Supplementary Information

PLP-independent racemization: mechanistic and mutational studies of *O*-ureidoserine racemase (DcsC)

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Content:

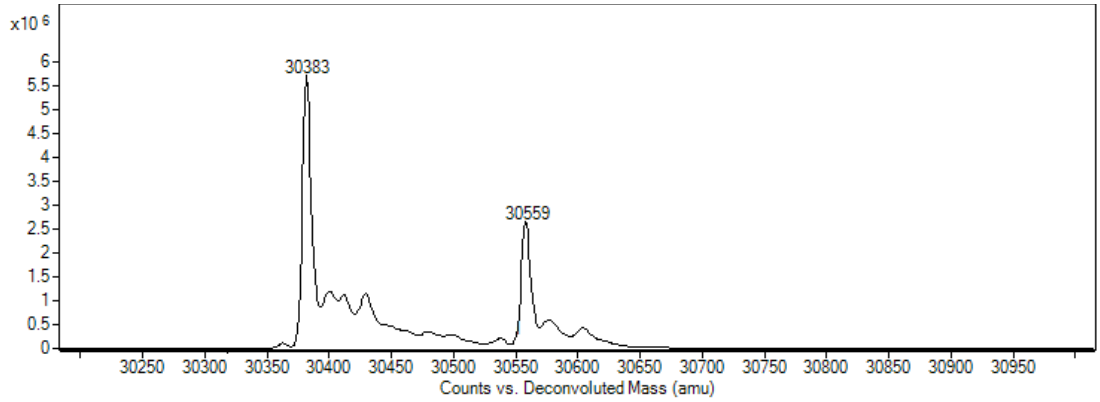


Figure S1. Electrospray mass spectra of inhibited Native DcsC with racemic inhibitor **2**.

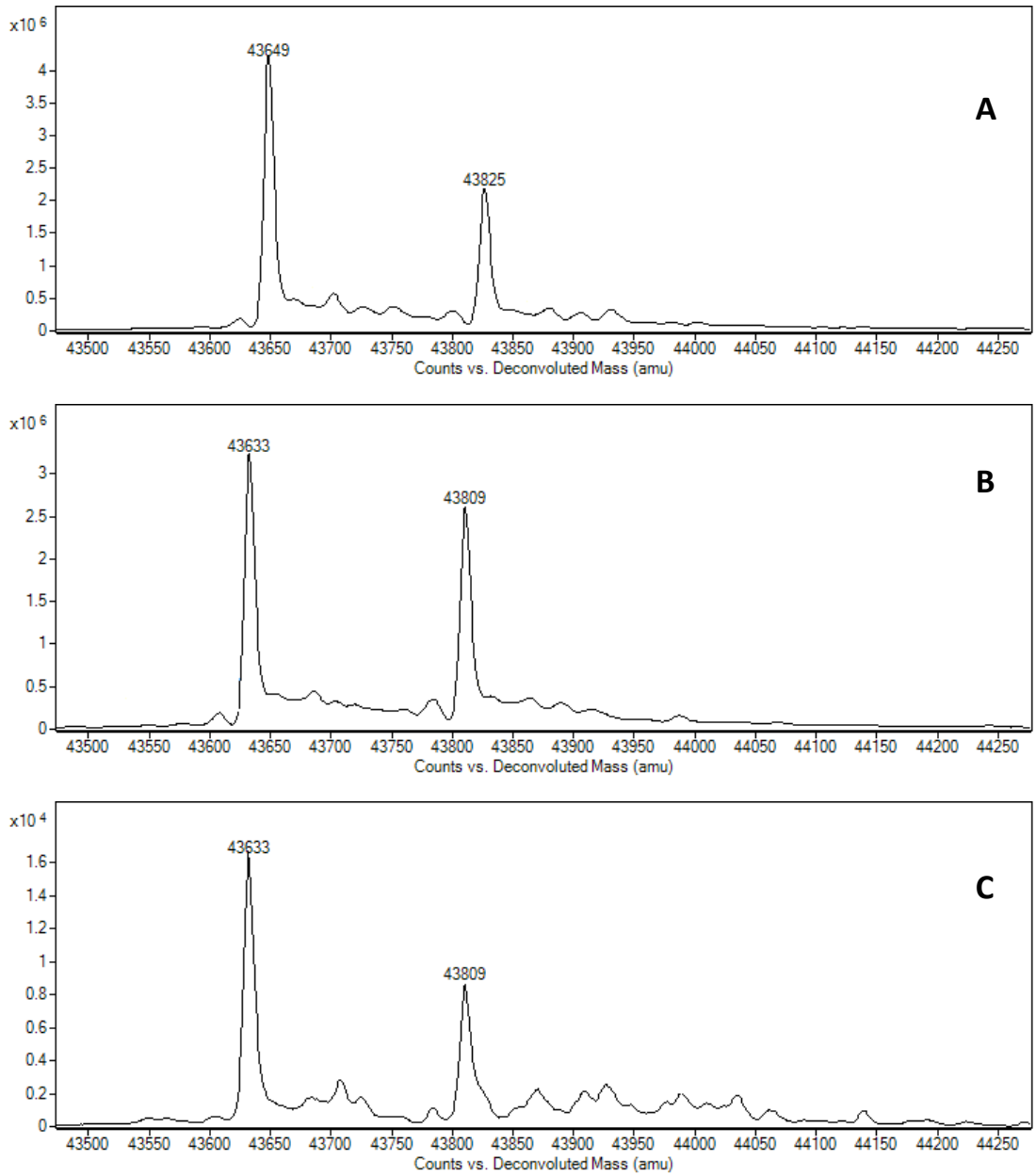
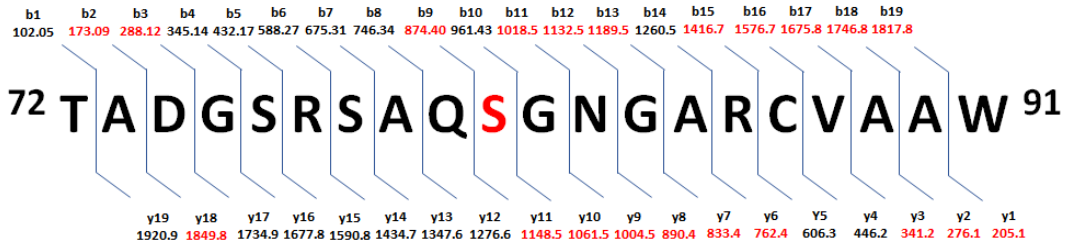
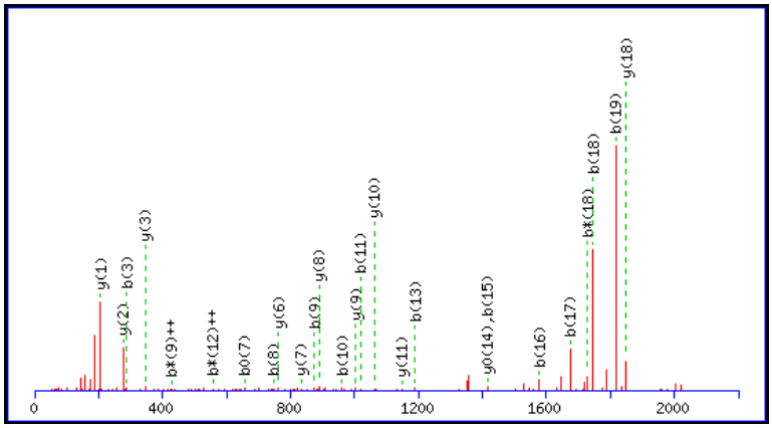


Figure S2. Electrospray mass spectra of inhibited SUMO-DcsC(**A**), M1-DcsC(**B**), M2-DcsC(**C**) with racemic inhibitor **2**.

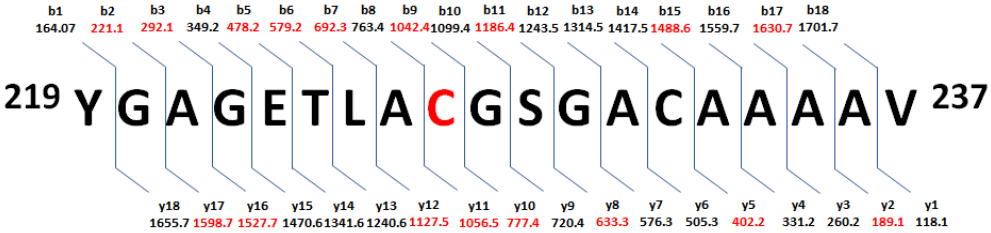
A

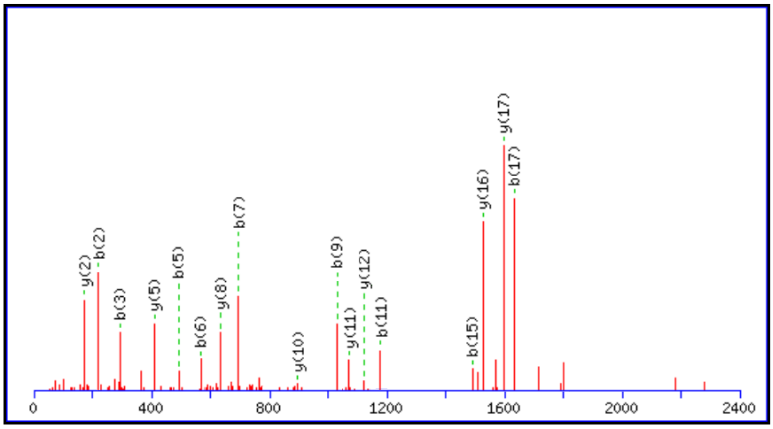
72 - 91 674.6565 2020.9477 2020.9133 0.0343 0 W.TADGSRSAQSGNGARCVAAW.A Carbamidomethyl (**C87**)





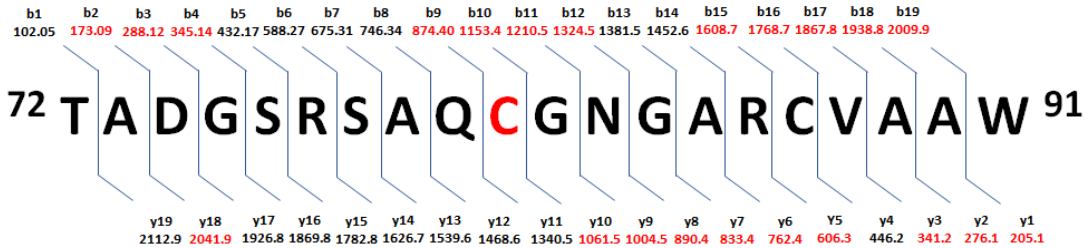
219 - 237 665.6338 1993.8796 1993.7993 0.0803 0 E.YGAGETLACGSGACAAAAV.L Inhibitor (**C81**)

