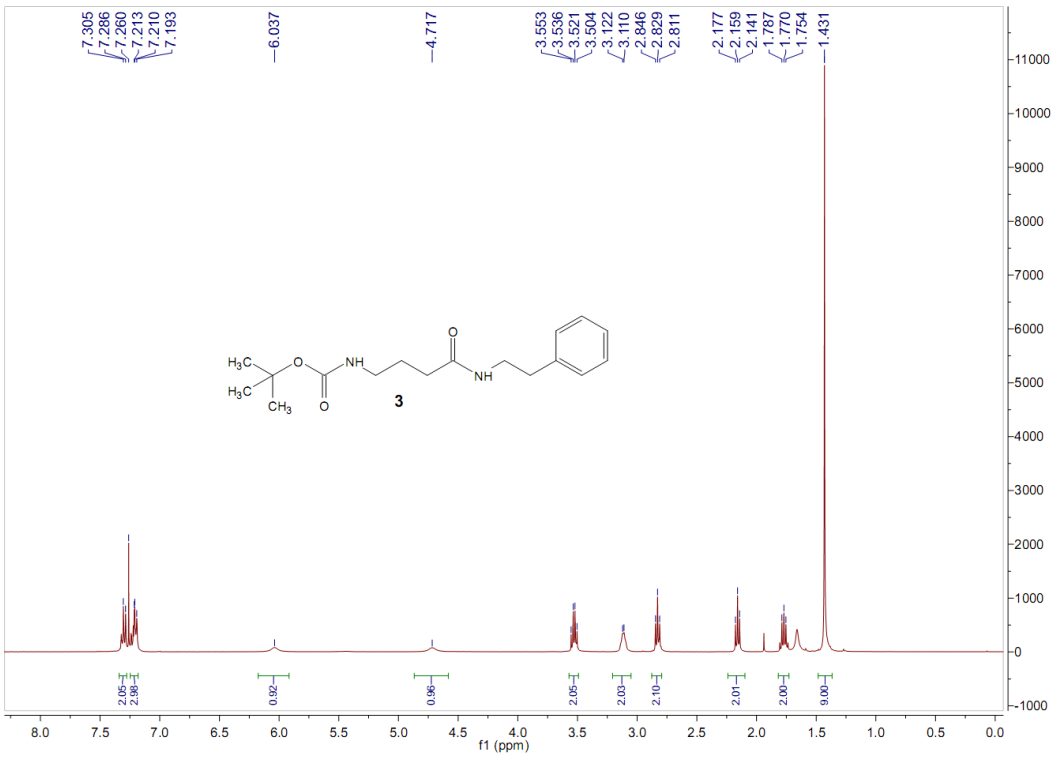
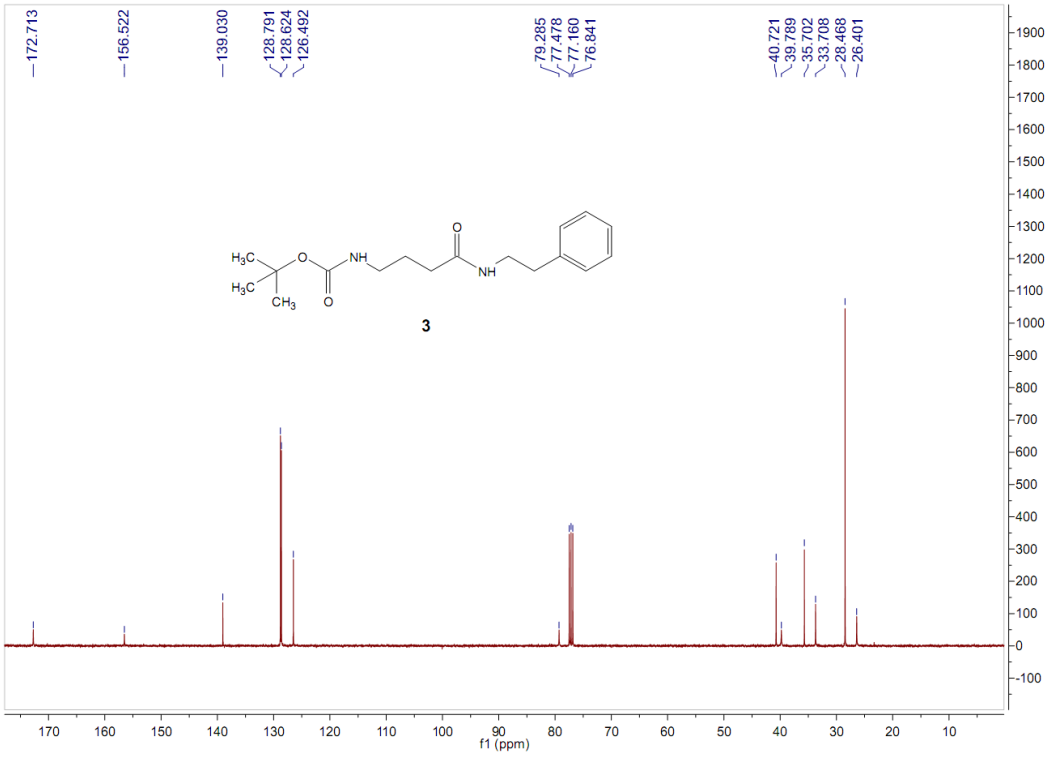
