Facile and Stabile Linkages through Tyrosine: Bioconjugation Strategies with the Tyrosine-Click Reaction

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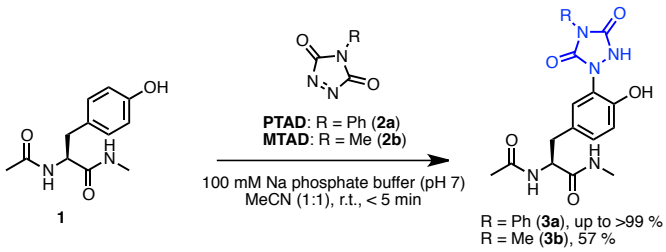
Supporting Information

1. Coupling of *N*-acyl tyrosine methylamide with PTAD or MTAD

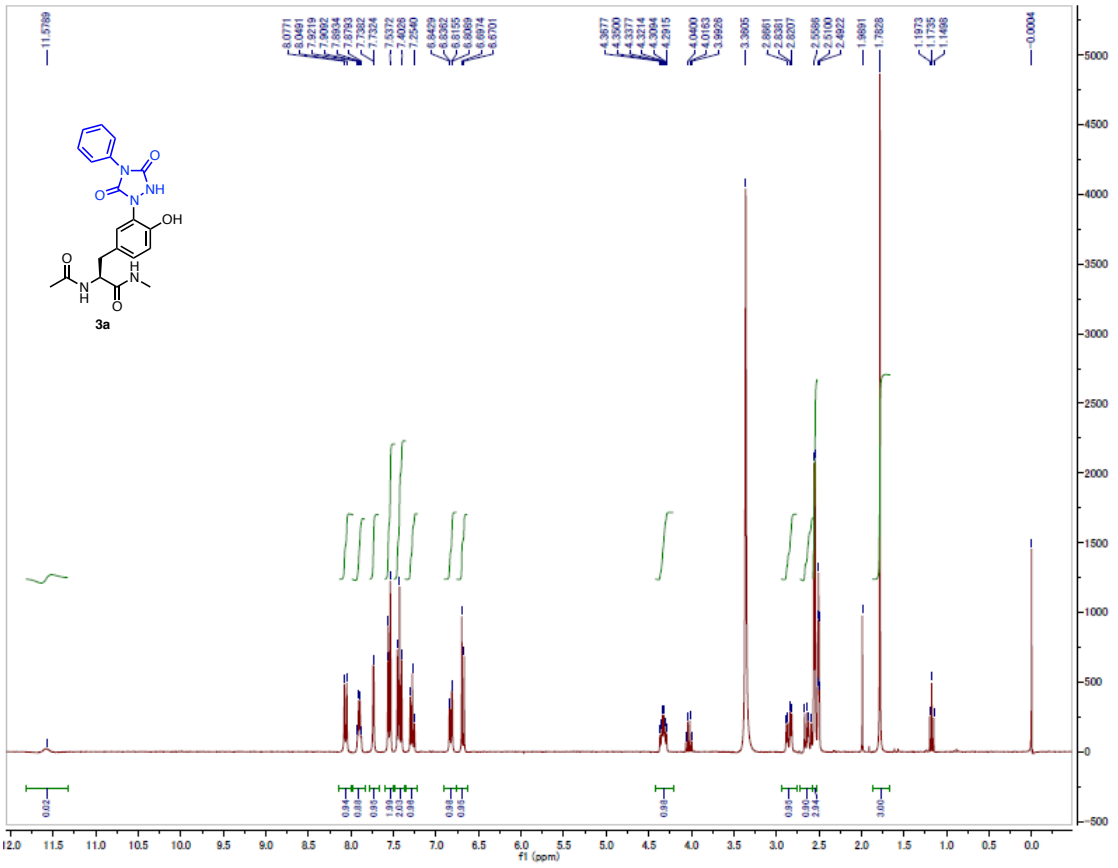


Compound (3a). To a solution of tyrosine **1** (14.2 mg, 0.060 mmol) in 100 mM pH 7.0 NaH_2PO_4/Na_2HPO_4 buffer (1.5 mL) - MeCN (1.5 mL) was added the 0.5 M solution of PTAD **2a** (0.132 mL, 0.066 mmol) in MeCN at room temperature. The resulting solution was stirred at room temperature for 30 min. The reaction mixture was acidified with 12N HCl (0.249 mL) and then concentrated *in vacuo*. The obtained crude material was purified by flash column chromatography ($CHCl_3$/MeOH) to give **3a** (16.0 mg, 65%) as a white solid.

^{1}H NMR (300 MHz, DMSO-d6): δ 11.57 (br, 1H), 8.06 (d, *J* = 8.4 Hz, 1H), 7.90 (q, *J* = 4.3 Hz, 1H), 7.74 (d, *J* = 1.7 Hz, 1H), 7.63 – 7.51 (m, 2H), 7.43 (t, *J* = 7.8 Hz, 2H), 7.34 – 7.21 (m, 1H), 6.83 (dd, *J* = 8.2, 2.0 Hz, 1H), 6.68 (d, *J* = 8.2 Hz, 1H), 4.33 (m, 1H), 2.85 (dd, *J* = 13.5, 5.1 Hz, 1H), 2.63 (dd, *J* = 13.7, 9.2 Hz, 1H), 2.55 (d, *J* = 4.5 Hz, 3H), 1.78 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d6): δ172.64, 170.02, 153.90, 150.86, 148.44, 135.37, 129.22, 129.02, 126.96, 126.48, 126.03, 122.72, 117.74, 55.47, 38.23, 26.51, 23.56. HRMS: calcd for $C_{20}H_{22}N_5O_5$ (MH^+) 412.1615, found 412.1615.

Compound (3b). The compound **3b** was prepared from tyrosine **1** (14.2 mg, 0.060 mmol) and 0.5 M solution of MTAD **2b** (0.438 mL, 0.132 mmol), and was obtained as white amorphous solid (11.9 mg, 57%).

^{1}H NMR (300 MHz, DMSO-d6): δ 10.51 (br, 1H), 8.05 (d, *J* = 8.4 Hz, 1H), 7.86 (q, *J* = 4.4 Hz, 1H), 7.21 (s, 1H), 7.02 (dd, *J* = 1.9, 8.4 Hz, 1H), 6.77 (d, *J* = 8.3 Hz, 1H), 4.28 (m, 1H), 2.92 (s, 3H), 2.82 (dd, *J* = 13.8, 4.8 Hz, 1H), 2.60 (dd, *J* = 13.5, 9.9 Hz, 1H), 2.53 (d, *J* = 4.5 Hz, 3H), 1.75 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d6): δ172.28, 169.81, 154.68, 153.39, 152.07, 150.78, 130.59, 129.42, 124.27, 117.14, 54.98, 37.38, 26.20, 25.44, 23.22. HRMS: calcd for $C_{15}H_{19}N_5O_5$ (MH^+) 350.1459, found 350.1460.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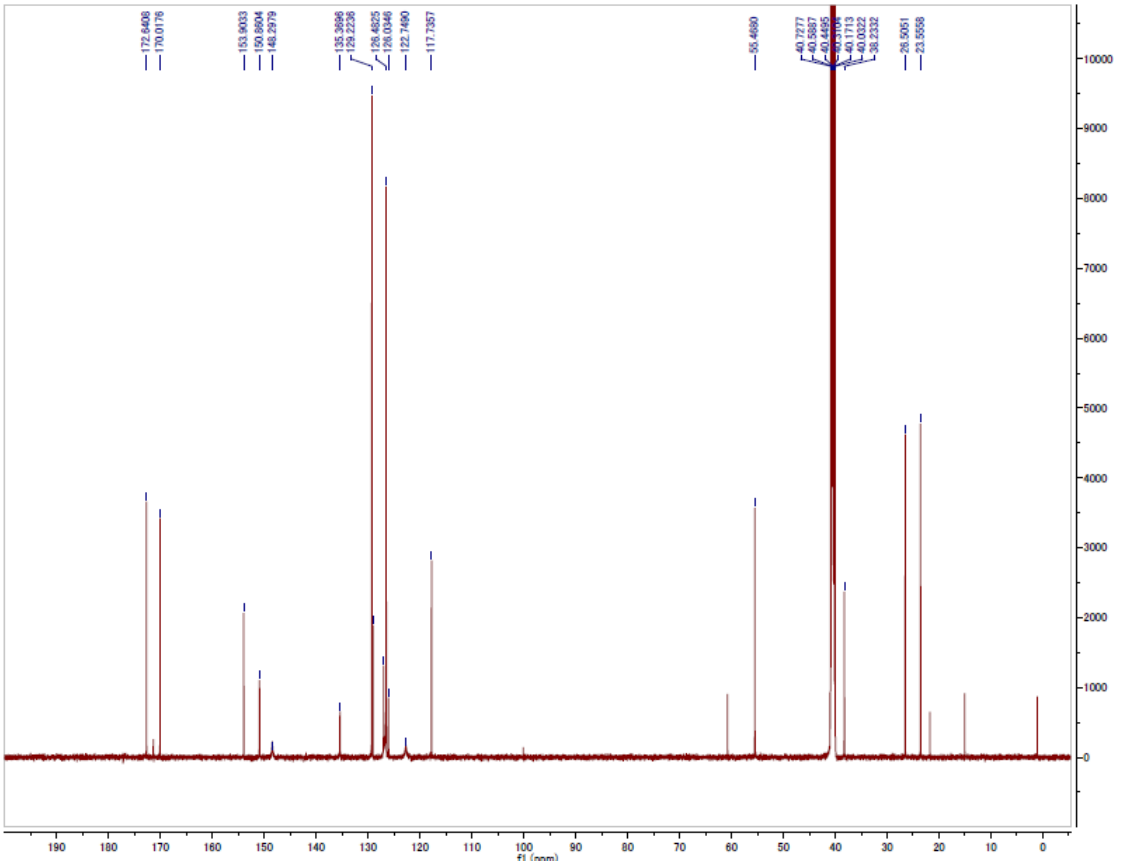
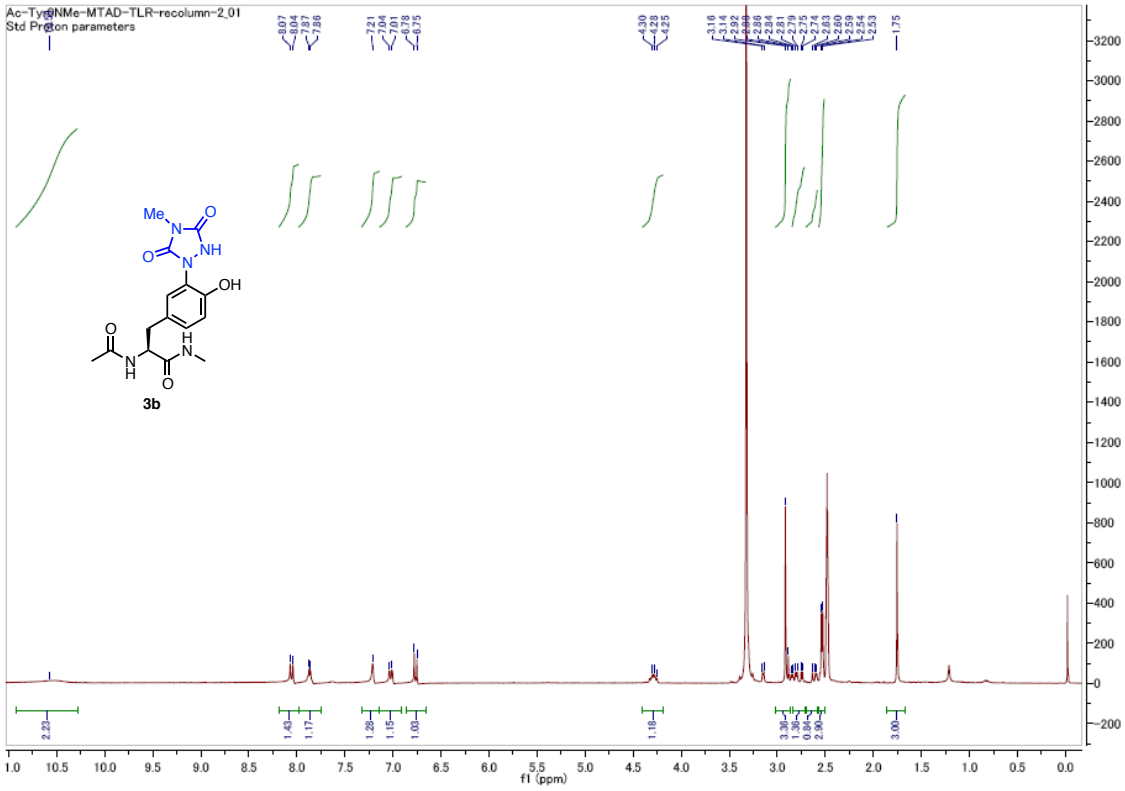
3a
fl (ppm)

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NH
N
OH
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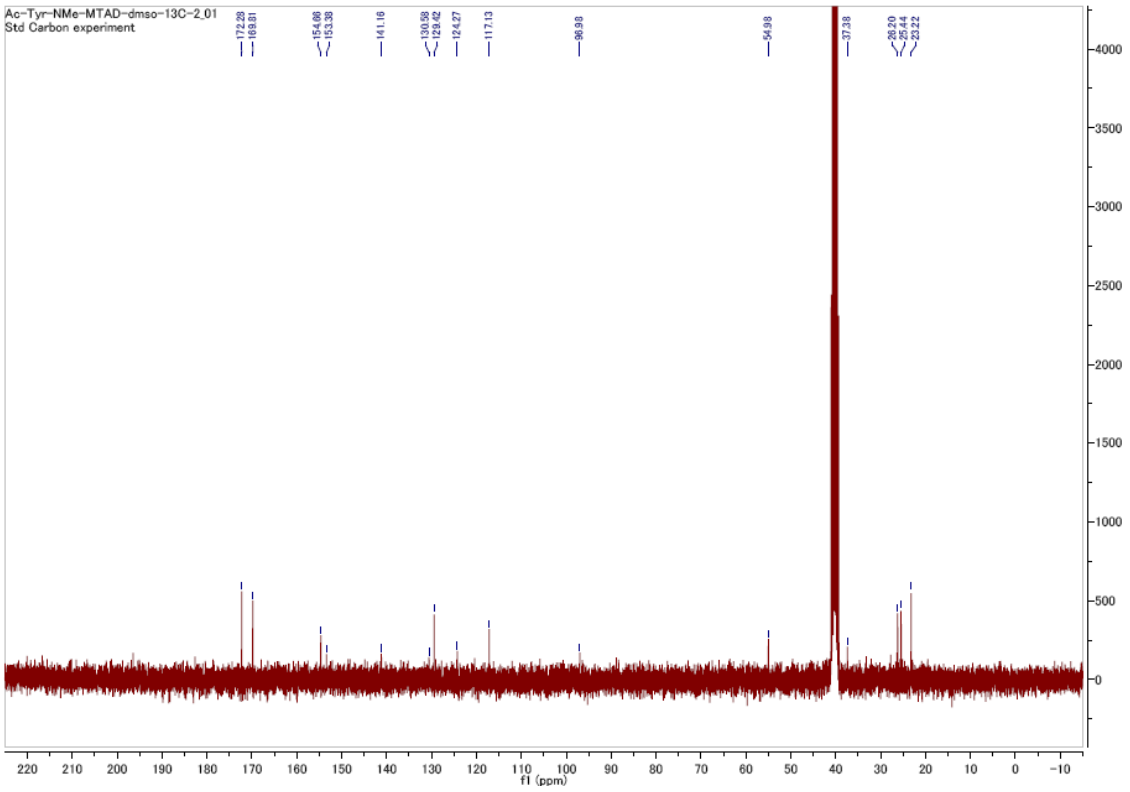
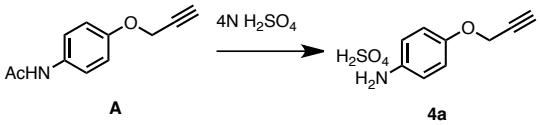
N
H
H
N
O
3b
f1 (ppm)



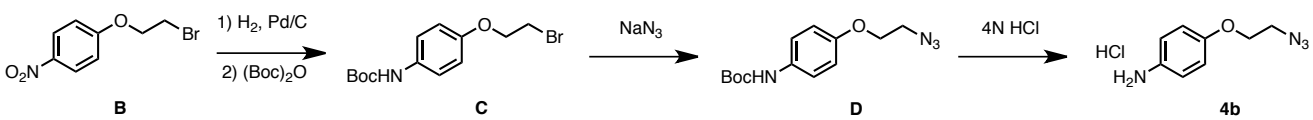
2. Synthesis of the PTAD analogs

2-1. Synthesis of aniline derivatives.



4-Propargyloxy aniline sulfate (**4a**)**.** A solution of *N*-(4-(propargyloxy)phenyl)acetamide **A** (650 mg, 3.44 mmol) in 4 M H_2SO_4 (10 mL) was stirred under reflux for 3 h. Obtained white crystals were filtered and washed with Et_2O to give **4a** (577 mg, 68%).

^{1}H NMR (300 MHz, DMSO-d6): δ 8.18 (br, 2H), 6.97-6.90 (m, 4H), 4.73 (d, J = 3.0 Hz, 2H), 3.55 (t, J = 3.0 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d6): δ153.92, 134.71, 120.94, 117.17, 80.61, 79.20, 57.06. HRMS: calcd for $C_9H_{10}NO$ (MH^+) 148.0757, found 148.0754.



4-*N*-Boc-(2-bromoethoxy)benzene (C). A suspension of 1-(2-bromoethoxy)-4-nitrobenzene **B** (1.00 g, 4.06 mmol) and 10% Pd/C (100 mg) in THF (20 mL) was stirred at room temperature for 3 h under a hydrogen atmosphere. Hydrogen was replaced with argon, and a solution of $(Boc)_2O$ (708 mg, 4.06 mmol) in THF (5 mL) was added. After overnight, the catalyst was removed by passing through Celite. After evaporation, the obtained solids were washed with Hexane/Et_2O to give **C** (742 mg, 58%) as white solid.

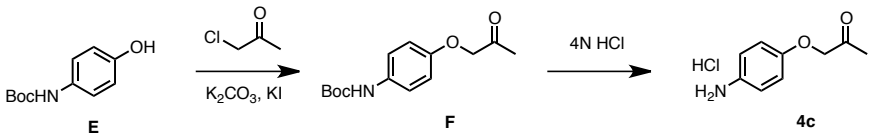
^{1}H NMR (300 MHz, $CDCl_3$): δ 7.28-7.25 (m, 2H), 6.87-6.84 (m, 2H), 6.41 (br, 1H), 4.25 (t, J = 6.0 Hz, 2H), 3.61 (t, J = 6.0 Hz, 2H), 1.51 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ154.36, 153.39, 132.52, 120.80, 115.66, 80.67, 68.65, 29.53, 28.69. HRMS: calcd for $C_{13}H_{18}BrNNaO_3$ (MNa^+) 338.0362, found 338.0366.

4-*N*-Boc-(2-azidoethoxy)benzene (D). A suspension of compound **C** (1.64 g, 5.19 mmol) and NaN_3 (1.68 g, 25.9 mmol) in DMF (25 mL) was stirred at 50 ^{0}C for 3 h. Then, EtOAc and water were added. The organic layer was separated and washed once with water. The resulting aqueous layer was extracted once with EtOAc. The combined organic layer was dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by short silica gel chromatography (Hexane/EtOAc) and washing with Hexane/Et_2O to give **D** (1.24 g, 86%) as white crystals.

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.29-7.26 (m, 2H), 6.87-6.84 (m, 2H), 6.43 (br, 1H), 4.11 (t, J = 6.0 Hz, 2H), 3.57 (t, J = 6.0 Hz, 2H), 1.51 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ154.51, 153.41, 132.42, 120.75, 115.36, 80.63, 67.64, 50.49, 28.67. HRMS: calcd for $C_{13}H_{18}N_4NaO_3$ (MNa^+) 301.1271, found 301.1258.

4-Propargyloxy aniline sulfate (4a). A solution of *N*-(4-(propargyloxy)phenyl)acetamide **A** (650 mg, 3.44 mmol) in 4 M H_2SO_4 (10 mL) was stirred under reflux for 3 h. Obtained white crystals were filtered and washed with Et_2O to give **4a** (577 mg, 68%).

^{1}H NMR (300 MHz, DMSO-d6): δ 8.18 (br, 2H), 6.97-6.90 (m, 4H), 4.73 (d, J = 3.0 Hz, 2H), 3.55 (t, J = 3.0 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d6): δ153.92, 134.71, 120.94, 117.17, 80.61, 79.20, 57.06. HRMS: calcd for $C_9H_{10}NO$ (MH^+) 148.0757, found 148.0754.



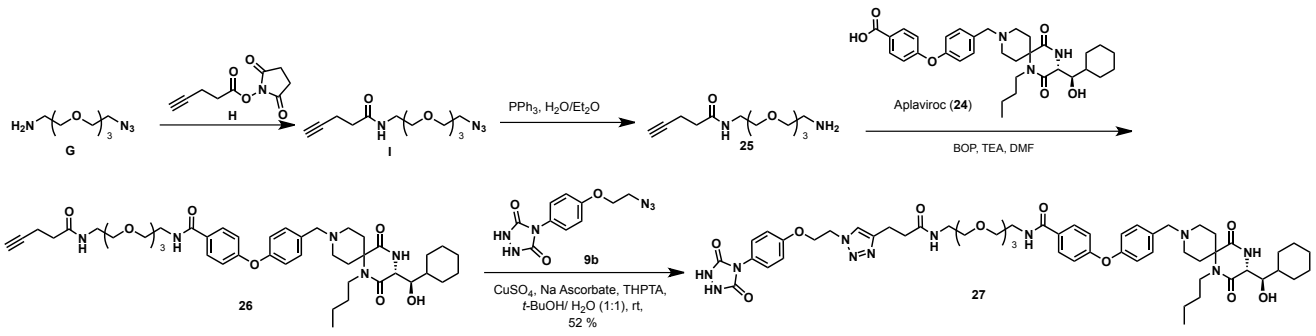
4-*N*-Boc-(2-oxopropoxy)benzene (F). To a suspension of 4-*N*-Boc-aminophenol **E** (4.00 g, 18.2 mmol), K_2CO_3 (3.05 g, 22.1 mmol) and KI (1.22 g, 7.36 mmol) in acetone (40 mL) was added chloroacetone (0.703 mL, 8.83 mmol) under reflux. After 2 h, additional chloroacetone (0.703 mL, 8.83 mmol) was added. The resulting suspension was stirred under reflux for 2 h. Then, EtOAc and water were added. The organic layer was separated and washed once with water. The resulting aqueous layer was extracted once with EtOAc. The combined organic layer was dried over $MgSO_4$, and concentrated *in vacuo*. The generated white solids were washed with Hexane/Et_2O to give **F** (1.63 g, 83%).

^{1}H NMR (300 MHz, DMSO-d6): δ 9.13 (br, 1H), 7.33-7.30 (m, 2H), 6.82-6.78 (m, 2H), 4.71 (s, 2H), 2.13 (s, 3H), 1.45 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ206.25, 153.96, 153.37, 132.78, 120.81, 115.23, 80.65, 73.72, 28.65, 26.92. HRMS: calcd for $C_{14}H_{20}NNaO_4$ (MNa^+) 288.1206, found 288.1199.

4-(2-Oxopropoxy)aniline hydrochloride (4c). A solution of compound **F** (400 mg, 1.51 mmol) in 4 M HCl/dioxane (10 mL) was stirred at room temperature for 3 h. Solvent was removed *in vacuo* and resulting pale brown solids were washed with EtOAc to give **4c** (303 mg, quant.).

^{1}H NMR (300 MHz, DMSO-d6): δ 10.3 (br, 2H), 7.35-7.32 (m, 2H), 7.02-6.99 (m, 2H), 4.87 (s, 2H), 2.16 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d6): δ204.68, 158.20, 125.42, 116.32, 73.17, 27.20. HRMS: calcd for $C_9H_{12}NO_2$ (MH^+) 166.0863, found 166.0867.

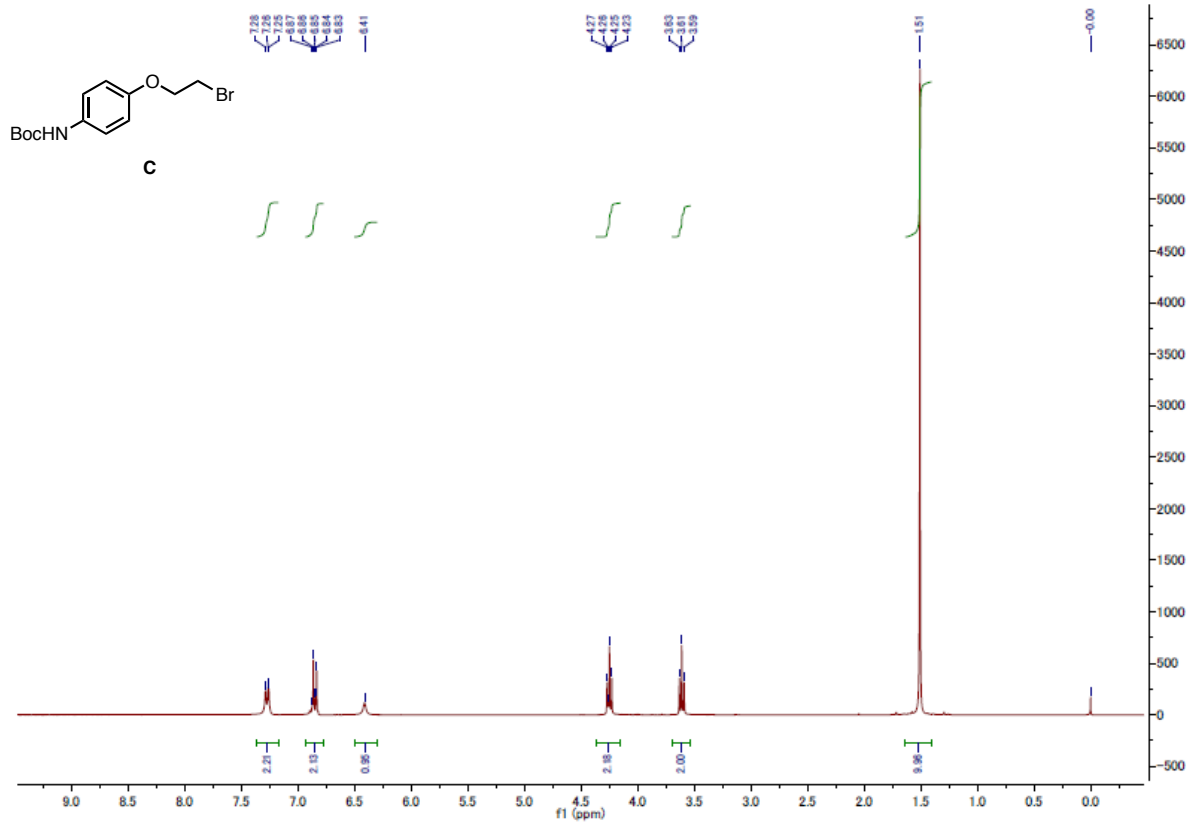
2-2. Synthesis of Aplaviroc derivatives

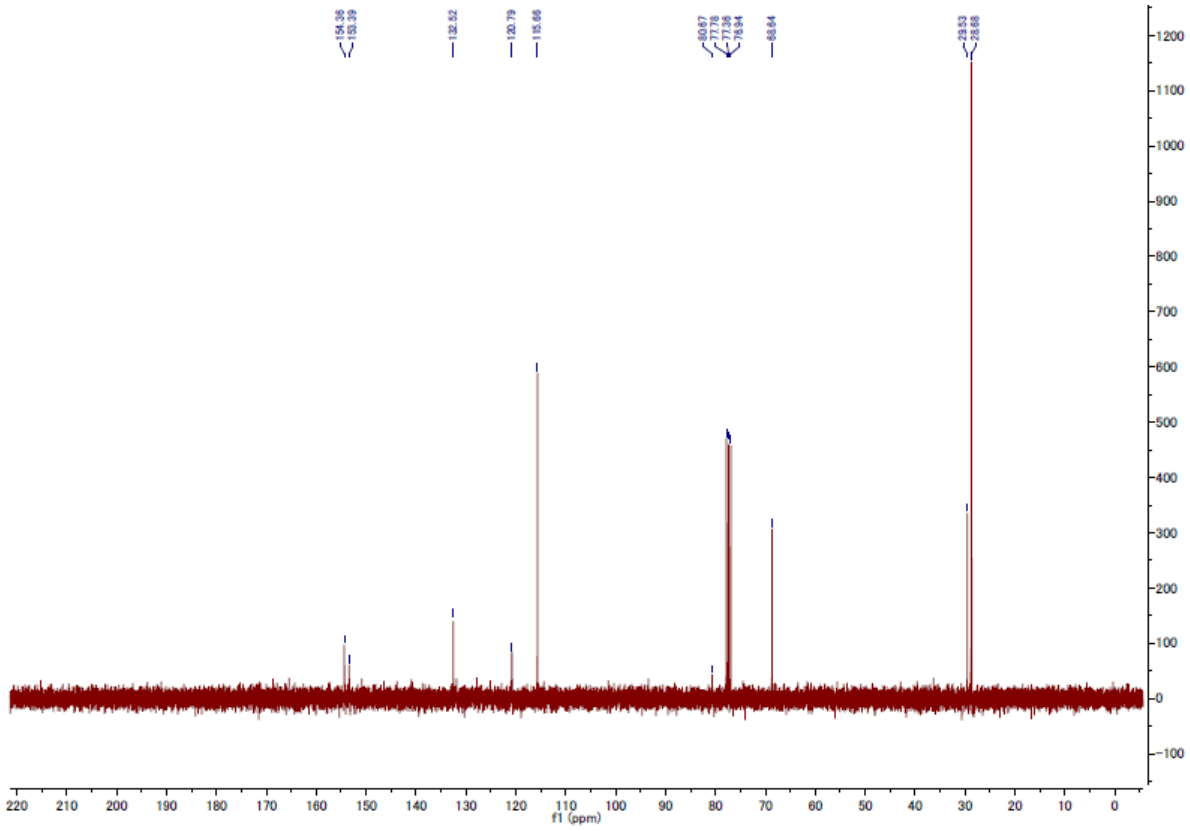


Compound I:

To a solution of azido-amine **G** (Aldrich, 468 mg, 2.391 mmol) in acetonitrile (5 mL) was added pentynoic acid succinimide ester **H** (474 mg, 2.391 mmol) at room tempareture and stirred for 12 hours. Then, dichloromethane were added and was separated and washed 0.5 M HCl, sat.$NaHCO_3$ aq. and brine. Combined organic layer was dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica gel chromatography (EtOAc) to give **I** (620 mg, 87%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.30 (br s, 1H), 3.75-3.57 (m, 10H), 3.60-3.57 (m, 2H), 3.52-3.47 (m, 2H), 3.49-3.41 (m, 2H), 2.57-2.53 (m, 2H), 2.45-2.41 (m, 2H), 2.04 (t, J = 5.3 Hz, 1H), ^{13}C NMR (125 MHz, MeOD-d4): δ173.09, 82.78, 72.70, 70.67, 70.65, 70.62, 70.60, 70.56, 70.52, 70.48, 70.42, 70.32, 70.12, 69.60, 61.26, 50.81, 48.19, 39.48, 35.47. HRMS: calcd for $C_{13}H_{22}N_4O_4$ (MH^+) 299.1714, found 299.1715.