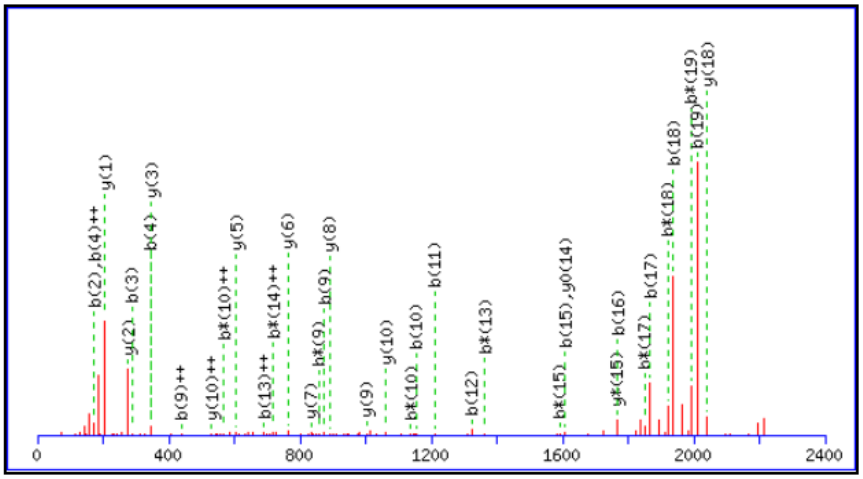




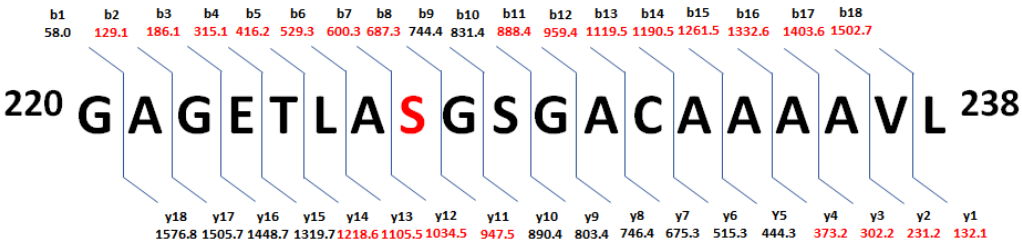
B

72 - 91 738.6553 2212.9441 2212.9338 0.0103 0 W.TADGSRSAQCGNGARCVAAW.A Inhibitor (**C81**), Carbamidomethyl (**C87**)





220 - 238 817.3961 1632.7776 1632.7777 -0.0001 0 Y.GAGETLASGSGACAAAAVL.M Carbamidomethyl (**C87**)



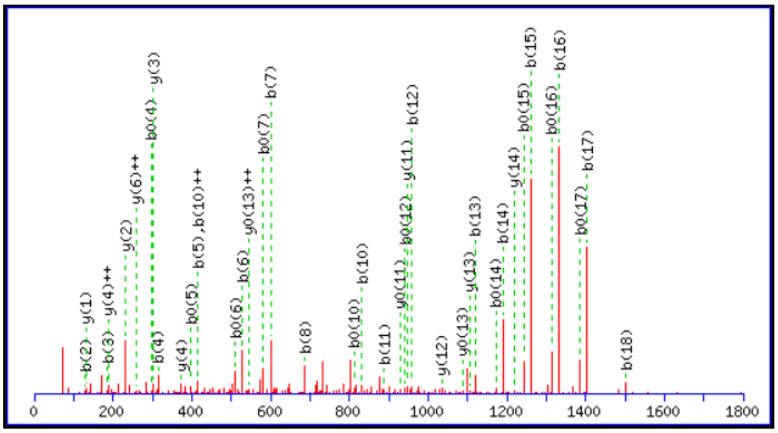


Figure S3. MS-MS sequencing data of inhibited M1-DcsC (A) and M2-DcsC mutants (B) by racemic inhibitor **2**, indicating the site of inhibition.

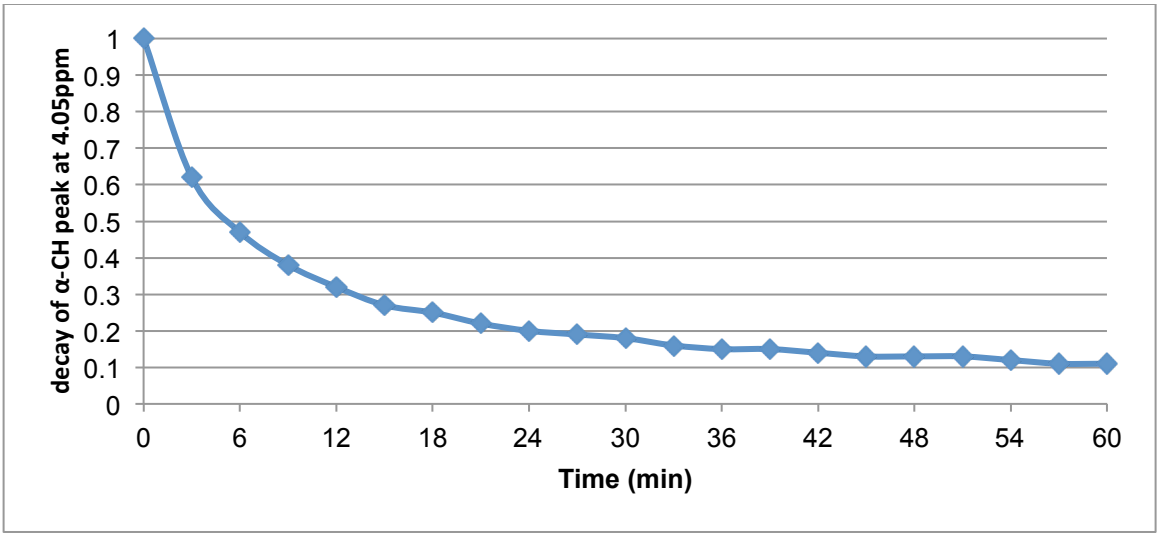


Figure S4. ^{1}H NMR assay of SUMO-DcsC, exemplarily shown for substrate **1a**. The decay of the α-CH peak at 4.05ppm is recorded over time.

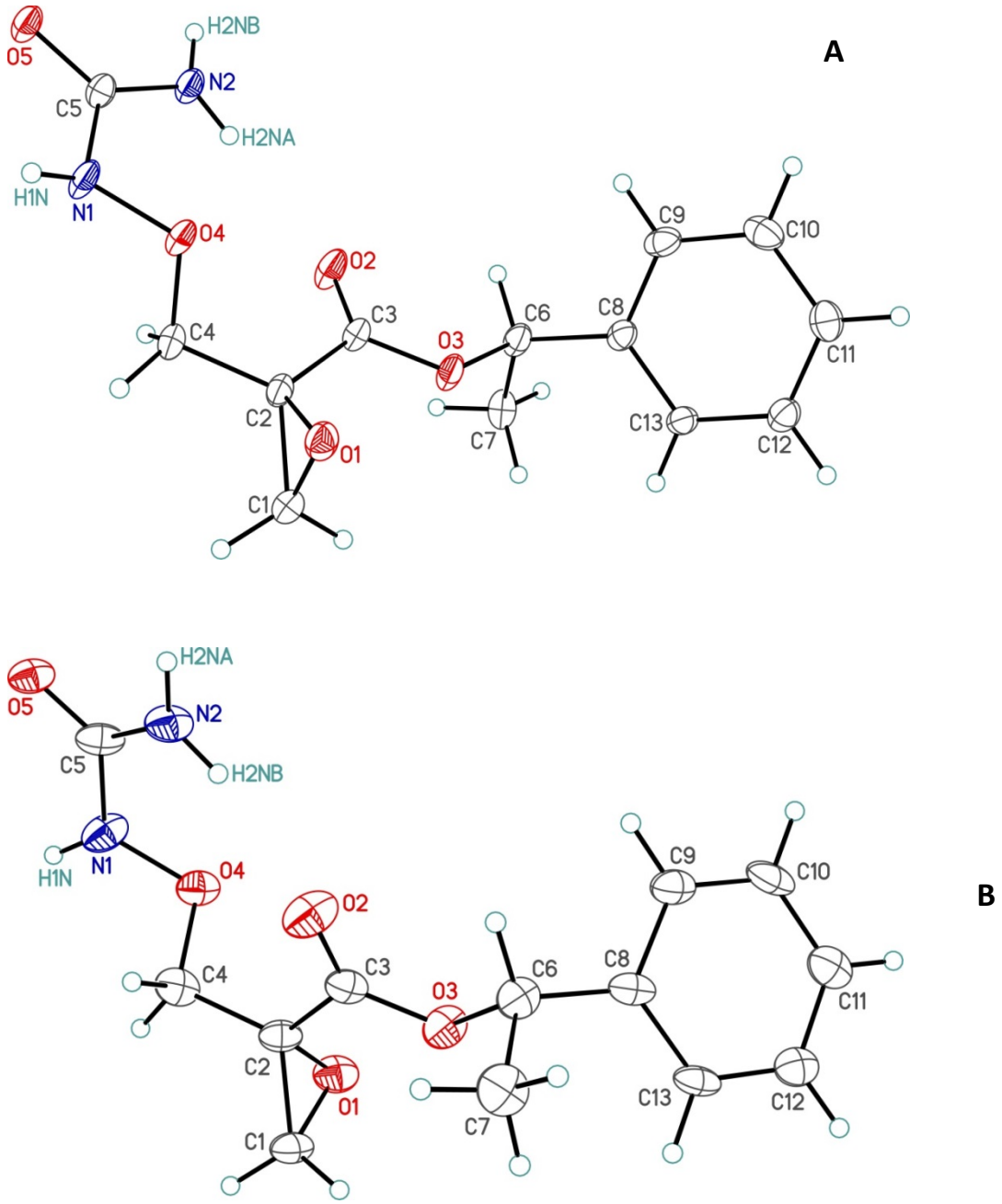


Figure S5. X-ray diffraction structure of chiral inhibitors. ORTEP diagram (30 % probability level) (A: **7a**, B: **7b**).

