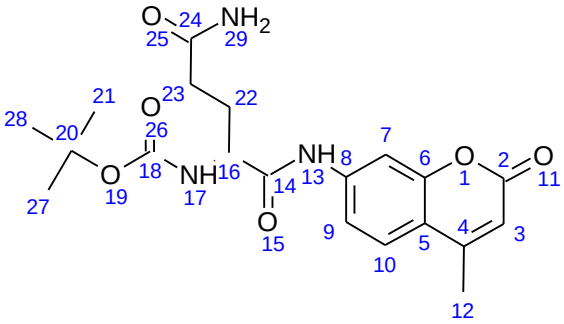
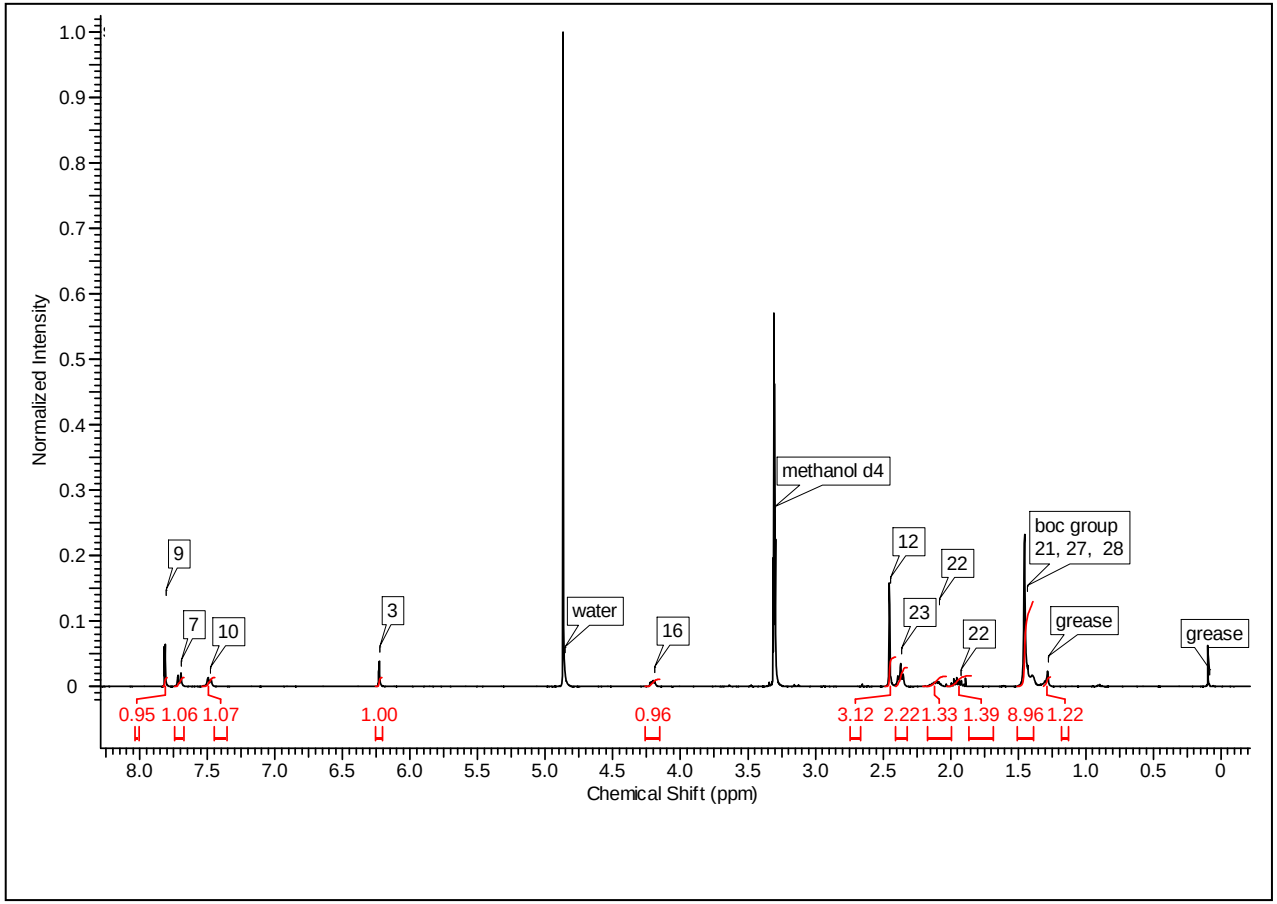
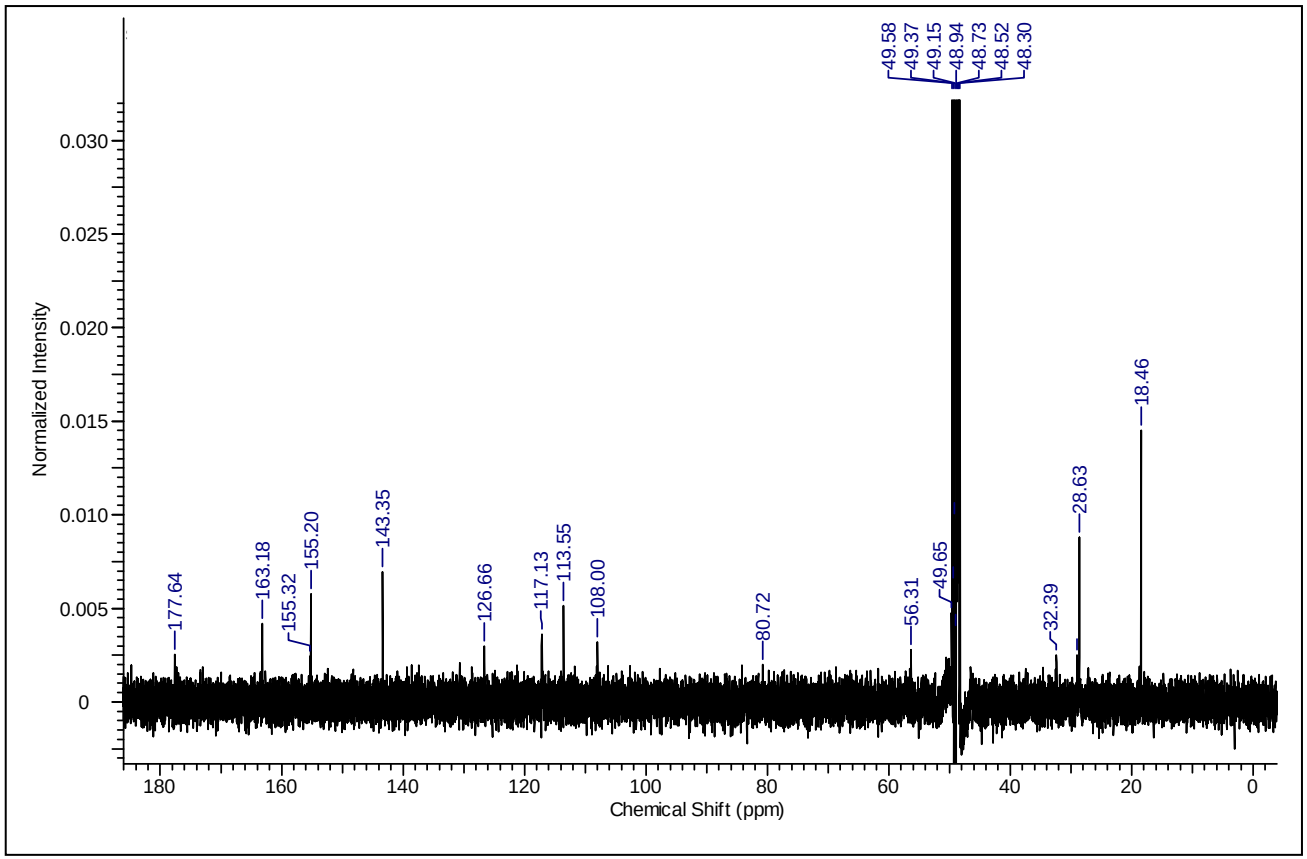
Appendix