3. NMR-charts of the PTAD analogs

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115.66
80.87
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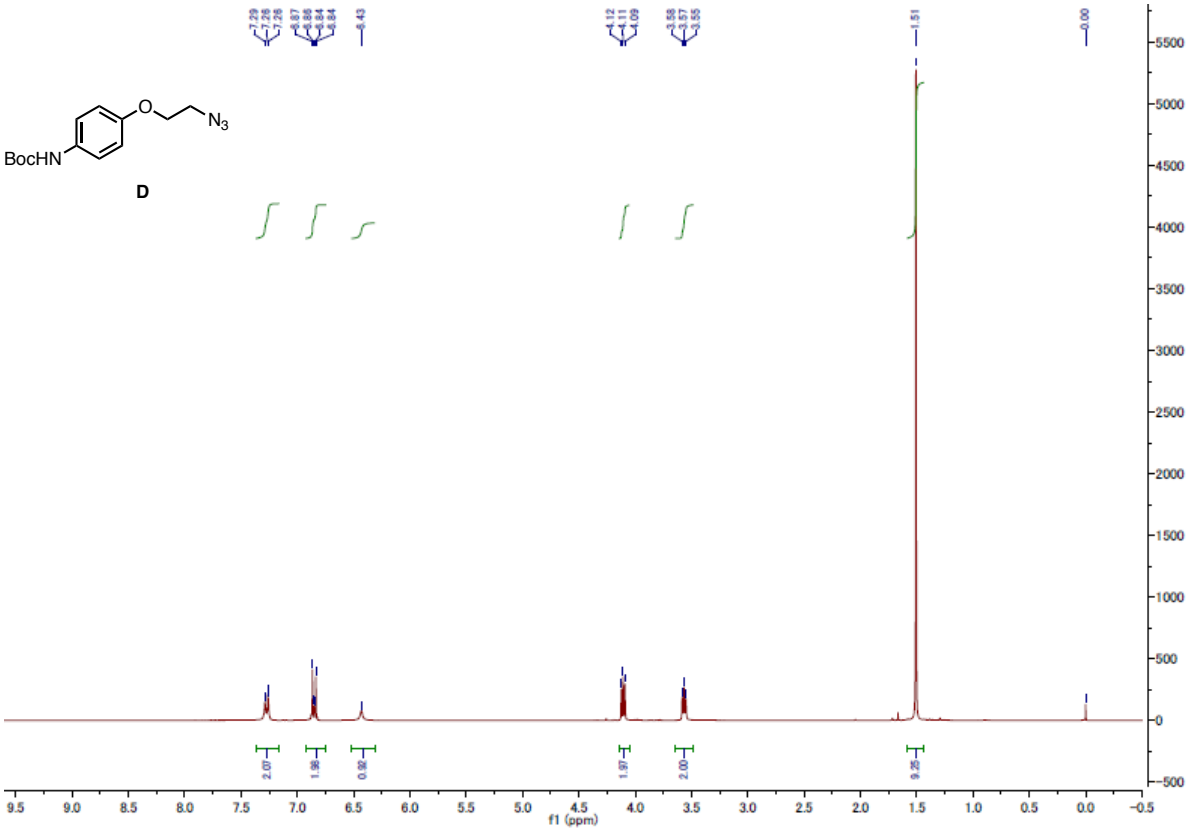
77.36
76.94
68.64
28.53
28.48
f1 (ppm)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6.84
6.43
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4.09
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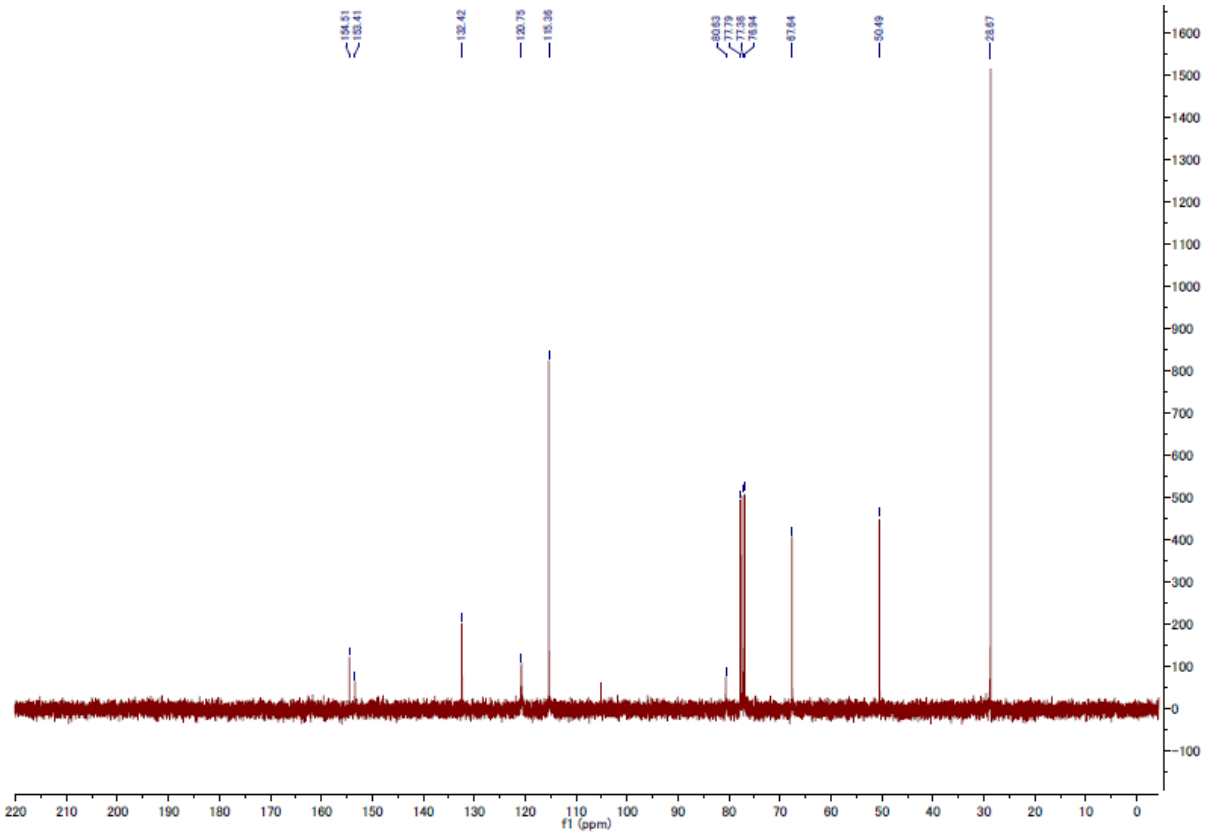
f1 (ppm)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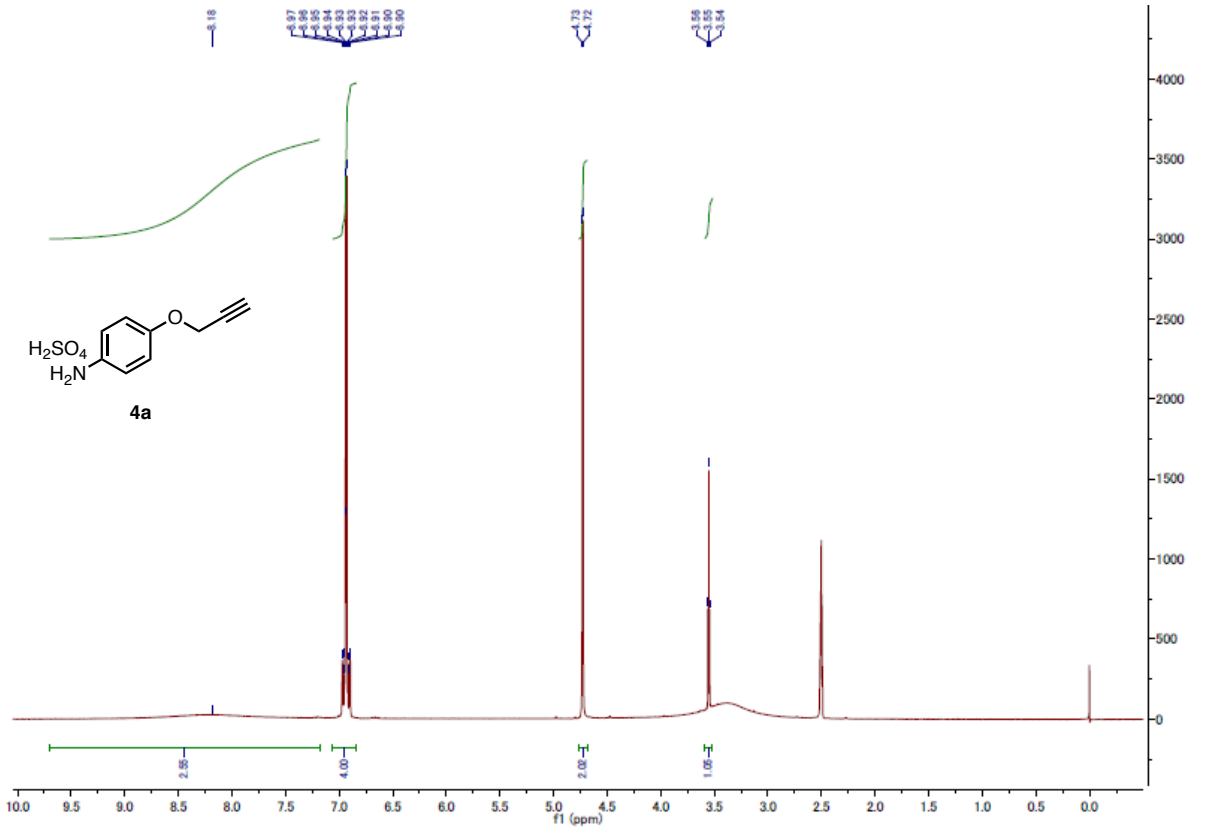
67.64
50.49
28.67
f1 (ppm)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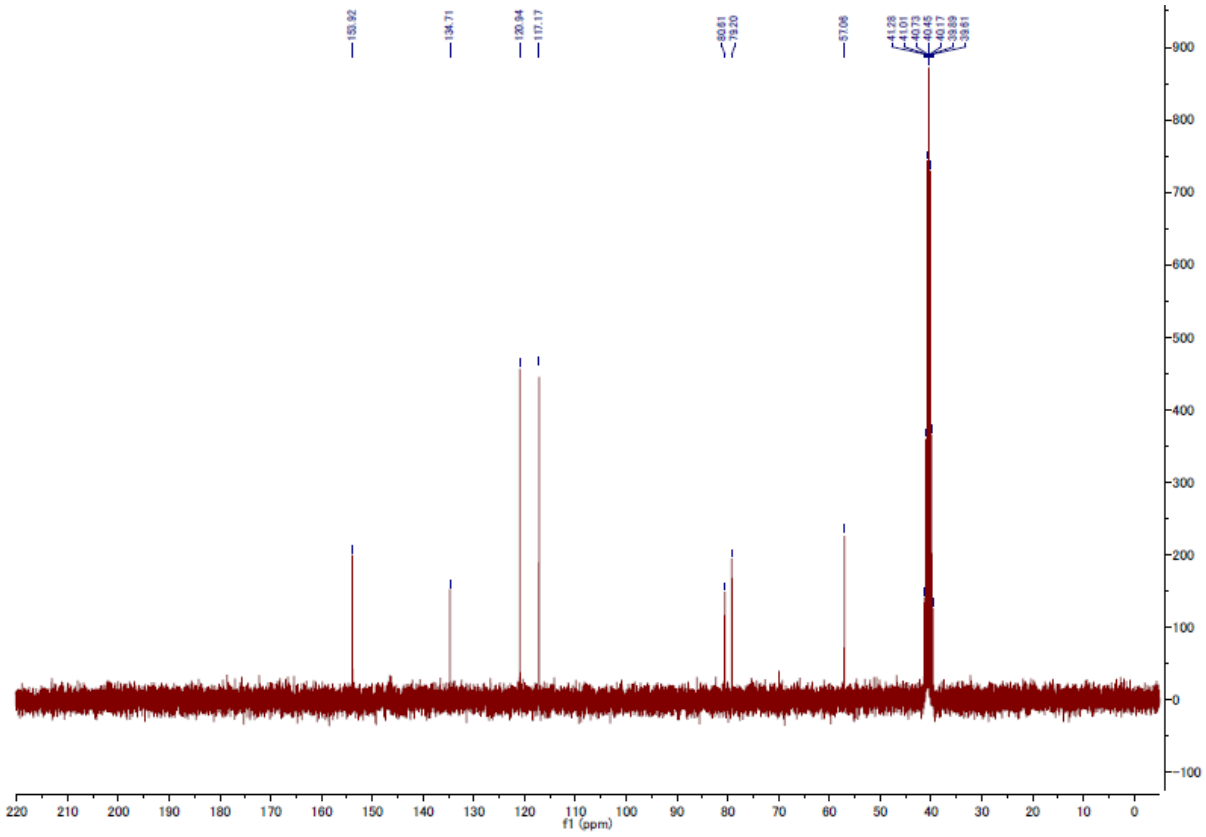
f1 (ppm)