Table S1. X-ray data summary for structures **7a** and **7b**

	7a	**7b**
A. Crystal data		
Formula	$C_{13}H_{16}N_2O_5$	
Formula weight (g/mol)	280.28	
Crystal dimensions (mm)	0.25 ´ 0.13 ´ 0.03	0.25 ´ 0.25 ´ 0.05
Crystal system	monoclinic	monoclinic
Space group	$P2_1$ (No. 4)	$P2_1$ (No. 4)
Unit cell parameters	9857 reflections with 5.46°<2θ< 147.08°	3325 reflections with 5.48°<2q< 138.66°
a (Å)	5.92380 (10)	5.9509 (12)
b (Å)	7.17870 (10)	7.1789 (12)
c (Å)	16.1585 (3)	16.121 (4)
β (deg)	90.8223 (8)	90.821 (18)
V (Å^3)	687.07 (2)	688.6 (3)
Z	2	2
ρ_{calcd} (g cm^{-3})	1.355	1.352
μ (mm^{-1})	0.886	0.884
B. Data Collection and Refinement Conditions		
Diffractometer	Bruker D8/APEX II CCD[1]	
Radiation (λ [Å])	Cu Kα (1.54178) (microfocus source)	
Temperature (deg C)	–100	-100
Scan type	w and f scans (1.0°) (5 s exposures)	
Data collection 2θ limit (deg)	147.90	135.31
Total data collected	4927 (-7 ≤ h ≤ 7, -8 ≤ k ≤ 8, -20 ≤ l ≤ 20)	3111 (-7 ≤ h ≤ 7, -8 ≤ k ≤ 8, -19 ≤ l ≤ 18)
Independent reflections	2754 (R_{int} = 0.0174)	2076 (R_{int} = 0.0492)
Number of observed reflections (NO)	2674 [$F_o^2 \geq 2s(F_o^2)$]	1582 [$F_o^2 \geq 2s(F_o^2)$]
Structure solution method	intrinsic phasing (*SHELXT-2014*[2])	
Refinement method	full-matrix least-squares on F^2 (*SHELXL–2014*[3])	
Absorption correction method	Gaussian integration (face-indexed)	
Range of transmission factors	1.0000–0.7597	1.0000–0.5229
Data/restraints/parameters	2754 / 0 / 194	2076 / 0 / 182
Extinction coefficient (x)[4]	0.0037(11)	
Flack absolute structure parameter[5]	0.08(7)	0.1(5)
Goodness-of-fit (S)[g] [all data]	1.025	1.097
Final R indices[6]		
R_1 [$F_o^2 \geq 2s(F_o^2)$]	0.0268	0.0830
wR_2 [all data]	0.0729	0.2556
Largest difference peak and hole	0.171 and –0.137 e Å^{-3}	0.369 and –0.422 e Å^{-3}

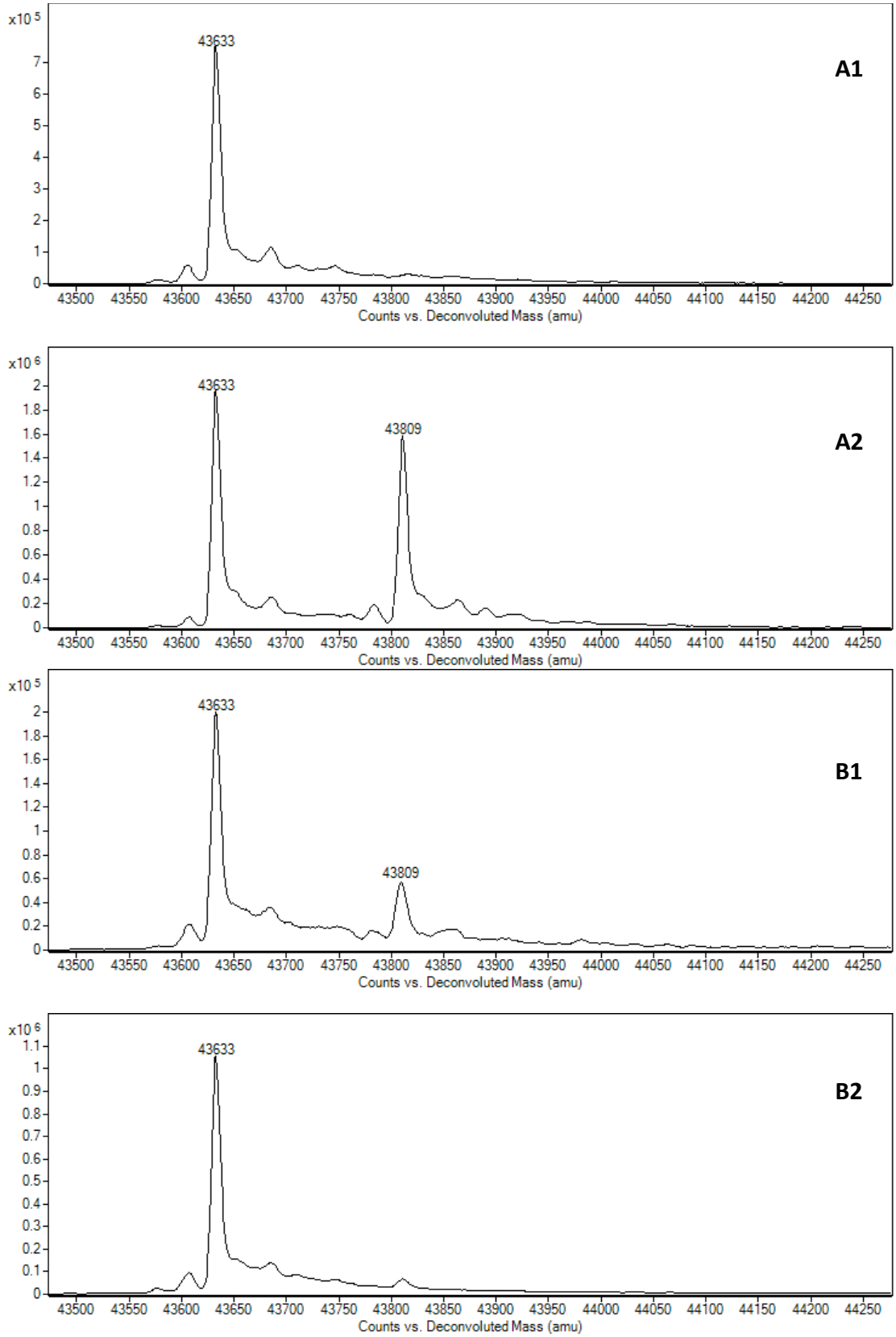
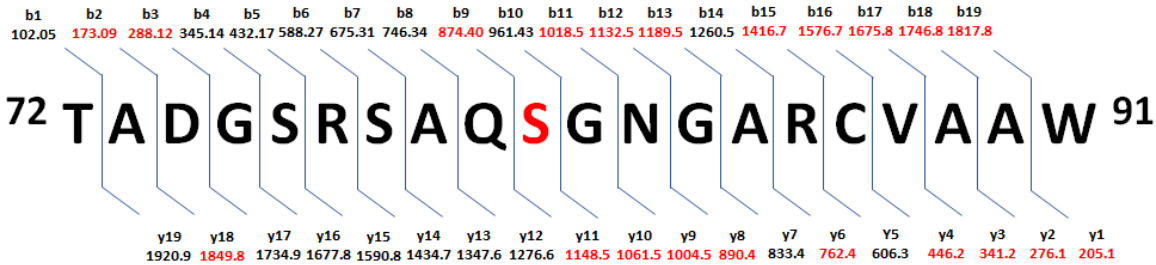
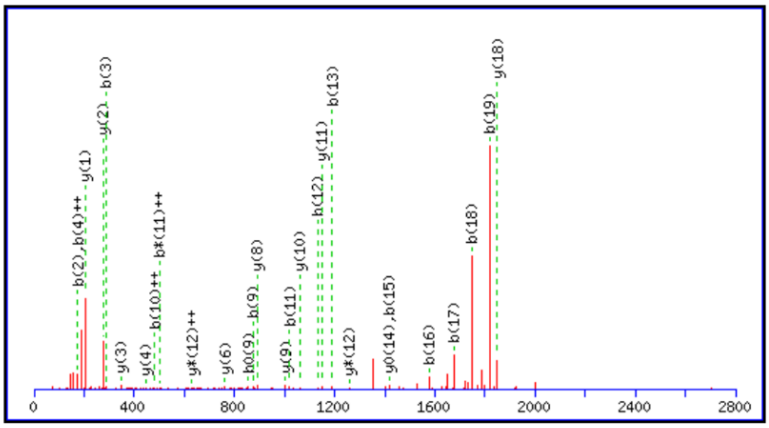


Figure S6. Electrospray mass spectra of inhibited M1-DcsC mutants (A1: **2a**, A2: **2b**) and M2-DcsC mutants (B1: **2a**, B2: **2b**). The mass of inhibitors is 176 Da.

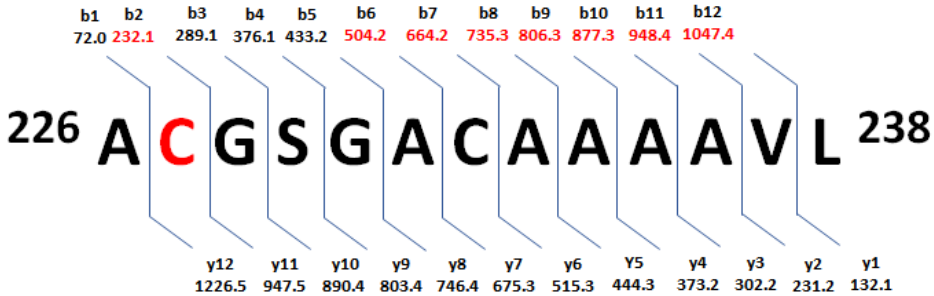
A1

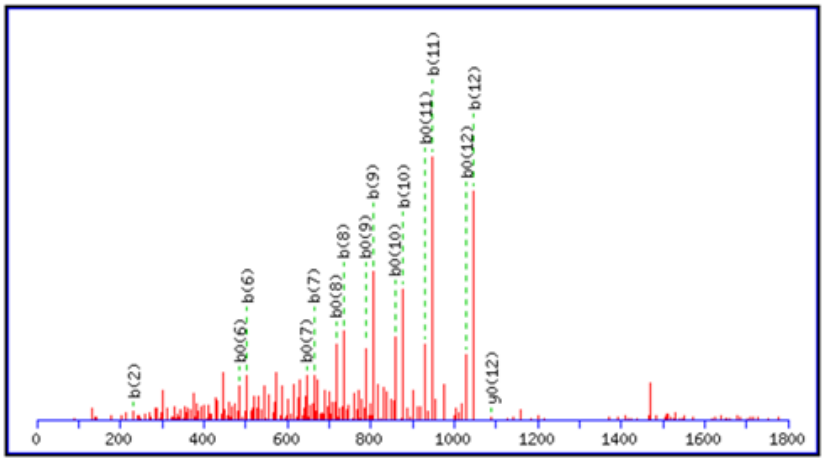
72 - 91 674.6555 2020.9447 2020.9133 0.0313 0 W.TADGSRSAQSGNGARCVAAW.A Carbamidomethyl (**C87**)





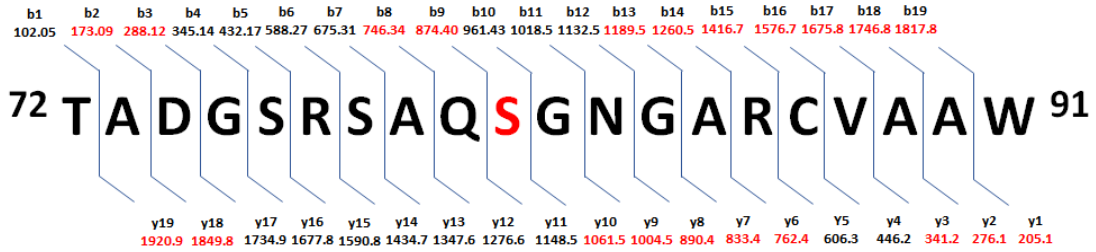
226 - 238 1178.5057 1177.4984 1177.5220 -0.0235 0 L.ACGSGACAAAAVL.M Carbamidomethyl (**C227**, **C232**)