BocGlnAMC, **68**

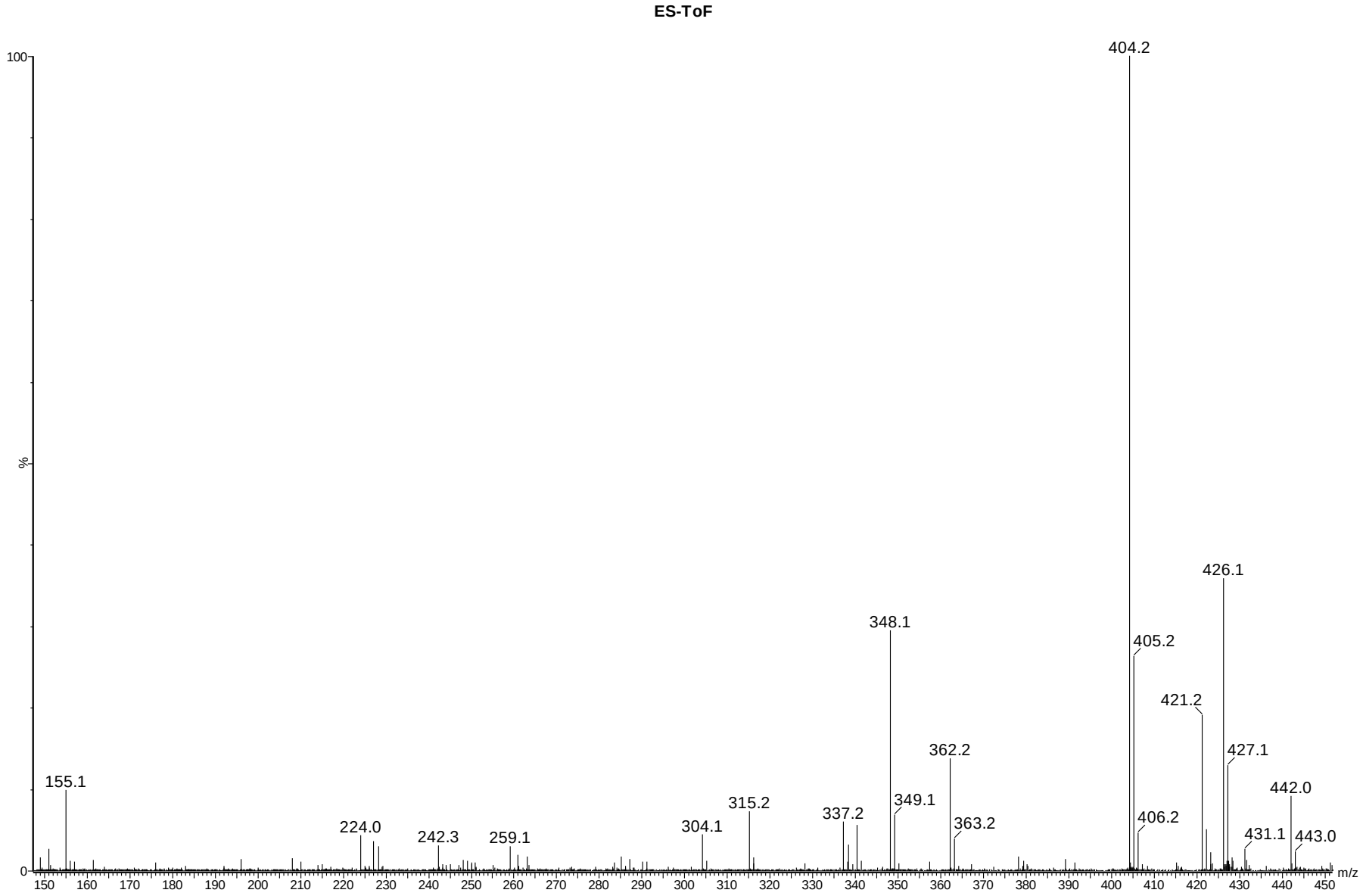




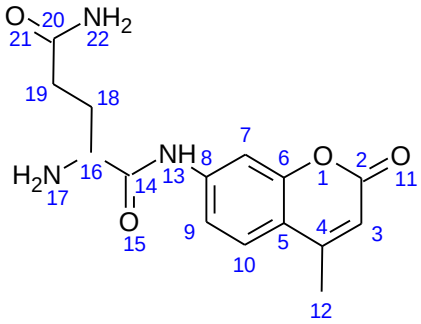
^{1}H NMR for BocGlnAMC

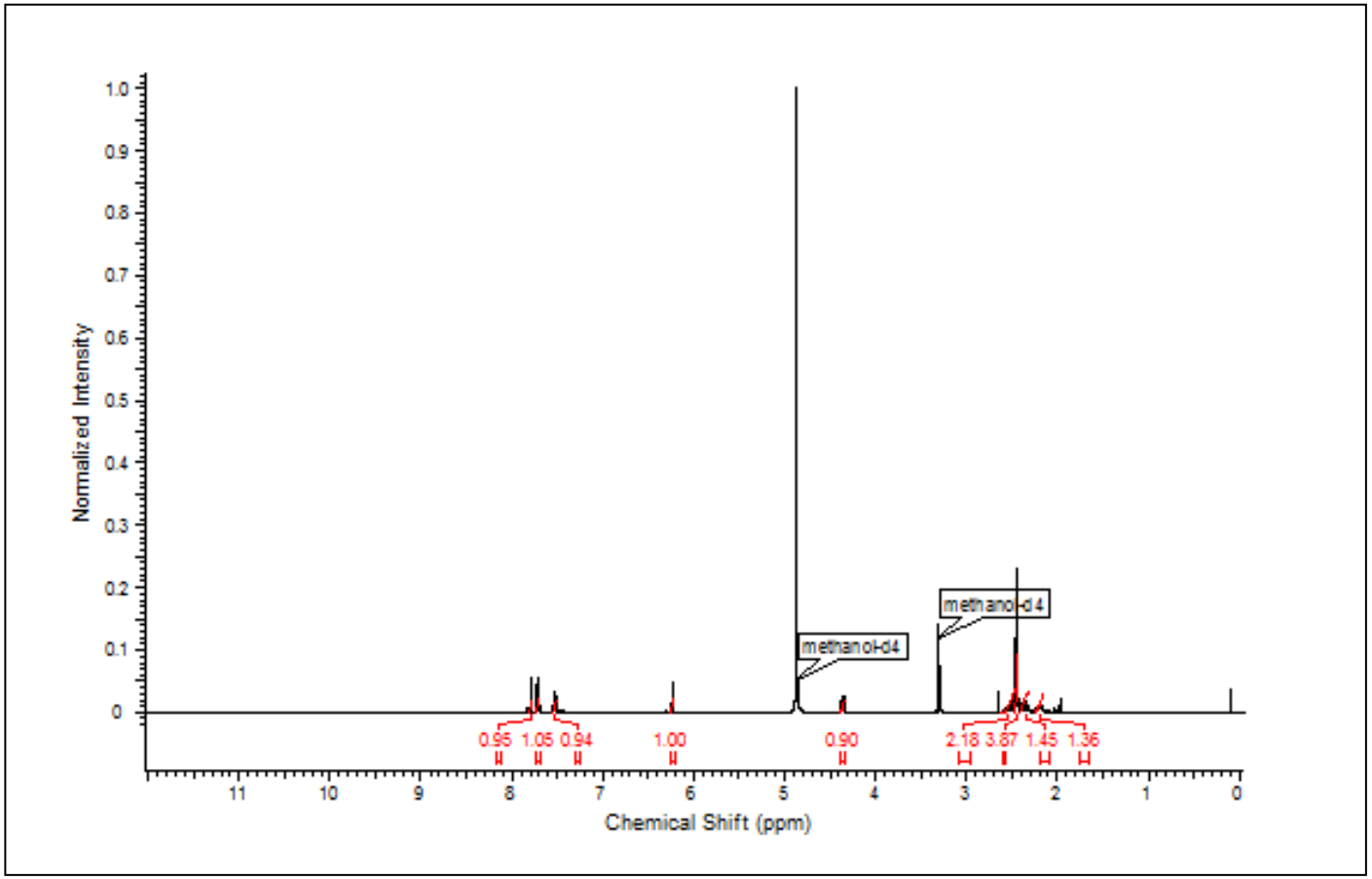


^{13}C NMR for BocGlnAMC

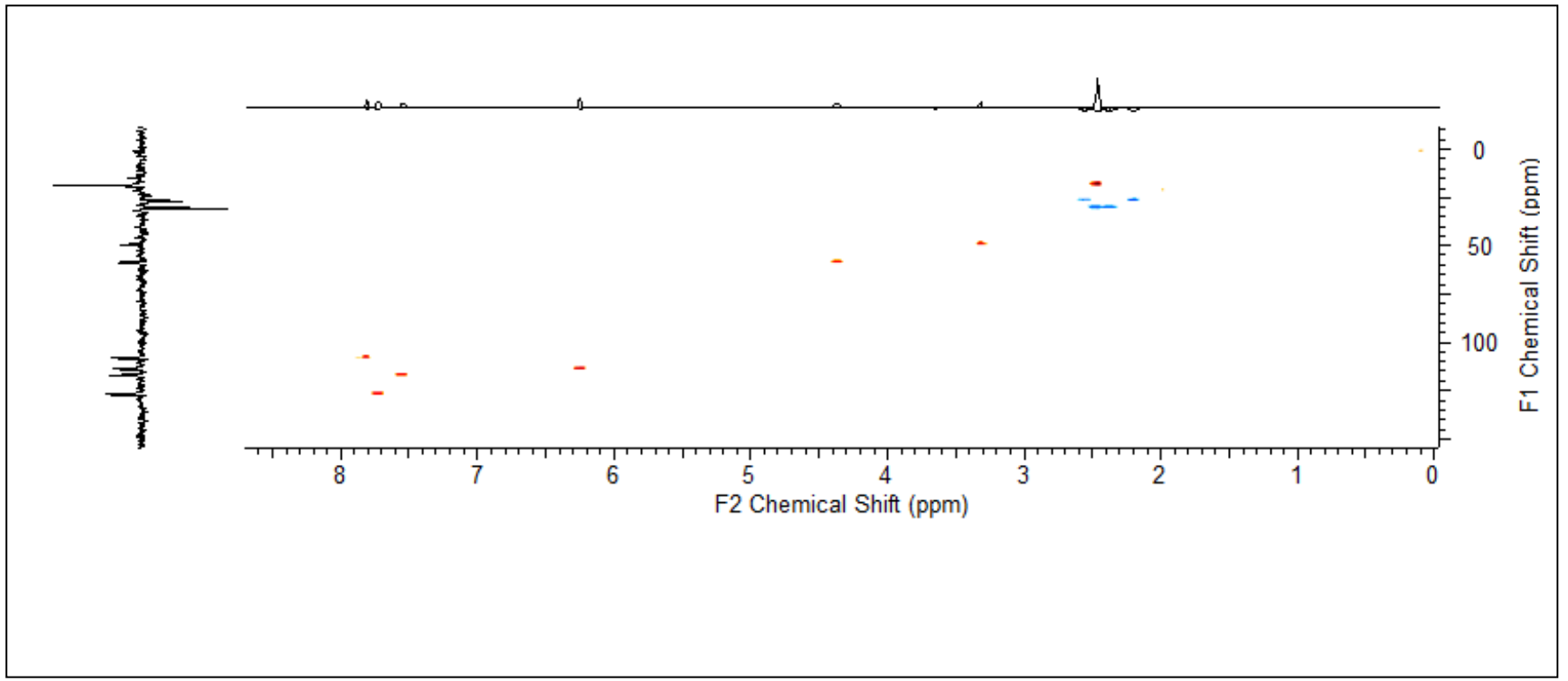

ES-ToF
100
%
0
404.2
426.1
348.1
405.2
421.2
362.2
427.1
155.1
442.0
315.2
349.1
337.2
224.0
304.1
363.2
406.2
242.3
259.1
431.1
443.0
150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 400 410 420 430 440 450
m/z