H_2SO_4
H_2N
O

4a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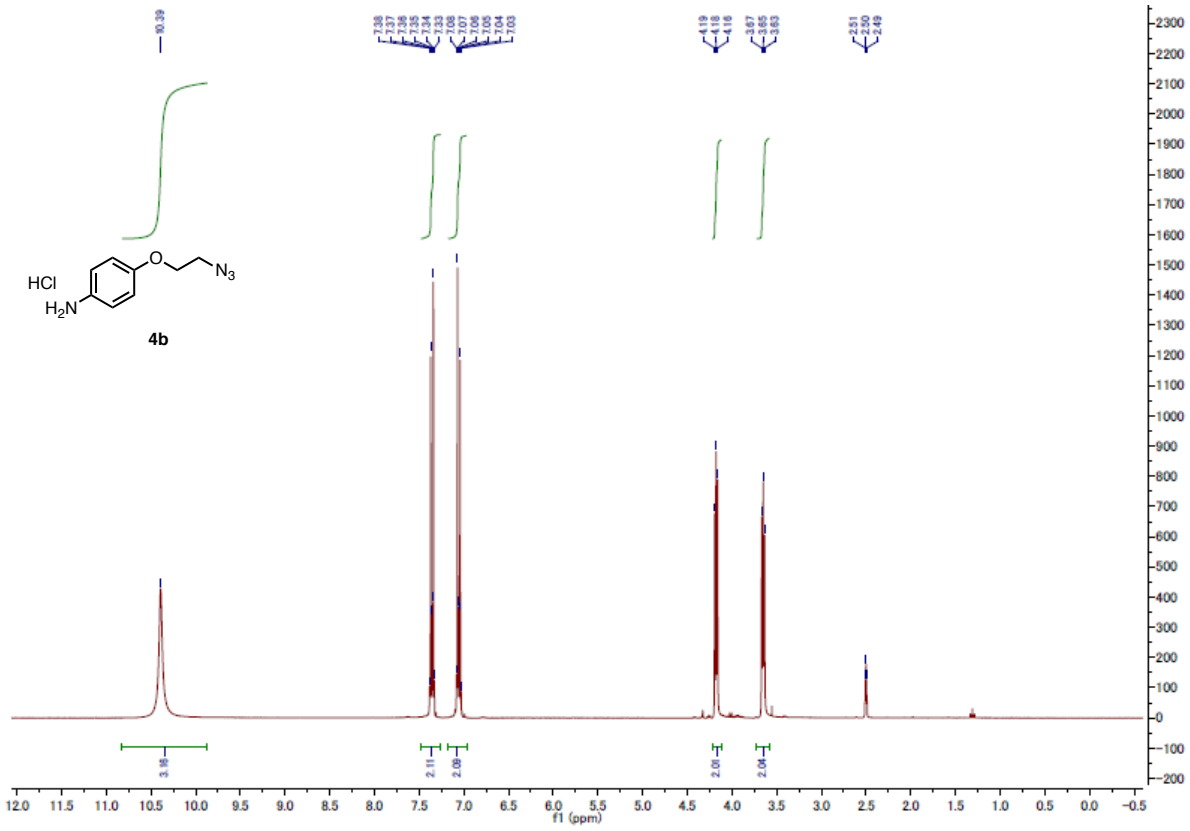
39.61
f1 (ppm)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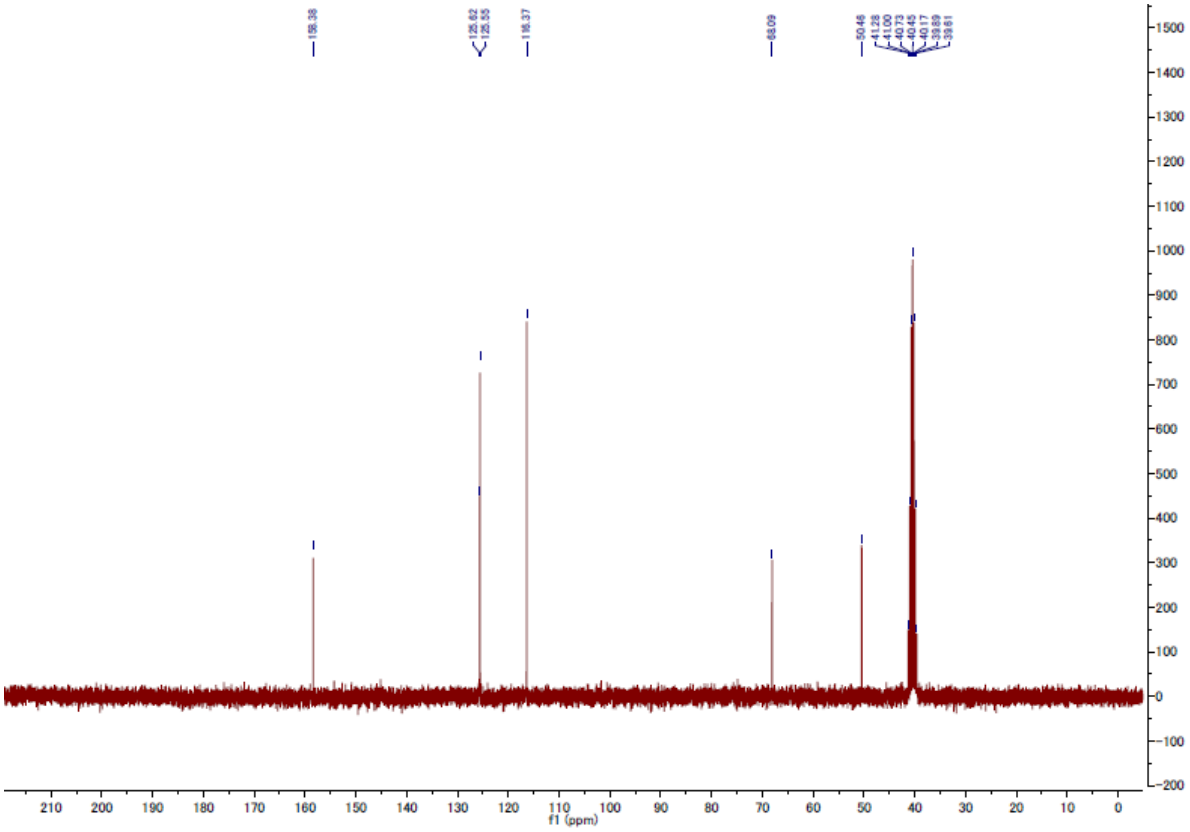
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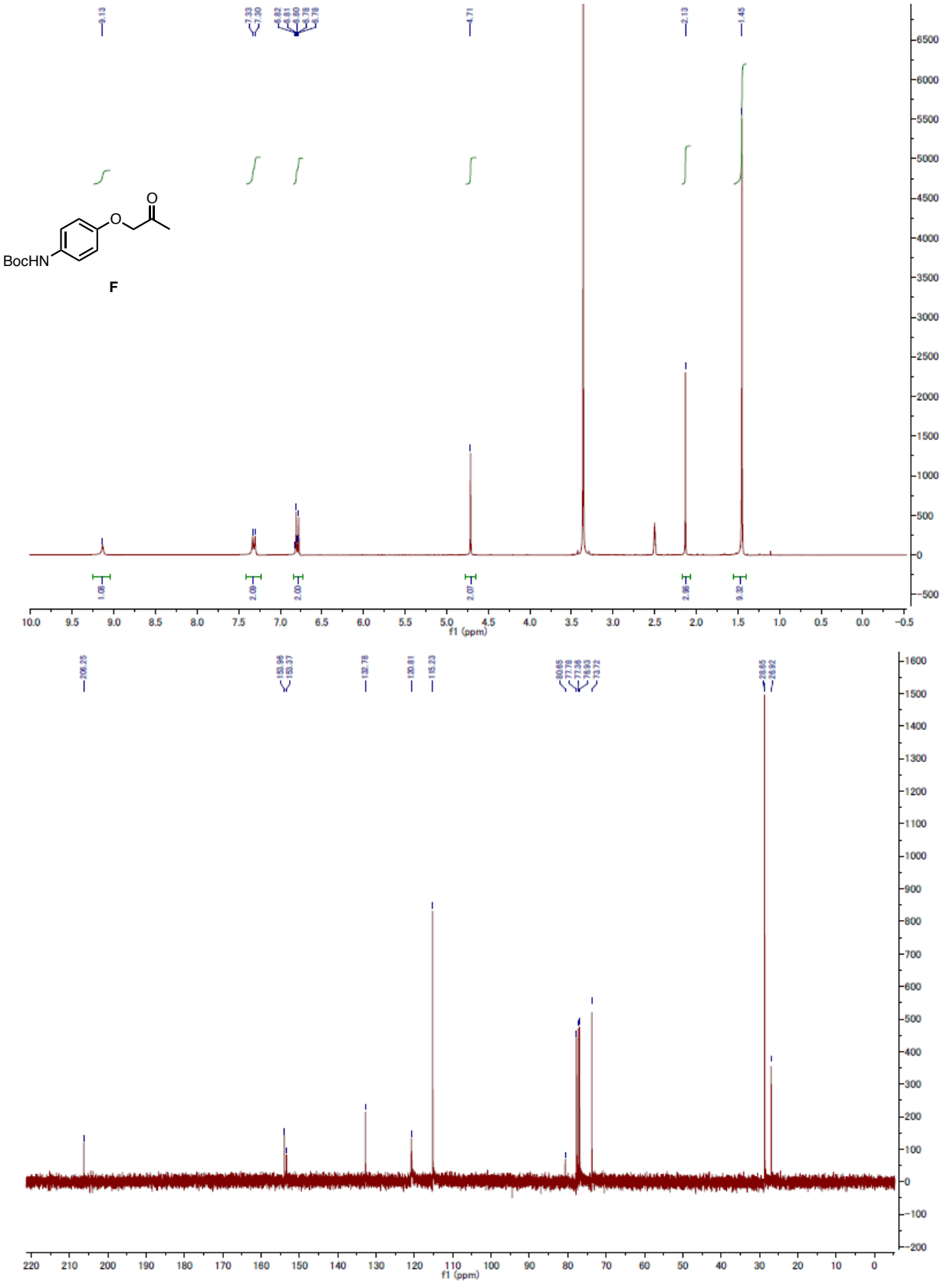
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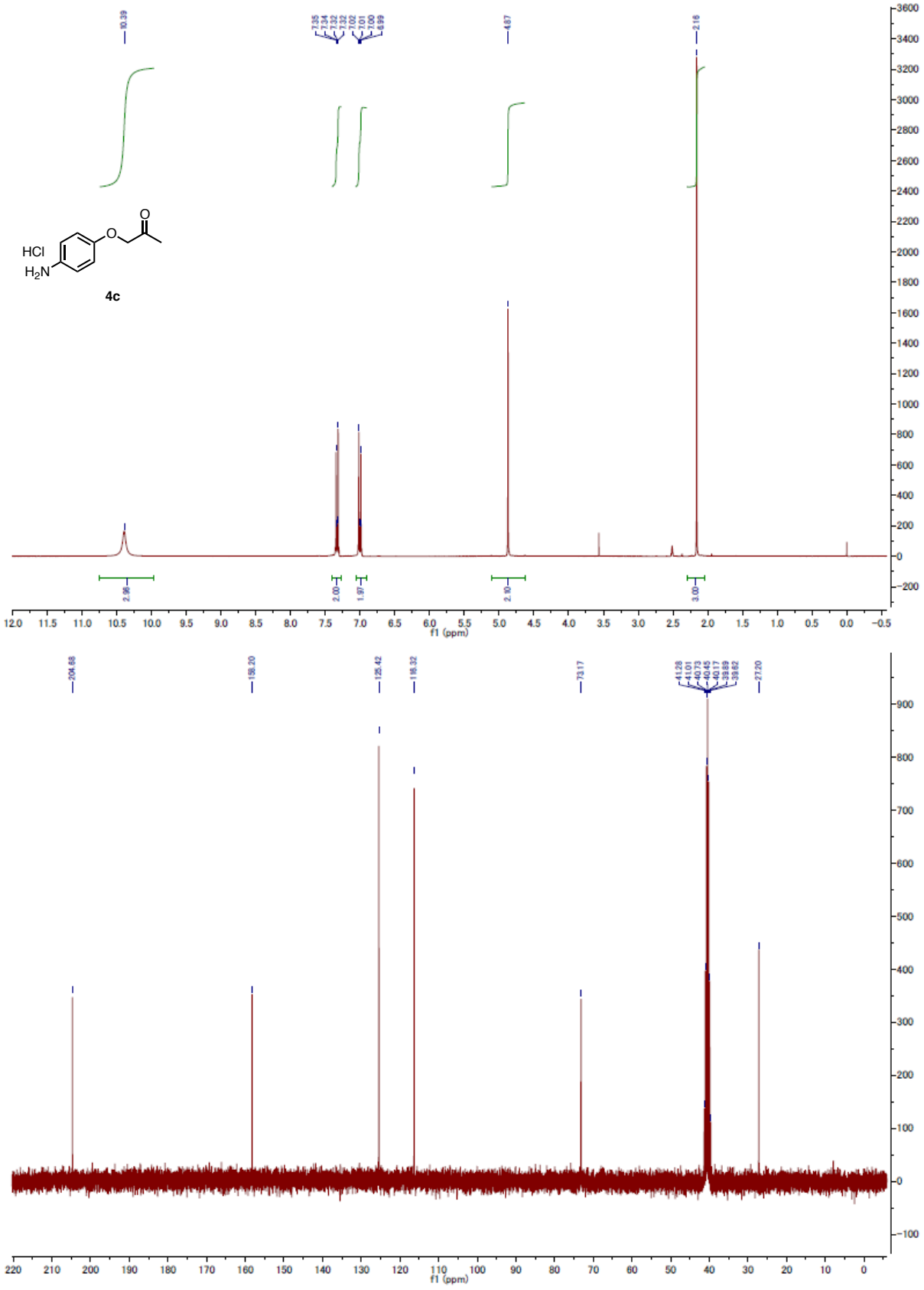
f1 (ppm)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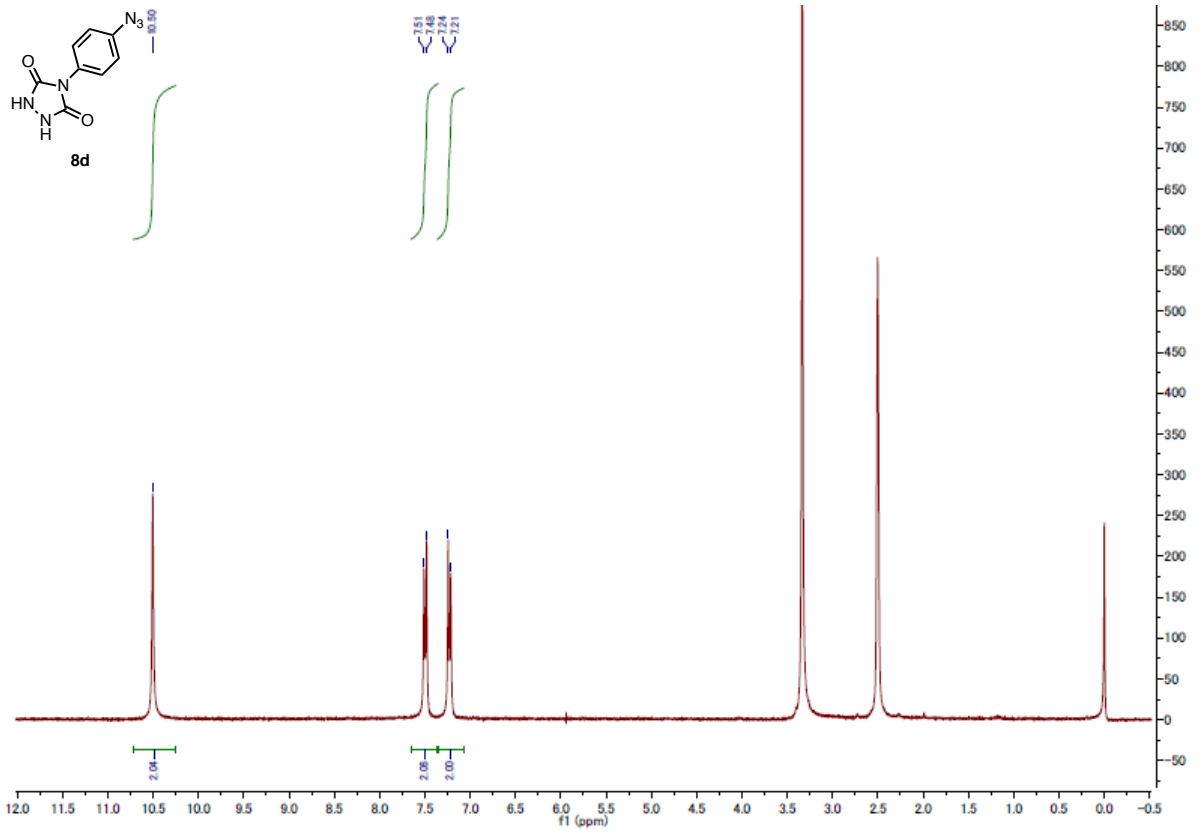
f1 (ppm)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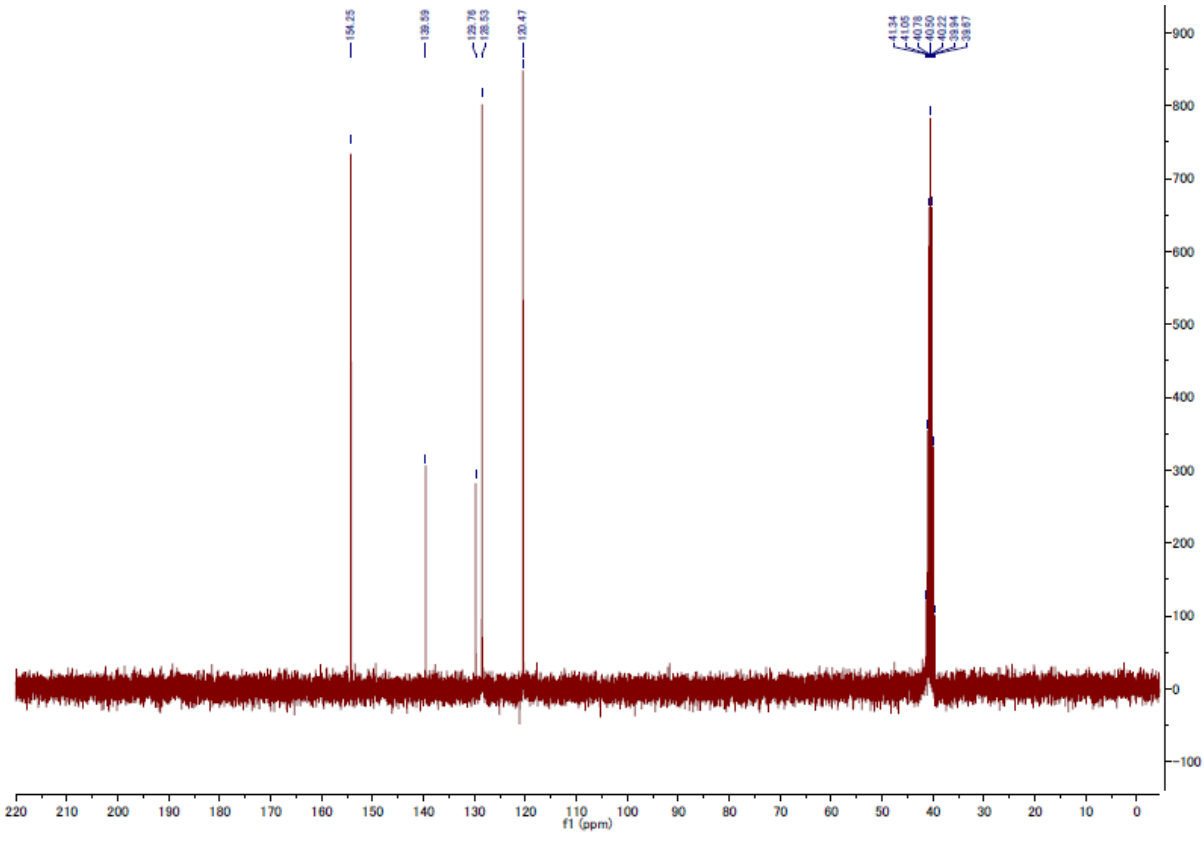
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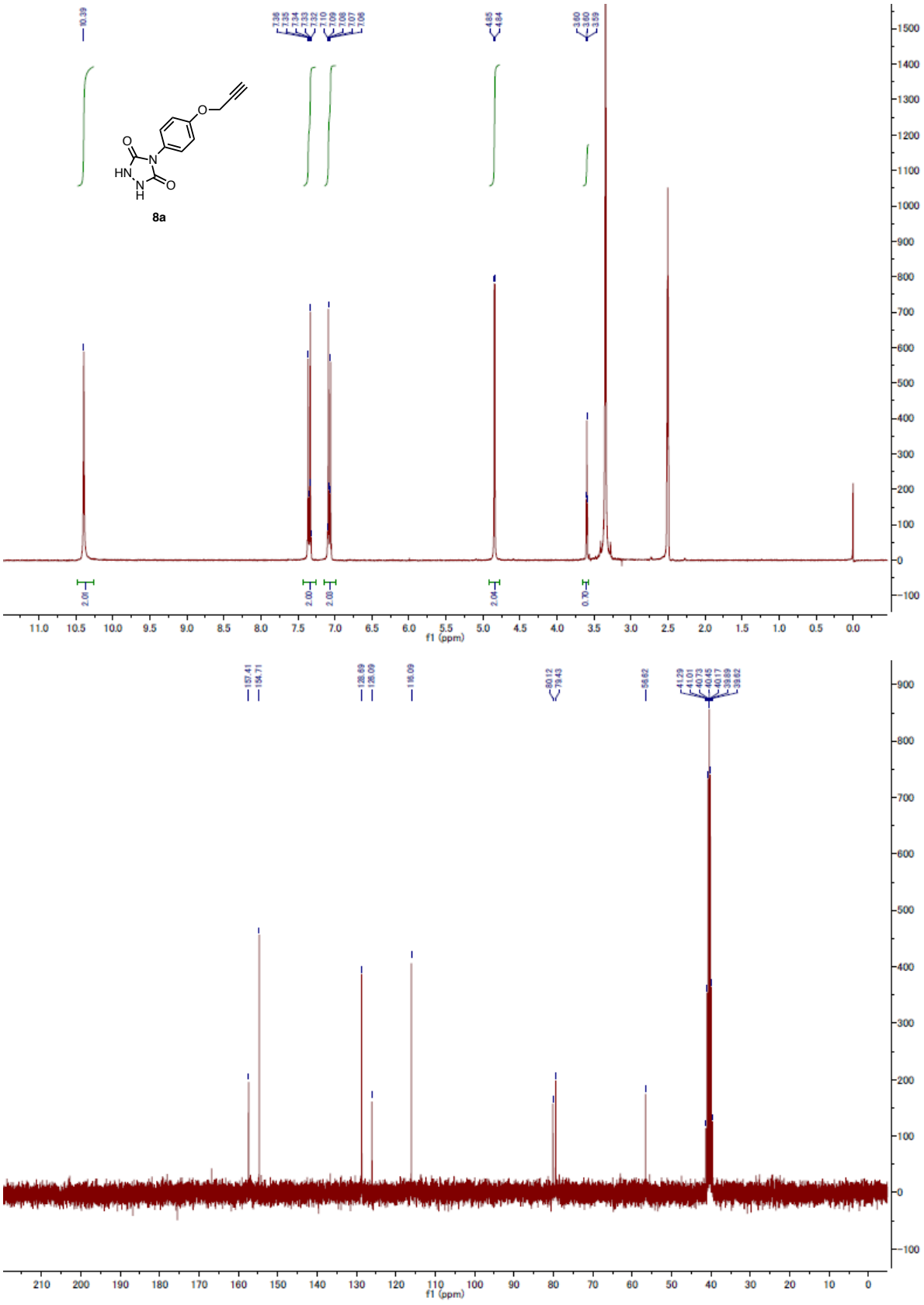
f1 (ppm)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7.07
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3.60
3.59
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2.00
2.00
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f1 (ppm)
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154.71
128.89
128.09
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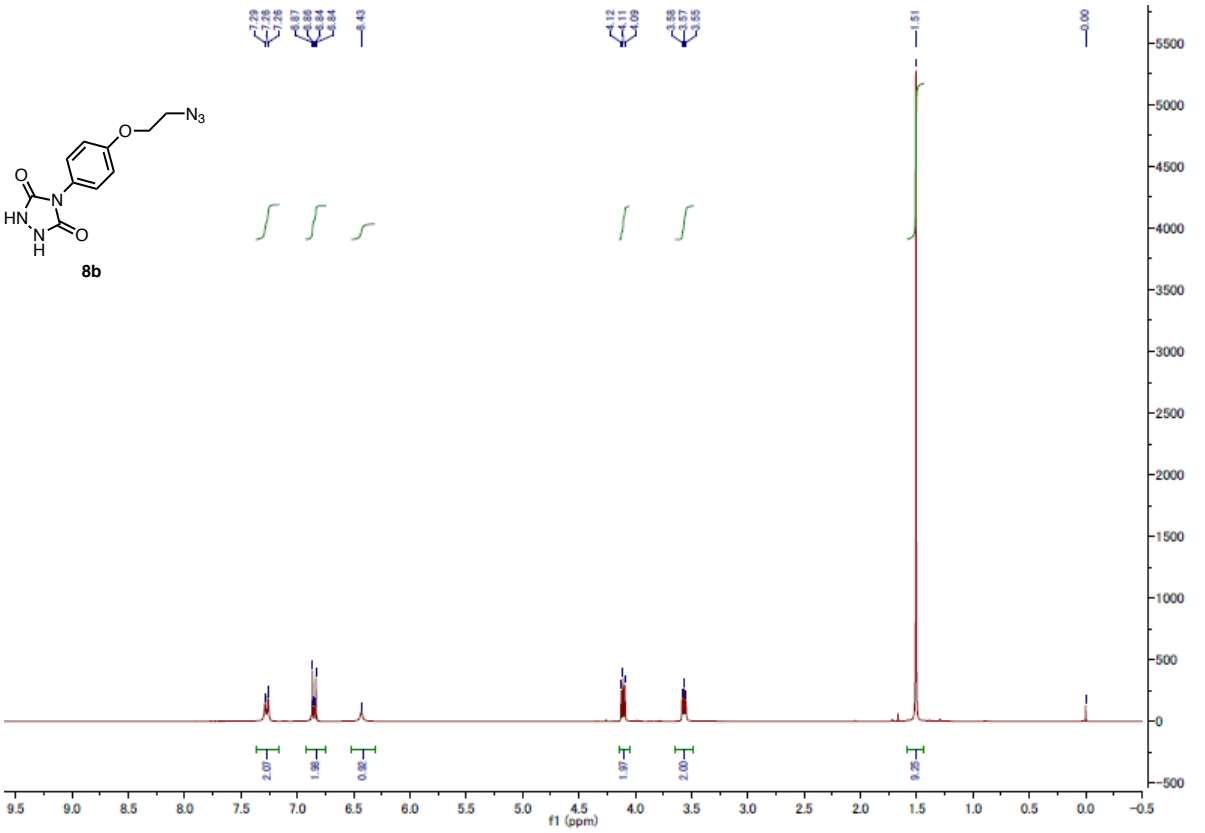
f1 (ppm)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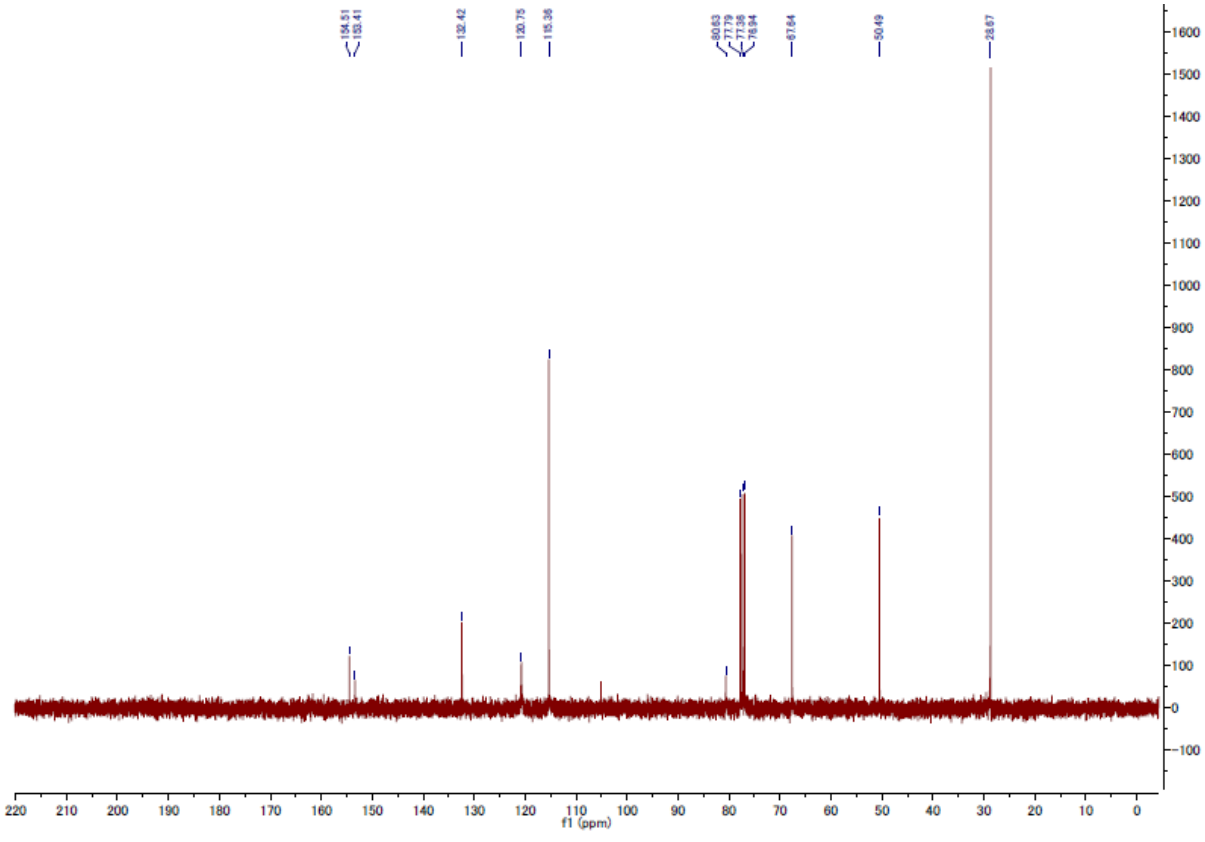
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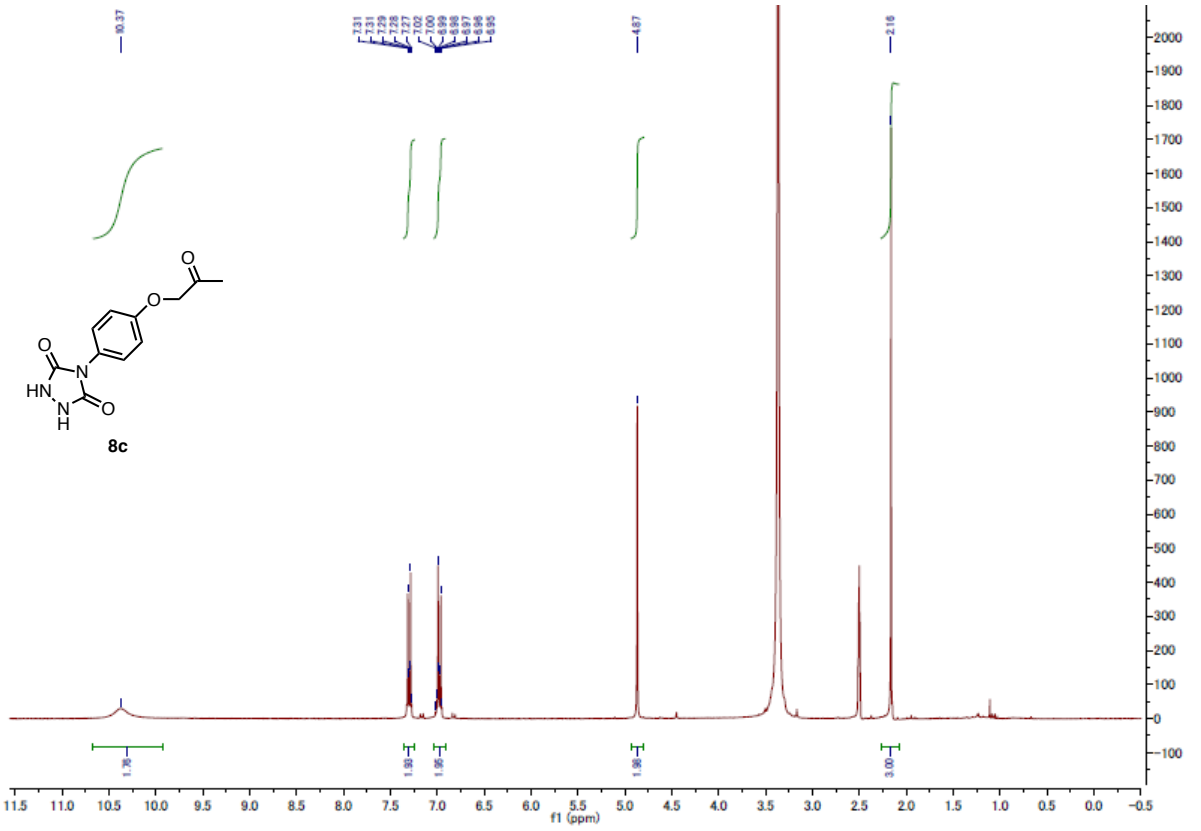
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6.96
6.95
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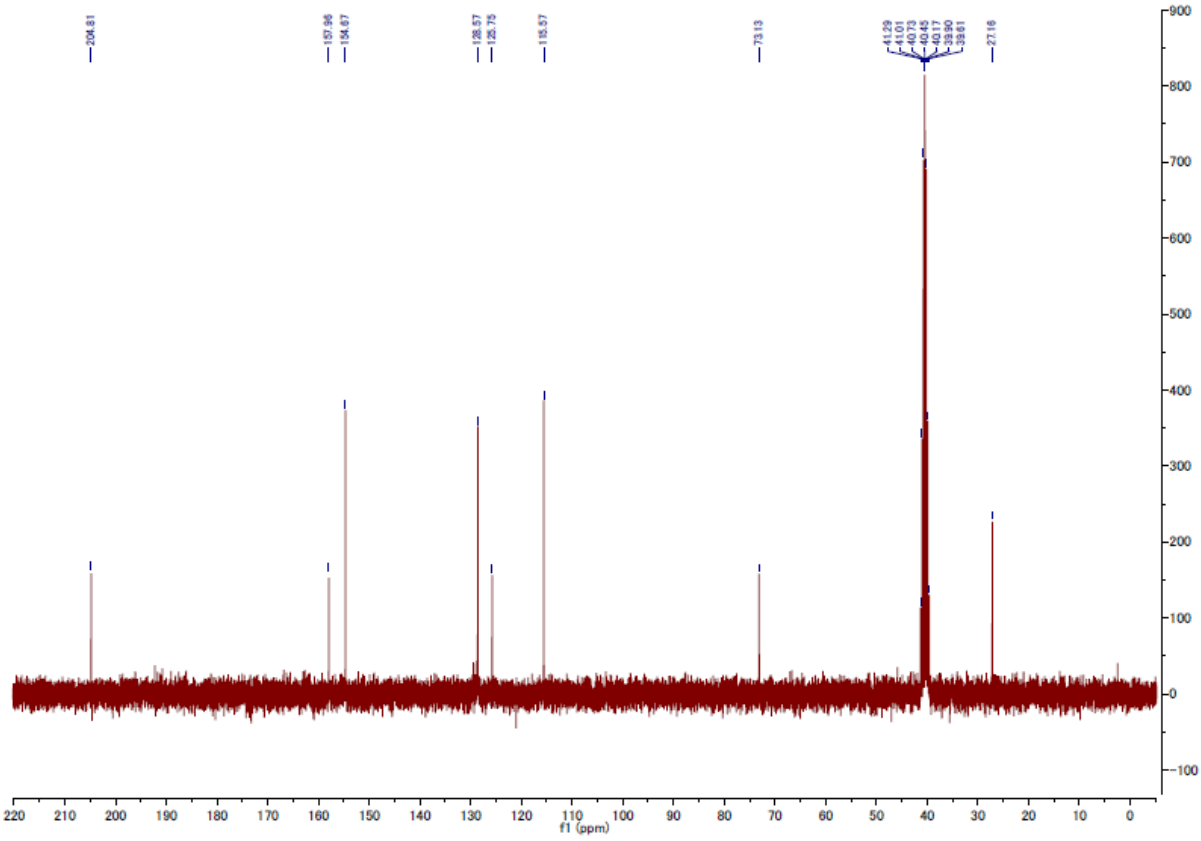
8c
f1 (ppm)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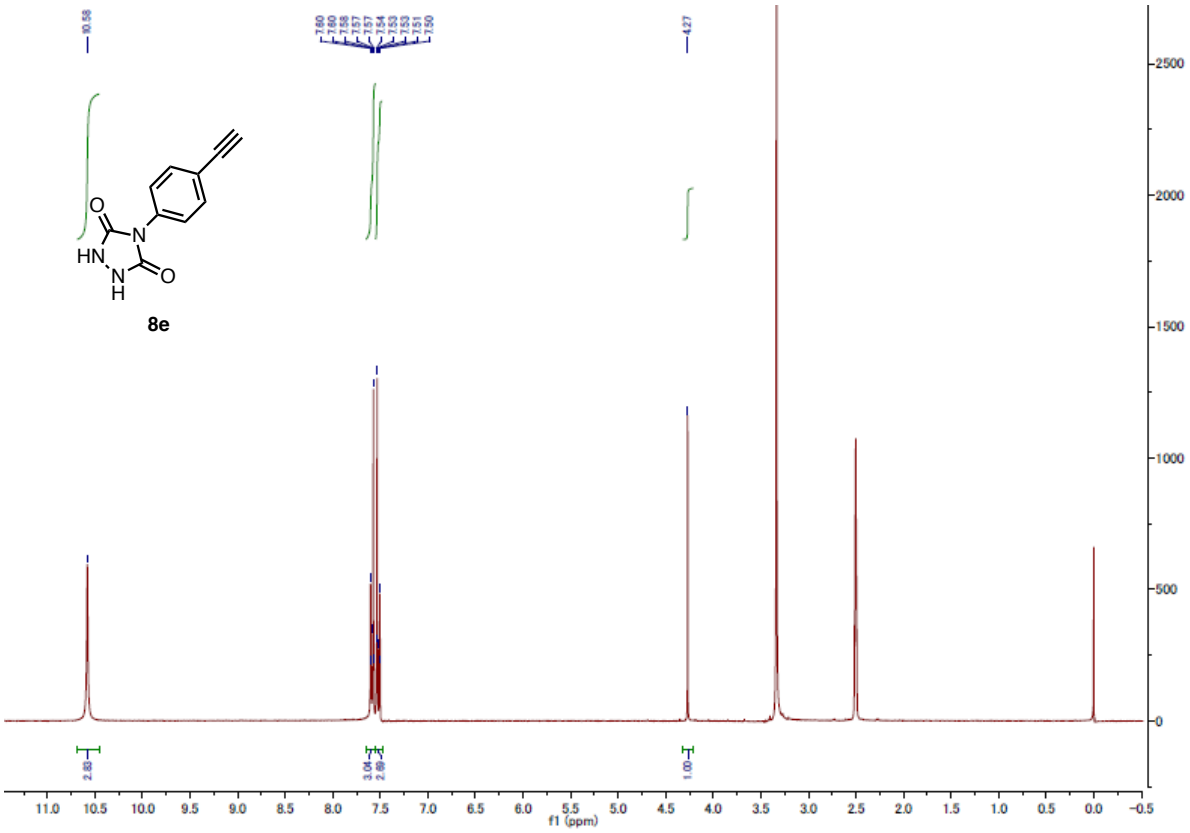
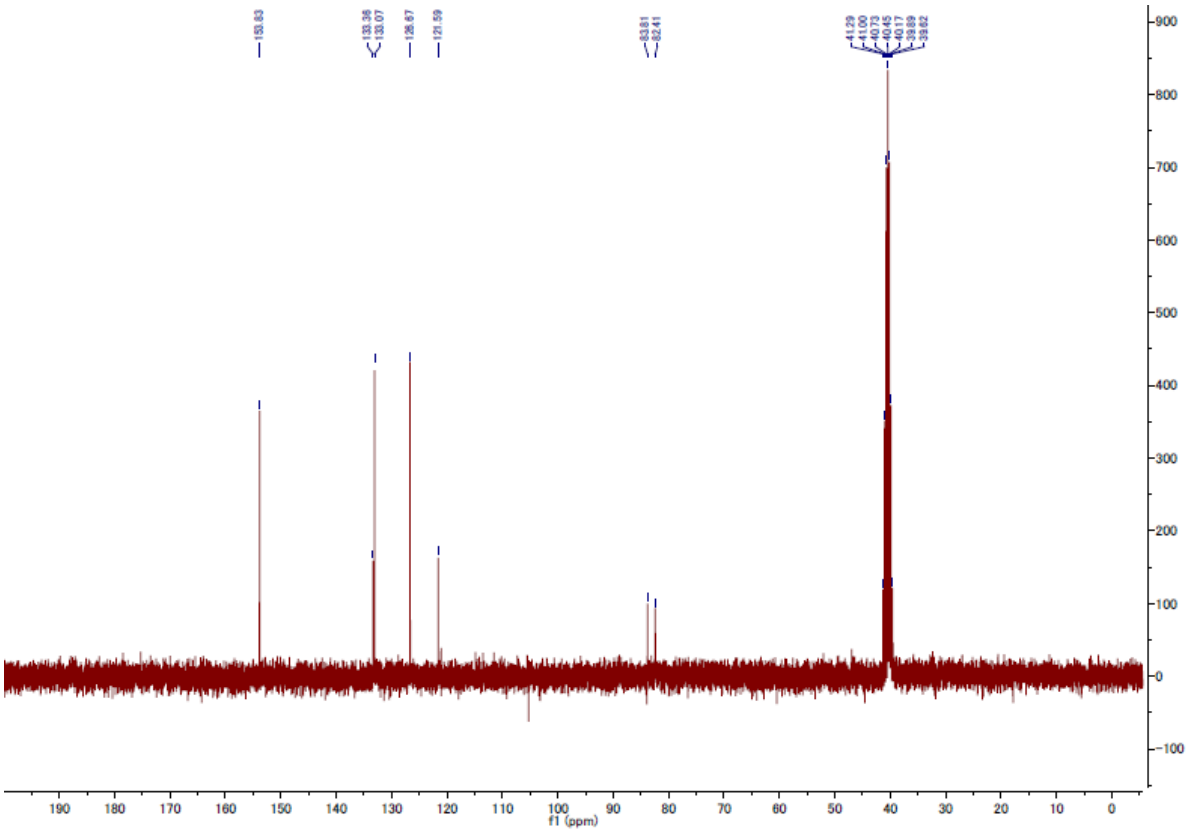
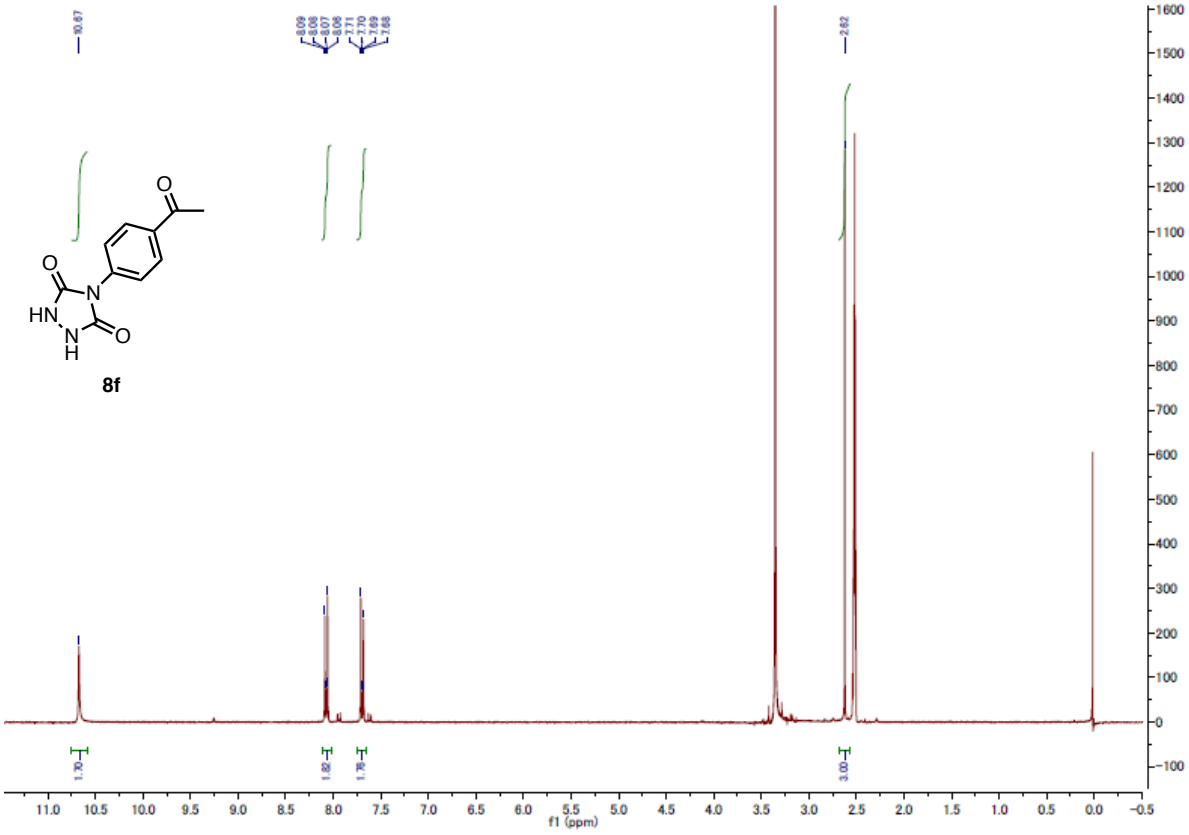
39.61
27.16
f1 (ppm)

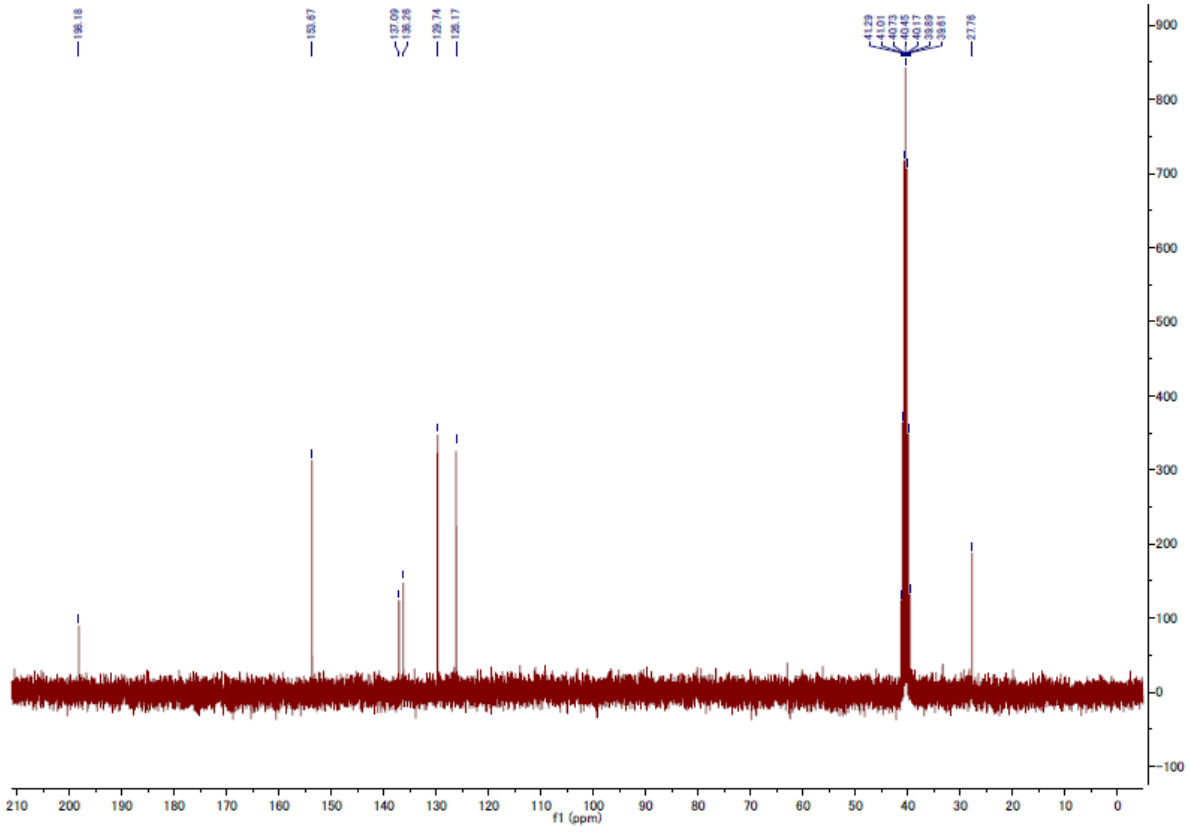

10.58
7.60
7.60
7.58
7.57
7.57
7.54
7.53
7.53
7.51
7.50
4.27
2.83
3.04
2.89
1.00
f1 (ppm)