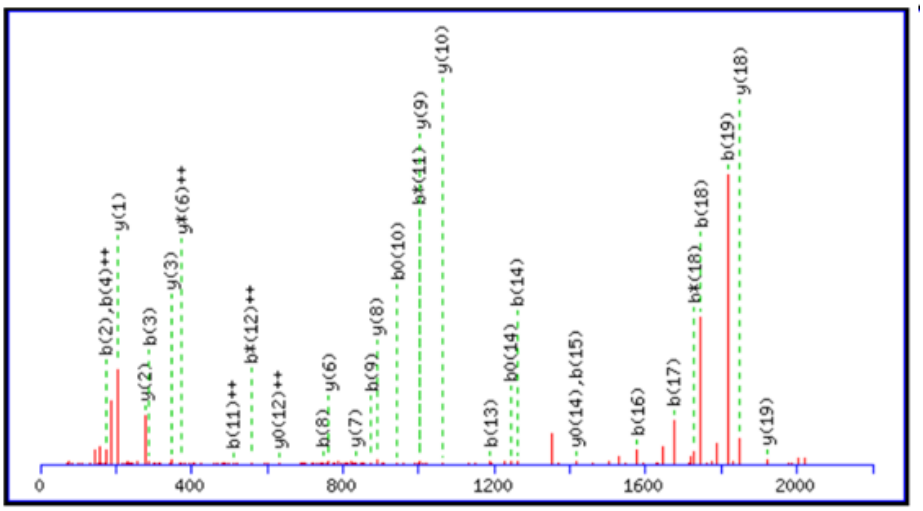




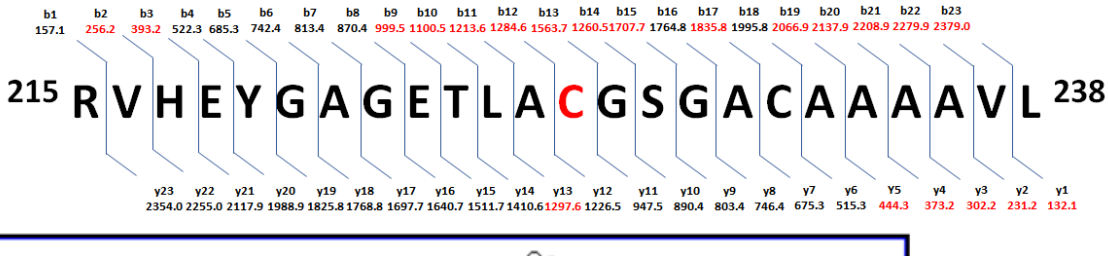
A2

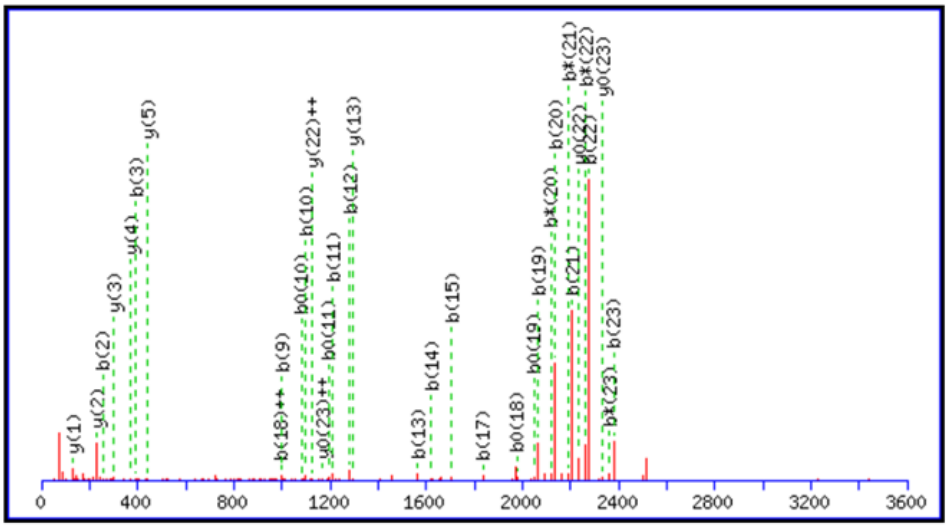
72 - 91 674.6558 2020.9456 2020.9133 0.0322 0 W.TADGSRSAQSGNGARCVAAW.A Carbamidomethyl (**C87**)





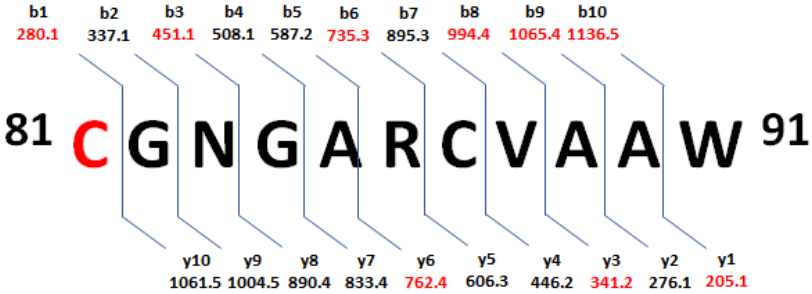
215 - 238 837.3826 2509.1260 2509.1326 -0.0066 0 L.RVHEYGAGETLACGSGACAAAAVL.M Inhibitor (**C227**), Carbamidomethyl (**C232**)

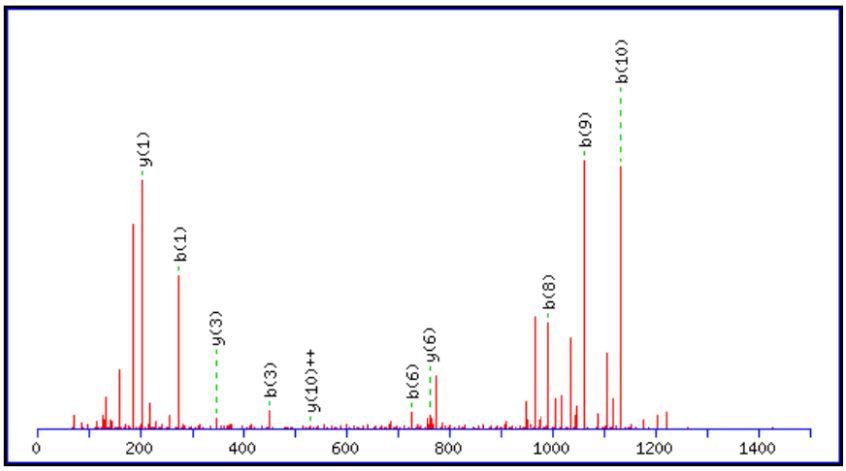




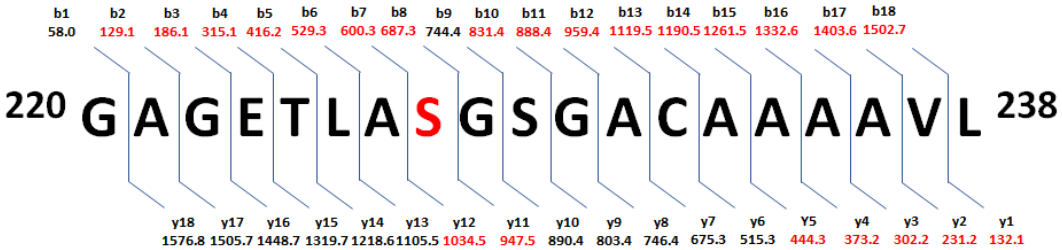
B1

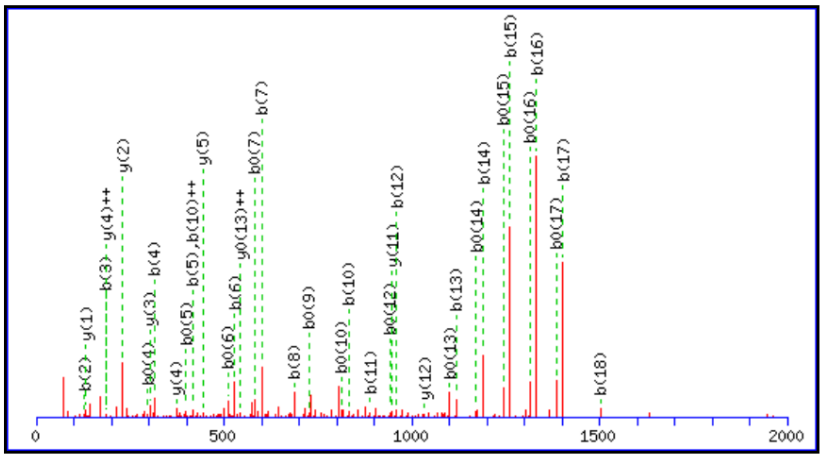
81 - 91 611.2751 1220.5356 1220.5179 0.0177 0 Q.CGNGARCVAAW.A Inhibitor (C81)





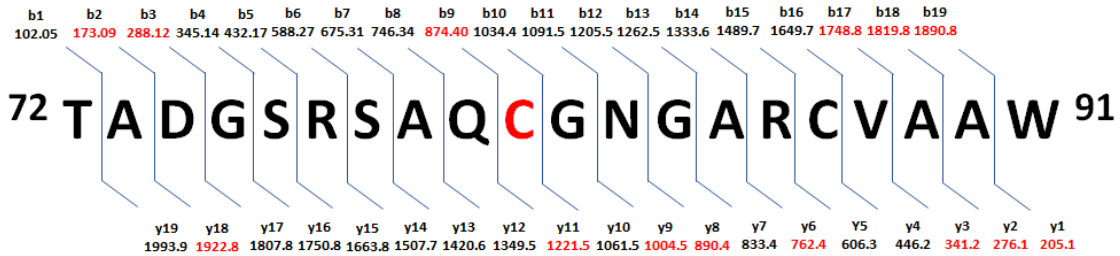
220 - 238 817.4003 1632.7860 1632.7777 0.0083 0 Y.GAGETLASGSGACAAAAVL.M

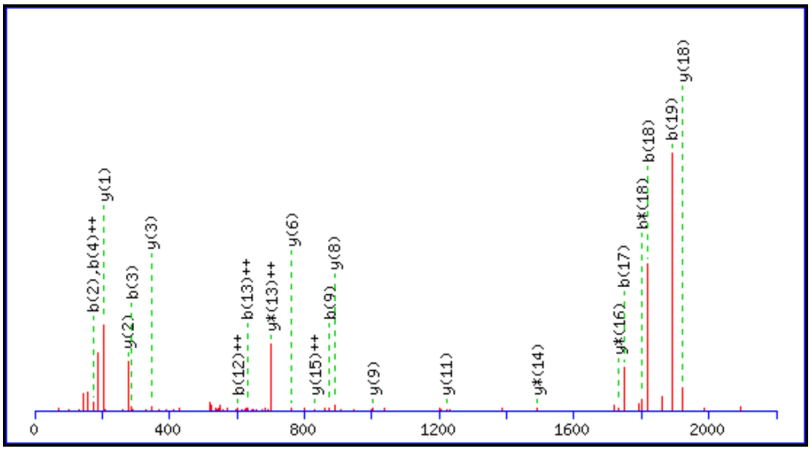




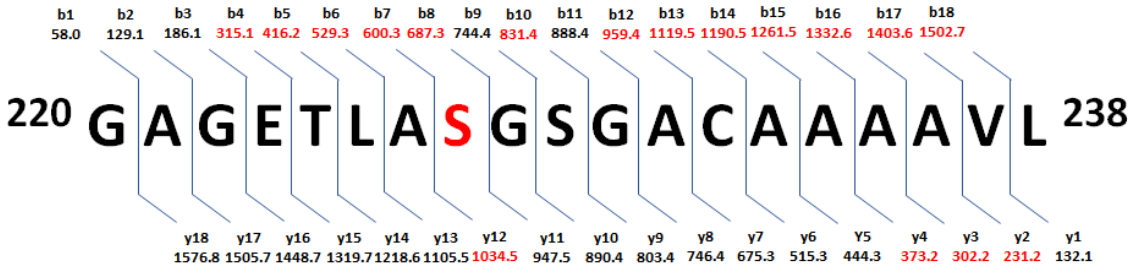
B2

72 - 91 698.9785 2093.9137 2093.9120 0.0017 0 W.TADGSRSAQCGNGARCVAAW.A Carbamidomethyl (**C81**, **C87**)