NH_2GlnAMC, **69**

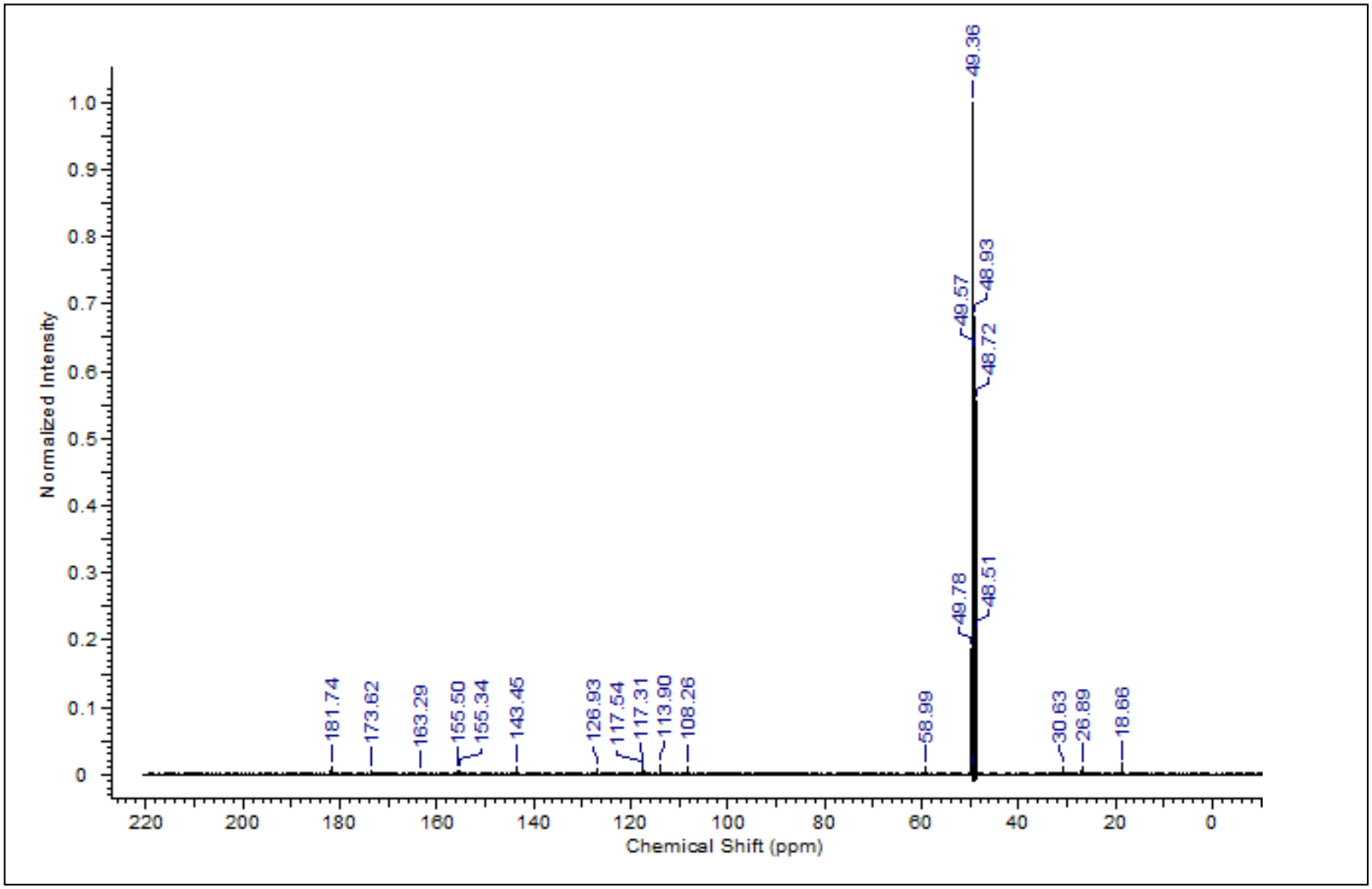




^{1}H NMR NH_2GlnAMC

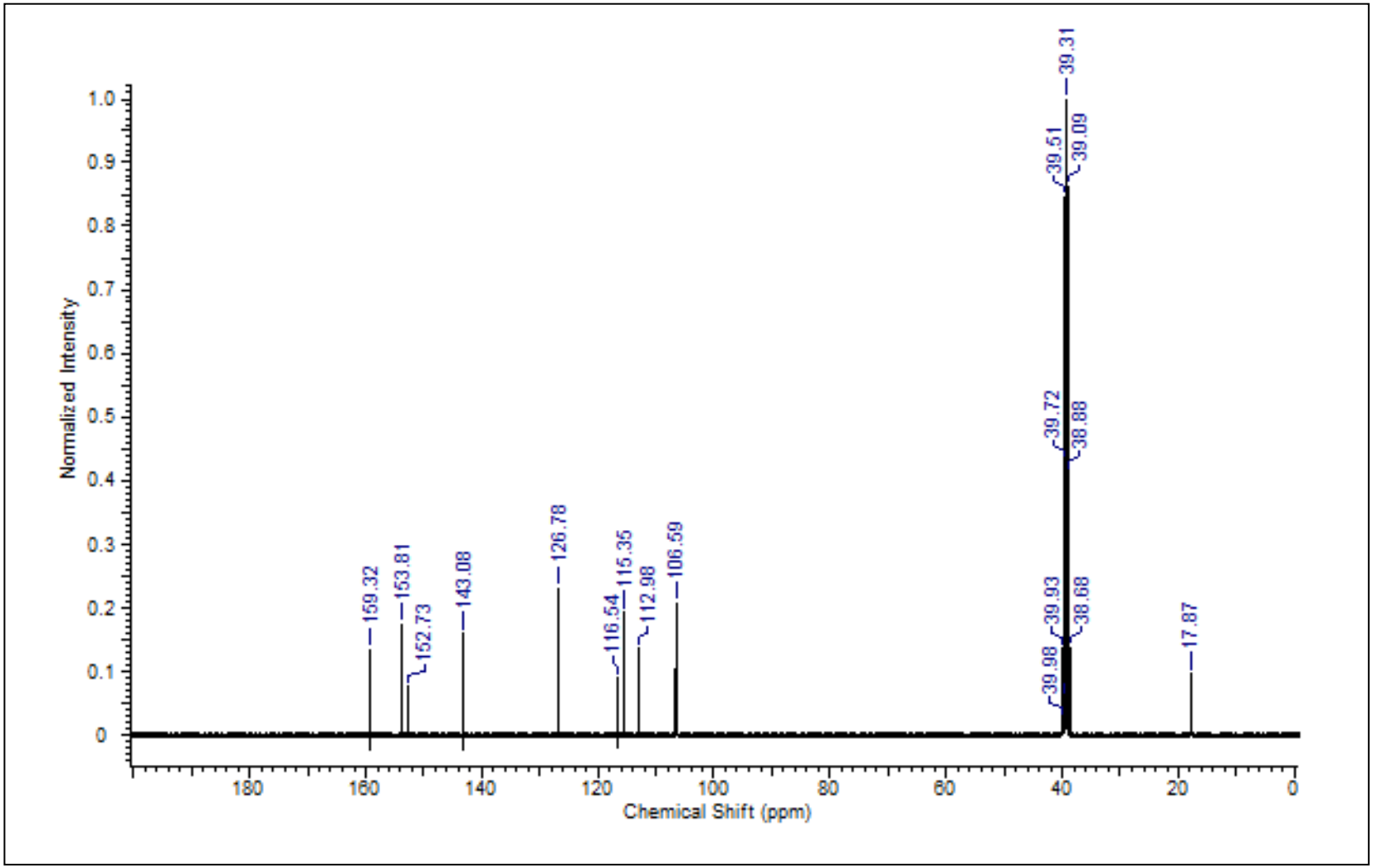


HSQC - NH_2GlnAMC



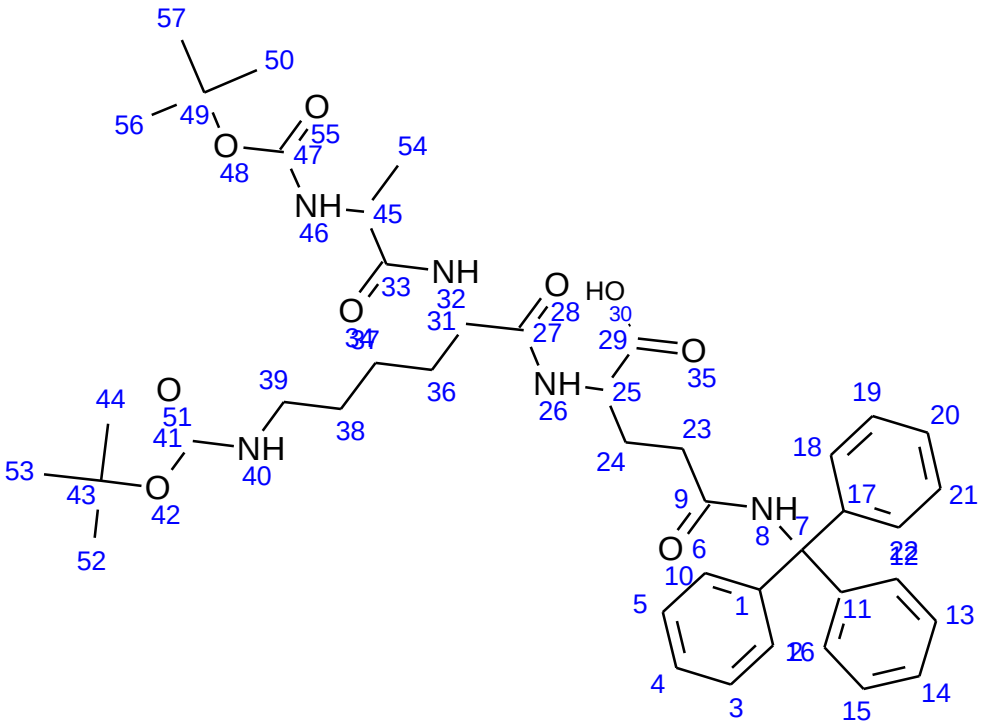
^{13}C NMR NH_2GlnAMC

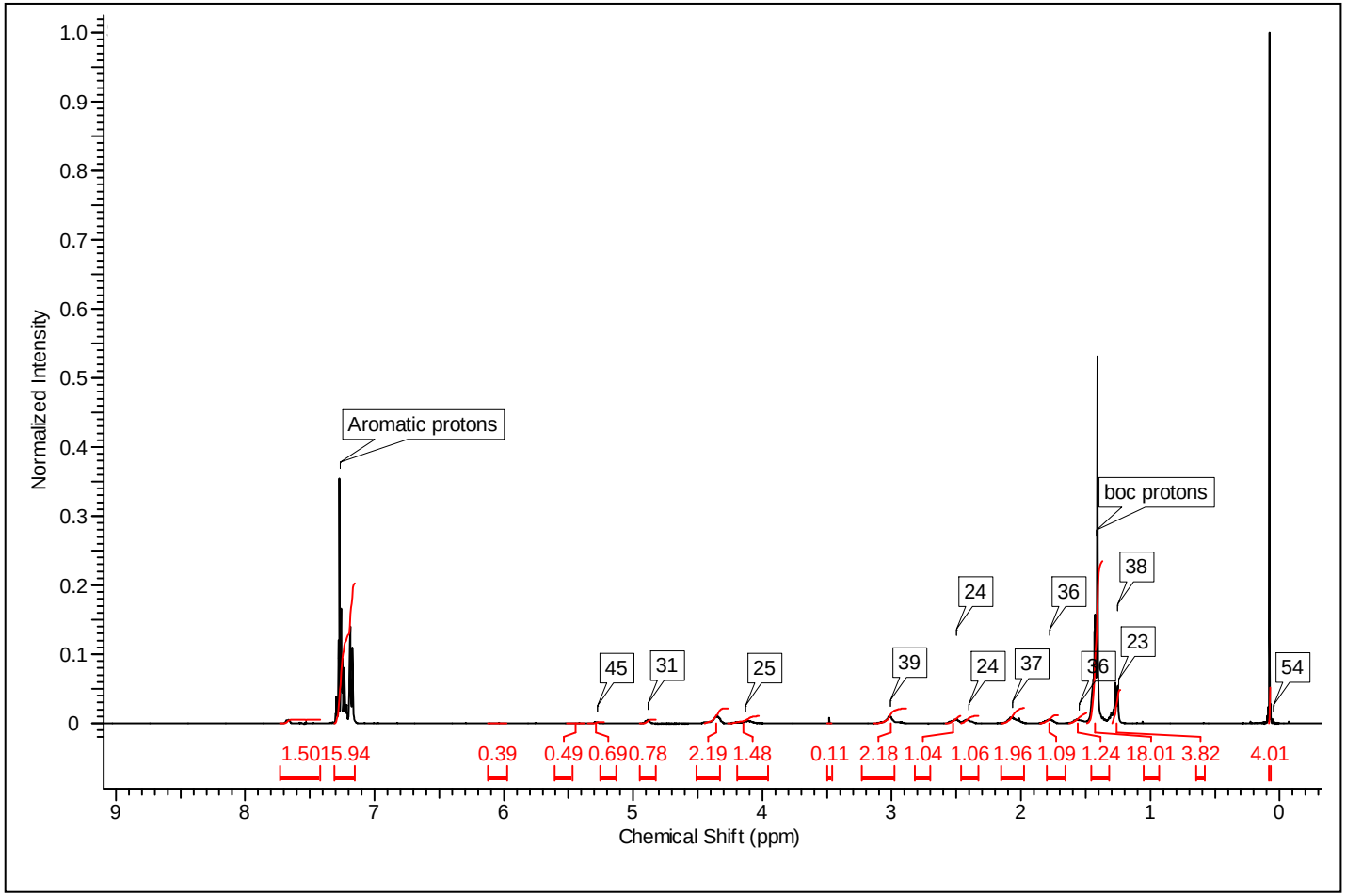
Azidomethylcoumarin **67**, proton and COSY spectrum in main body of thesis, figure 32 and 33 respectively.



^{13}C NMR Azidomethylcoumarin

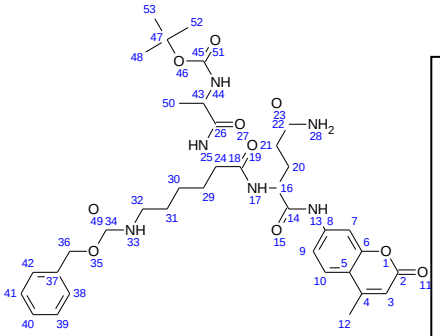
BocAlaLys(Boc)Gln(trt)OH – point of reference