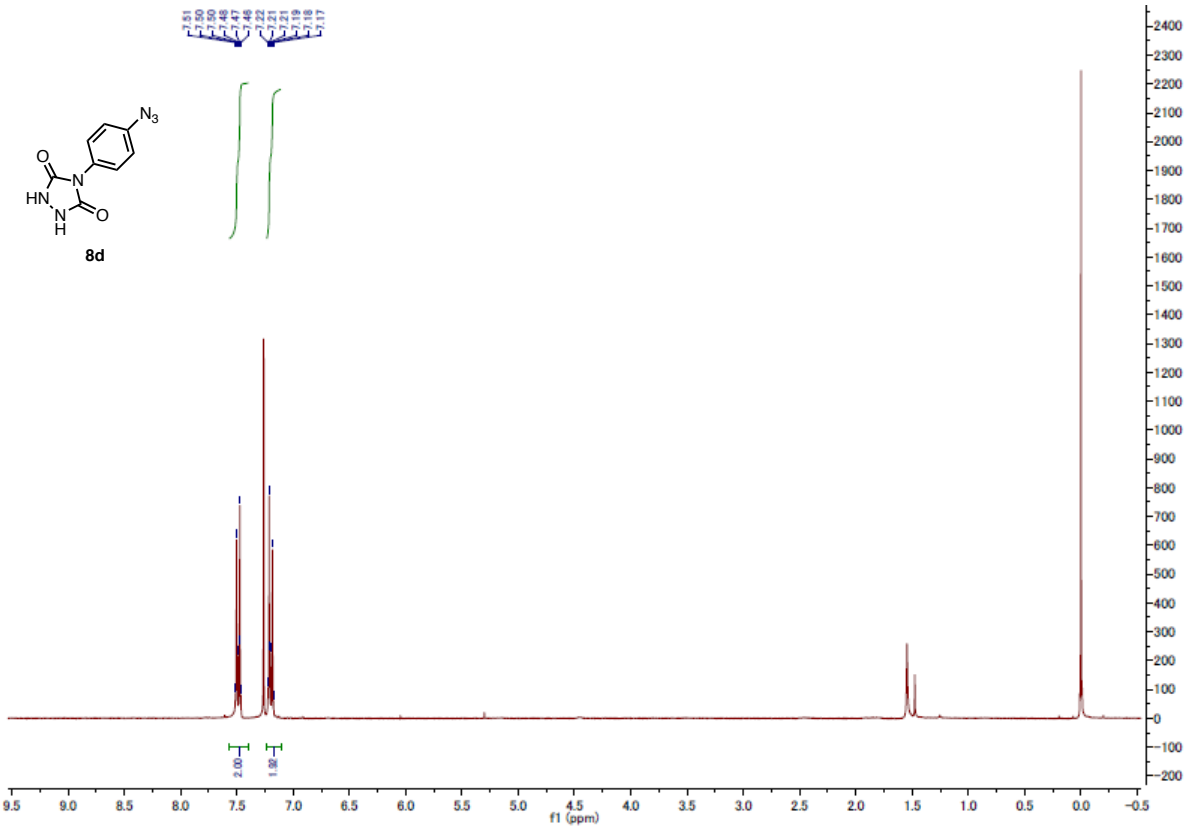
8e

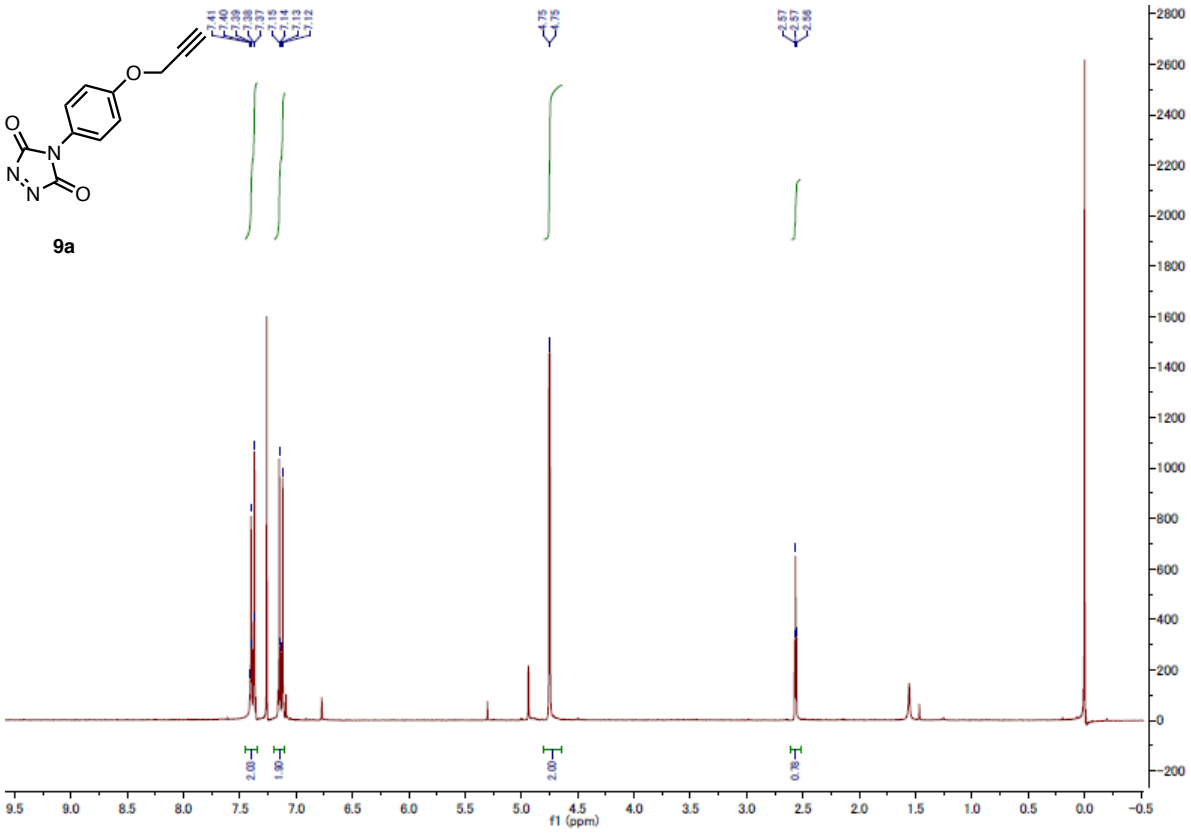

153.83
133.36
130.07
128.67
121.59
83.61
82.41
41.23
41.00
40.73
40.45
40.17
39.89
39.62
f1 (ppm)

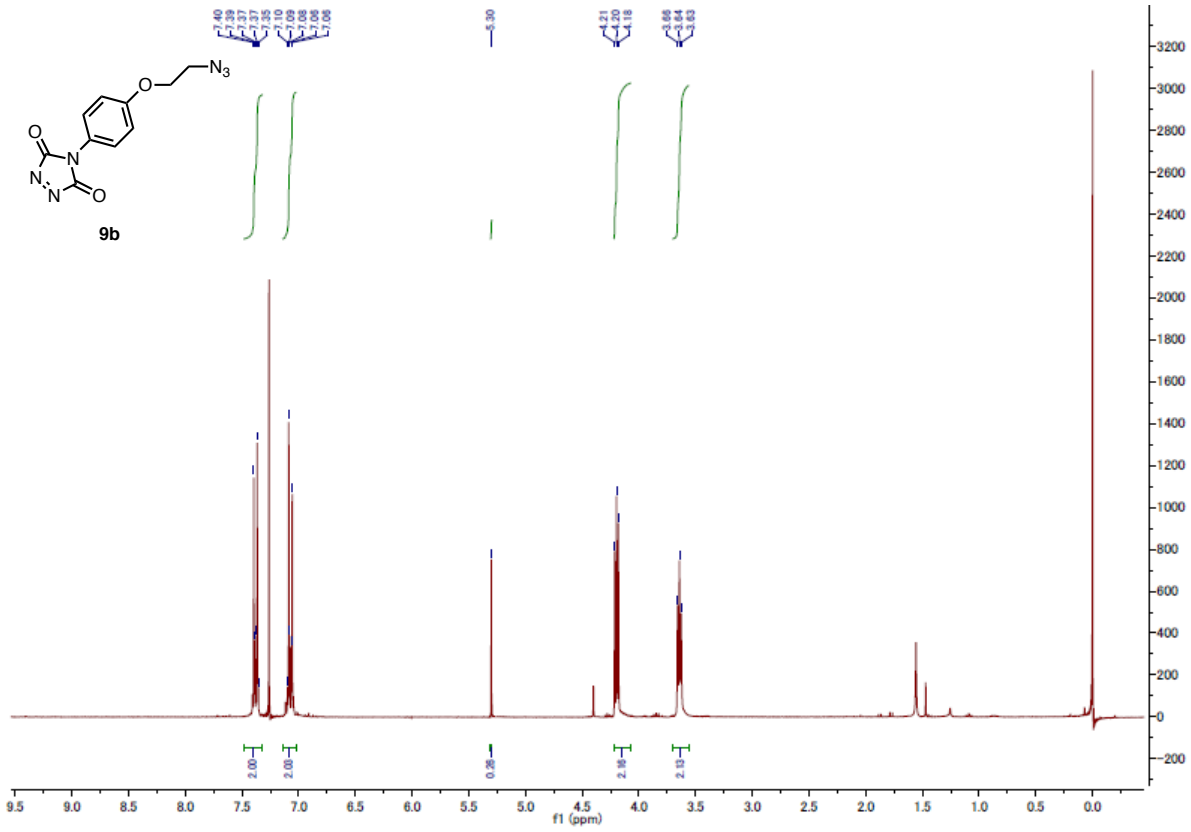

10.67
8.09
8.08
8.07
8.06
7.71
7.70
7.69
7.68
2.62
1.70
1.82
1.78
3.00
f1 (ppm)

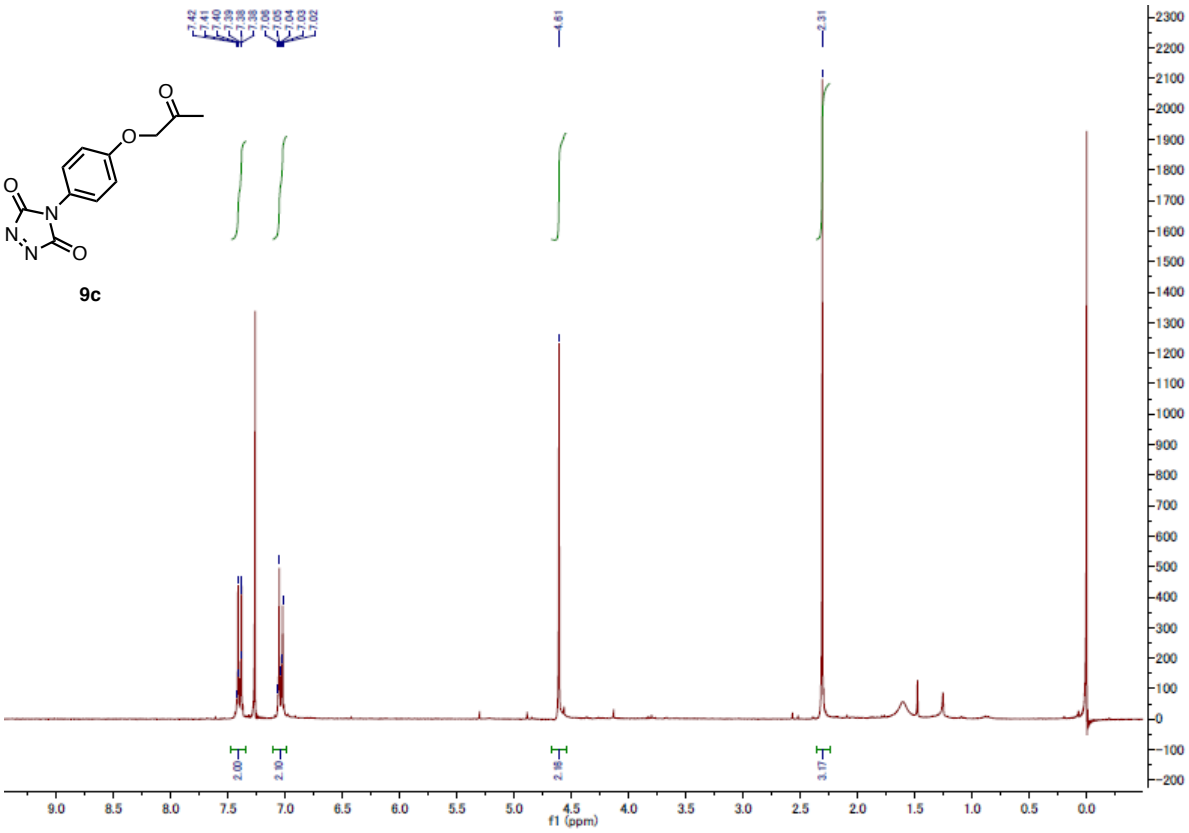
8f

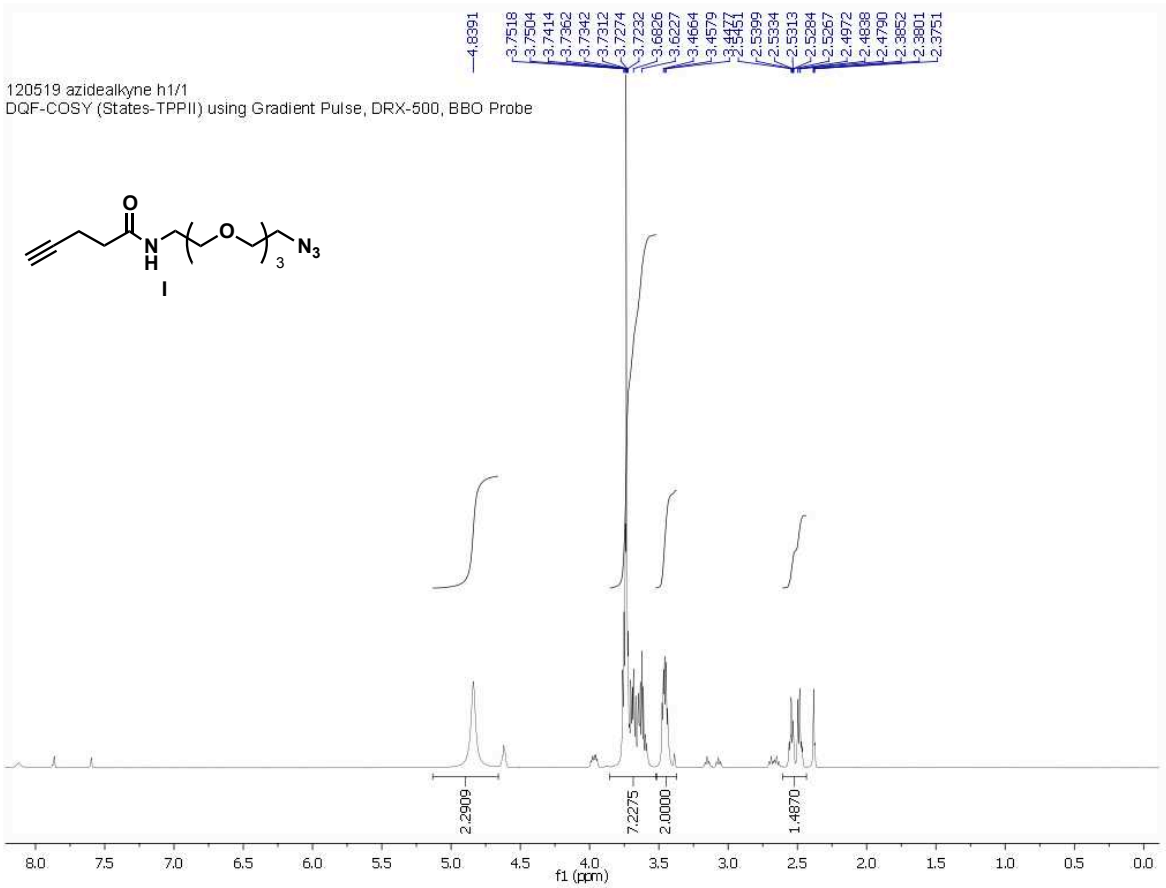

196.18
153.67
137.09
136.26
129.74
126.17
41.29
41.01
40.73
40.45
40.17
39.89
39.61
27.76
f1 (ppm)

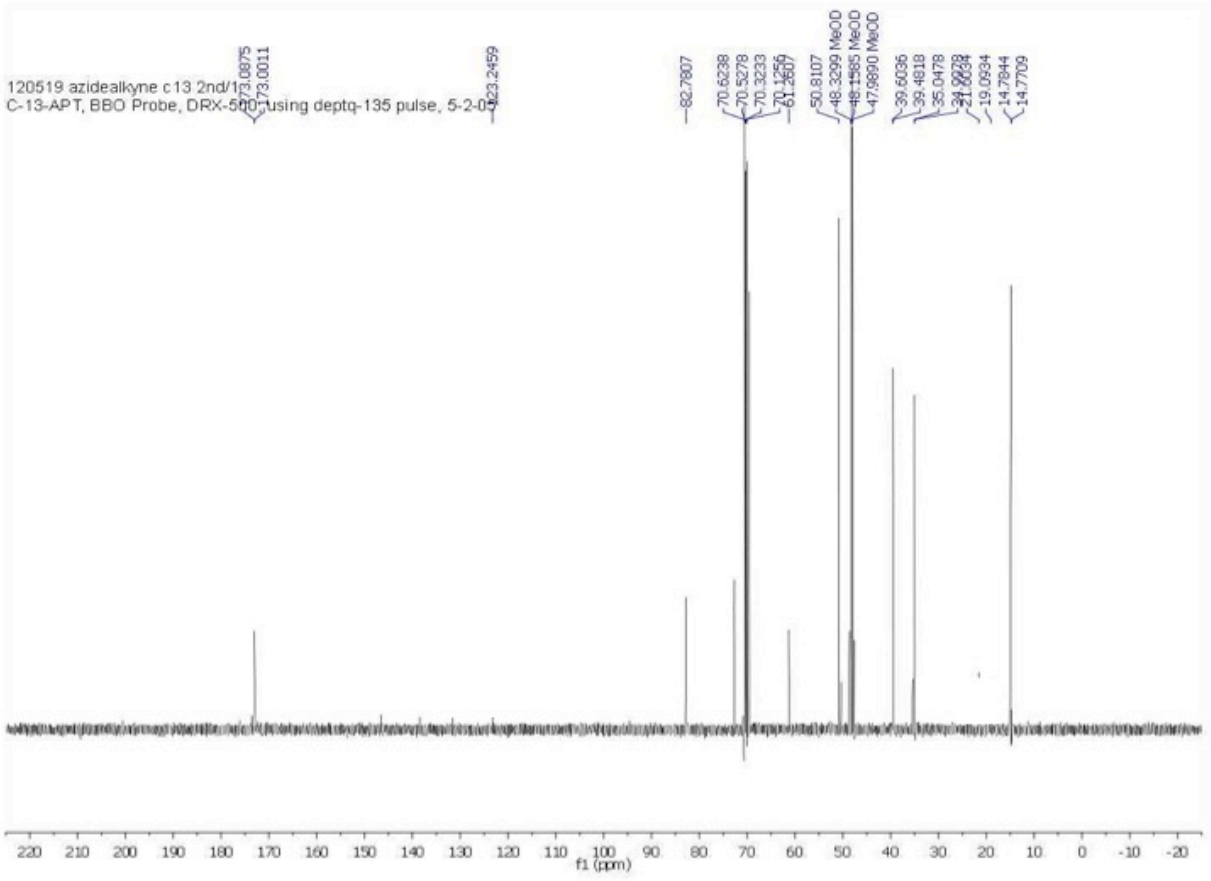

8d
f1 (ppm)

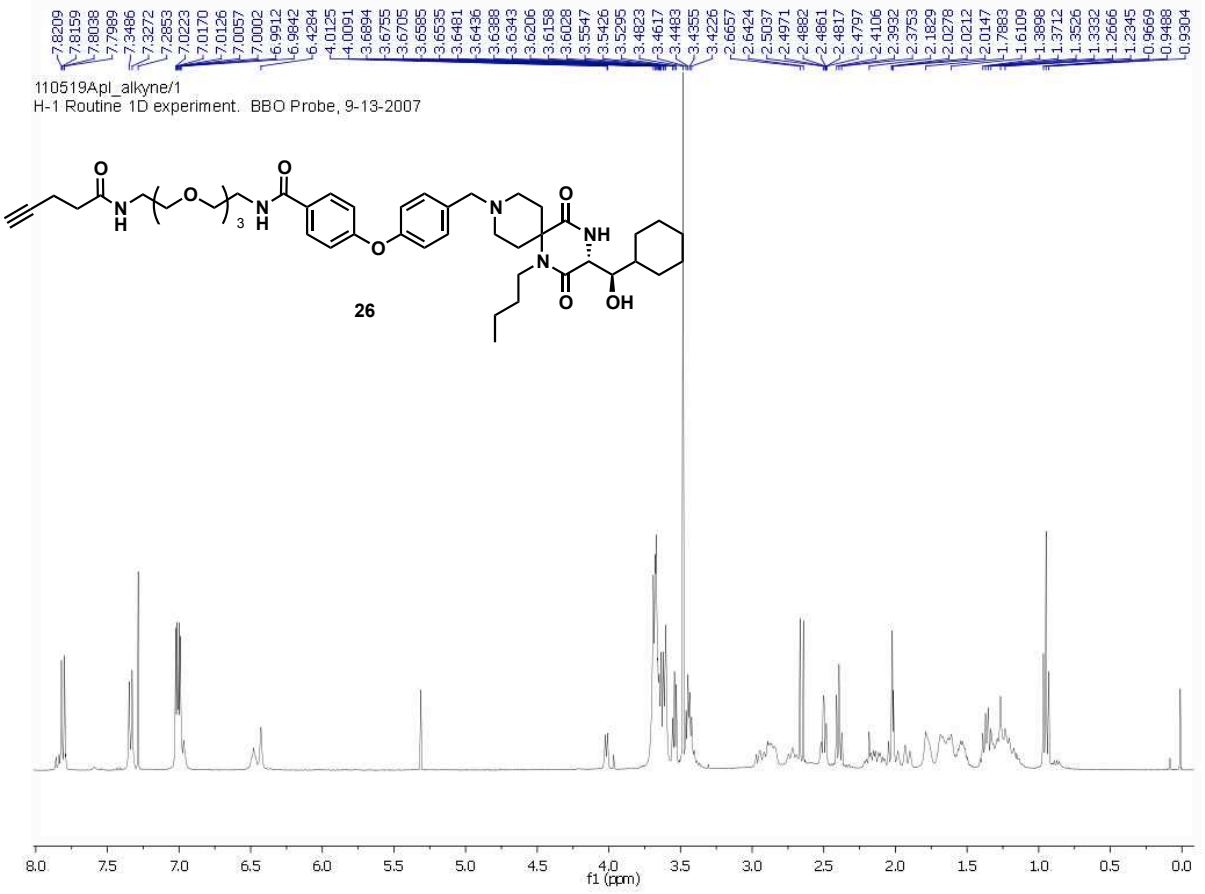

9a
f1 (ppm)

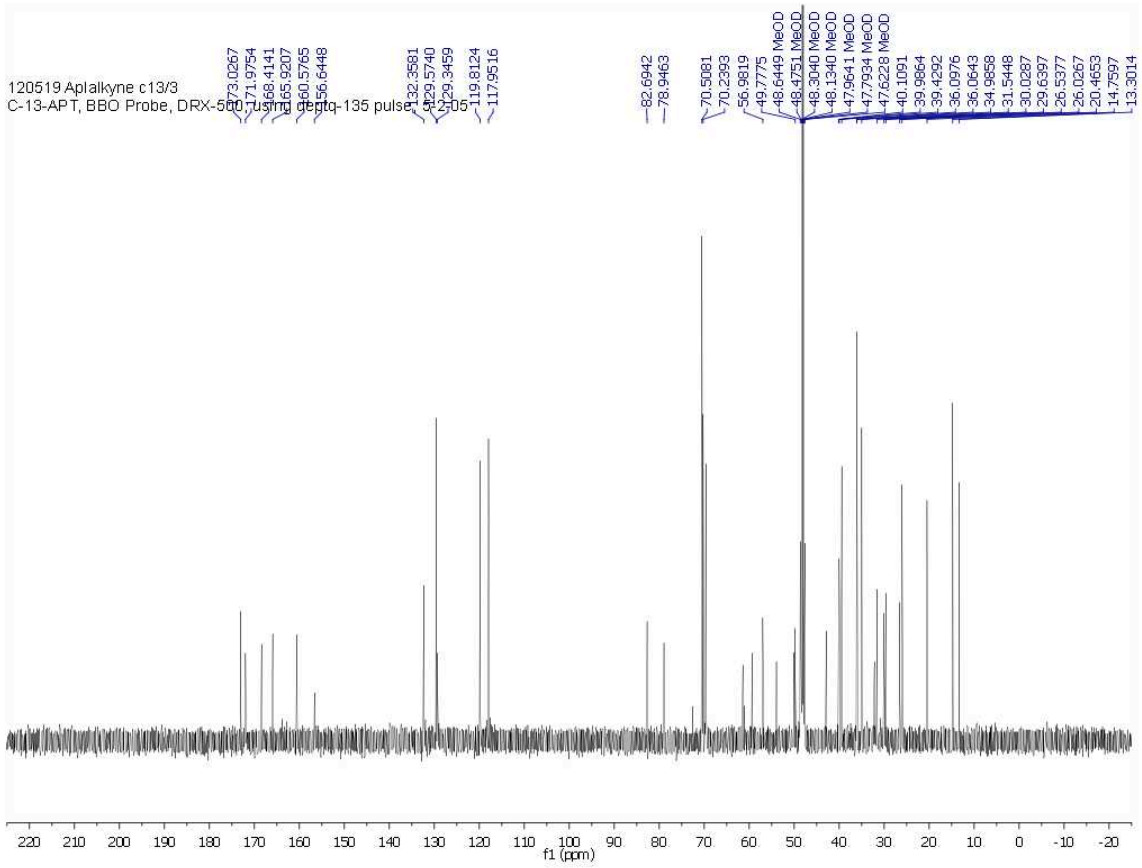

9b
f1 (ppm)

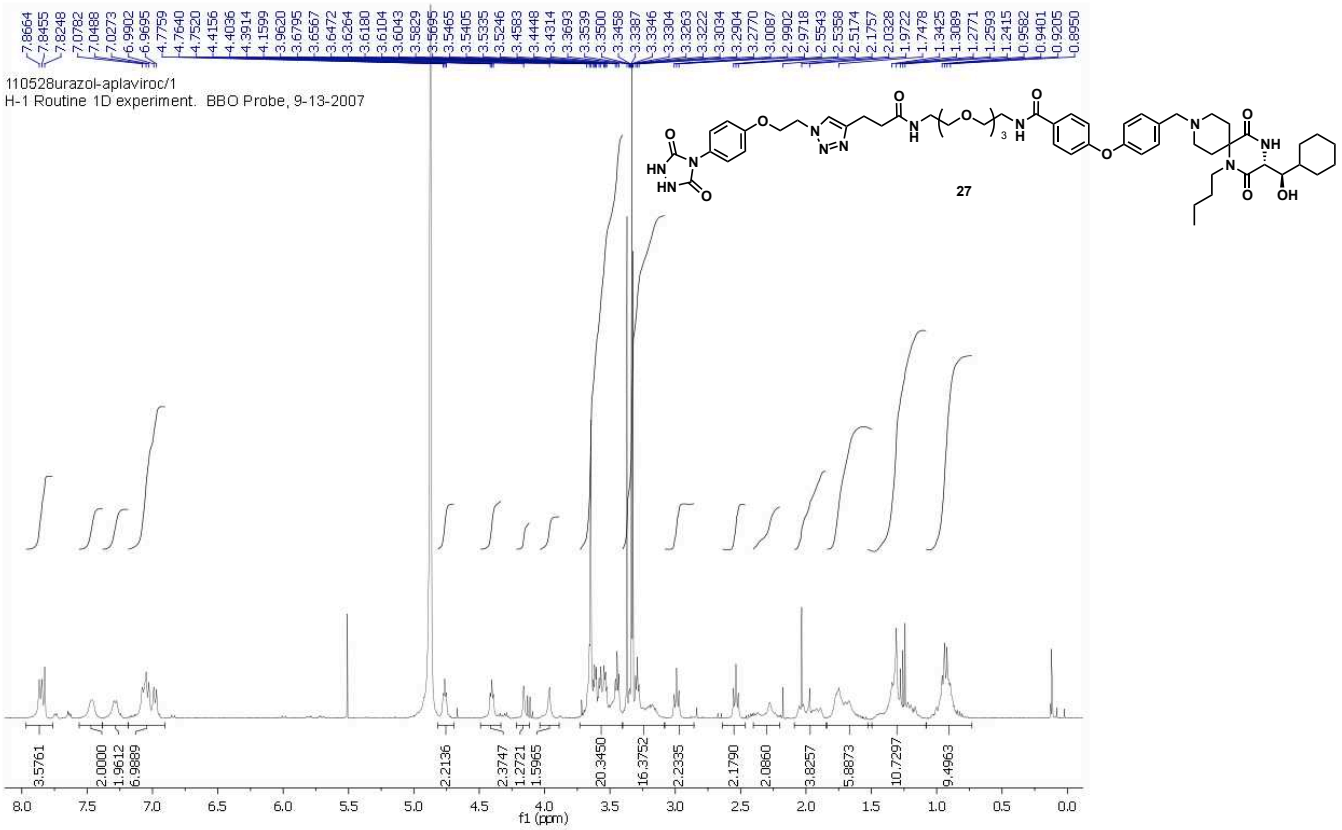

9c
7.42
7.41
7.40
7.39
7.38
7.06
7.04
7.03
7.02
4.61
2.31
2.00
2.10
2.16
3.17
f1 (ppm)

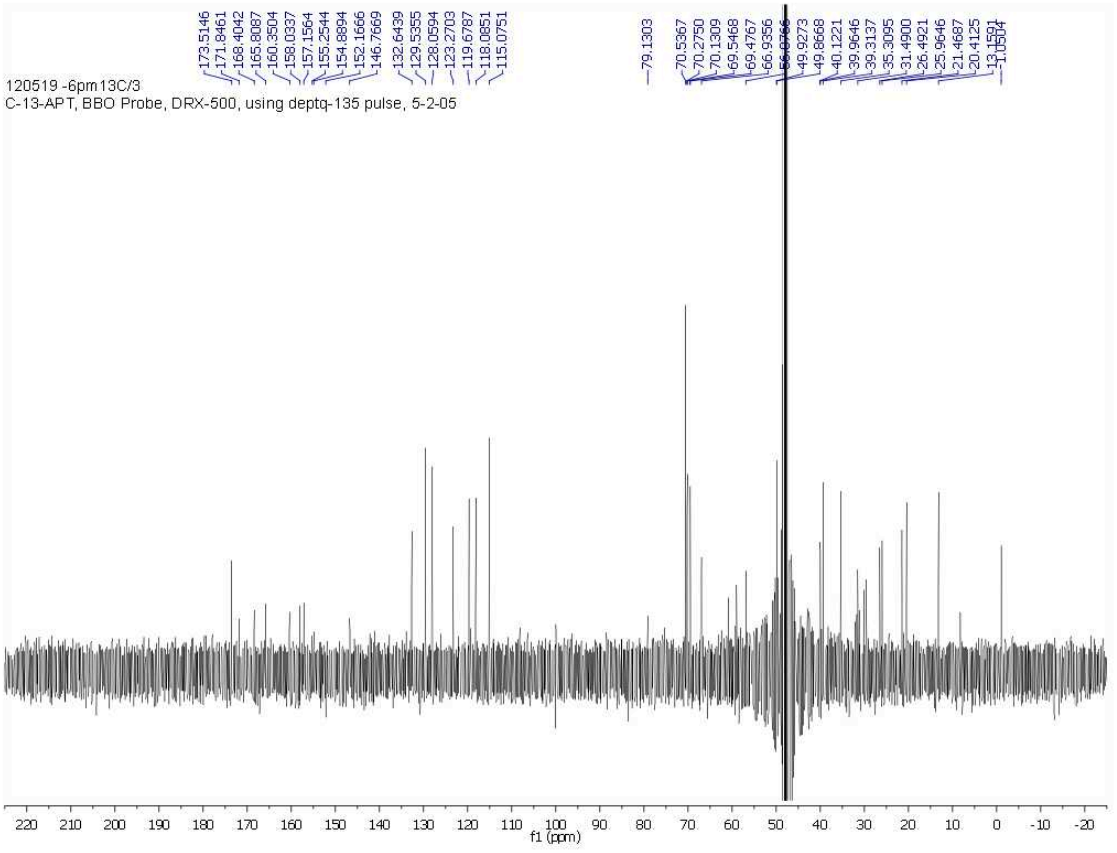

120519 azidealkyne h1/1
DQF-COSY (States-TPPI) using Gradient Pulse, DRX-500, BBO Probe
4.8391
3.7518
3.7504
3.7414
3.7362
3.7342
3.7312
3.7274
3.7232
3.6826
3.6227
3.4664
3.4579
3.3451
2.5399
2.5334
2.5313
2.5284
2.5267
2.4972
2.4838
2.4790
2.3852
2.3801
2.3751
2.2909
7.2275
2.0000
1.4870
f1 (ppm)