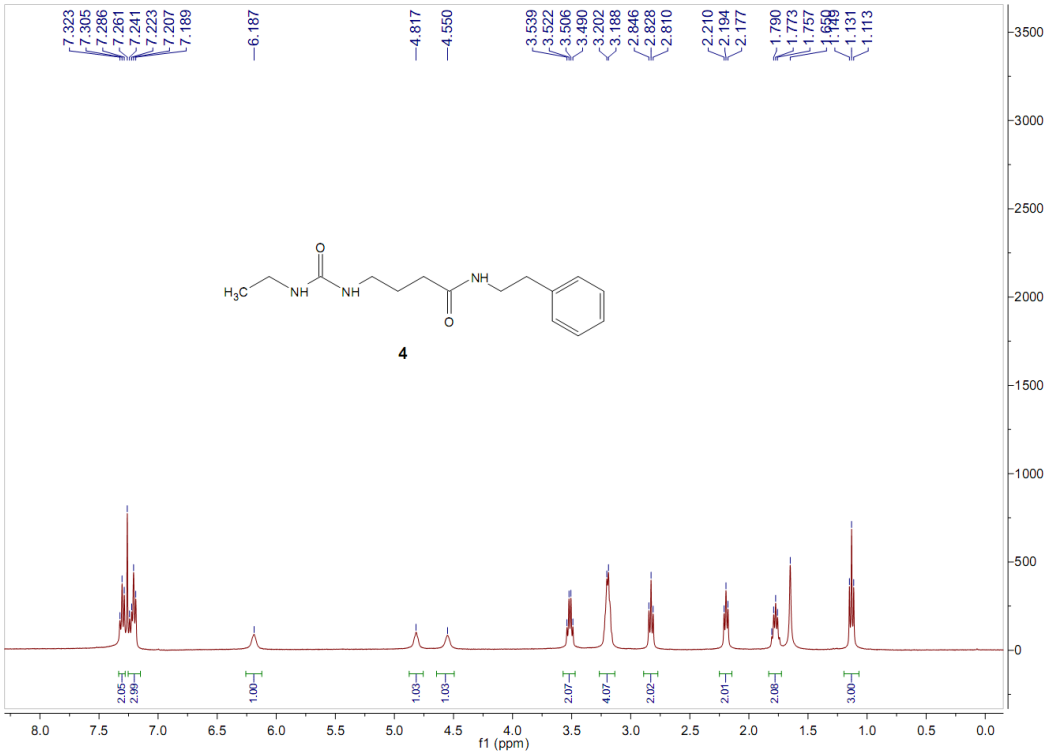