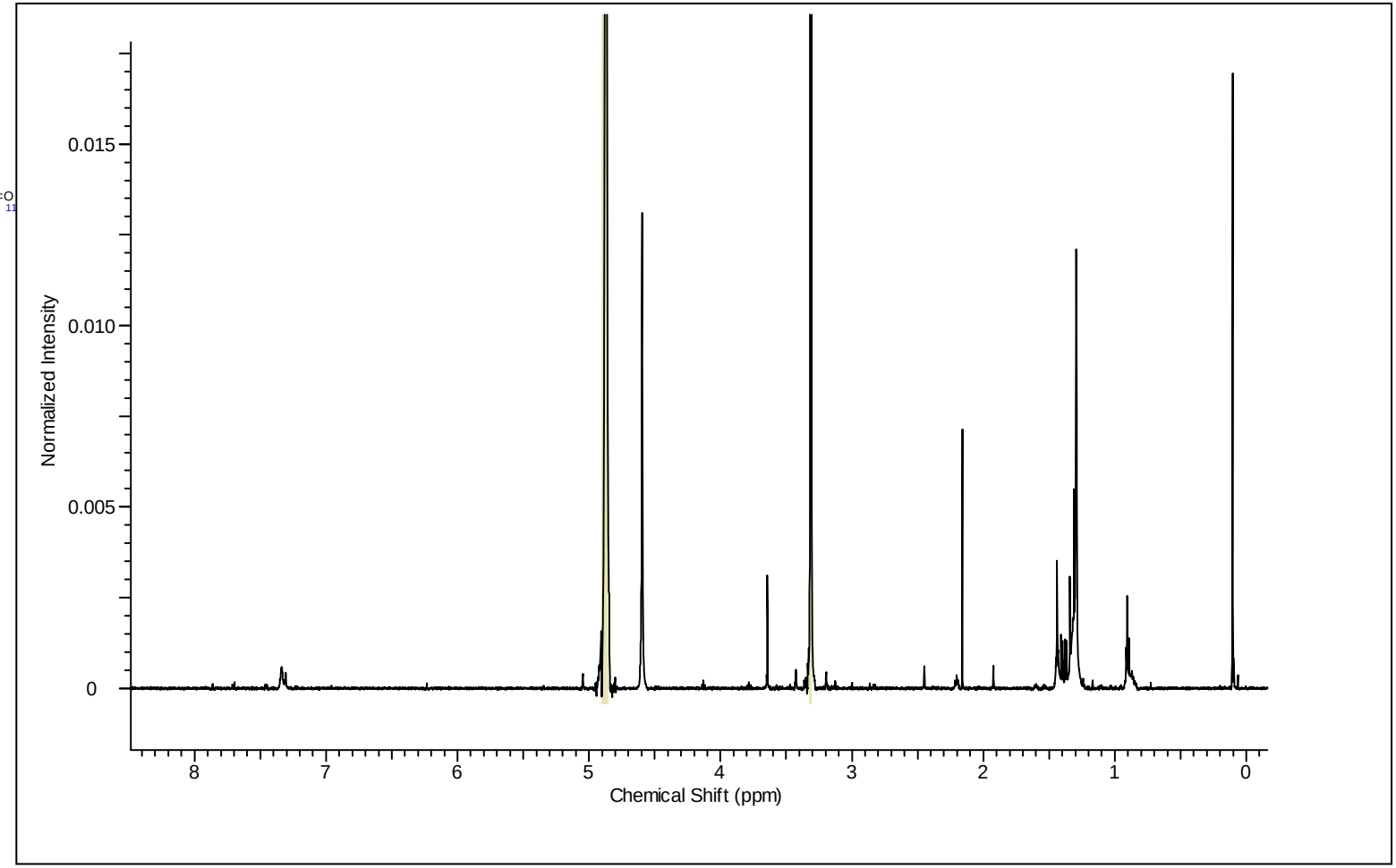


^{1}H NMR for BocAlaLys(Boc)Gln(trt)OH

BocAlaLys(Z)GlnAMC, **70**

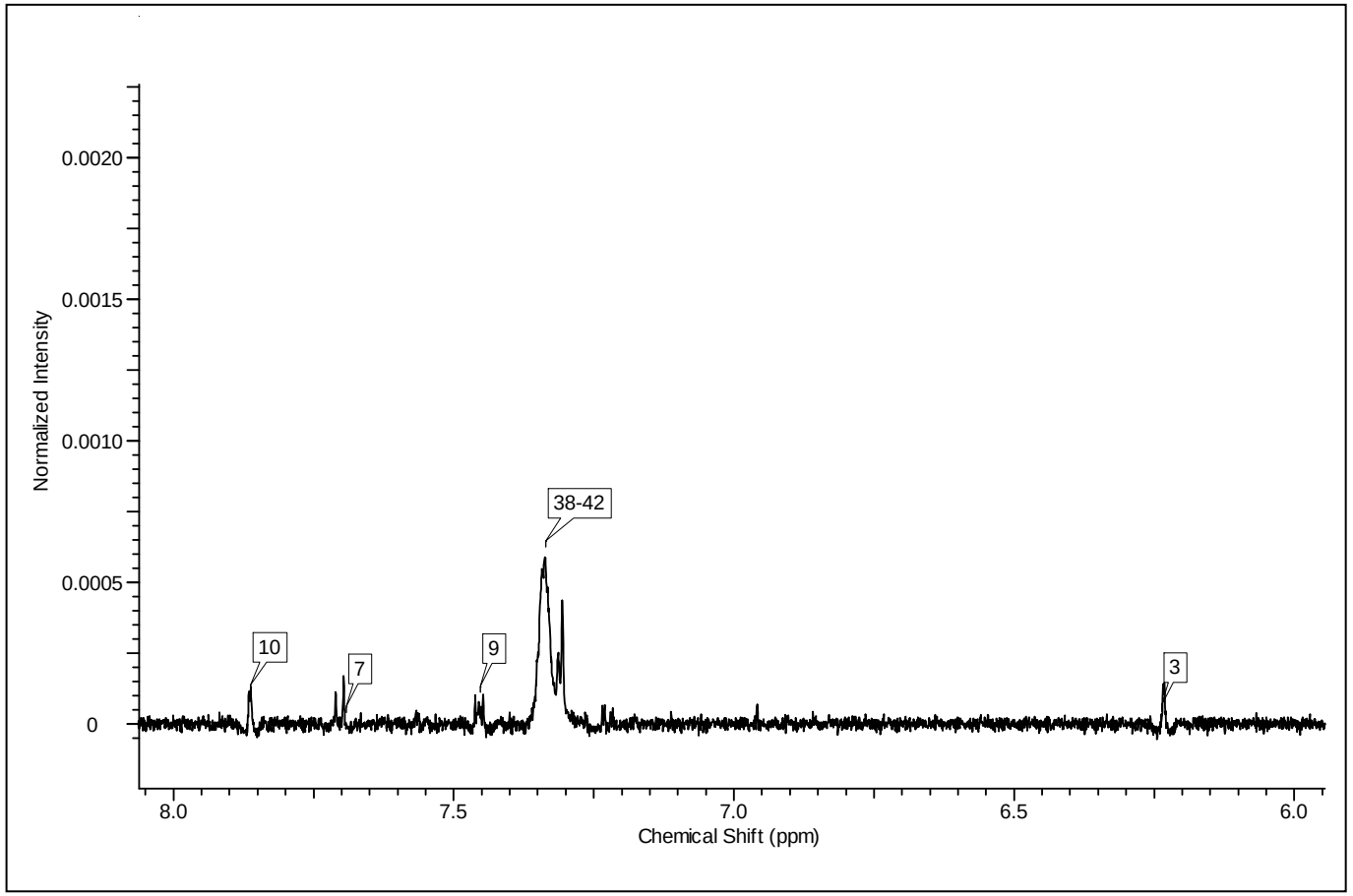


^{1}H NMR for
BocAlaLys(Z)GlnAMC



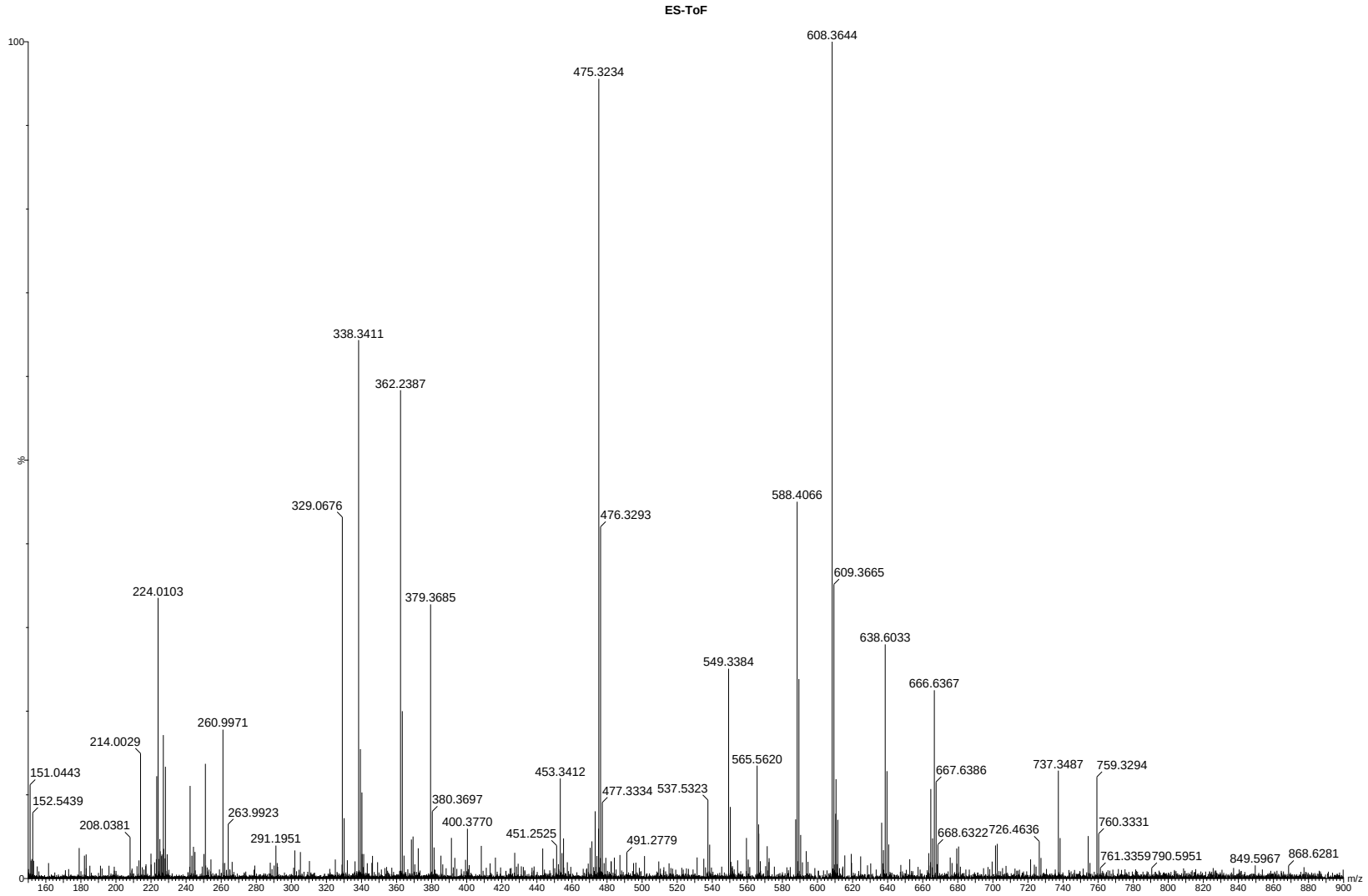
The NMR spectrum is also presented as zoomed shots, due to low amount of compound yield.

The main point of investigation for this compound was the positioning of peaks between 6-8 ppm. The single peak labelled ‘3’ is indicative of no free AMC being present in the sample. Due to large amounts of water in this hygroscopic sample, the integration values weren’t accurate in the proton NMR, furthermore due to poorly concentrated NMR sample of purified compound gaining a carbon NMR was impossible without an extended NMR experiment, however this method was deemed unsuitable for this unstable fluorogenic product. Therefore structure elucidation was completed using mass spectroscopy and elemental analysis, these techniques were selected as they require small amounts of the purified product for analysis.