120519 azidealkyne c13 2nd/1
C-13-APT, BBO Probe, DRX-500, using deptq-135 pulse, 5-2-0
173.0875
173.0011
123.2459
82.7807
70.6238
70.5278
70.3233
70.1256
61.2607
50.8107
48.3299 MeOD
48.1585 MeOD
47.9890 MeOD
39.6036
39.4818
35.0478
19.0934
14.7844
14.7709
f1 (ppm)


110519Apl_alkyne/1
H-1 Routine 1D experiment. BBO Probe, 9-13-2007
26
f1 (ppm)

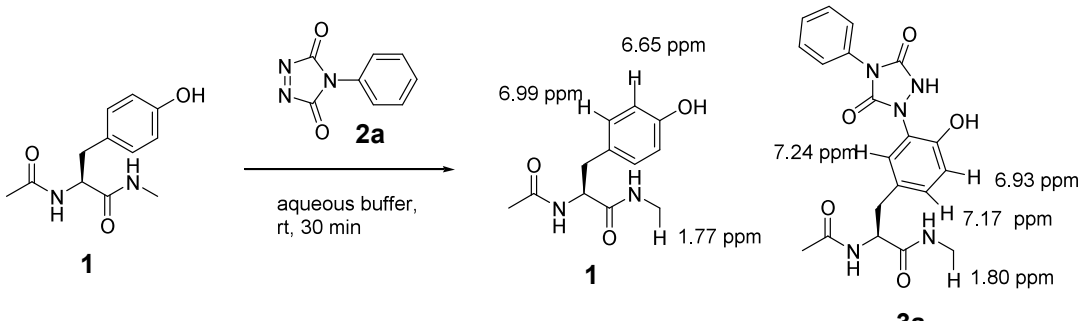

120519 Aplalkyne c13/3
C-13-APT, BBO Probe, DRX-500 tusmg dept-q-135 pulse 42-05
f1 (ppm)

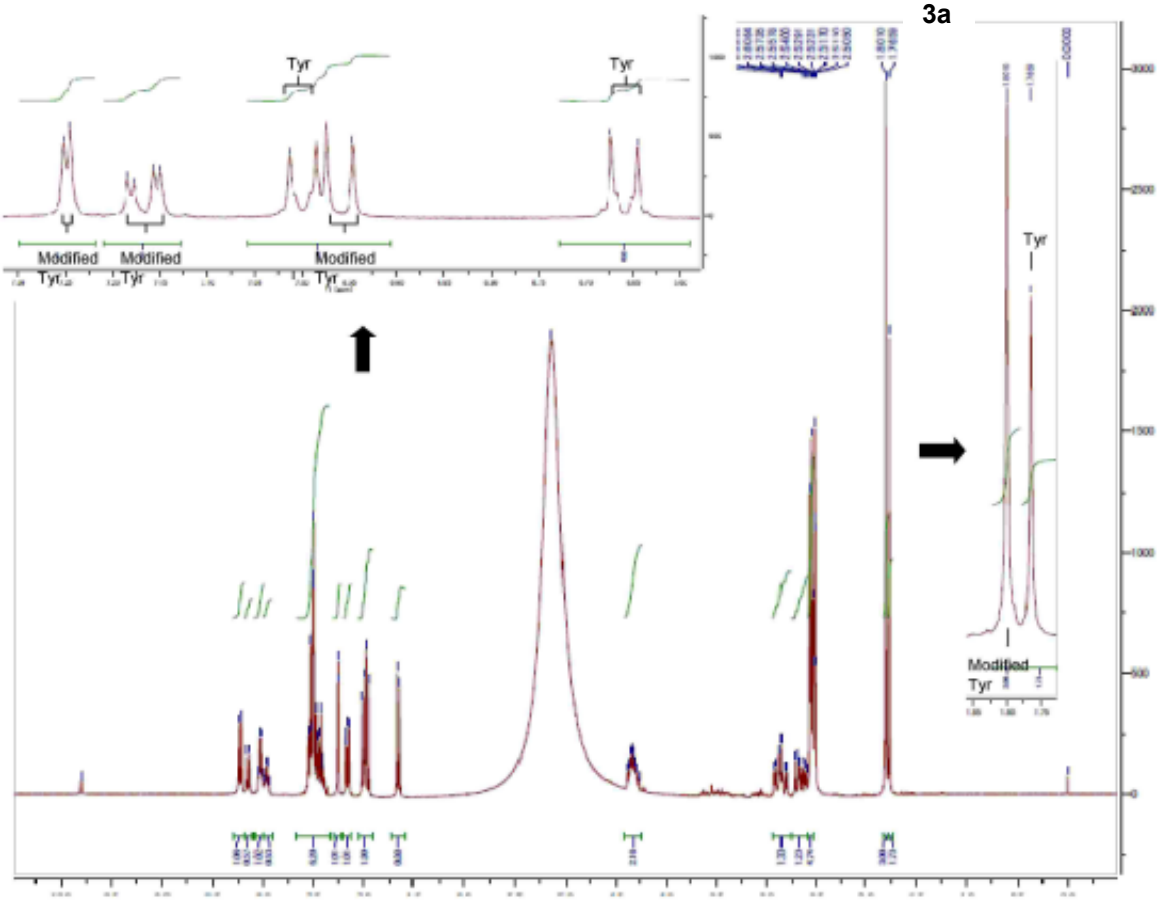

110528urazol-aplaviroc/1
H-1 Routine 1D experiment. BBO Probe, 9-13-2007
27
f1 (ppm)


120519 -6pm13C/3
C-13-APT, BBO Probe, DRX-500, using deptq-135 pulse, 5-2-05
f1 (ppm)

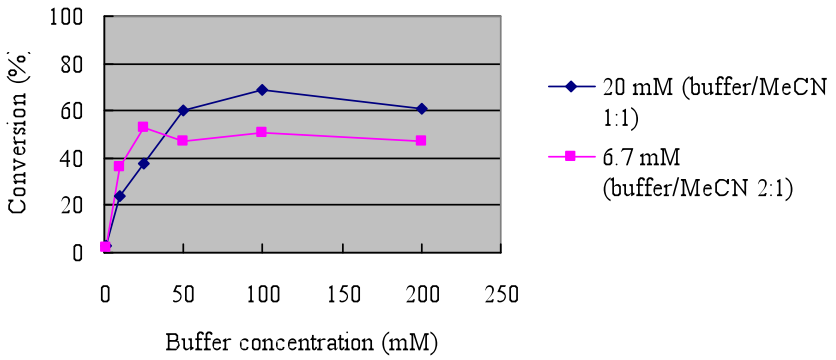
4. The reactions of tyrosine derivative with PTAD analogs

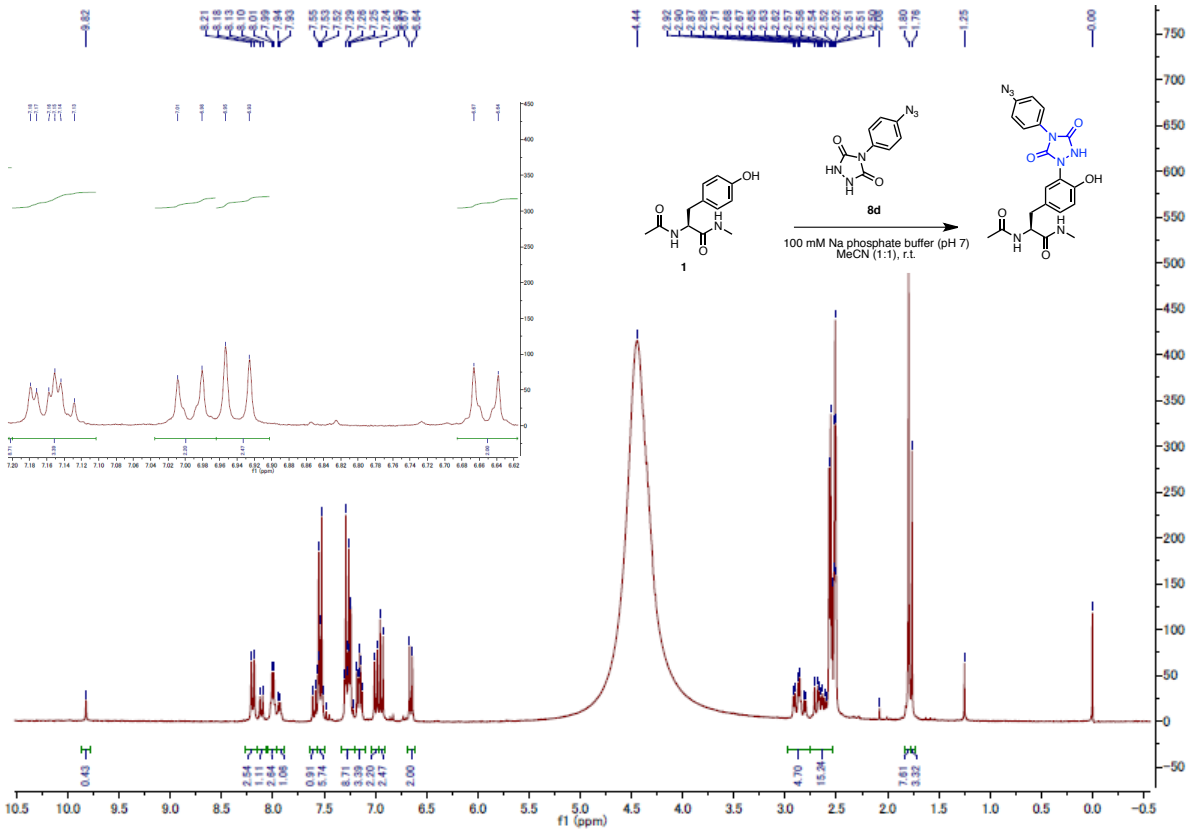
^{1}H NMR of the mixture of the reaction

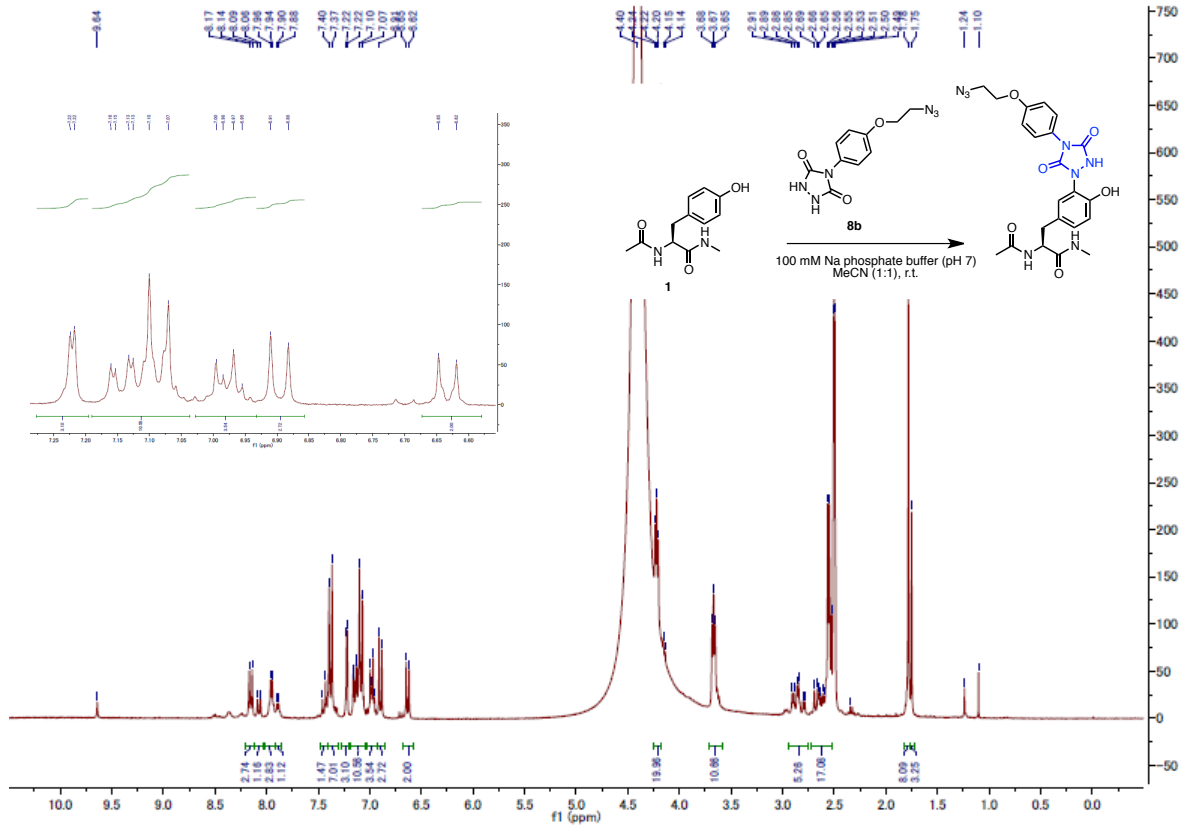


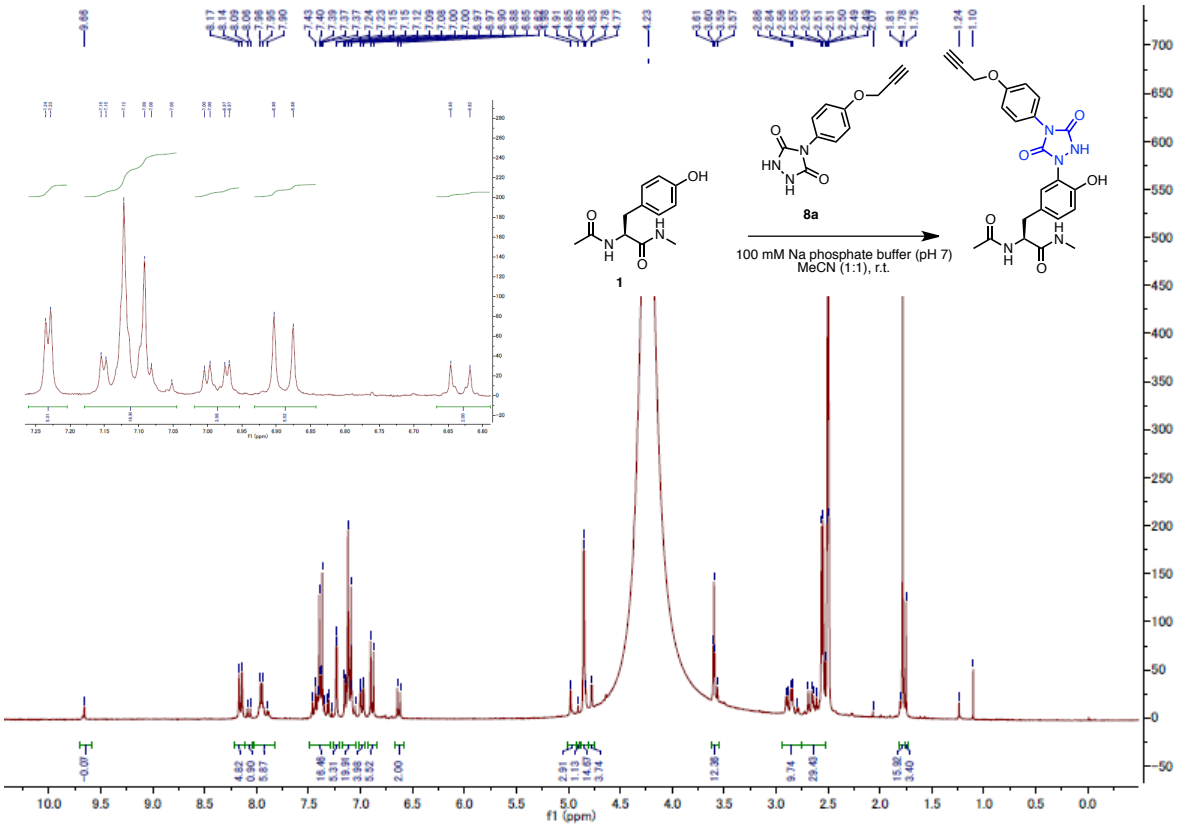


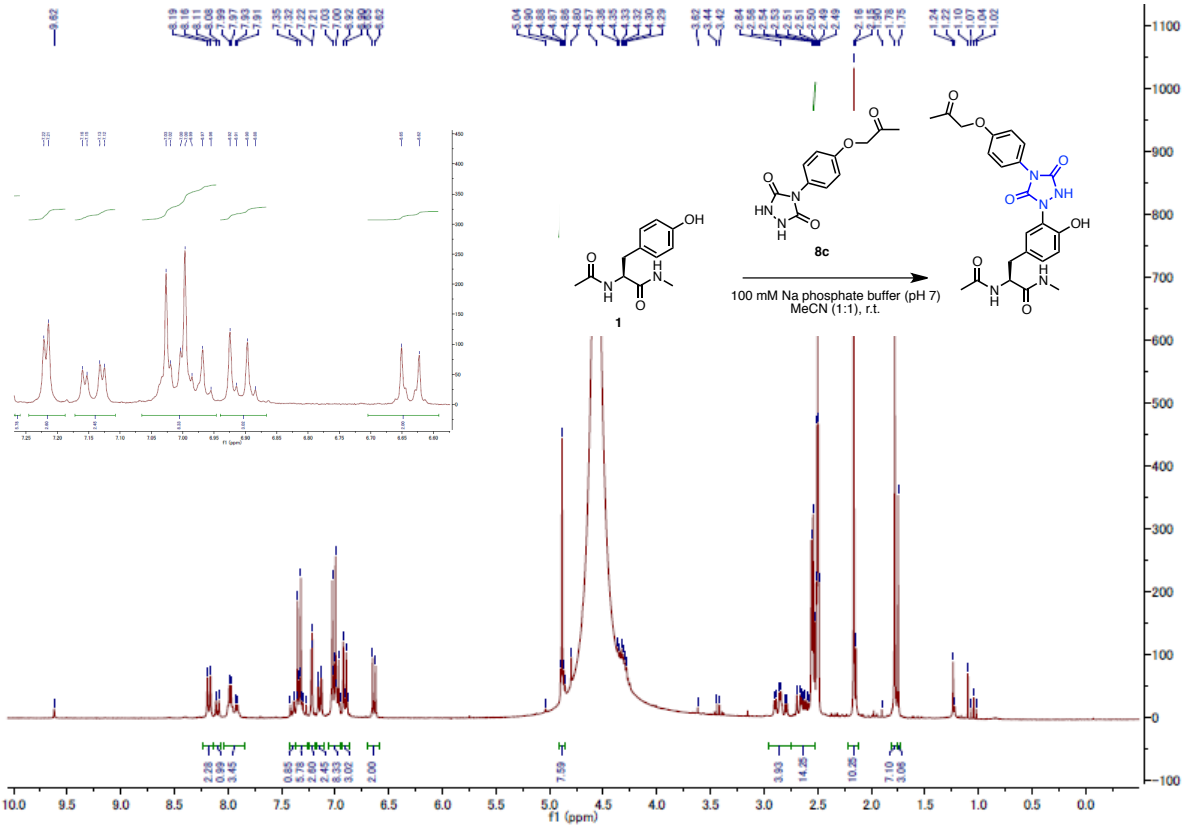
Effect of buffer concentration TLR in pH 7phpsphate buffer/MeCN

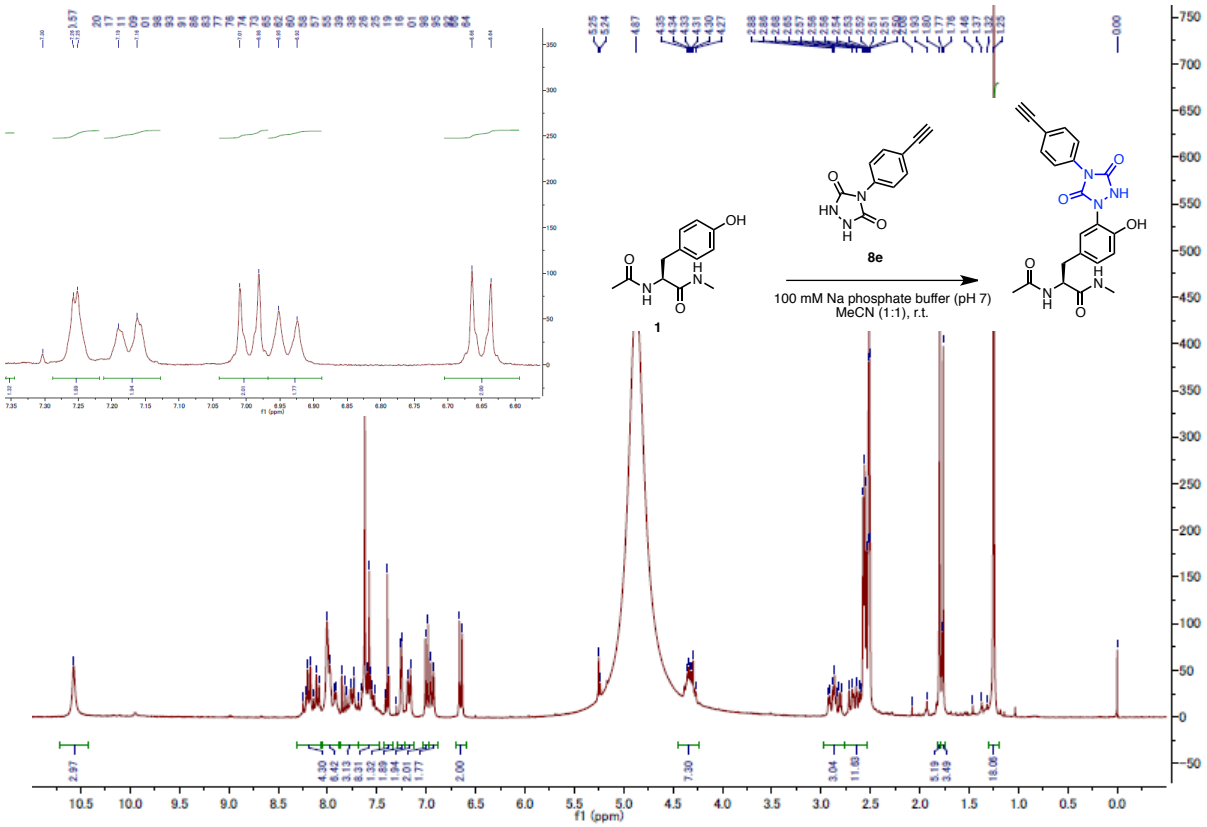



1
8d
100 mM Na phosphate buffer (pH 7)
MeCN (1:1), r.t.
f1 (ppm)


1
8b
100 mM Na phosphate buffer (pH 7)
MeCN (1:1), r.t.
f1 (ppm)

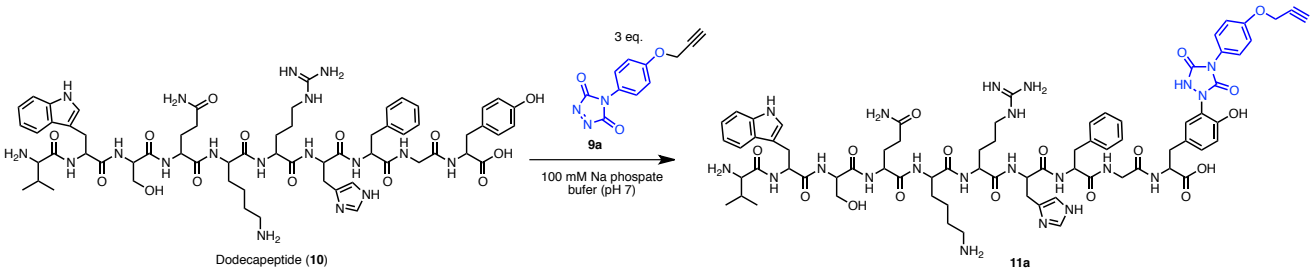

1
8a
100 mM Na phosphate buffer (pH 7)
MeCN (1:1), r.t.
f1 (ppm)

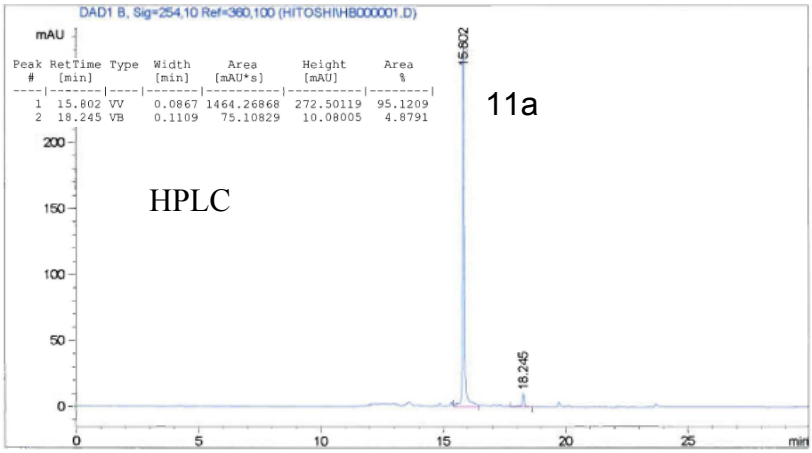

1
8c
100 mM Na phosphate buffer (pH 7)
MeCN (1:1), r.t.
f1 (ppm)


8e
1
100 mM Na phosphate buffer (pH 7)
MeCN (1:1), r.t.
f1 (ppm)

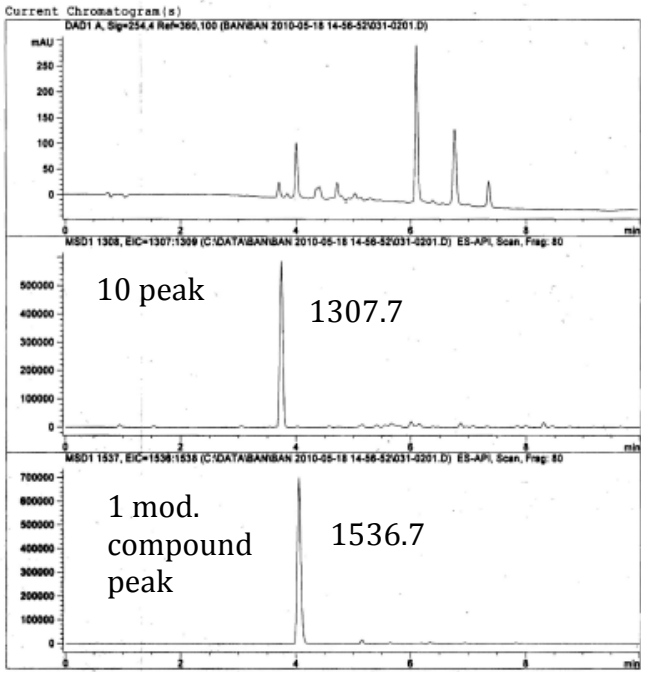
5. Peptide modification with PTAD analogs

5-1 HPLC and MS chart

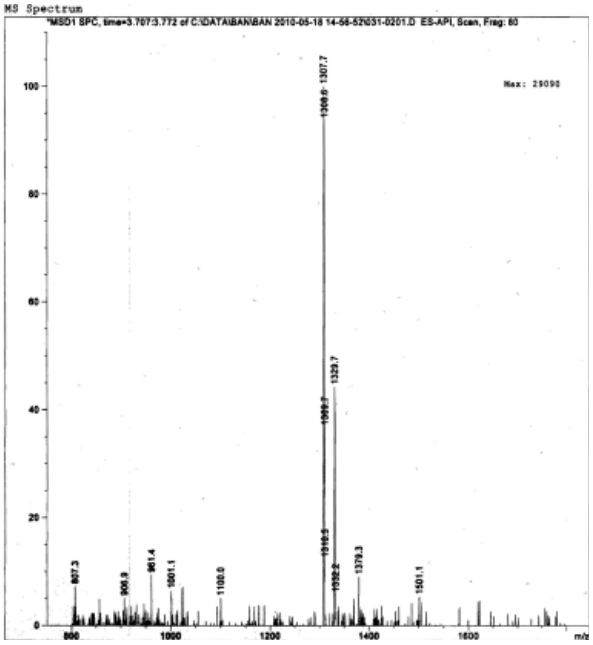




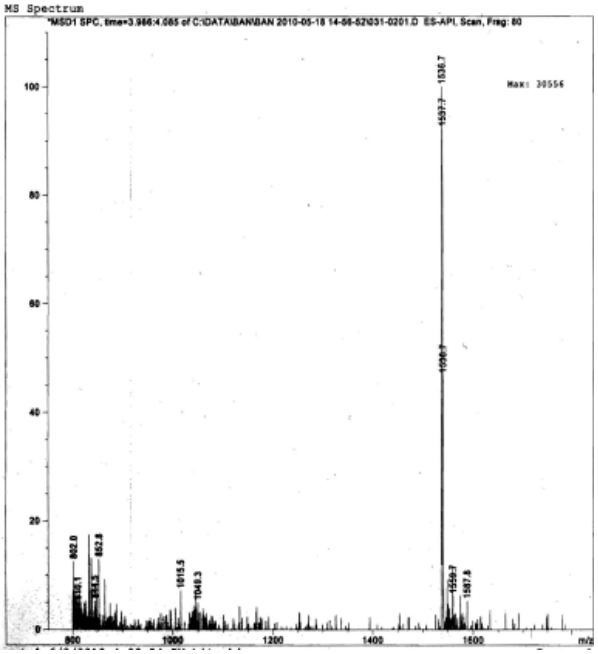
Crude reaction LC/MS

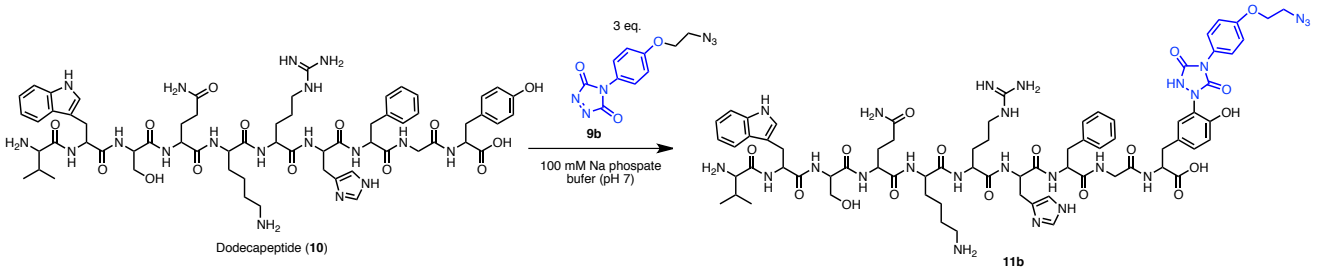


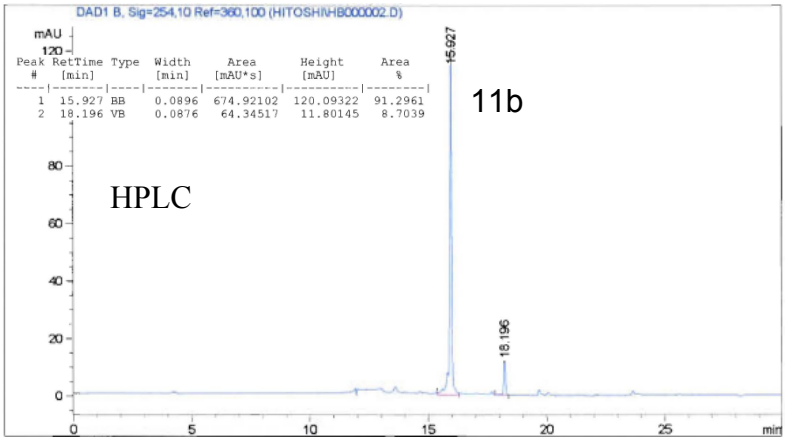
MS of peptide (10)