220 - 238 817.4019 1632.7892 1632.7777 0.0115 0 Y.GAGETLASGSGACAAAAVL.M Carbamidomethyl (**C232**)



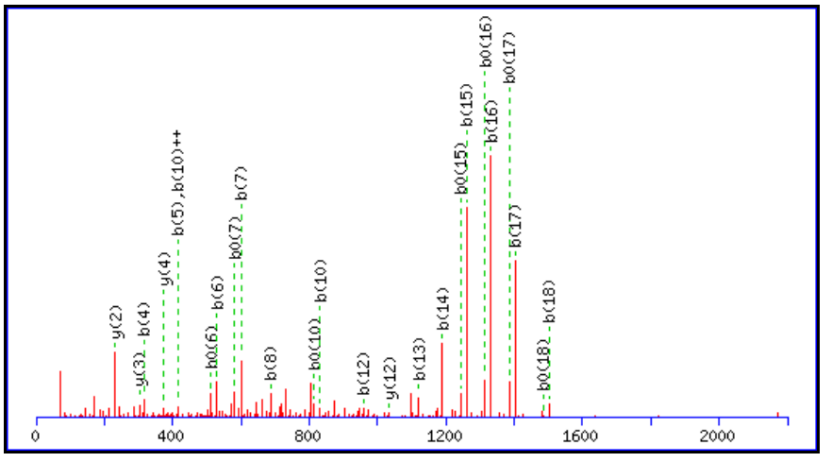


Figure S7. MS-MS sequencing data of mutant DcsC. A1: M1-DcsC with **2a**, A2: M1-DcsC with **2b**, B1: M2-DcsC with **2a**, B2:

M2-DcsC with **2b.**

```
           1        10        20        30        40        50        60        70        80        90       100       110       120       130
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
     DcsC  MIRMRTPSTLPFTKMHGAGNDFVYL-DLRDGPDPSPELCRALADRHKGYGCDLYLGIREPRSARAYAAFDI---------W-----TADGSRSAQCGNGARCVAAWAYRAGLARGPRFALDSPSGTHEYD
     DapF          HMIFAKGHGTQNDFYLLPDYDAELYLTAARYAALCDRRKGLGADGYLRYTTAGAAQAYGYLDSLPEGYRYTDWYMDYRNADGSAAQMCGNGYRYFAHYLRASGLEYRDEFYYGSLAGPRPYT
Consensus  ........h$iFaKgHGaqNDFYlL.DlraeldlsaarcaALaDRrKGlGaDgYLr!rearaArAYaalDi.........W.....nADGSaaaqCGNGaRcfAawaraaGLarrdrFaldSlaGpreYd

           131      140       150       160       170       180       190       200       210       220       230       240       250       260
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
     DcsC  YLDADTFRYALAYPRFAPESIPLFGHDGEQDLYEADLGDGTRYRFAAYSMGNPH-AYIEYDDTATAPYAR-YGRAYQASGLFLPT-YNYGFARYESRDRYHLRYHEYGAGETLACGSGACAAA-AYLMRR
     DapF  CHHYEAAYADYSYD---------MGKANRLGAGEAYYG-GRRFHGLAYDYGNPHLACYDSQLTYDGLAALDYGAPYSFDGAQFPDGYNYEYLTAPYDGAYWMRYHERGYGETRSCGTGTYAAAYAALAAY
Consensus  chda#aaraalaYd.........mGhanrqdagEAdlG.GrRfrgaAYdmGNPH.Ac!#s#dTadalaAr.YGaaYqadGaqlPd.YNYefaraesrdaYh$RYHErGaGETraCGsGacAAA.AaLaar

           261      270       280       290       300       310
           |--------+---------+---------+---------+---------|
     DcsC  GRYDRNYSYYLPGGELRISWPDDAADYLMTGPAAFYYEGTFLHASY
     DapF  GSPTGTLTYHYPGGEYYYTYTD--ATSFLRGPSYLYARGDLADDWWNAMG
Consensus  GrpdrnlsYhlPGGElr!swpD..Adsl$rGPaalYarGdladasw....
```

Figure S8. Sequence similarity between DcsC and DapF. Red letters indicate high similarity, blue letters depict mediocre alterations.

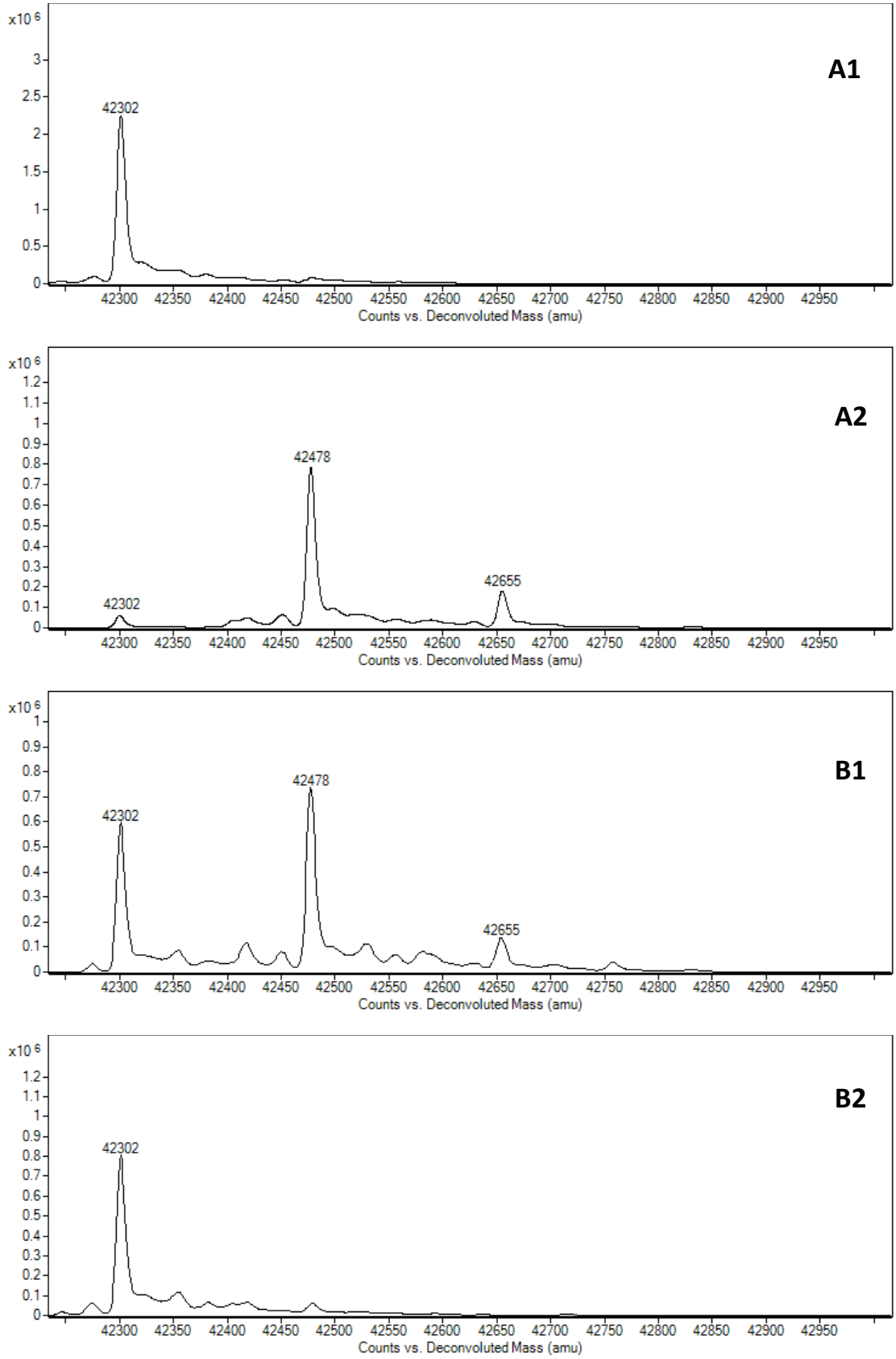
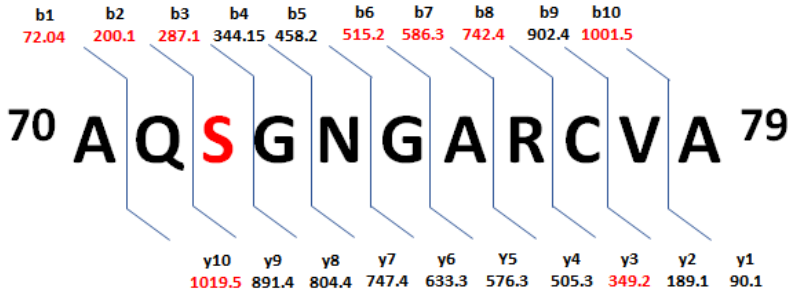
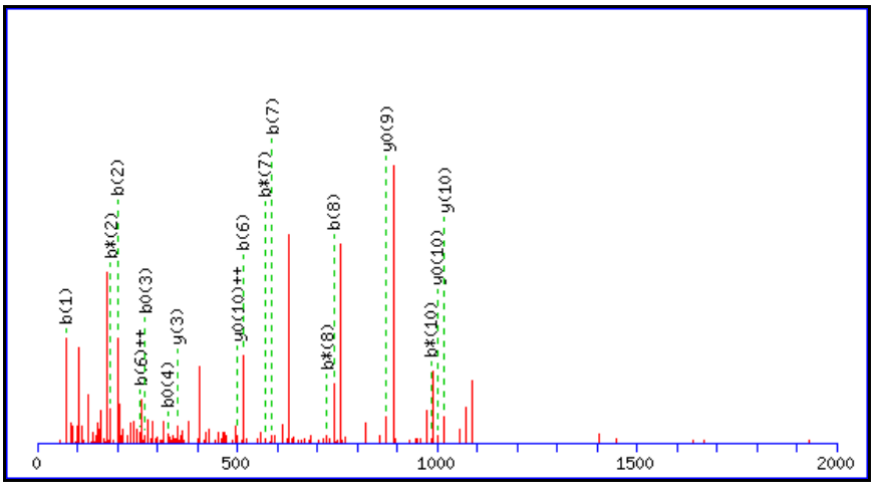


Figure S9. Electrospray mass spectra of inhibited M1-tDcsC mutants (**A1**:**2a**, **A2**: **2b**) and M2-tDcsC mutants (**B1**: **2a**, **B2**: **2b**).