^{1}H NMR for BocAlaLys(Z)GlnAMC

Mass spectrum – Formula = C37H48N6O10 Weight = 736.81 g/mol



Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Calc. Formula
737.3487	737.3510	-2.3	-3.1	16.5	56.2	0.0	C37 H49 N6 O10

Elements Used:

C: 37-37 H: 0-200 N: 0-10 O: 0-10

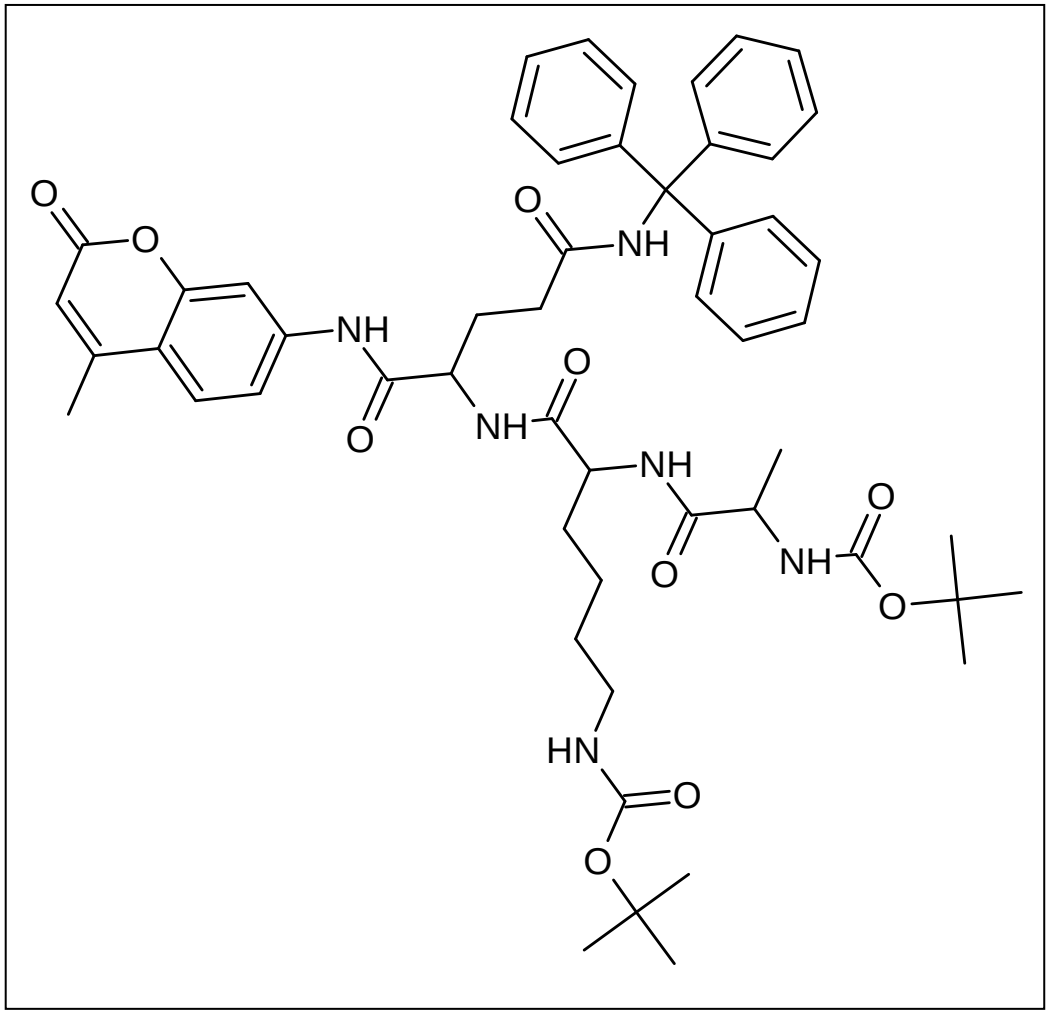
Formula calculated:

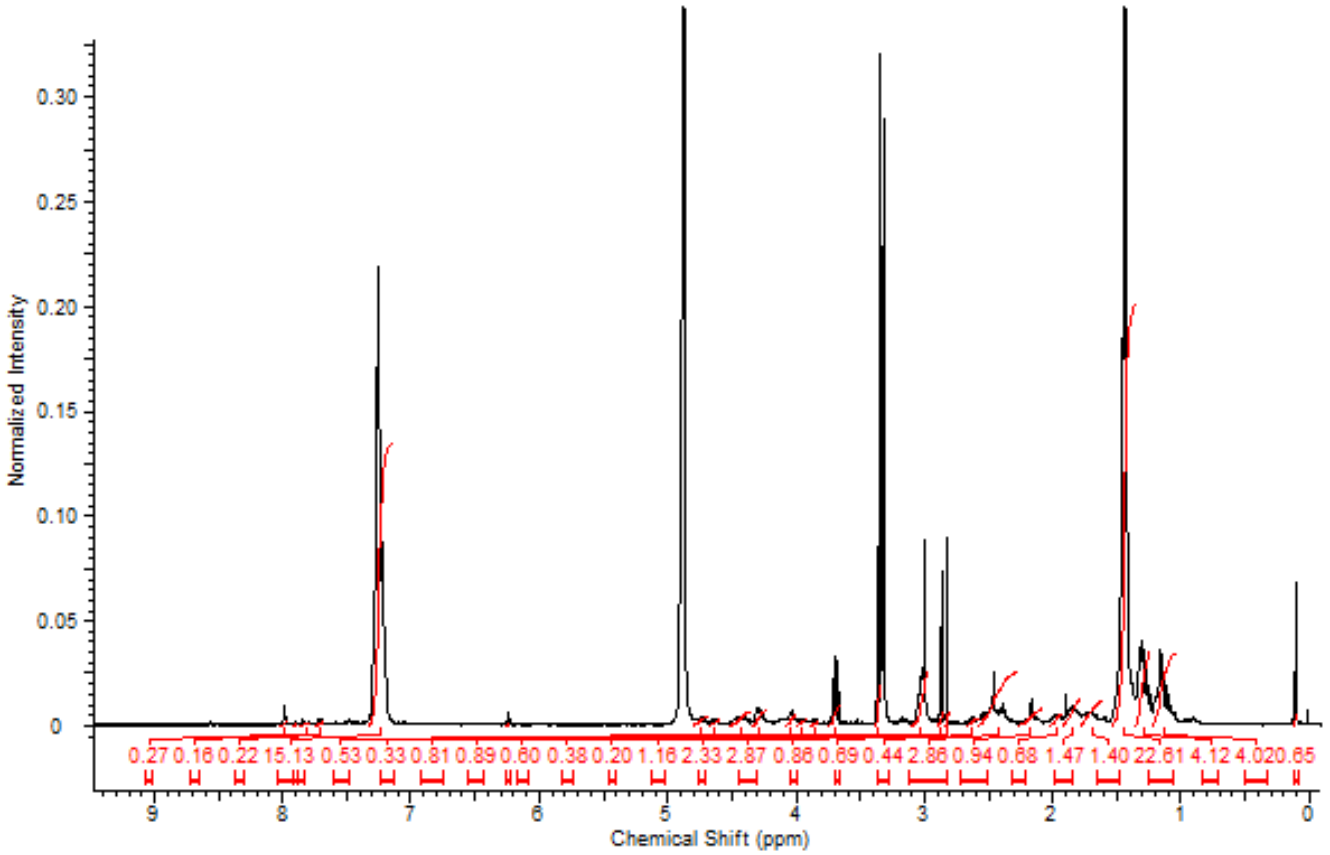
C37 H49 N6 O10

BocAlaLys(Boc)Gln(Trt)AMC, **71**

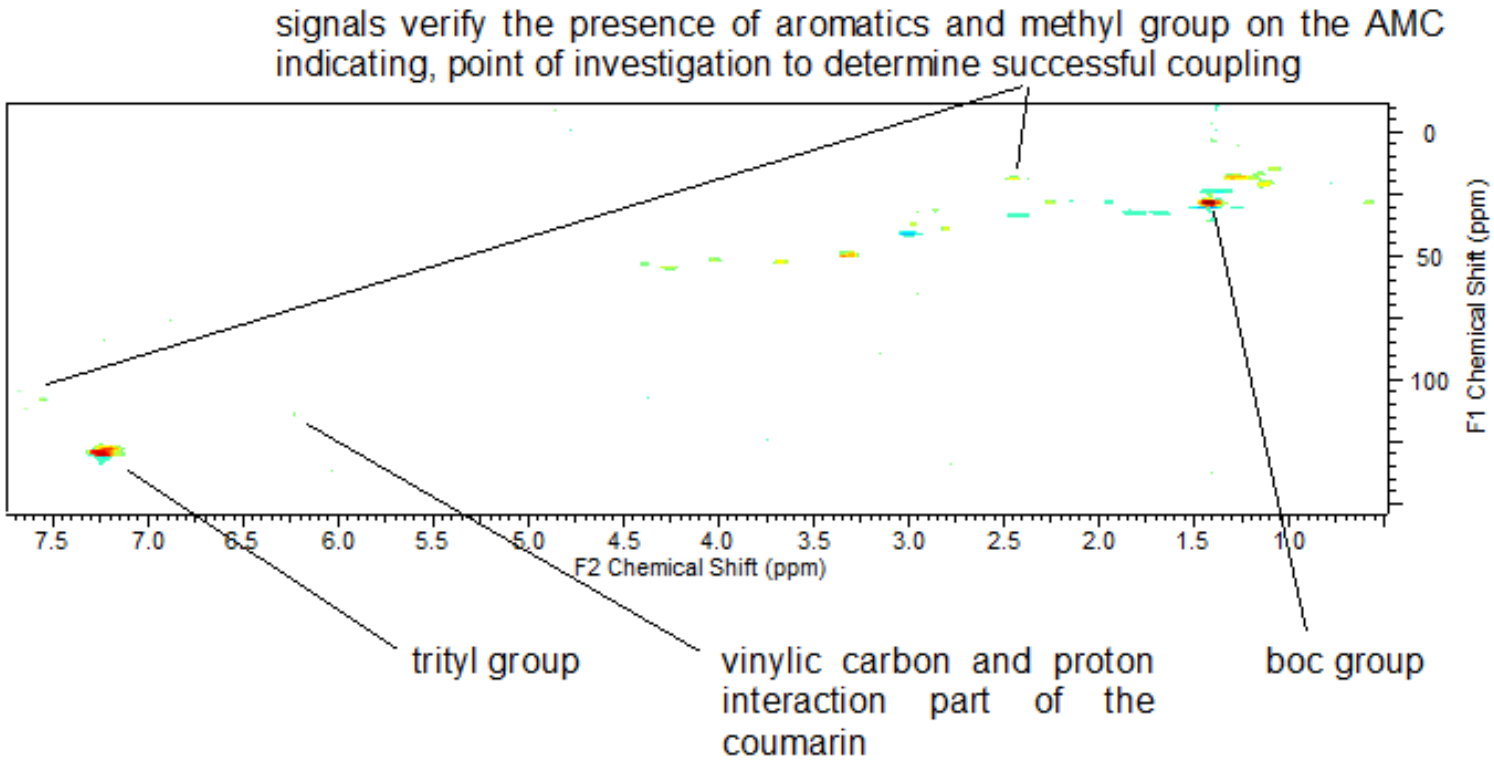
C53 H64 N6 O10

945.109g/mol




Normalized Intensity
0.30
0.25
0.20
0.15
0.10
0.05
0
0.27 0.16 0.22 15.13 0.53 0.33 0.81 0.89 0.60 0.38 0.20 1.16 2.33 2.87 0.86 0.69 0.44 2.86 0.94 0.68 1.47 1.40 22.61 4.12 4.02 0.65
9
8
7
6
5
4
3
2
1
0
Chemical Shift (ppm)

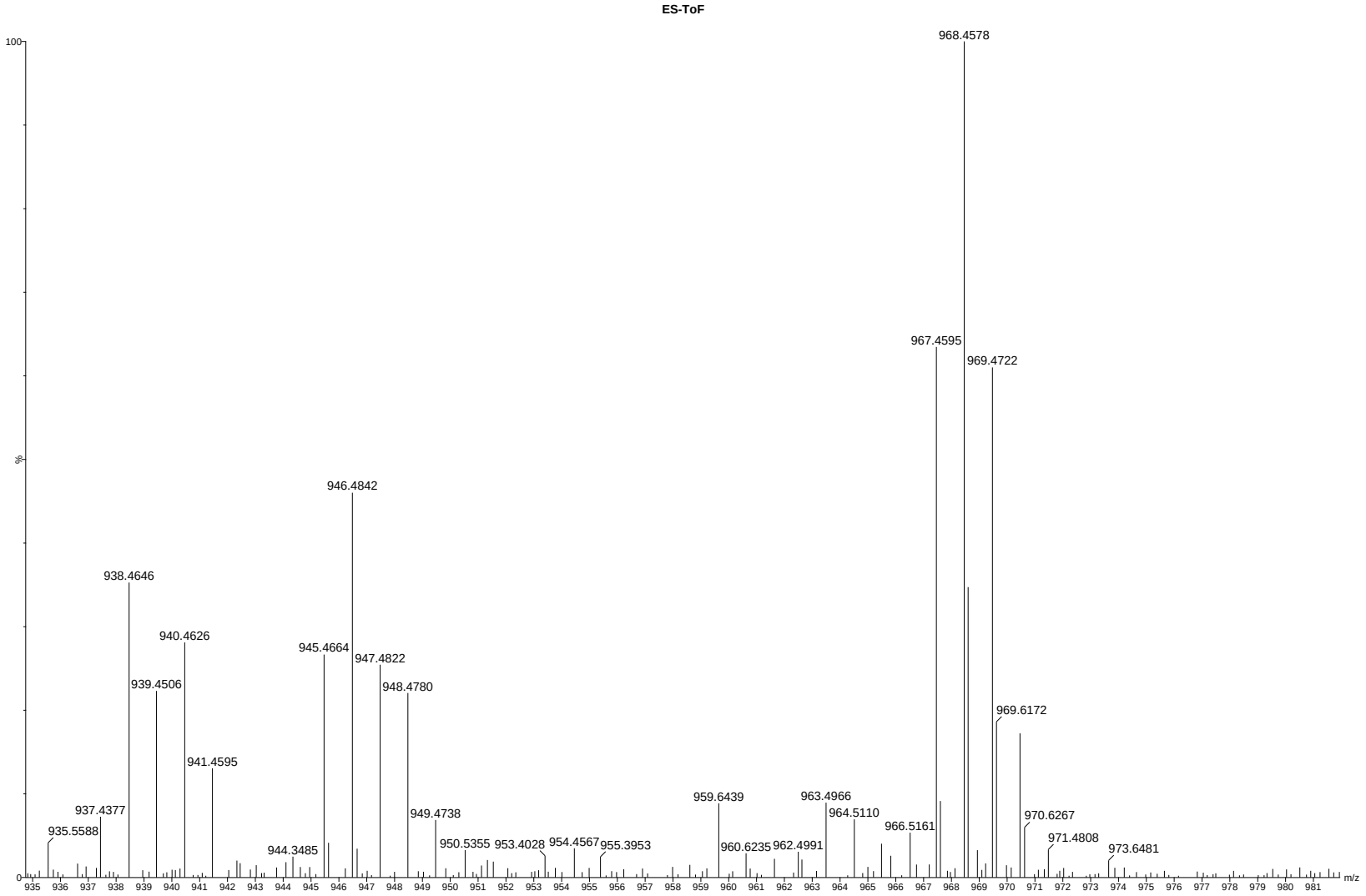
2D HSQC for BocAlaLys(Boc)Gln(Trt)AMC



The ^{13}C assignment has been reported using the HSQC projections of the molecule because with reduced overall yield and large number of carbons in the molecule, a good C spectral analysis would only have been possible with an extended run > 18 hrs. From previous NMR experiments of amino-acid coumarin based compounds it is known that this prolonged NMR experimentation isn't suitable for decomposition prone compounds such as this unstable fluorogenic substrate, hence the shorter HSQC experiment was used to express the ^{13}C assignments.

^{13} C NMR (100 MHz, MeOD) δ ppm,16.61, 17.31, 18.43, 19.91, 23.10, 27.45, 28.12, 28.41, 29.93, 30.35, 30.83, 31.31, 32.6, 33.24, 33.33, 36.46, 37.10, 38.35, 40.23, 49.05, 49.20, 51.56, 54.27, 111.13, 117.25, 121.35, 122.05, 127.04, 128.18, 128.21, 133.5.

Mass spectroscopy



Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Calc. Formula
967.4595	967.4582	1.3	1.3	24.5	71.2	0.0	C53 H64 N6 O10 Na