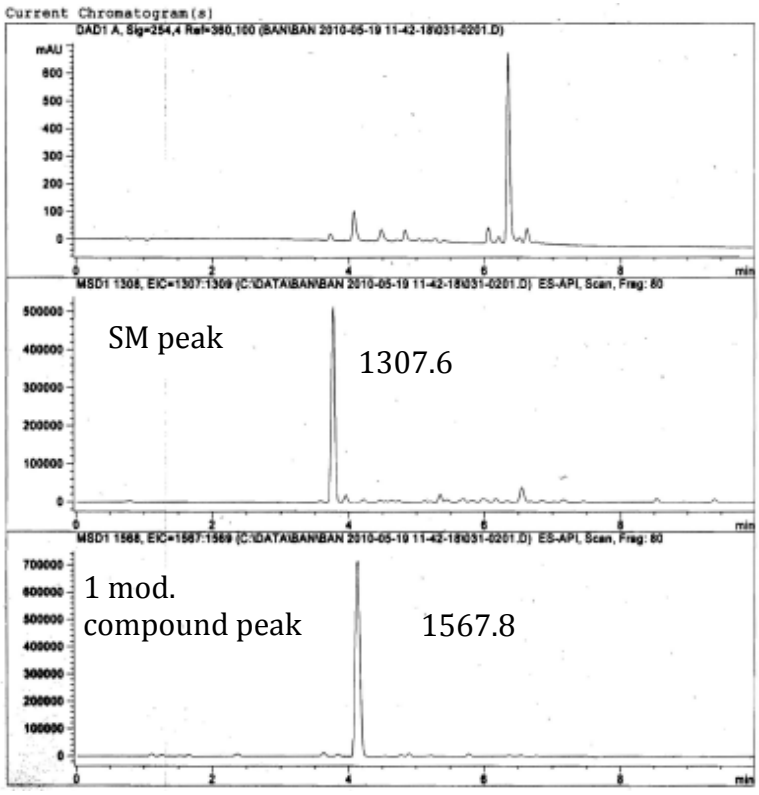
MS of modified peptide (11a)



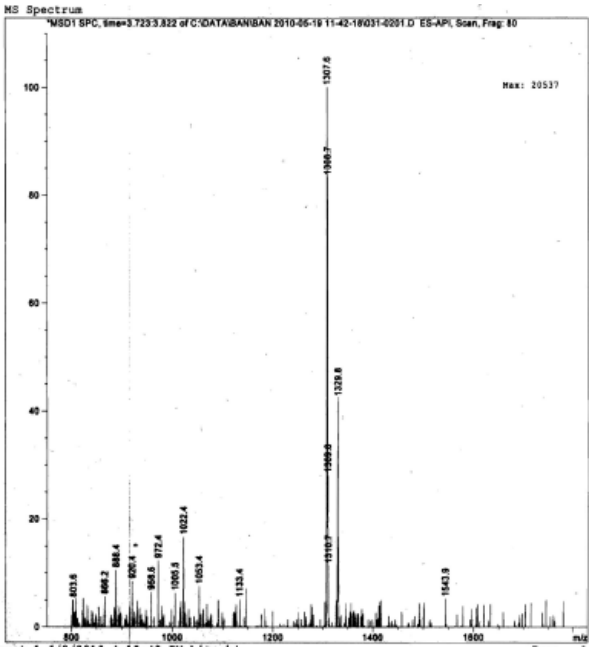




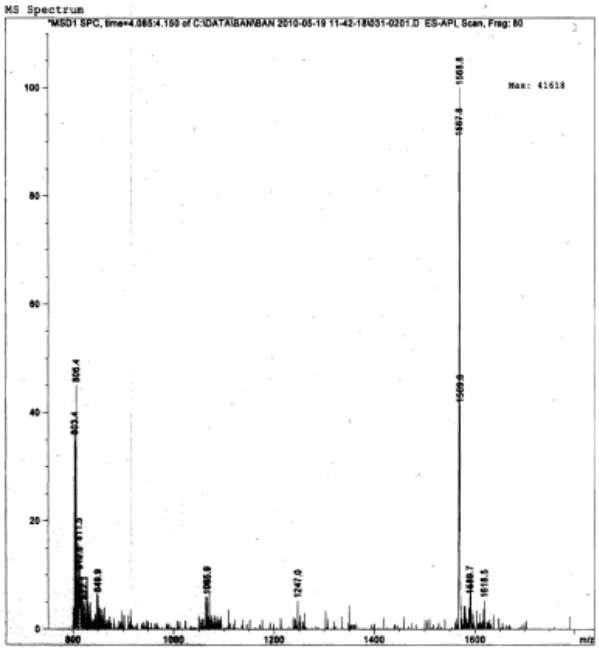
Crude reaction LC/MS

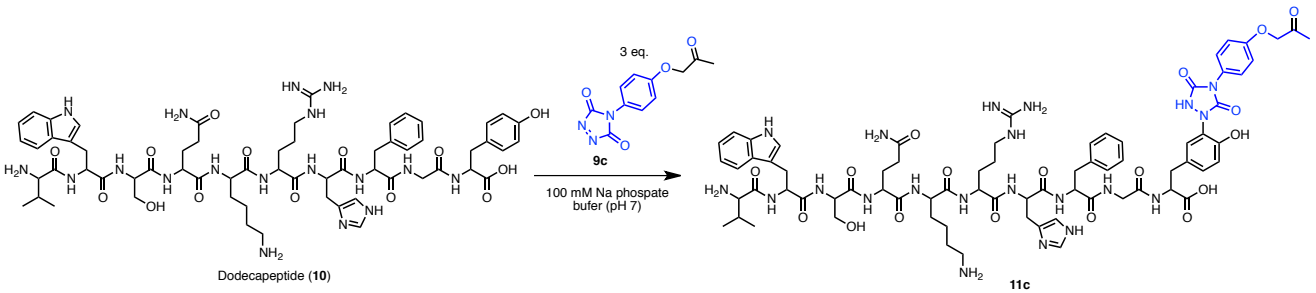


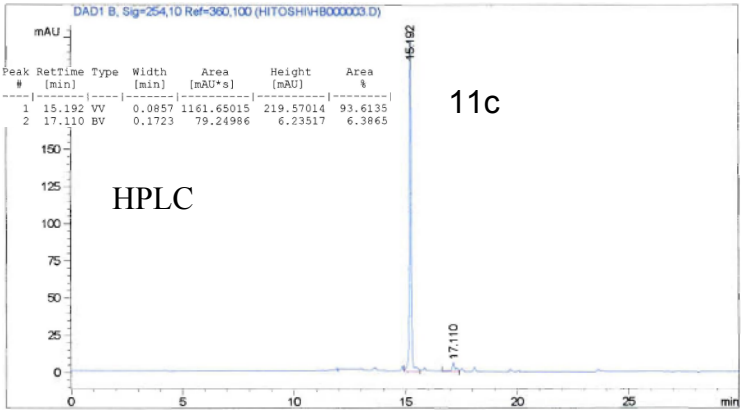
MS of peptide (10)



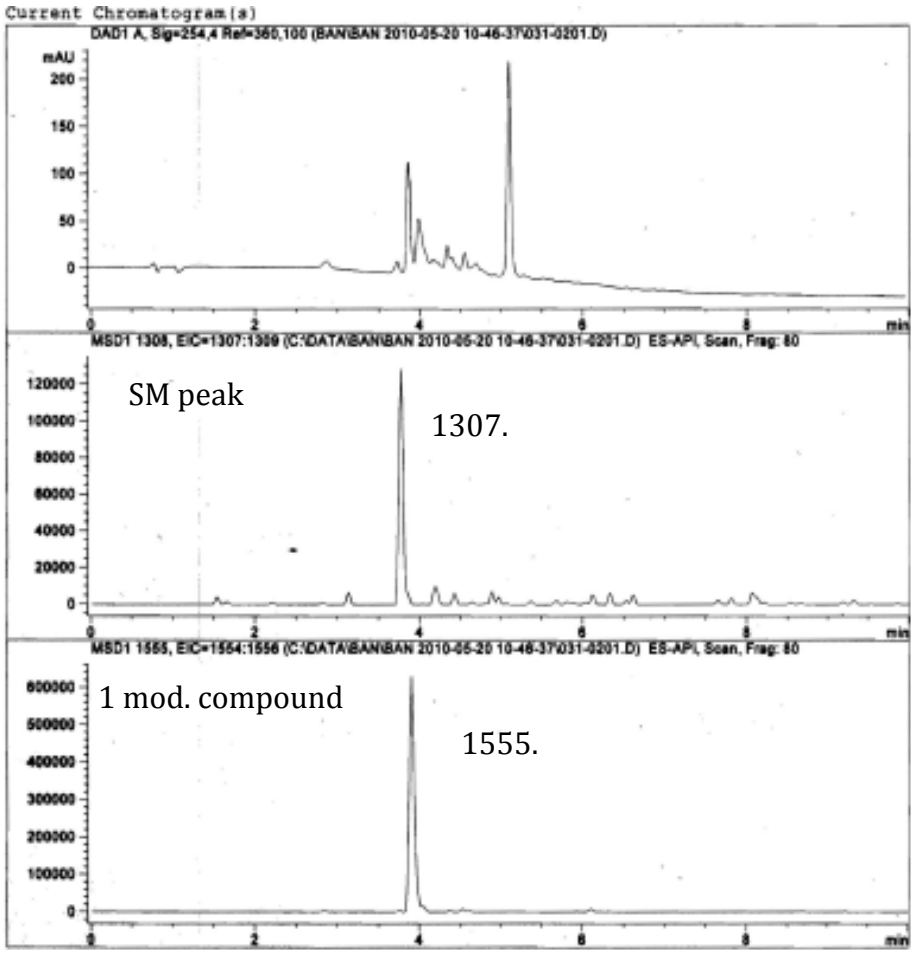
MS of modified peptide (11b)



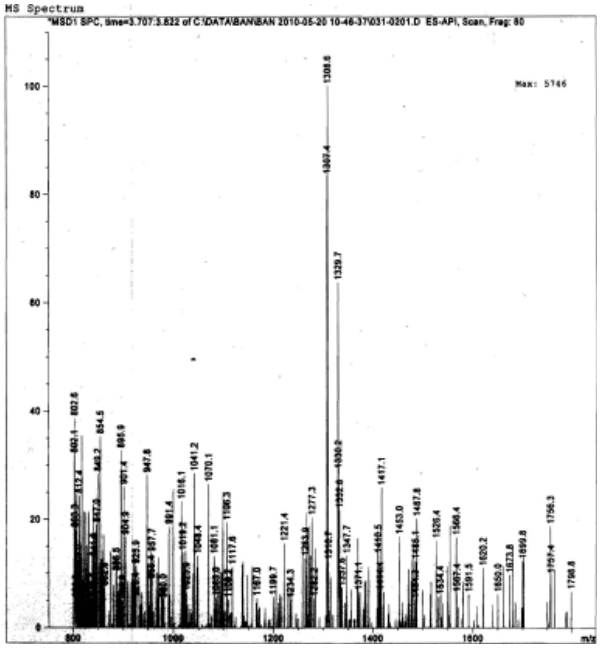




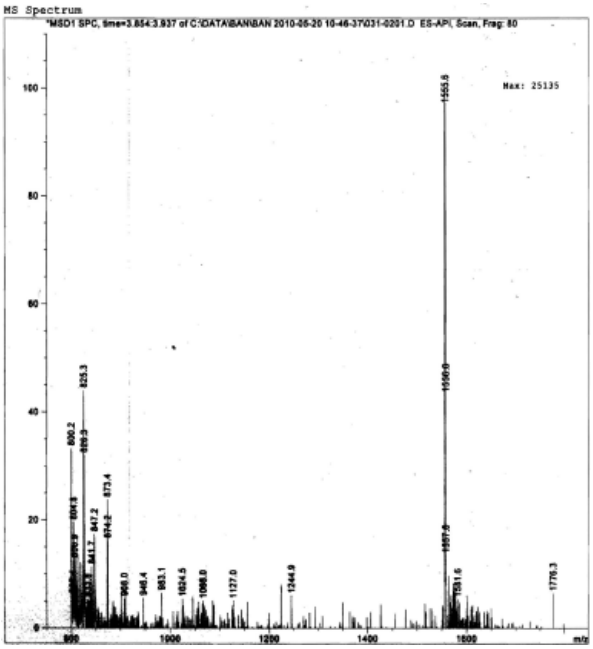
Crude reaction LC/MS



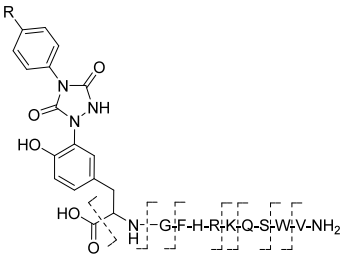
MS of decapeptide (**10**)

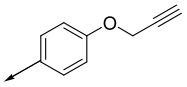


MS of 1 modified peptide (**11c**)

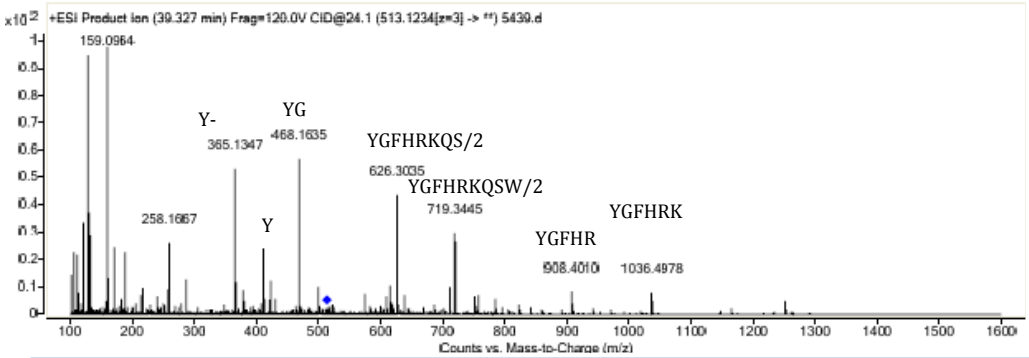


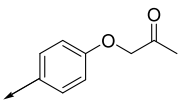
5-2. MS/MS analysis of labeling peptides



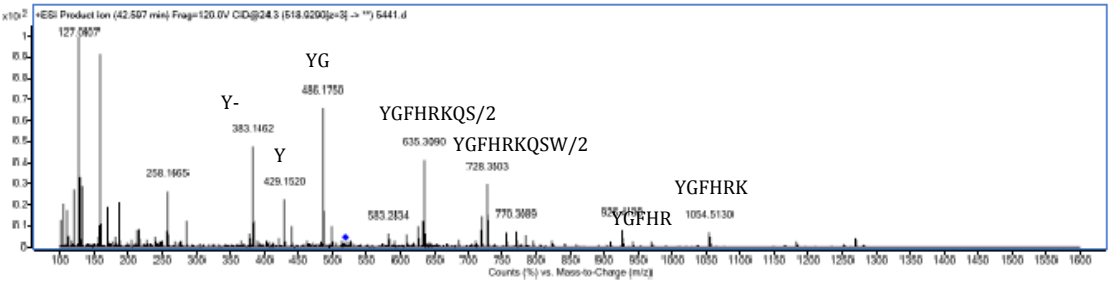


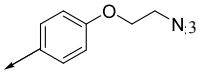
11a



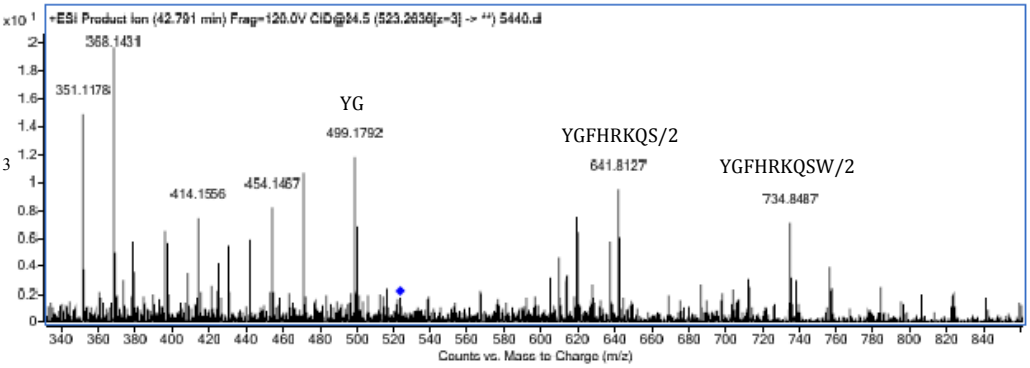


11c



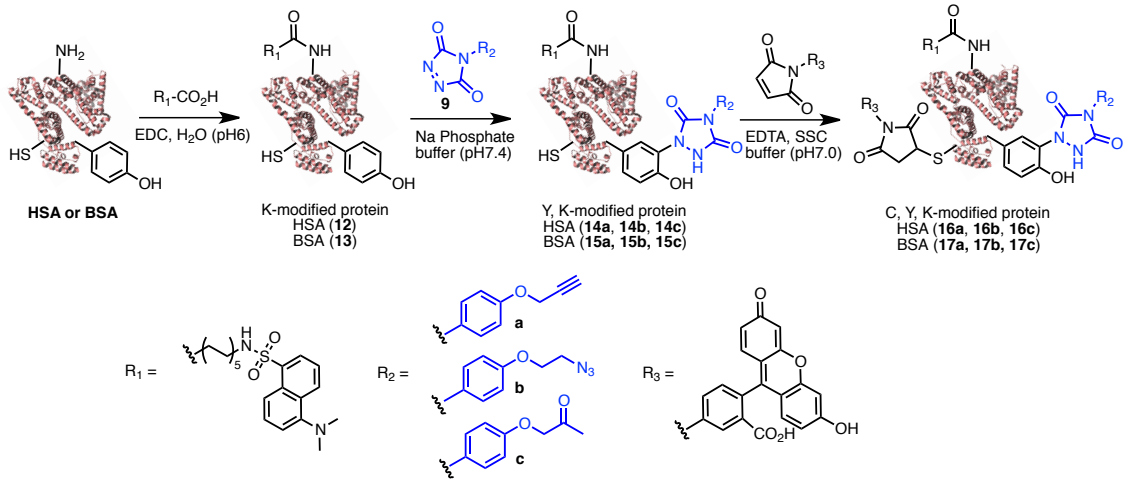


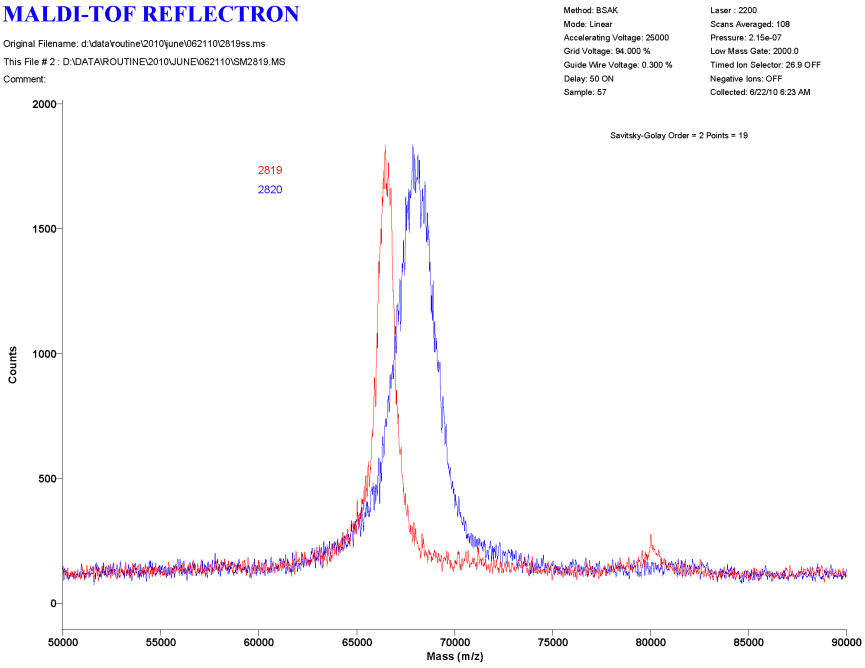
11b



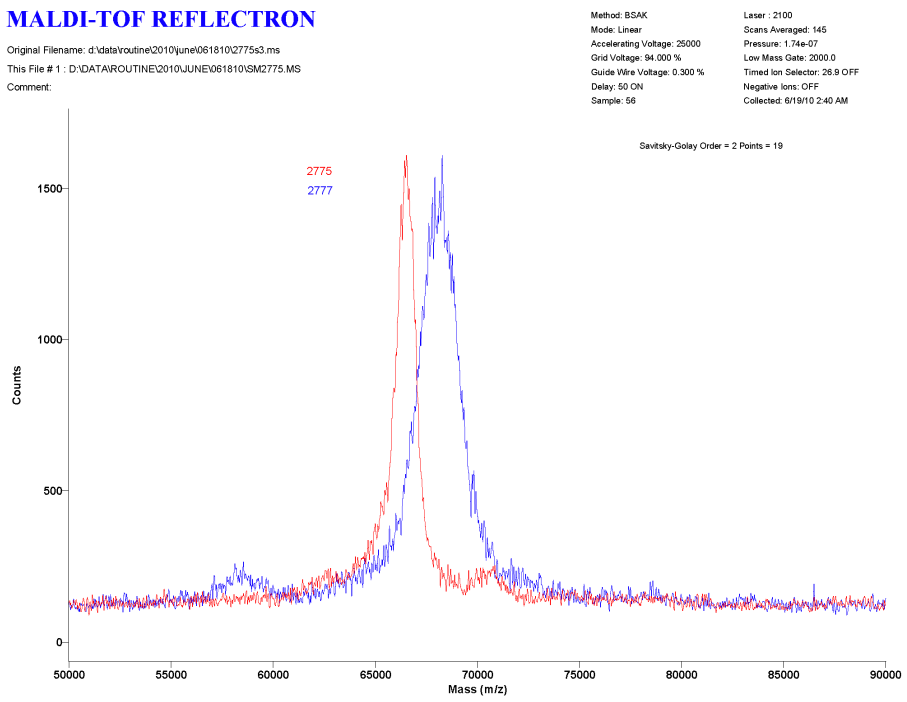
6. Albumin modification through three different orthogonal reactions

1) Reaction of albumins with dansyl derivative



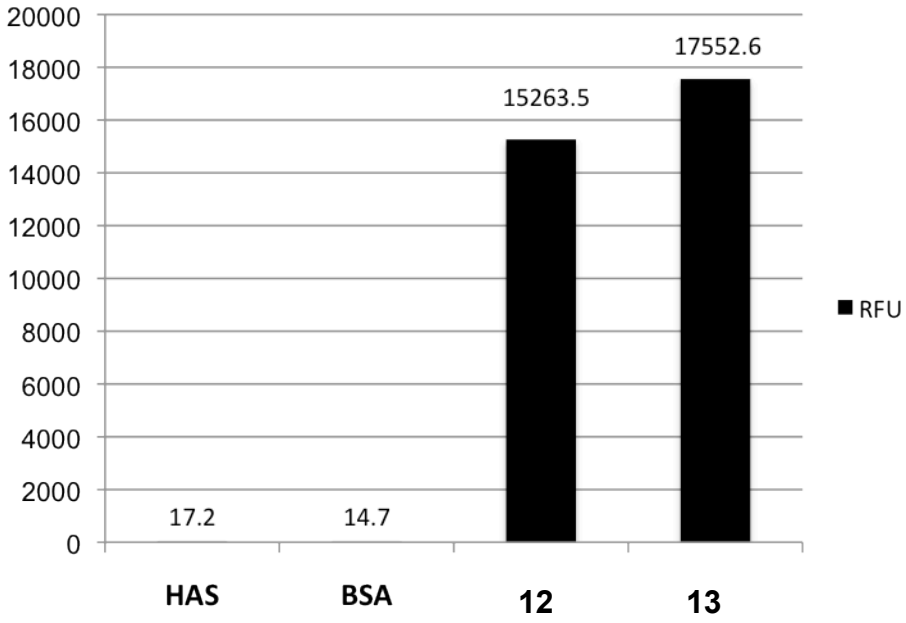


Overlay of MALDI-TOF MS charts of HSA (red) and **12** (blue); molecular weight increase 1592.

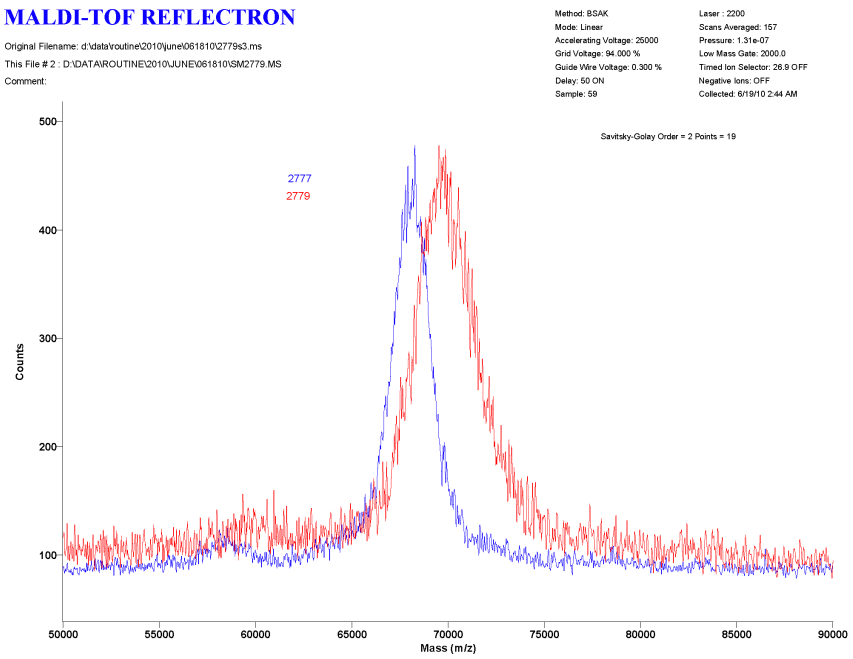


Overlay of MALDI-TOF MS charts of BSA (red) and **13** (blue); molecular weight increase 1706.

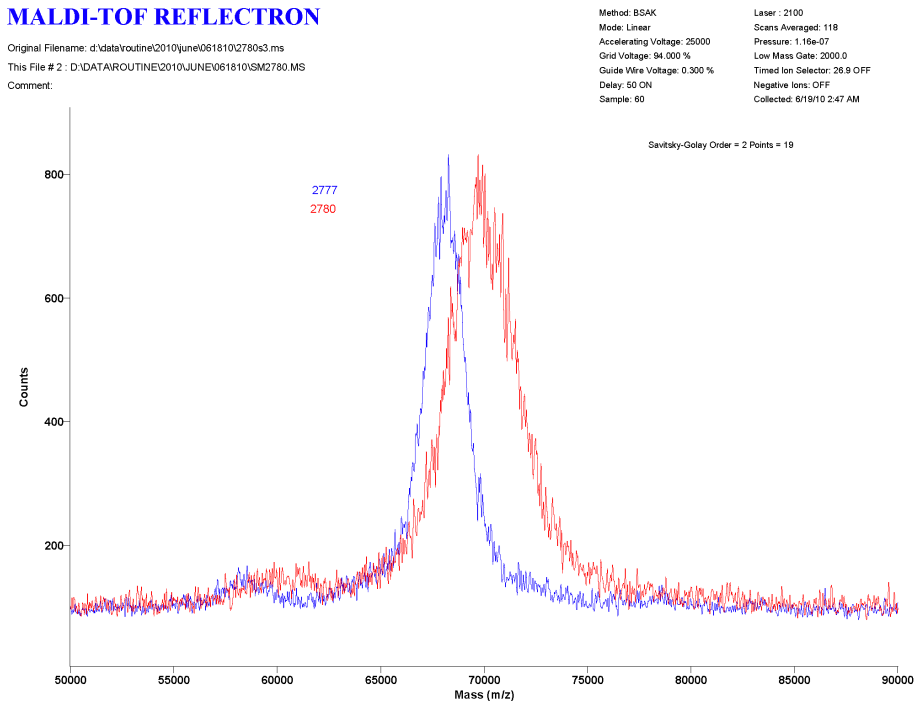
Fluorescence intensity of dansyl moiety of HSA, BSA, **12**, and **13**.



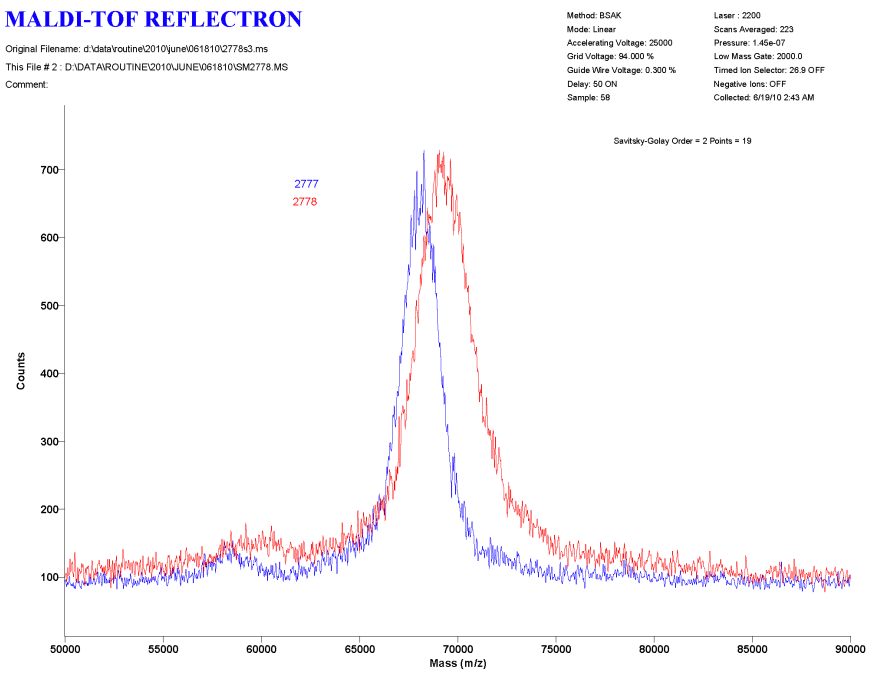
2) Reaction of dansyl-albumins with PTAD derivative.