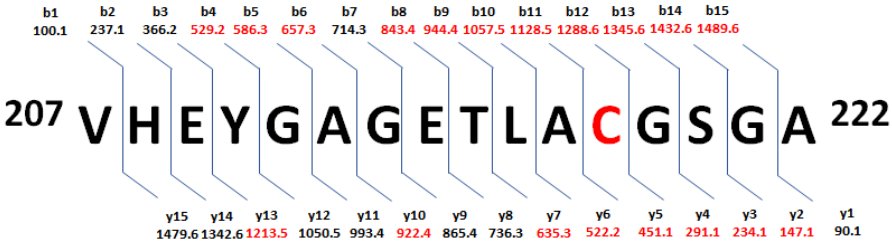
A1

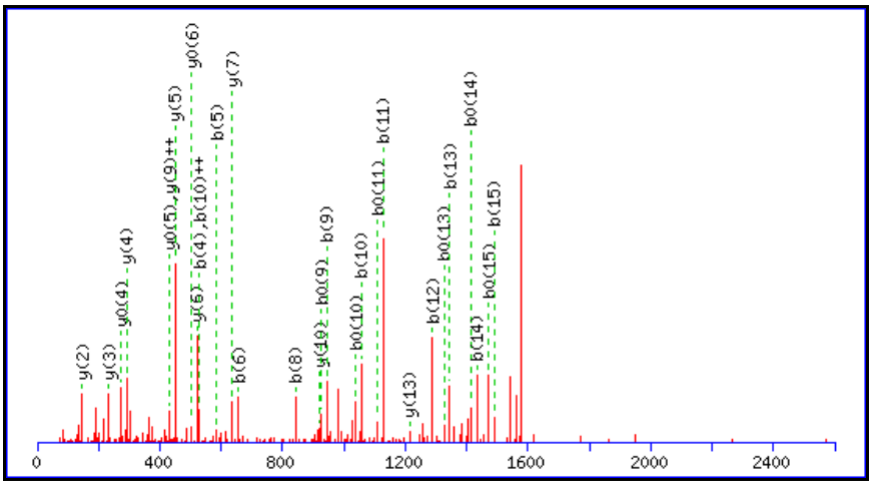
70 - 79 545.7897 1089.5648 1089.4985 0.0663 0 S.AQSGNGARCVA.A Carbamidomethyl (**C78**)





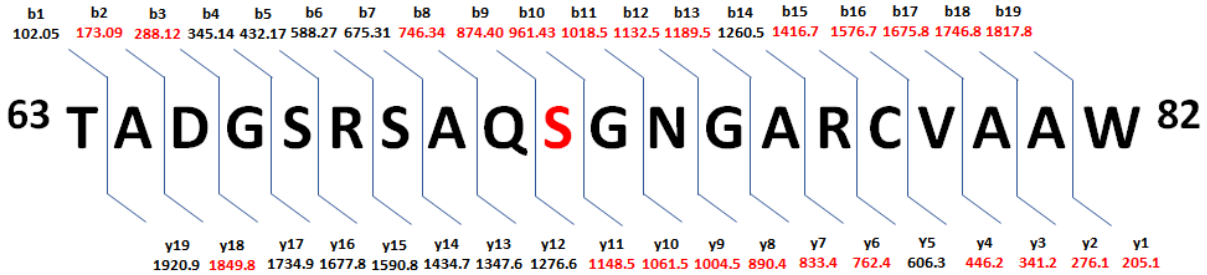
207 - 232 789.8521 1577.6896 1577.6780 0.0116 0 R.VHEYGAGETLACGSGA.C Carbamidomethyl (**C218**)

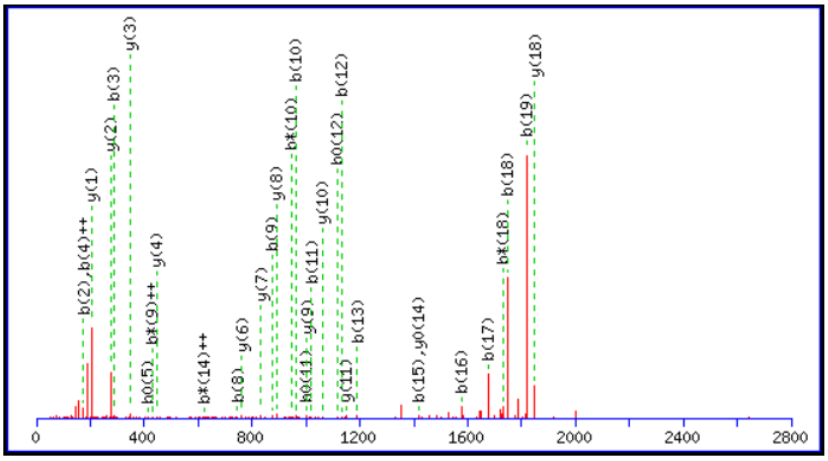




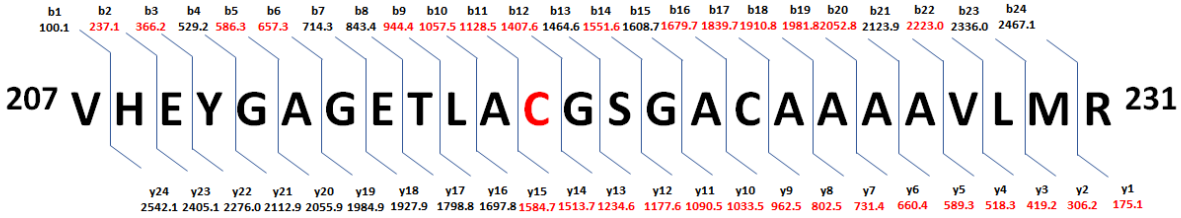
A2

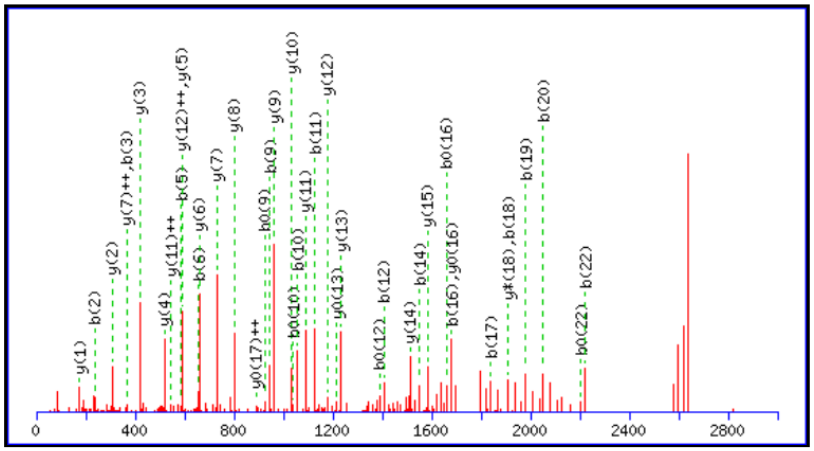
63 - 82 1011.4595 2020.9044 2020.9133 -0.0089 0 W.TADGSRSAQSGNGARCVAAW.A Carbamidomethyl (C78)





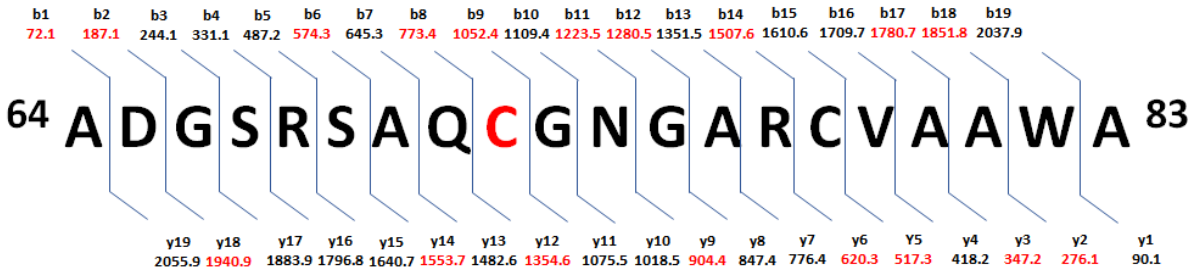
207 - 231 881.0576 2640.1510 2640.1730 -0.0221 0 R.VHEYGAGETLACGSGACAAAAVLMR.R Inhibitor (**C218**), Carbamidomethyl (**C223**)

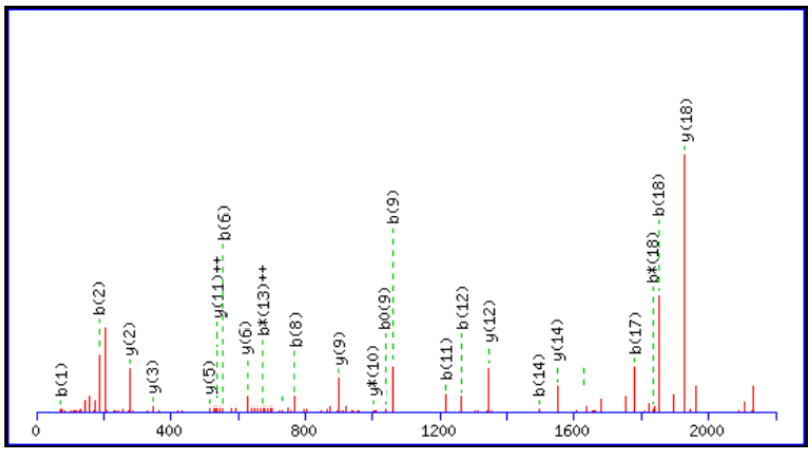




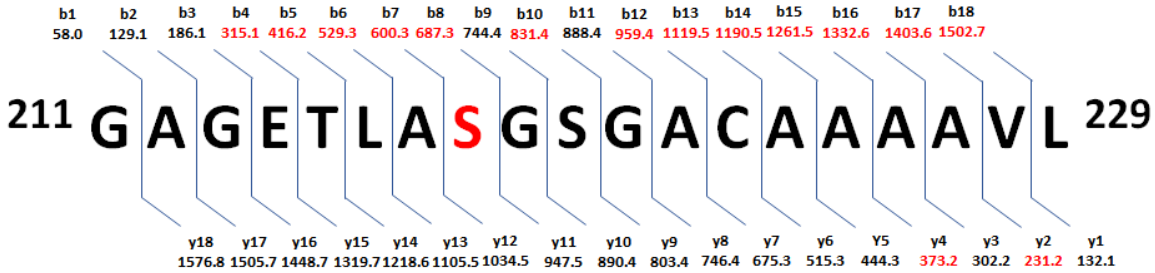
B1

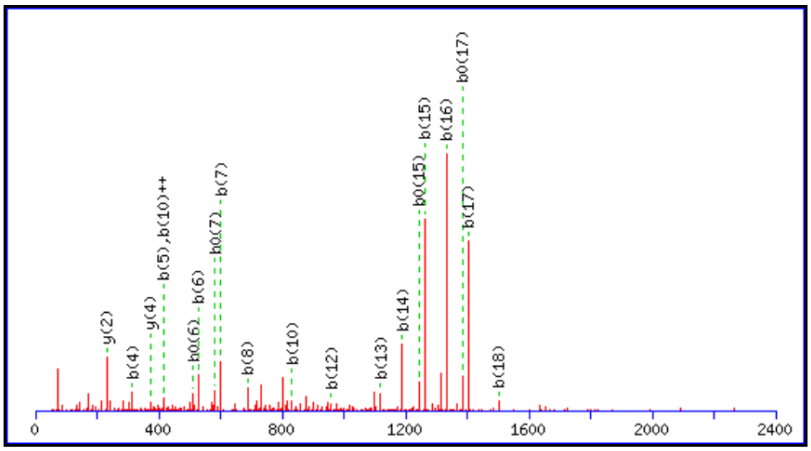
64 - 83 709.6388 2125.8946 2125.9018 0.0072 0 T.ADGSRSAQCGNGARCVAAWA.V Inhibitor (**C72**)