171.338
156.979
141.450
128.543
128.404
126.105
60.871
39.291
37.132
36.213
33.357
31.164
14.676
1d
f1 (ppm)

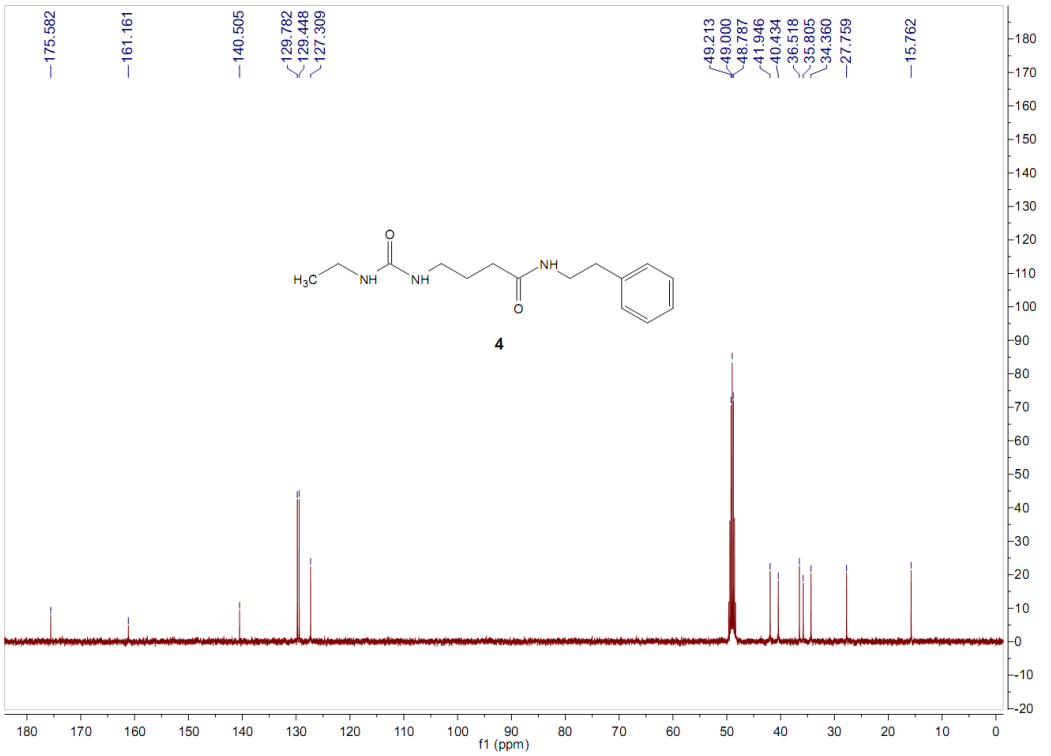

8.109
7.617
7.598
7.388
7.367
7.260
7.226
7.208
7.190
7.146
7.144
7.127
7.054
5.972
5.298
4.112
4.100
4.082
4.064
3.621
3.606
3.589
3.574
3.158
3.142
3.006
2.989
2.972
2.179
2.162
2.145
1.804
1.787
1.770
1.245
1.228
1.210
H3C
O
O
NH
O
NH
NH
2
1.02
1.00
1.07
1.17
1.11
1.01
0.93
2.07
2.10
2.02
2.13
2.00
2.20
3.14
f1 (ppm)

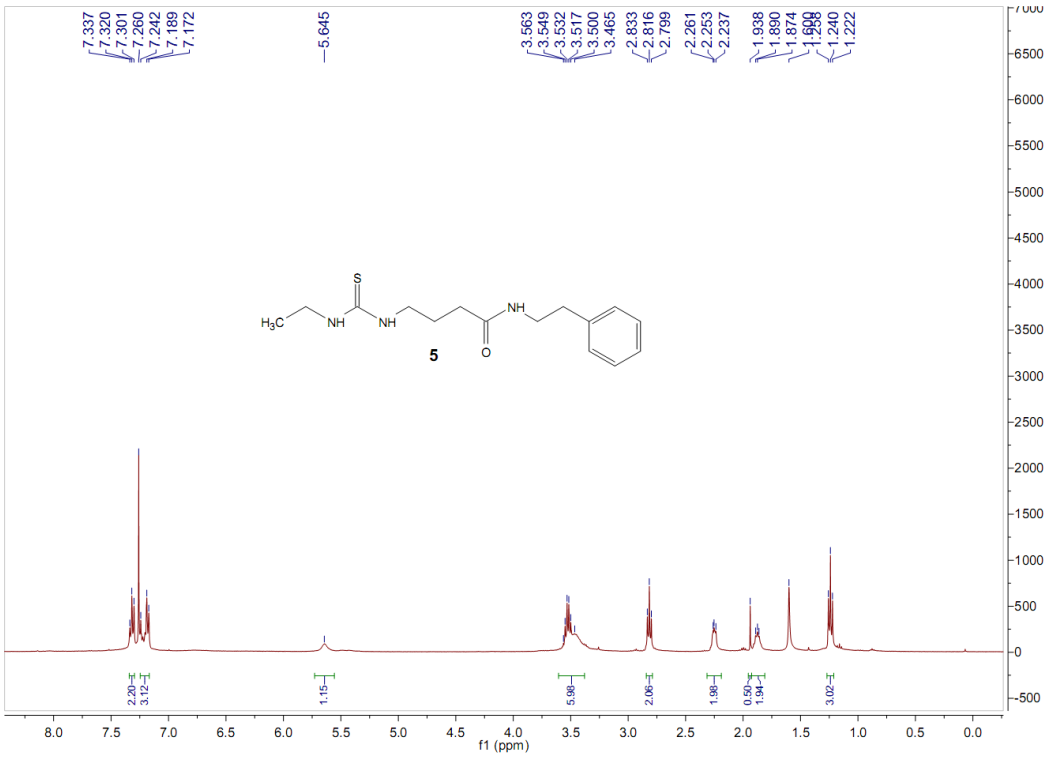

172.862
157.223
136.515
127.381
122.321
121.958
119.235
118.605
112.615
111.443
77.478
77.160
76.842
60.823
40.293
39.924
33.671
26.020
25.267
14.680
H3C
O
O
NH
O
NH
NH
2
f1 (ppm)