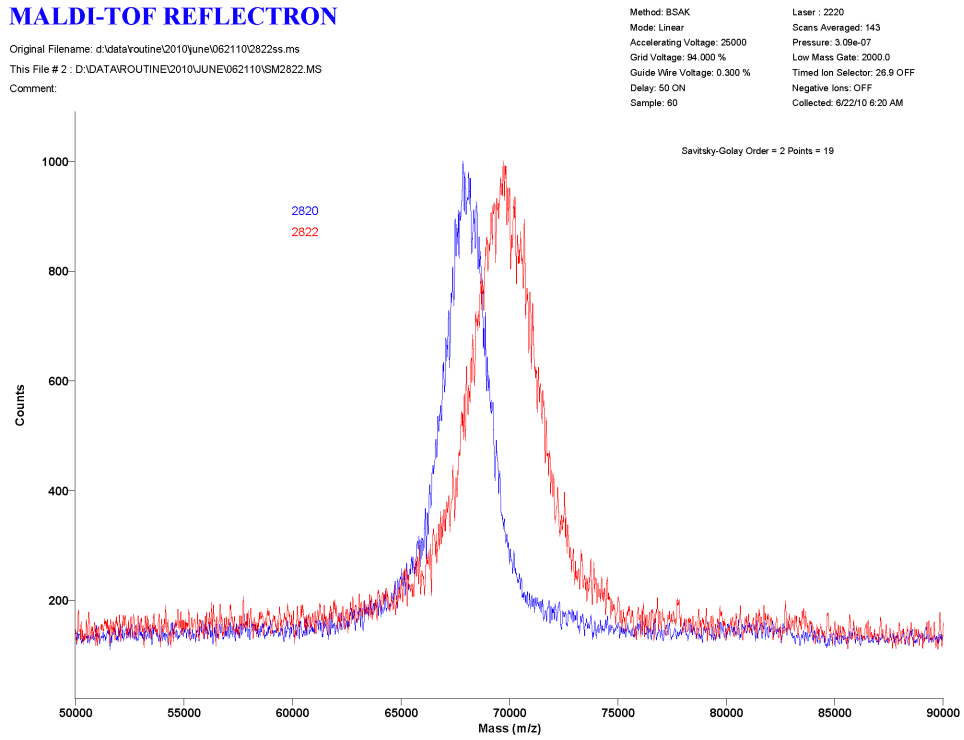
Overlay of MALDI-TOF MS charts of **12** (blue) and **14a** (red). Molecular weight increase 1540.



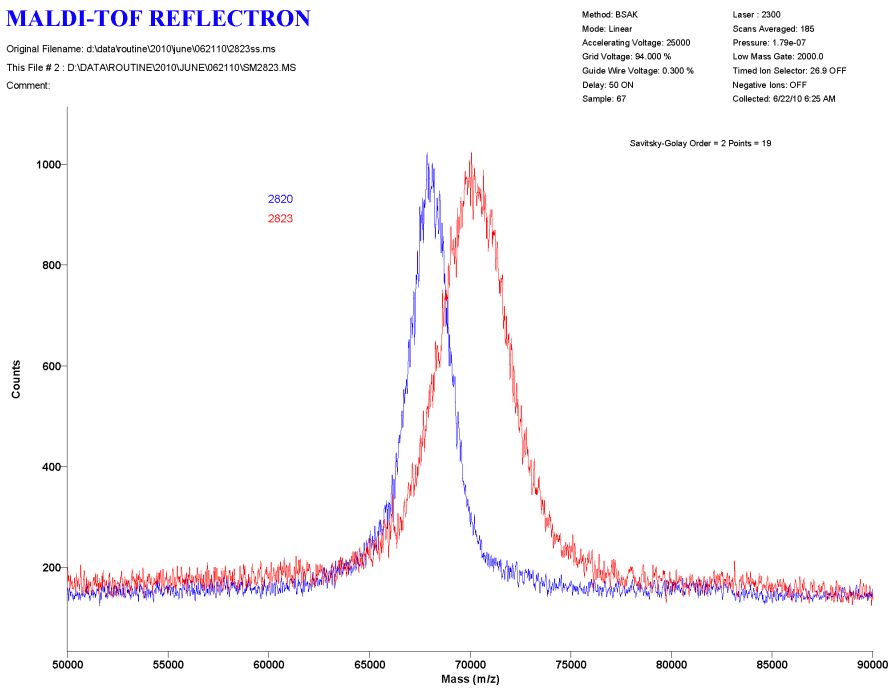
Overlay of MALDI-TOF MS charts of **12** (blue) and **14b** (red). Molecular weight increase 1681.



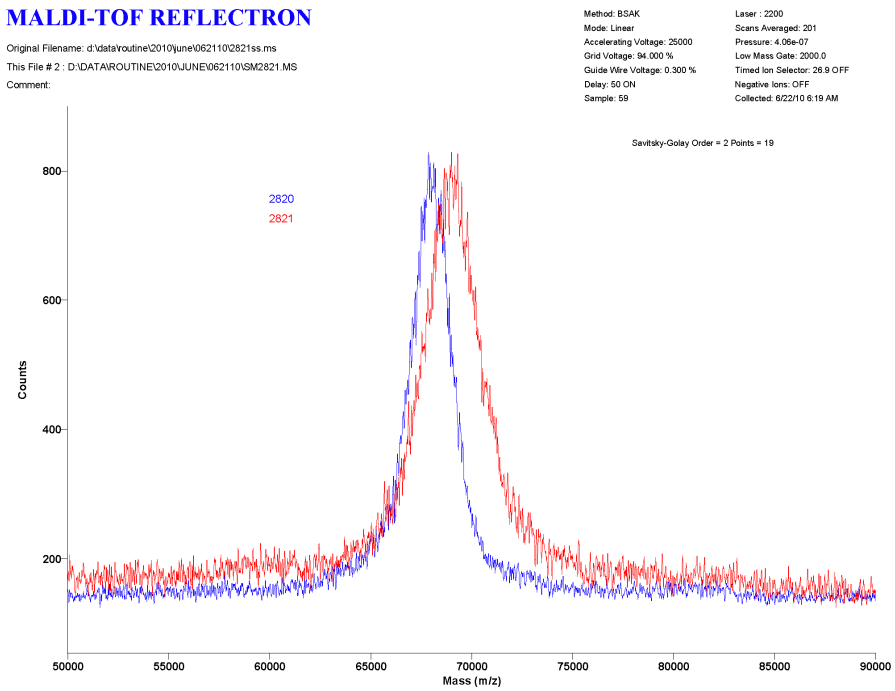
Overlay of MALDI-TOF MS charts of **12** (blue) and **14c** (red). Molecular weight increase 1130.



Overlaid MALDI-TOF MS chart of **13** (blue) and **15a** (red). Molecular weight increase 1708.

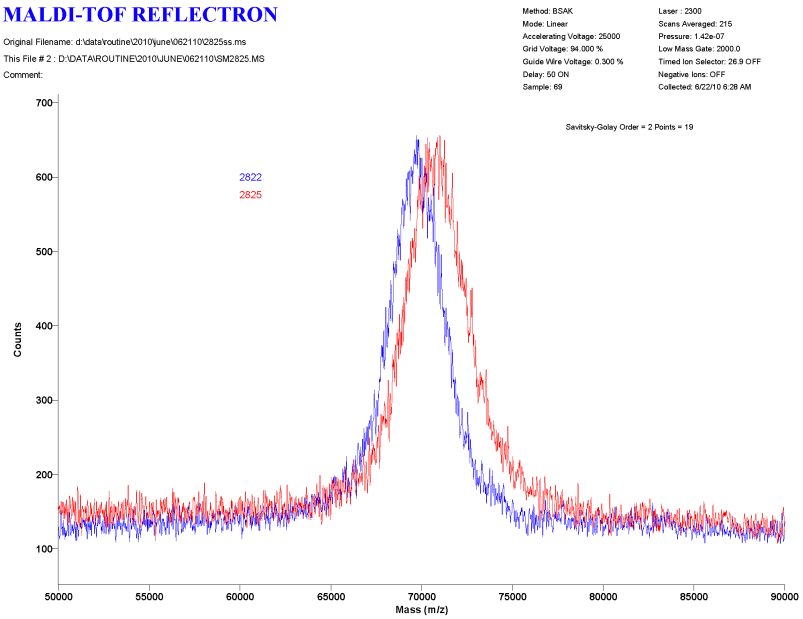


Overlay of MALDI-TOF MS charts of **13a** (blue) and **15b** (red). Molecular weight increase 1681.

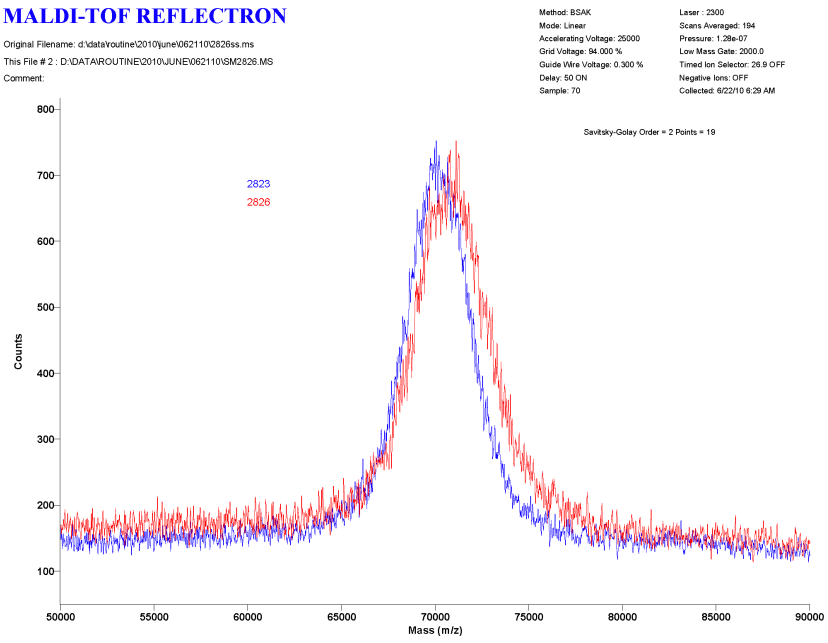


Overlay of MALDI-TOF MS charts of **13a** (blue) and **15c** (red). Molecular weight increase 969.

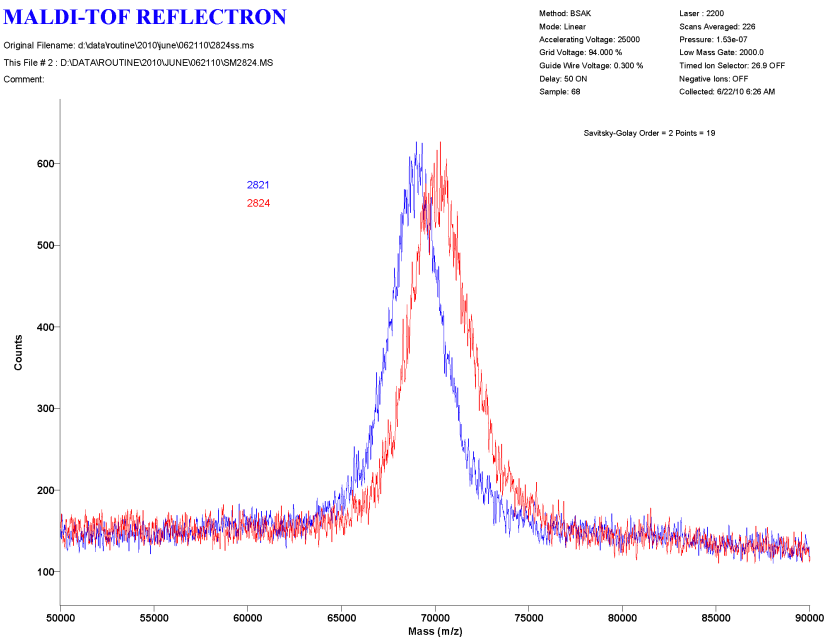
3) Reaction of dual labeled albumins with fluorescein-5-maleimide.



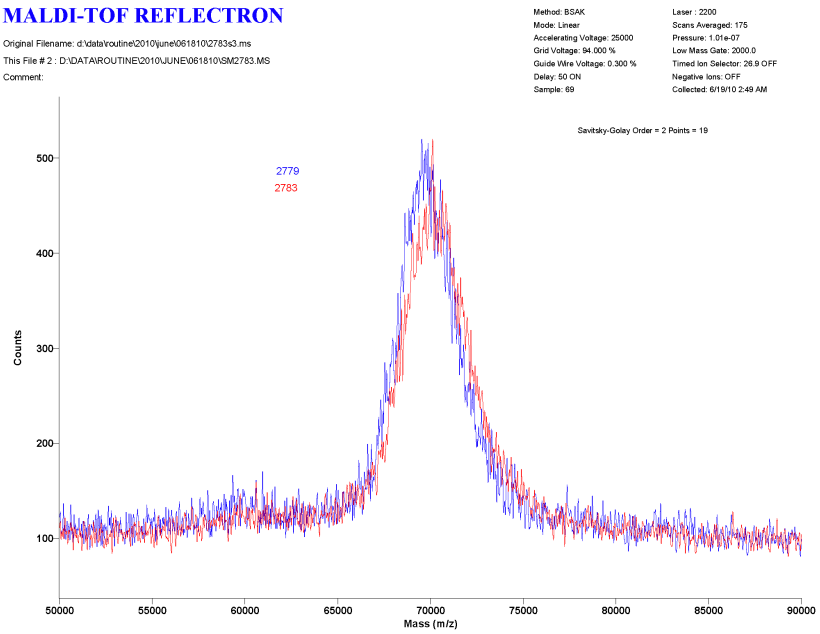
Overlay of MALDI-TOF MS charts of **14a** (blue) and **16a** (red). Molecular weight increase 966.



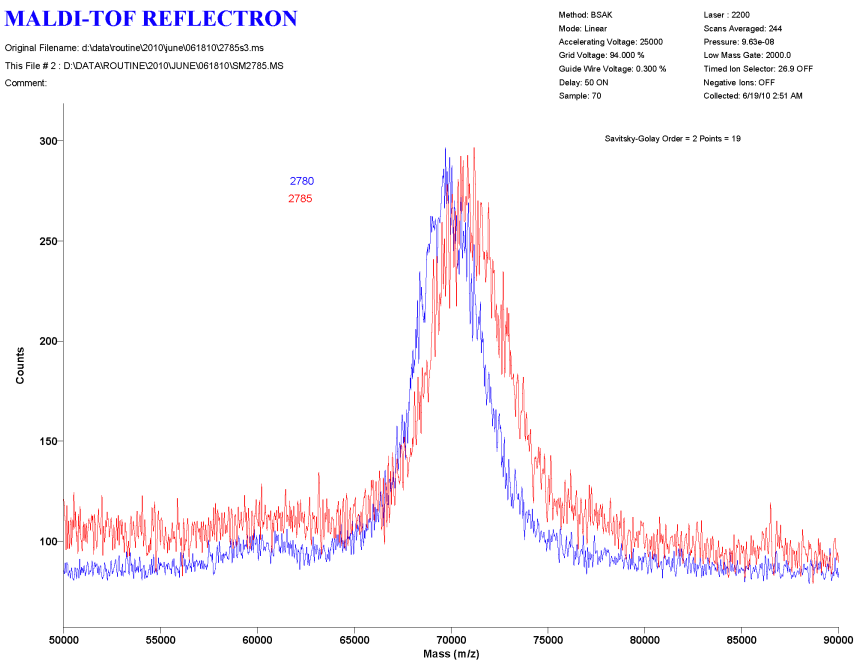
Overlay of MALDI-TOF MS charts of **14b** (blue) and **16b** (red). Molecular weight increase 568.



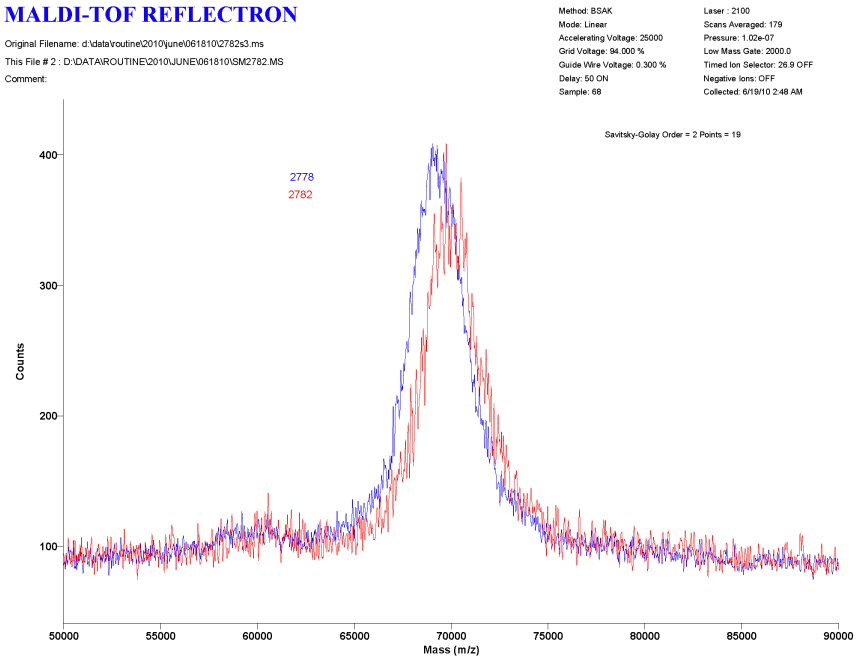
Overlay of MALDI-TOF MS charts of **14c** (blue) and **16c** (red). Molecular weight increase 1142.



Overlay of MALDI-TOF MS charts of **15a** (blue) and **17a** (red). Molecular weight increase 492.

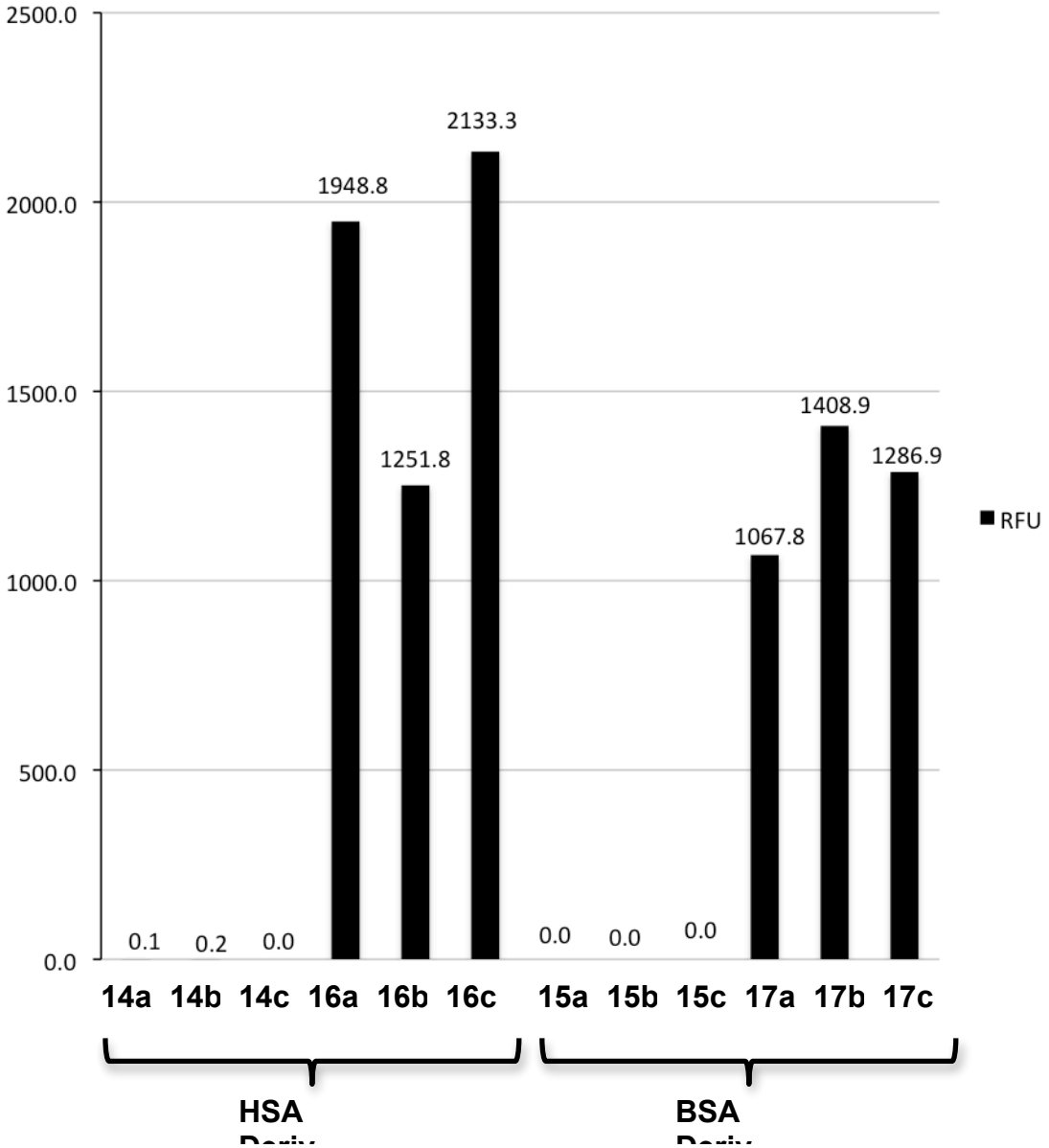


Overlay of MALDI-TOF MS charts of **15b** (blue) and **17b** (red). Molecular weight increase 755.

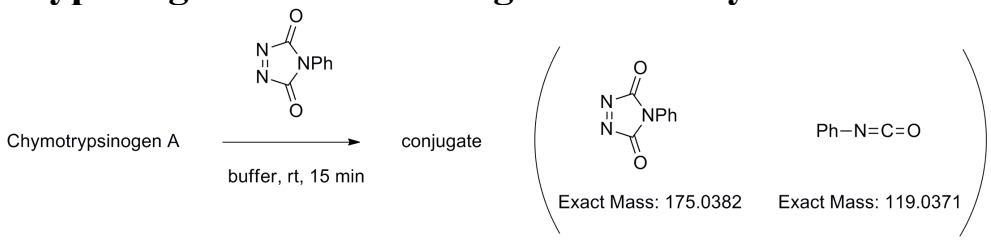


Overlay of MALDI-TOF MS charts of **15c** (blue) and **17c** (red). Molecular weight increase 682.

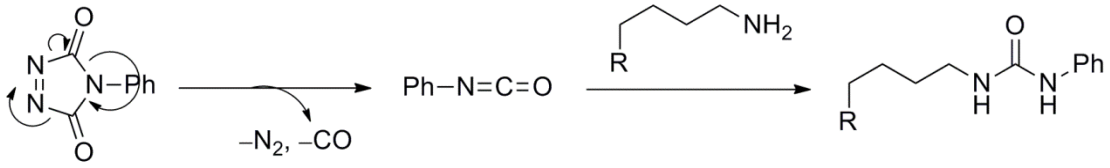
Fluorescence intensity of fluorescein moiety of **14a-c**, **15a-c**, **16a-c** and **17a-c**.



7. Chymotrypsinogen PTAD labeling buffer study



We noted an experimental artifact in our original PTAD labeling study of chymotrypsinogen (ref. 1, figure 3B), namely the addition of a mass of approximately 336 daltons to the protein. It is known that PTADs can decompose to form isocyanates[2] and a mass addition of 336 is consistent with isocyanate formation of the PTAD azide derivative used in the original study and its subsequent reaction with various nucleophilic functionalities present on proteins like amines and hydroxyls. The simplest PTAD shown above is expected to decompose to an isocyanate of mass 119 (see above). Reaction of isocyanate derivatives with a protein is expected to be promiscuous whereas direct reaction with PTADs is highly selective for the phenolic side chain of tyrosine as we have proven in many cases.[3] We suspect that with slow reacting proteins with no or poorly exposed/reactive tyrosine residues like chymotrypsinogen, decomposition of PTADs produces isocyanates that then promiscuously label the protein.



In our initial disclosure of PTAD labeling[1], we had documented that labeling is effective in a wide variety of buffered solutions, including 2-amino-2-hydroxymethyl-propane-1,3-diol or Tris buffered solution. Since Tris buffer contains a primary amine, we studied the potential of using Tris buffer as an isocyanate scavenger in PTAD labeling reactions of chymotrypsinogen. As shown in the ESI-MS charts that follow, chymotrypsinogen labeling in phosphate buffer revealed significant isocyanate labeling. Addition of as little as 5% volume Tris to the PBS solution, however, acted to significantly scavenge the isocyanate decomposition product, while addition of 25% volume Tris nearly completely removed detectable isocyanate-derived protein labeling as effectively as performing the reaction in Tris buffer itself. ***Thus for the PTAD labeling of particular proteins, an exploration of buffers, including mixed PBS/Tris buffered media is recommended to ensure tyrosine selective labeling when Tyr specificity is critical. PBS buffer is often fine.***

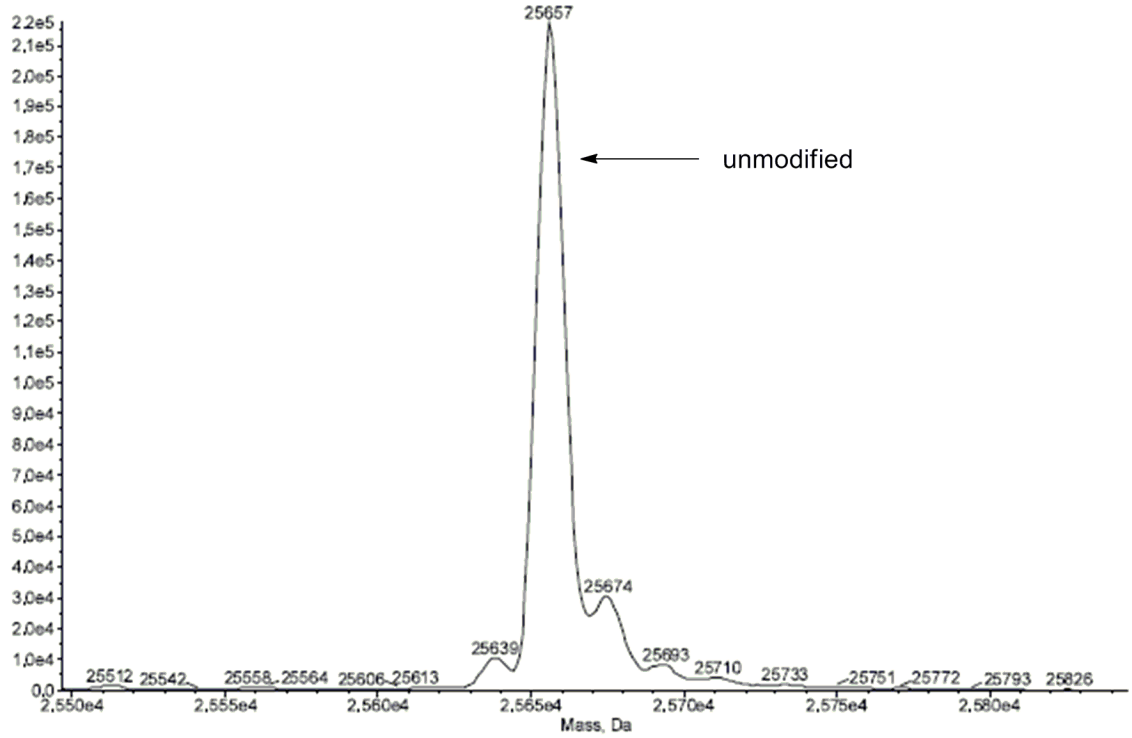
Study of Chymotrypsinogen labeling with PTAD in PBS/Tris buffered conditions:

To the 1.7 ml microcentrifuge tube was added chymotrypsinogen solution (60.6 μM protein in 98 μL mixed buffer prepared by volumetric mixing of PBS (10 mM, pH 7.4) and Tris (1.0 M, pH 7.4) buffers) followed by addition of PTAD (100 mM in DMF, 2 μL). The reaction mixture was allowed to stand at room temperature for 15 min before the unreacted small molecules were removed using Zeba spin desalting column (7k MWCO). ESI-MS was then obtained and are given below.

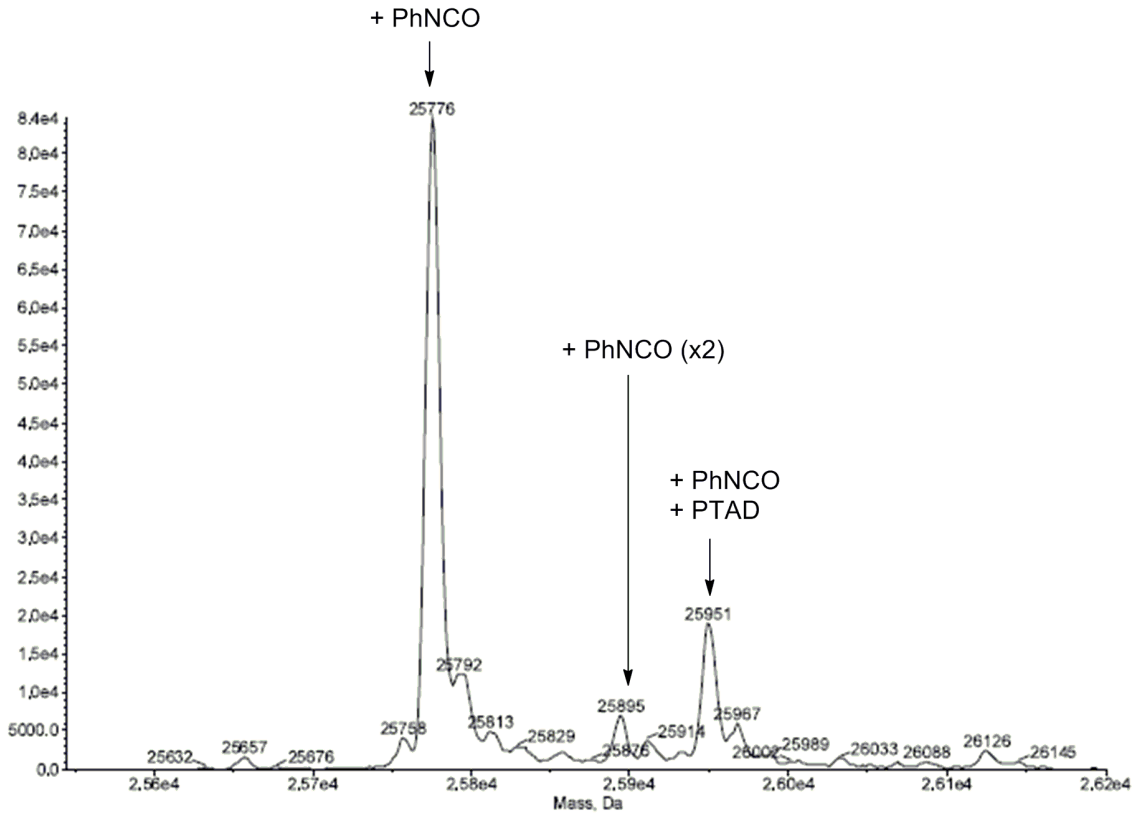
1. Ban, H., Gavrilyuk, J., and Barbas, C. F. (2010) Tyrosine Bioconjugation through Aqueous Ene-Type Reactions: A Click-Like Reaction for Tyrosine. *Journal of the American Chemical Society 132*, 1523-1525.
2. Wamhoff, H., and Wald, K. (1977) Zur Photolyse und Thermolyse von 4-Aryl-1,2,4-triazolin-3,5-dionen. *Chem. Ber.*, 110, 1699-1715.
3. We thank Drs Edmund Graziani and Qi-Yang Hu for also bringing the artifact at ref. 1, Fig. 3B to our attention and Qi-Yang Hu for additional discussion.