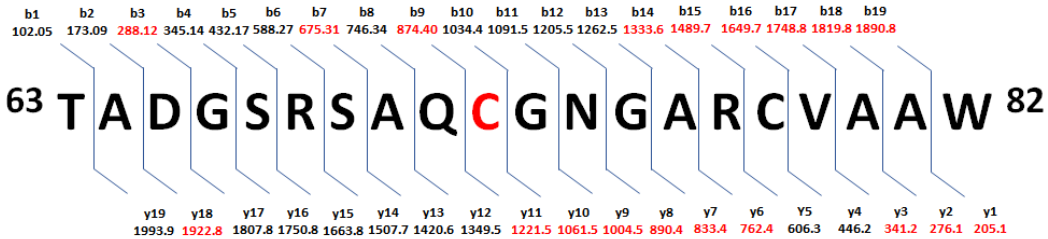
211 - 229 817.3963 1632.7780 1632.7777 0.0003 0 Y.GAGETLASGSGACAAAAVL.M Carbamidomethyl (**C223**)

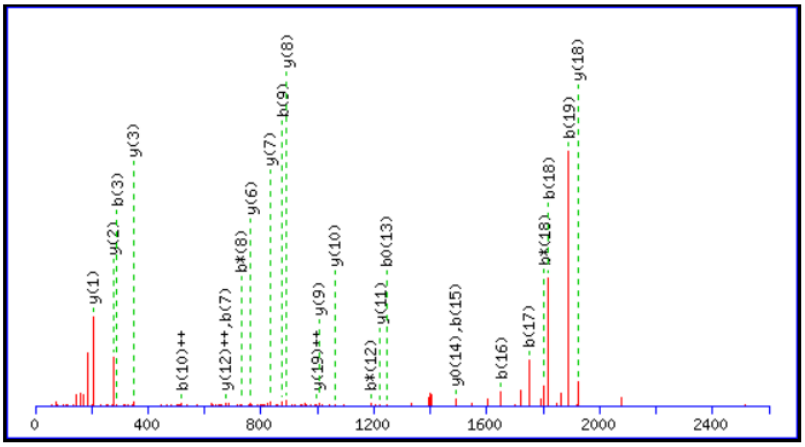




B2

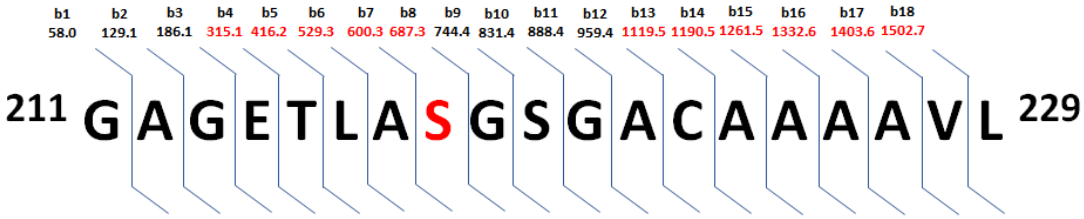
63 - 82 698.9808 2093.9206 2093.9120 0.0086 0 W.TADGSRSAQCGNGARCVAAW.A





B2

211 - 229 817.4042 1632.7938 1632.7777 0.0161 0 Y.GAGETLASGSGACAAAAVL.M Carbamidomethyl (**C223**)



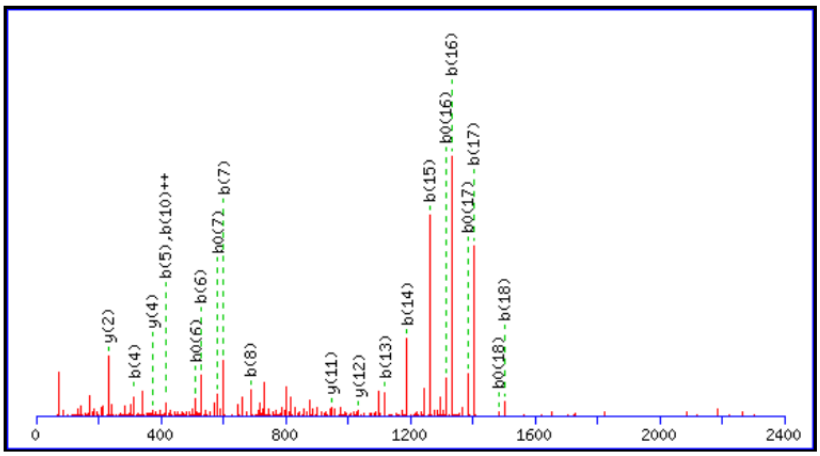
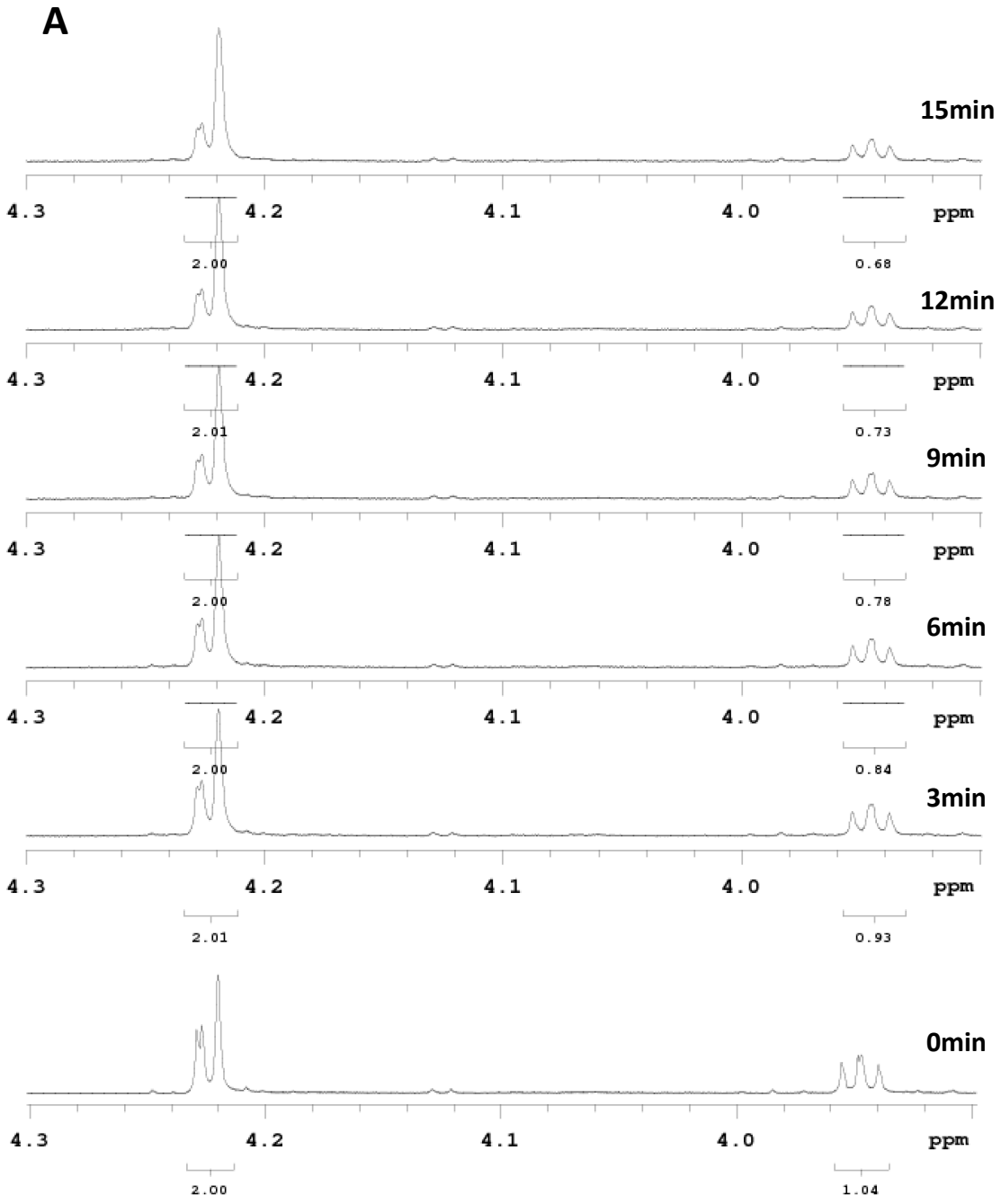
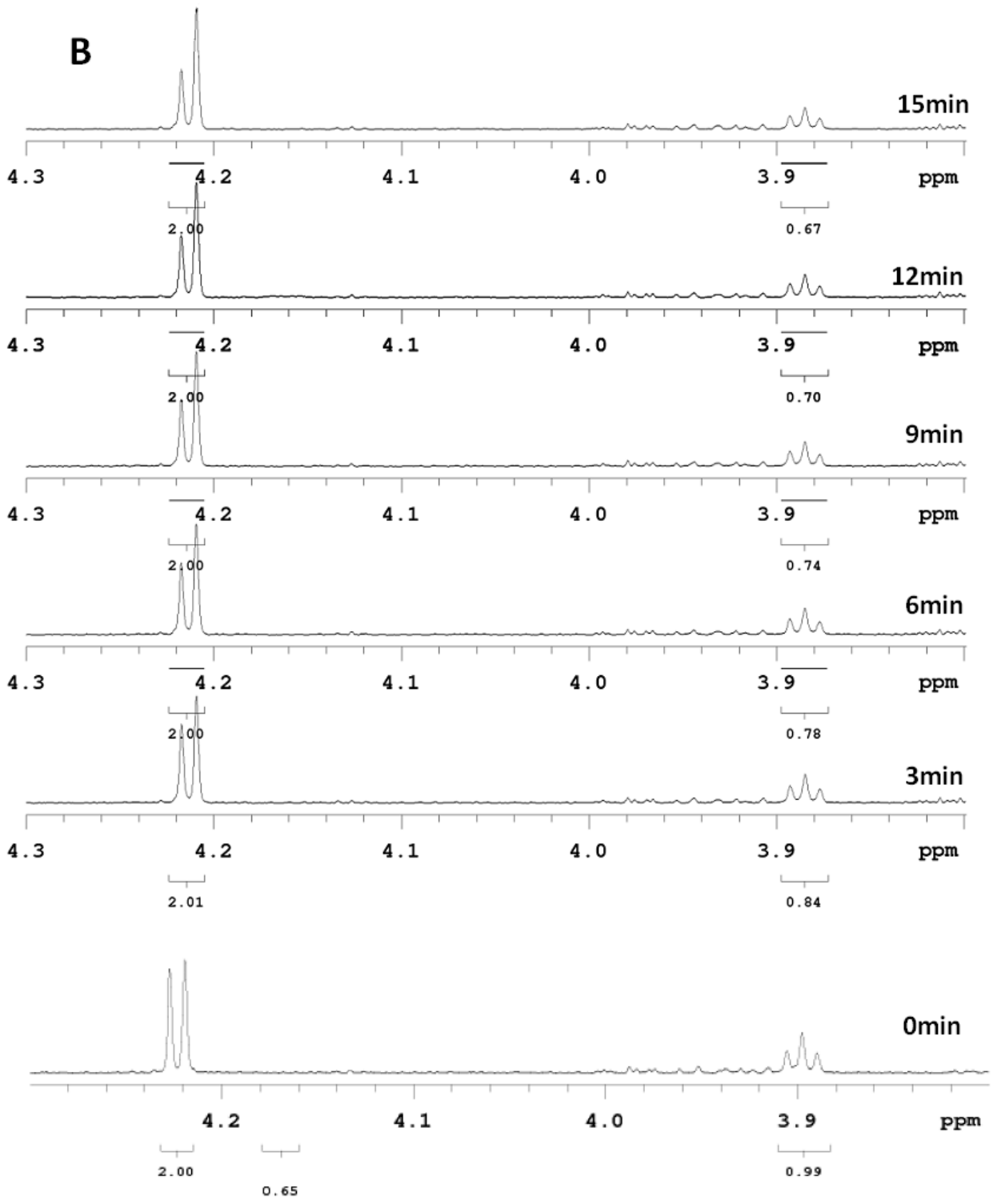
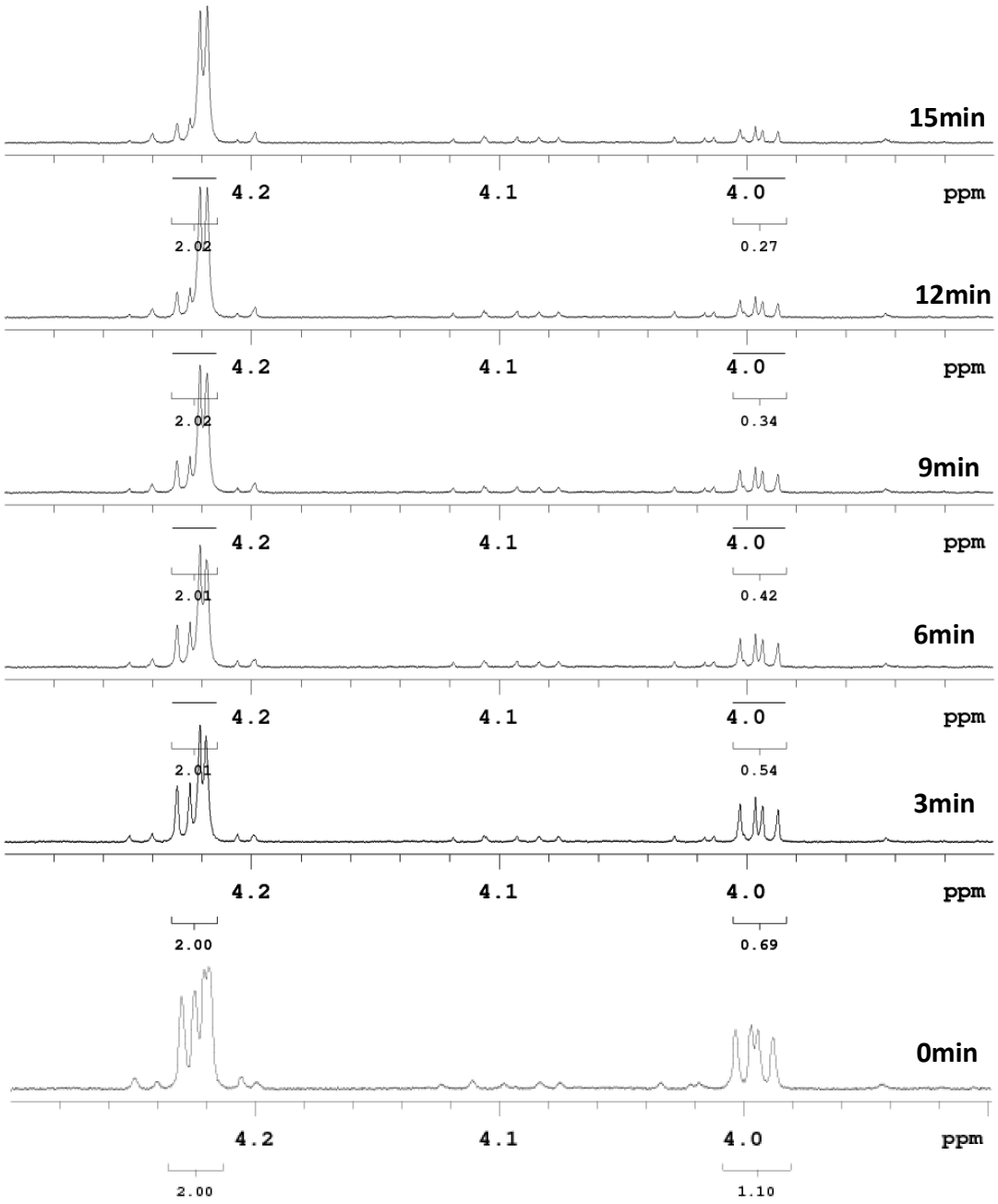