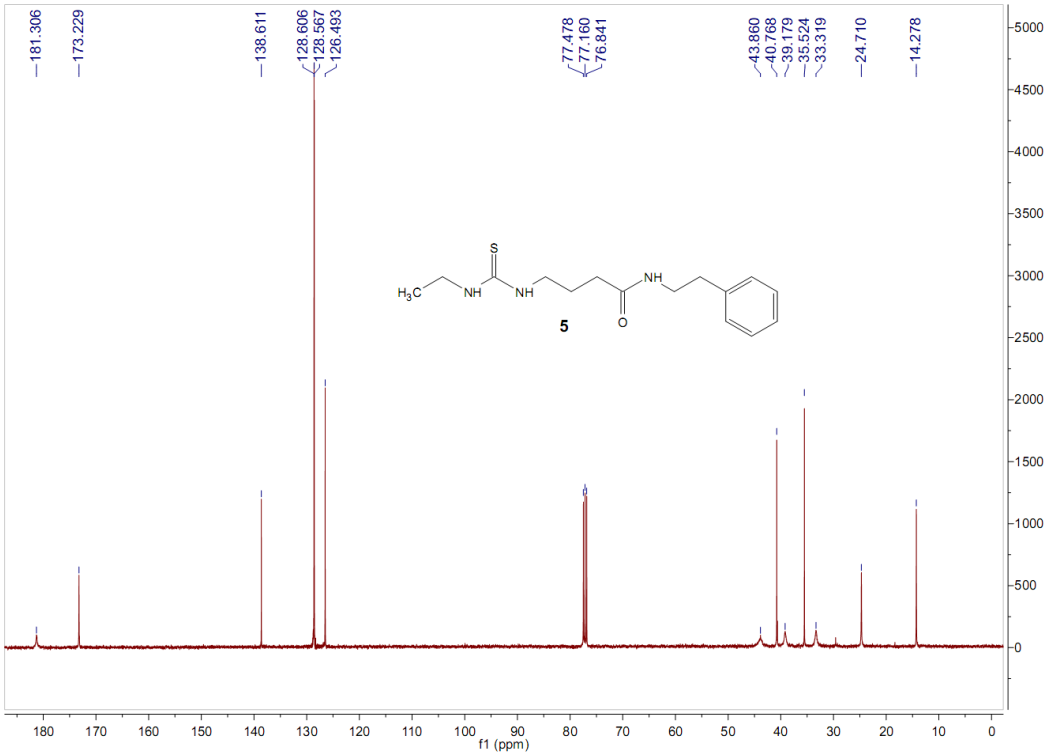

7.305
7.286
7.260
7.213
7.210
7.193
6.037
4.717
3.553
3.536
3.521
3.504
3.122
3.110
2.846
2.829
2.811
2.177
2.159
2.141
1.787
1.770
1.754
1.431
3
f1 (ppm)


172.713
156.522
139.030
128.791
128.624
126.492
79.285
77.478
77.160
76.841
40.721
39.789
35.702
33.708
28.468
26.401
3
f1 (ppm)


7.323
7.305
7.286
7.261
7.241
7.223
7.207
7.189
6.187
4.817
4.550
3.539
3.522
3.506
3.490
3.202
3.188
2.846
2.828
2.810
2.210
2.194
2.177
1.790
1.773
1.757
1.131
1.113
H3C
NH
NH
NH
O
O
4
f1 (ppm)


175.582
161.161
140.505
129.782
129.448
127.309
49.213
49.000
48.787
41.946
40.434
36.518
35.805
34.360
27.759
15.762
H3C
NH
NH
NH
O
O
4
f1 (ppm)


7.337
7.320
7.301
7.260
7.242
7.189
7.172
5.645
3.563
3.549
3.532
3.517
3.500
3.465
2.833
2.816
2.799
2.261
2.253
2.237
1.938
1.890
1.874
1.240
1.222
S
H3C
NH
NH
NH
O
5
2.20
3.12
1.15
5.90
2.06
1.98
0.50
1.94
3.02
f1 (ppm)


181.306
173.229
138.611
128.606
128.567
126.493
77.478
77.160
76.841
43.860
40.768
39.179
35.524
33.319
24.710
14.278
S
H3C
NH
NH
NH
O
5
f1 (ppm)