ESI-MS charts for Chymotrysinogen labeling with PTAD

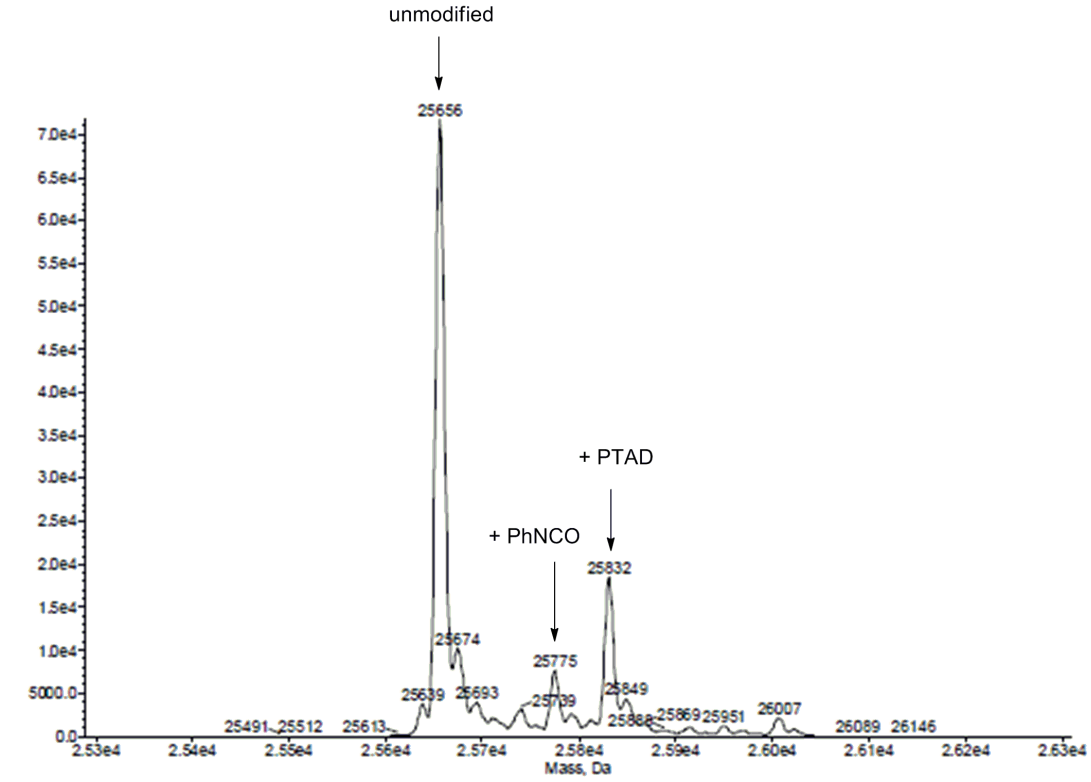
Unmodified protein



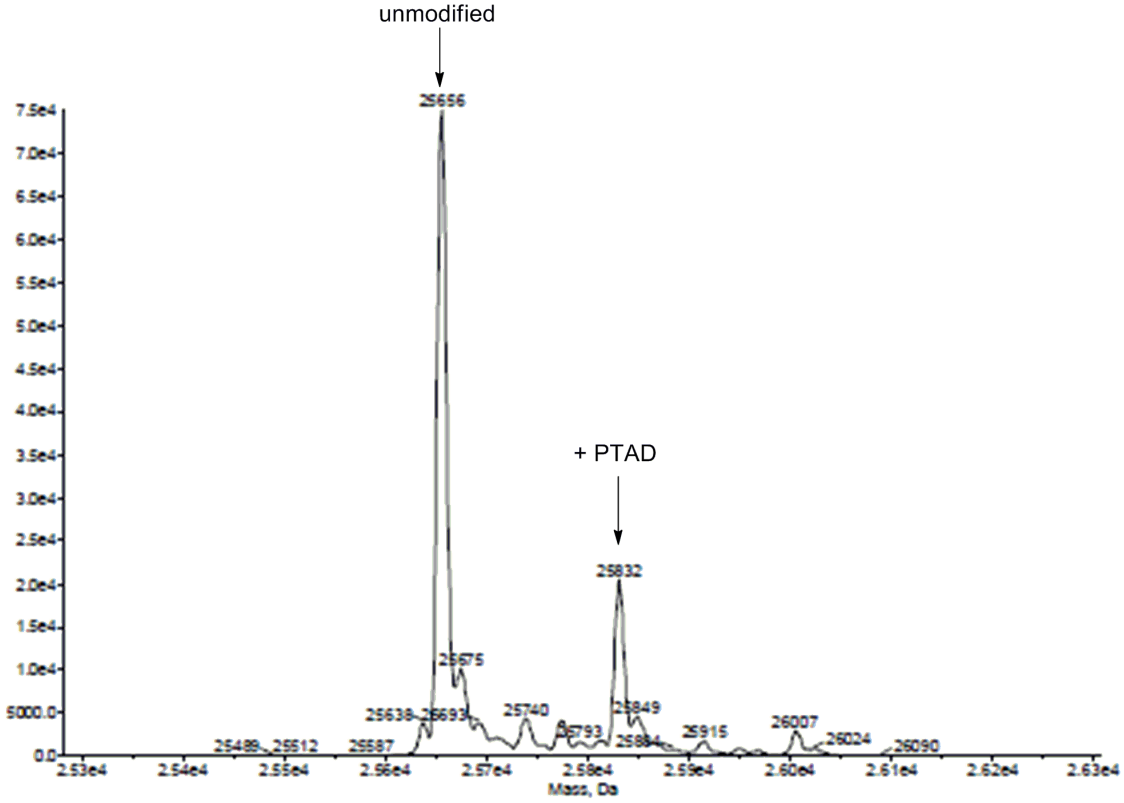
Labeling in PBS



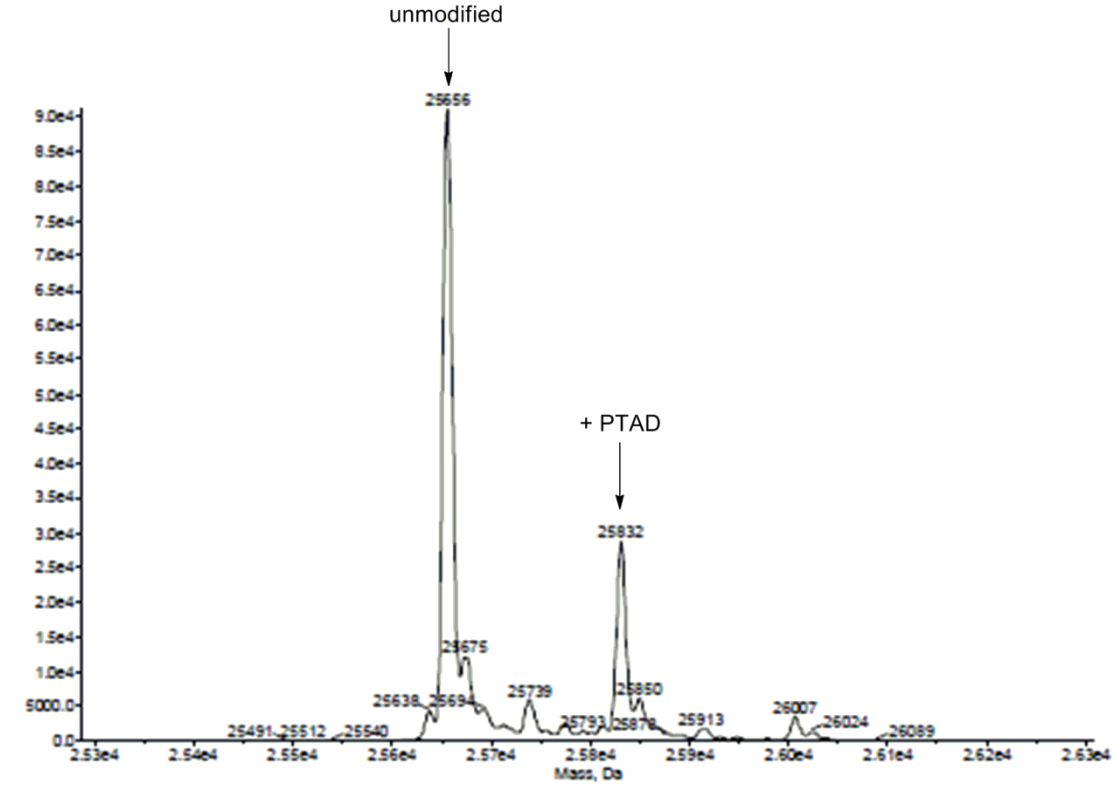
Labeling in PBS/Tris (95/5)



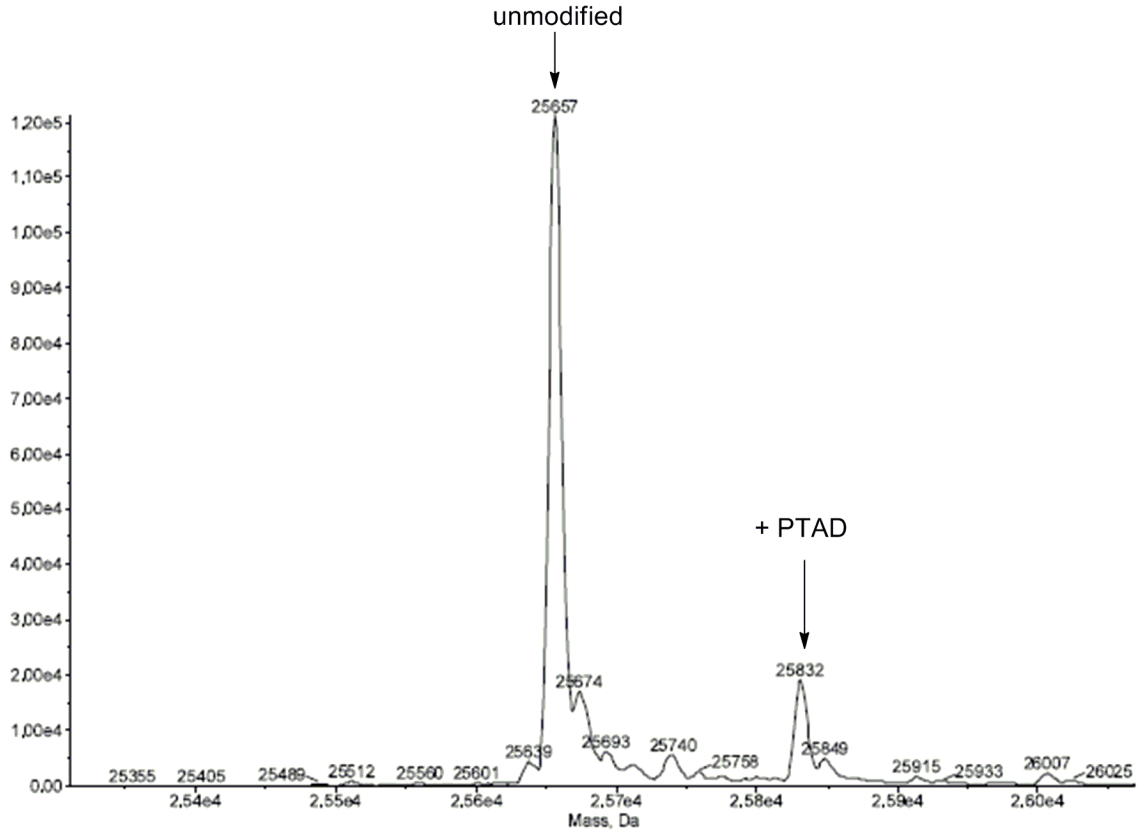
Labeling in PBS/Tris (85/15)



Labeling in PBS/Tris (75/25)

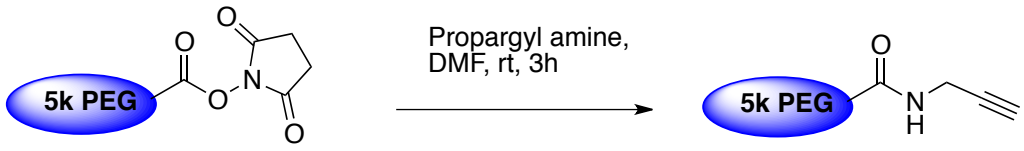


Labeling in Tris



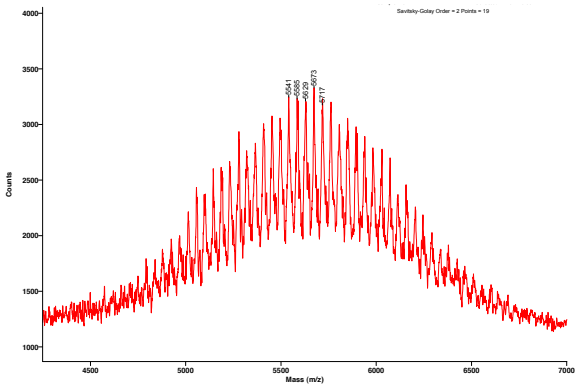
8. PTAD mediated PEGylation reaction

Preparation of PEG-PTAD

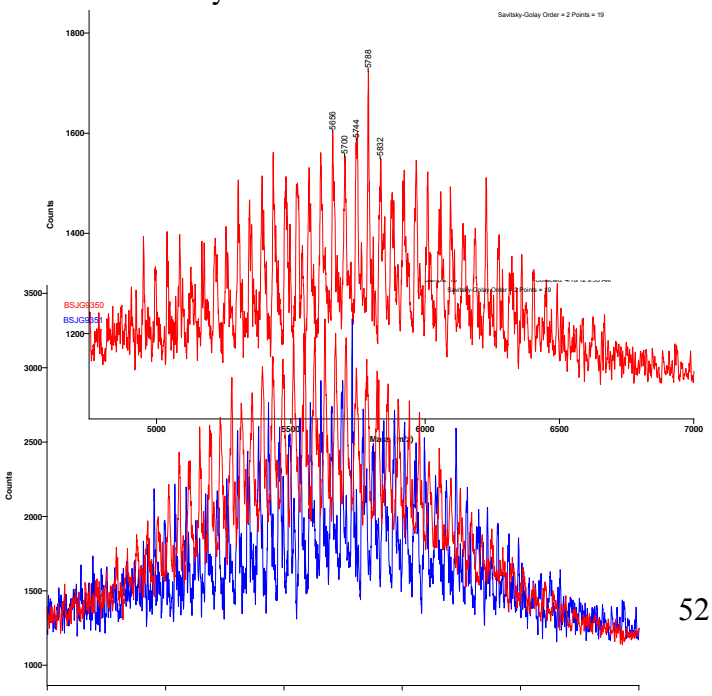


In the 1.5 ml Eppendorf tube were mixed 5k PEG-NHS (NOF Corporation, 98% end group reactivity) (38 ul of 50 mM solution in DMF, 1.89 umoles, 1 eq) and propargyl amine (1.89 ul of 1M solution in DMF, 1.89 umoles, 1 eq). The reaction mixture was vortexed gently and kept at room temperature for 3 hours with intermittent vortexing. Methyl amine (5 ul, neat) was added to the reaction to make sure all the activated ester groups were consumed; reaction was vortexed and kept at room temperature for 30 min. The product polymer was precipitated out with cold ether, centrifuged and ether decanted. The resulting white solid was washed with cold ether two times and dried. Isolated yield 9.1 mg, 93%. MALDI-TOF MW_{av}.= 5656.

PEG-NHS (starting material):



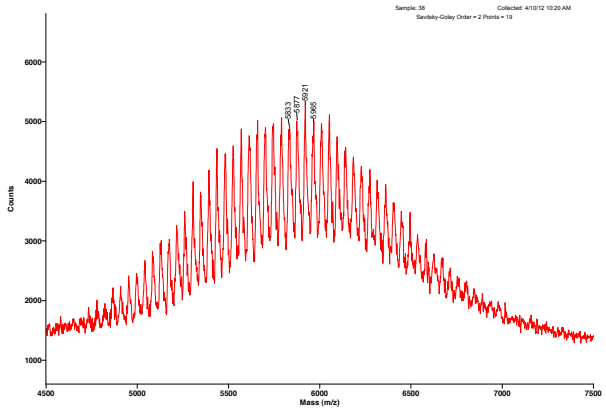
PEG-alkyne:



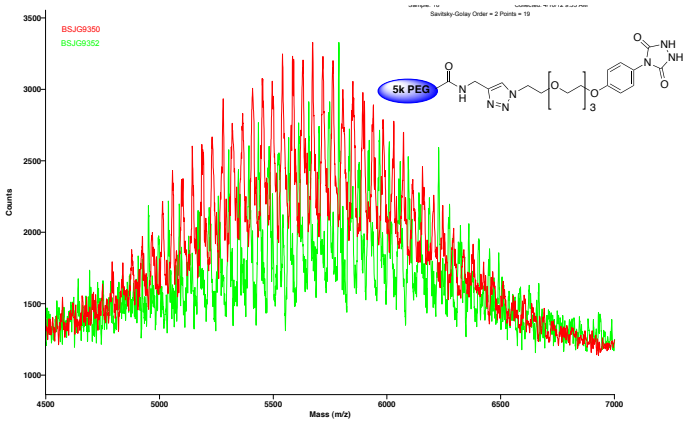
Overlay of PEG-NHS starting material (red) with PEG-alkyne (blue):

*Synthesis of PEG-urazole (**23**):* In the 1.5 ml Eppendorf tube were mixed 5k PEG-alkyne (NOF Corporation, 98% end group reactivity) (15 µl of 48 mM solution in DMF, 0.72 µmoles, 1 eq) and 1,2,4-triazolidine-3,5-dione azide **14** (30 µl of 24 mM solution in DMF, 0.72 µmoles, 1 eq) followed by addition of a small piece of copper wire and copper sulfate (0.72 µl, 100 mM solution in DI water). The reaction mixture was vortexed gently and kept at 37 ºC for 2 hrs with intermittent vortexing. Copper wire was removed and copper ions were scavenged from the reaction mixture using "CupriSorb" resin (Seachem) over night at room temperature. The Cuprisorb resin was filtered and product polymer was precipitated out with cold ether, centrifuged and ether decanted. The resulting white solid (**23**) was washed with cold ether two times and dried. Isolated yield 4.0 mg, 95%. MALDI-TOF $MW_{av.}$= 5921.

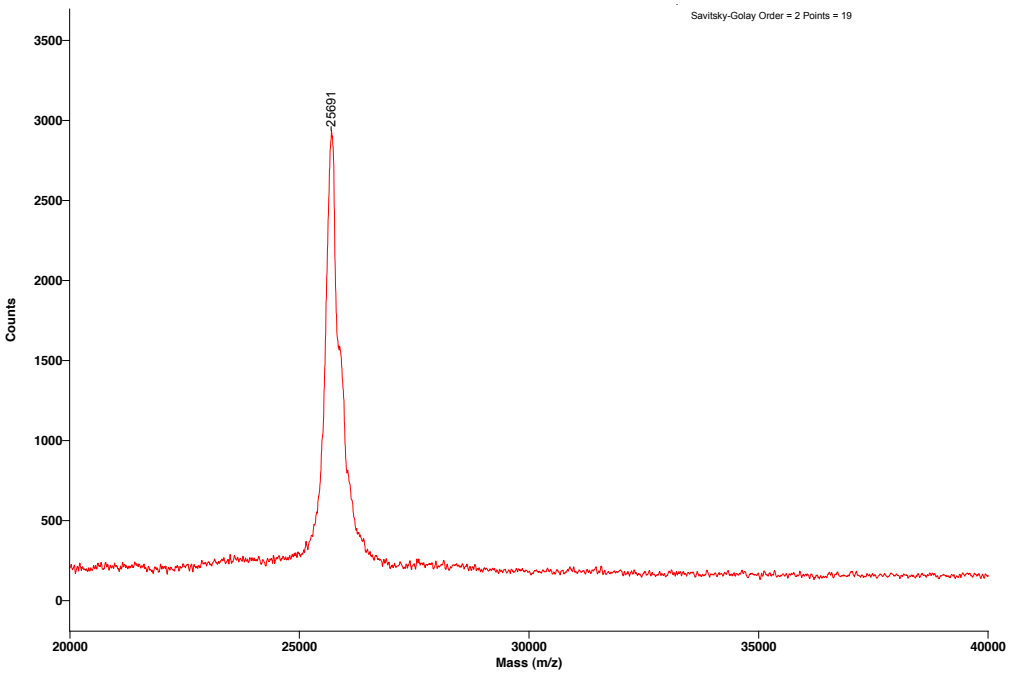
PEG-alkyne:



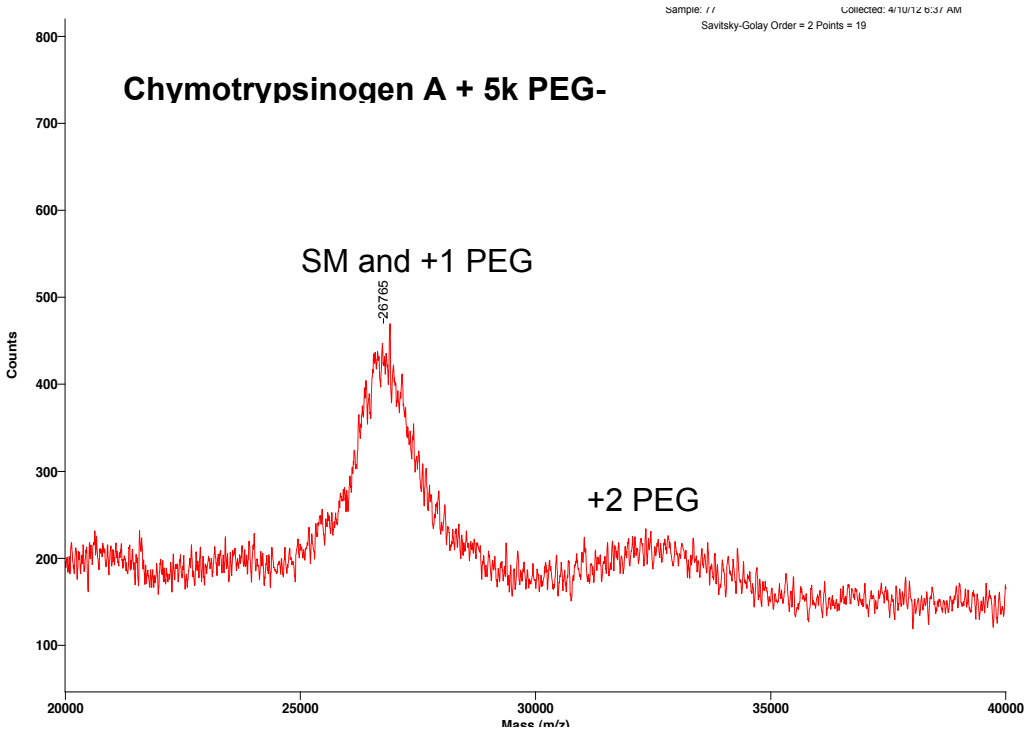
Overlay of PEG-NHS (red) with PEG-PTAD(green).



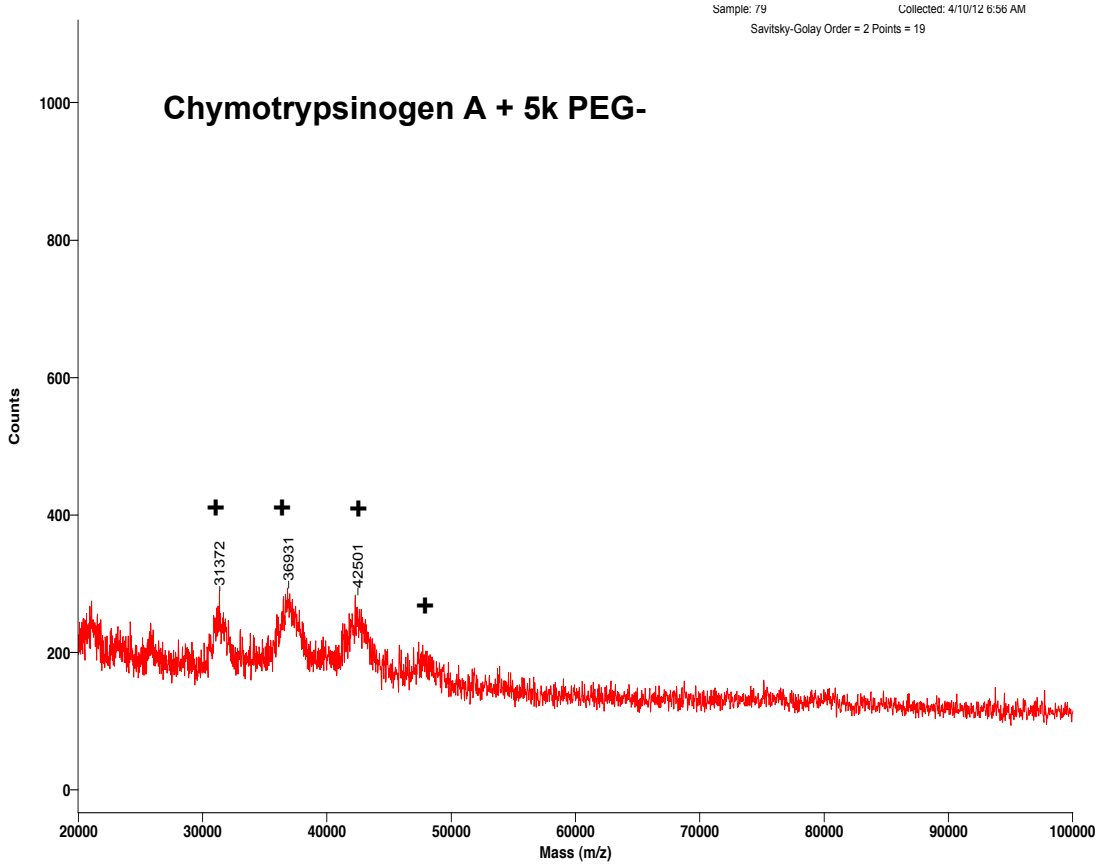
MALDI-TOF:
Chymotrypsinogen A:

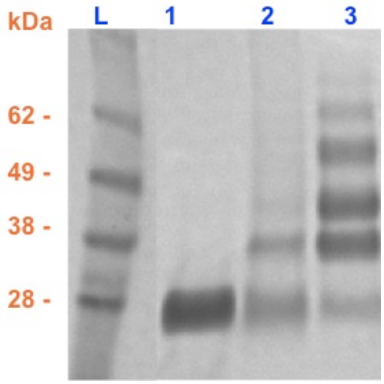


Chymotrypsinogen-PEG (PTAD)



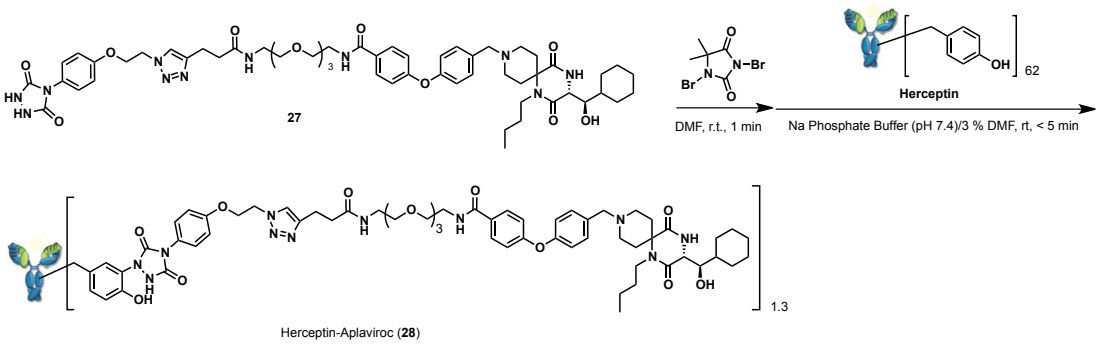
Chymotrypsinogen-PEG(NHS):



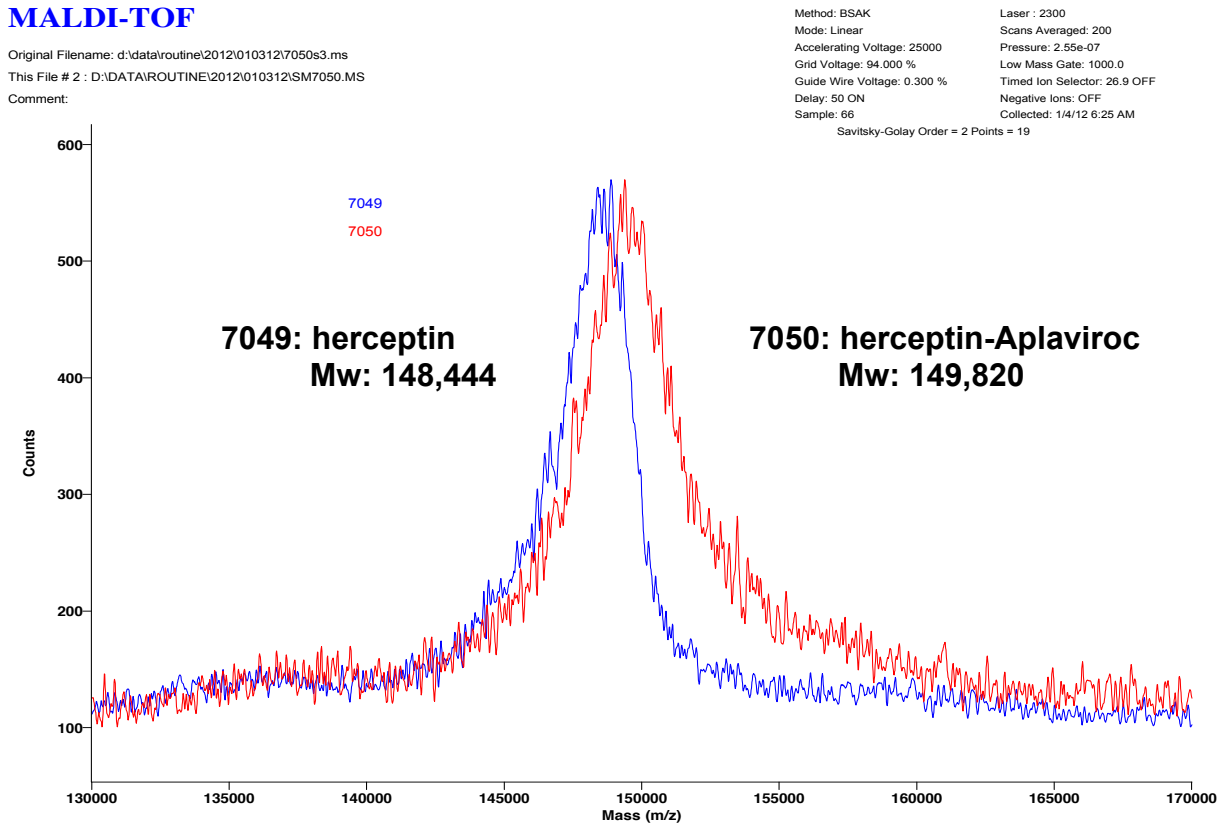


Comparison of products of reaction of Chymotrypsinogen A with 5-kDa PEG-PTAD and 5-kDa PEG-NHS. Products were separated on a NuPage 4-12% Bis-Tris gel (Invitrogen) and the gel was Coomassie stained: L, molecular weight ladder; lane 1, Chymotrypsinogen A; lane 2, reaction with 10 eq. PEG-PTAD; lane 3, reaction with 10 eq. PEG-NHS.

9. Trastuzumab (Herceptin) conjugation with Aplaviroc-PTAD



Herceptin-Aplaviroc (**28**)



10. Stability study in human plasma

HPLC charts of the analyzed compounds.