Elements Used:

C: 53-53 H: 0-200 N: 0-10 O: 0-10

Formula calculated:

C53 H64 N6 O10

Composition details of enzyme assays:

Well position	Component	Volume (µL)	Concentration (µM)
A	Enzyme	0	0
	Substrate	5	50
B	Enzyme	5	1.38
	Substrate	5	50
C	Enzyme	5	13.8
	Substrate	5	50

Table 201 - Composition details of the initial biological testing to investigate the optimum conditions needed for the detection assay. Enzyme: $3C^{pro}$, Substrate in deprotected form: AlaLysGlnAMC. Each well was made up to 100 µL by adding the buffer solution of 5% DMSO:PBS. Fluorescence readings of the plate were taken after adding all of the components and repeated. All samples were run in triplicates.

Well position	Component	Volume (µL)	Concentration (µM)
F	Enzyme	0	0
	Substrate	10	100
G	Enzyme	8	1.38
	Substrate	10	100
H	Enzyme	8	13.8
	Substrate	10	100

Table 21 - Composition details of the initial biological testing part 2, to investigate the optimum conditions needed for the detection assay. Enzyme: $3C^{pro}$, Substrate in deprotected form: AlaLysGlnAMC. Each well was made up to 100 µL by adding the buffer solution of 5% DMSO:PBS. Fluorescence readings of the plate were taken after adding all of the components and 10 minutes of incubation. Measurements were taken in triplicates.

Well plate position			
	1	2	3

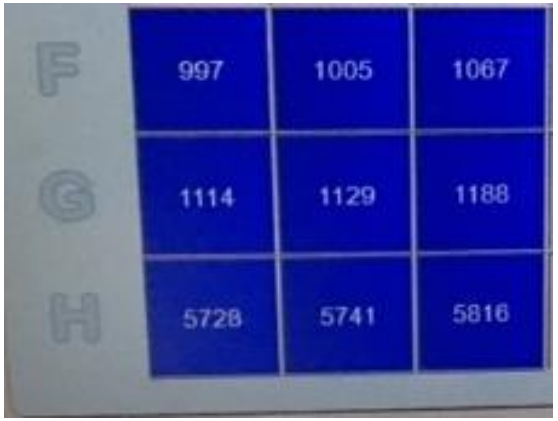


Photo of static fluorescent measurements recorded from initial biological testing.

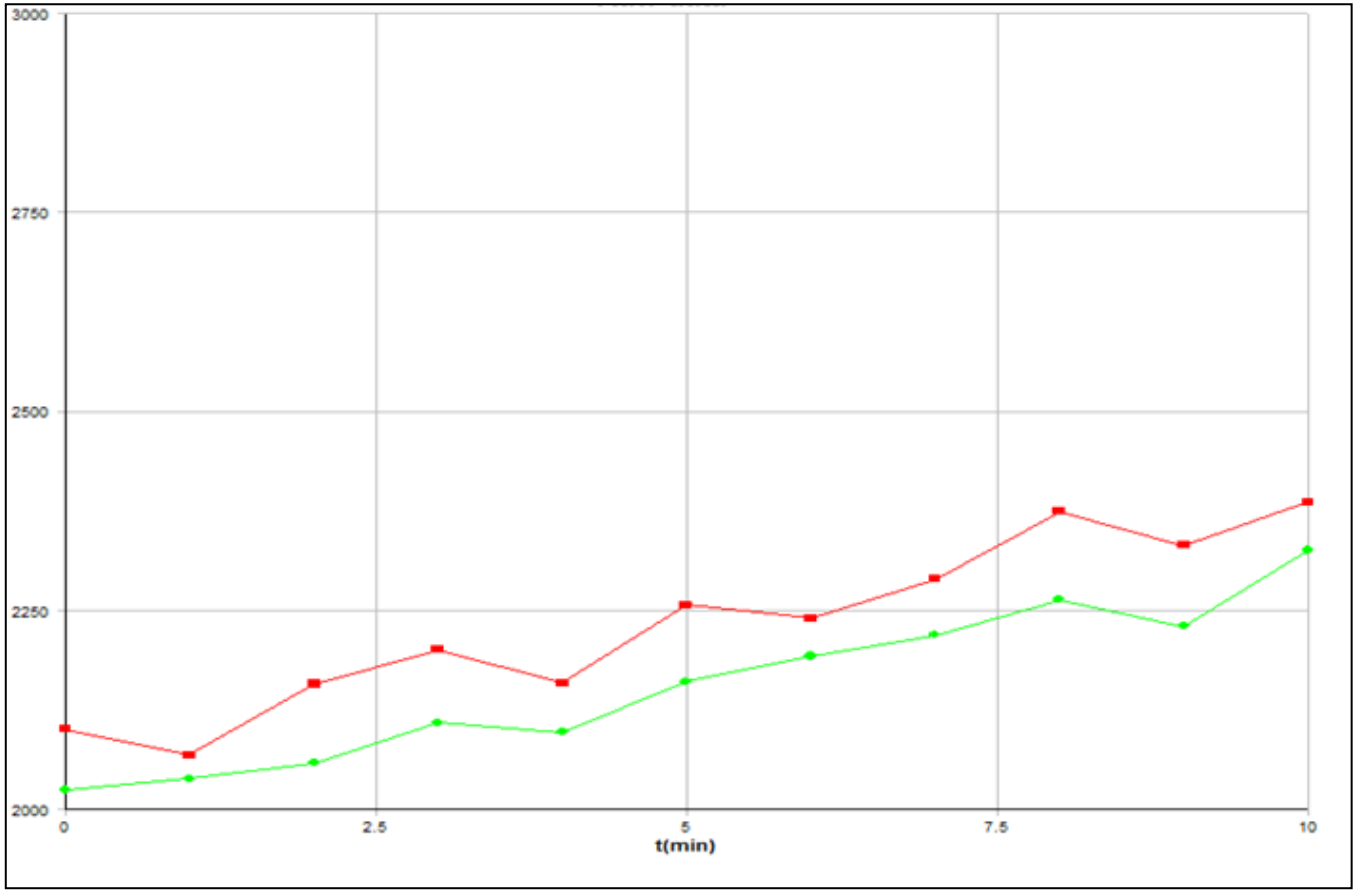
[S] (μM)	S (μL)	[E] (μM)	E (μL)	Multiple readings^{n}
100	10	0	0	n=3
100	10	13.8	8	n=3
150	10	0	0	n=2
150	10	13.8	8	n=2

Table 22 - Enzyme assay composition details, where S is ALQAMC and E is $3C^{pro}$.Composition details of the enzyme assays. Enzyme: $3C^{pro}$, Substrate in deprotected form: ALQAMC. Each well was made up to 100 μL by adding the buffer solution of 5% DMSO:PBS. Fluorescence readings of the plate were taken after adding all of the components and repeated after 15 minutes to observe any changes in the fluorescence intensity. All fluorescence measurements weren't recorded in triplicates due to limited amount of E.

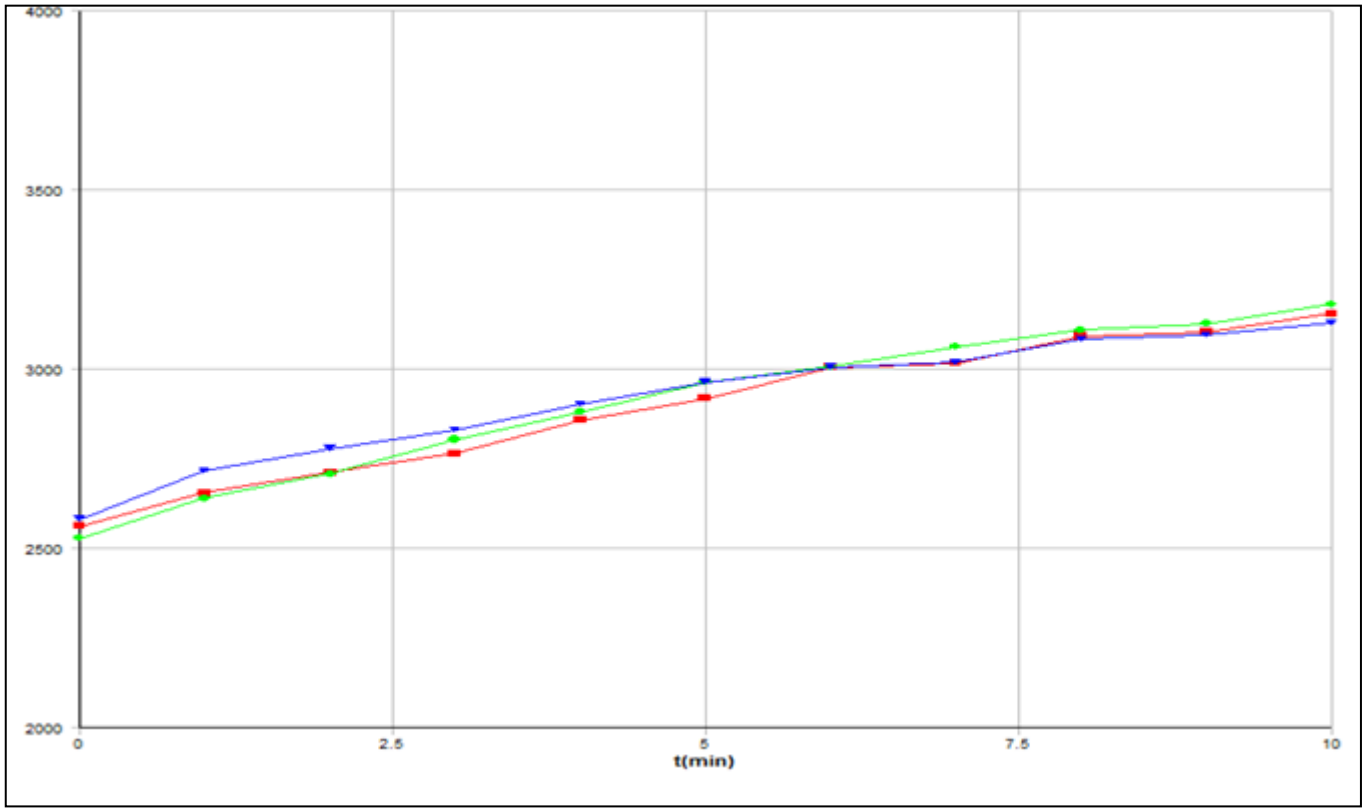
Enzyme (E)	**[E]** **µM / well**	**E** **µL / well**	**Substrate (S)**	**[S]** **µM / well**	**S** **µL / well**
Chymotrypsin type 2	10	8	AlaLysGlnAMC	100	10
Thrombin	10	8		100	10
TEV protease	10	8		100	10
Trypsin type 1	10	8		100	10
No enzyme Negative control	0	0		100	10

Table 23 - Details of components for selectivity testing assay. Enzyme: $3C^{pro}$, Substrate in deprotected form: AlaLysGlnAMC. Each well was made up to 100µL by adding the buffer solution of 5% DMSO:PBS. Fluorescence readings of the plate were taken over 10 minutes. Duration of fluorescence recordings was chosen to be 10 minutes long as this was enough to see a fluorescent reading in presence of the target enzyme.

Fluorescence raw data for 3C protease enzyme with novel detection probe AlaLysGlnAMC [S]:



[S] 80 micromolar



[S] 100 micromolar

Enzyme	Fluorescence changes
Chymotrypsin type 2	No significant fluorescent response recorded.
Thrombin	
TEV protease	
Trypsin type 1	

Table 24 - Fluorescence results from substrate fluorophore conjugate in the presence of other enzymes.

$[S_1]$ (μM)	S_1 (μL)	$[E_1]$ (unit)	E_1 (μL)	Buffer - PBS (μL)
20	1	1	1	98
40	1	1	1	98
60	1	1	1	98
80	1	1	1	98
100	1	1	1	98

Table 25 –Testing thrombin activity in presence of substrate Boc-VPA-AMC.