Figure S10. MS-MS sequencing data of mutant tDcsC. **A1**: M1-tDcsC with **2a**, **A2**: M1-tDcsC with **2b**, **B1**: M2-tDcsC with **2a**, **B2**: M2-tDcsC with **2b**.


A
15min
4.3
4.2
4.1
4.0
ppm
2.00
0.68
12min
2.01
0.73
9min
2.00
0.78
6min
2.00
0.84
3min
2.01
0.93
0min
2.00
1.04


B
15min
4.3
4.2
4.1
4.0
3.9
ppm
2.00
0.67
12min
2.00
0.70
9min
2.00
0.74
6min
2.00
0.78
3min
2.01
0.84
0min
2.00
0.65
0.99

C


15min
4.2
4.1
4.0
ppm
2.02
0.27
12min
4.2
4.1
4.0
ppm
2.02
0.34
9min
4.2
4.1
4.0
ppm
2.01
0.42
6min
4.2
4.1
4.0
ppm
2.01
0.54
3min
4.2
4.1
4.0
ppm
2.00
0.69
0min
4.2
4.1
4.0
ppm
2.00
1.10

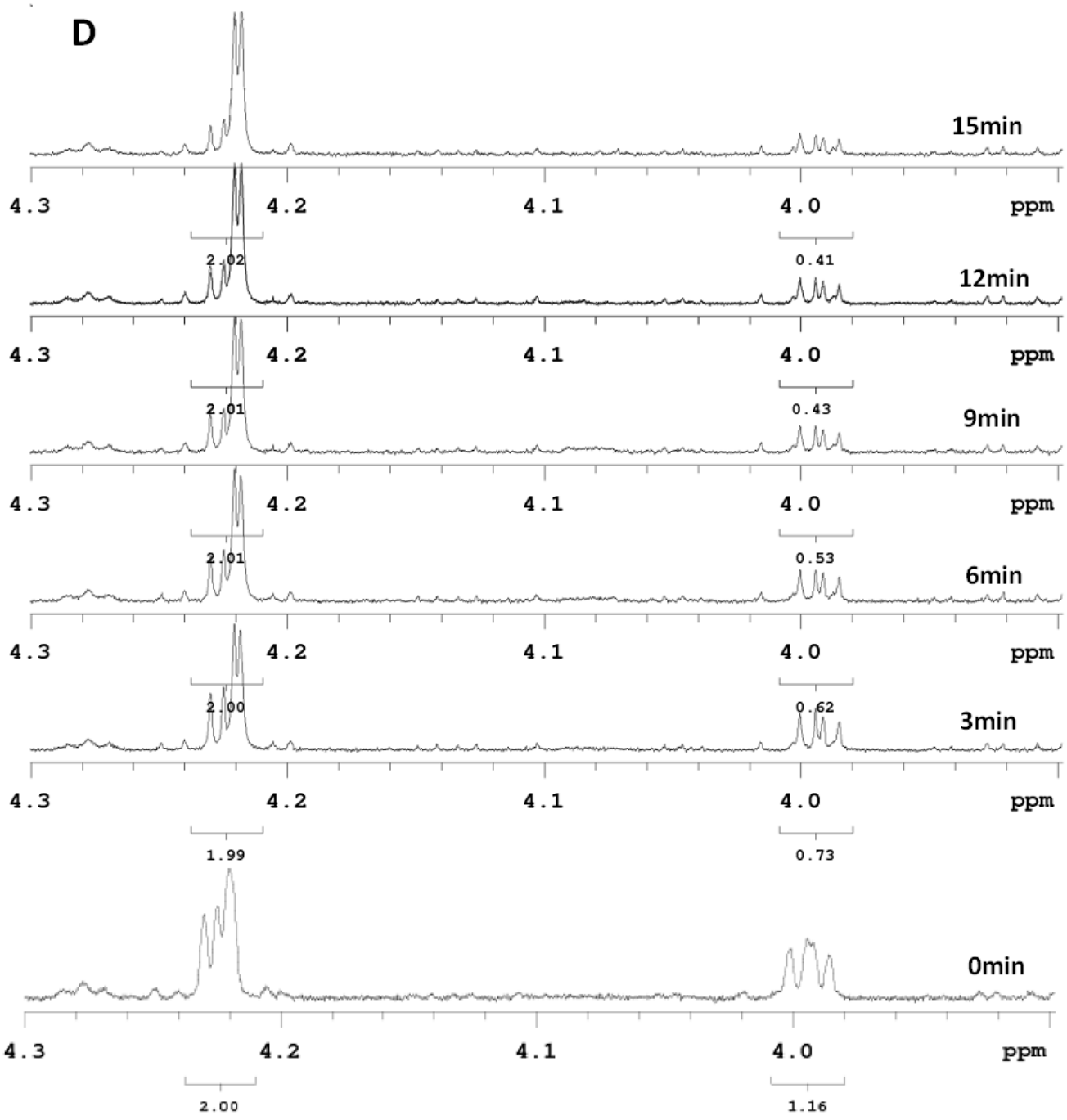
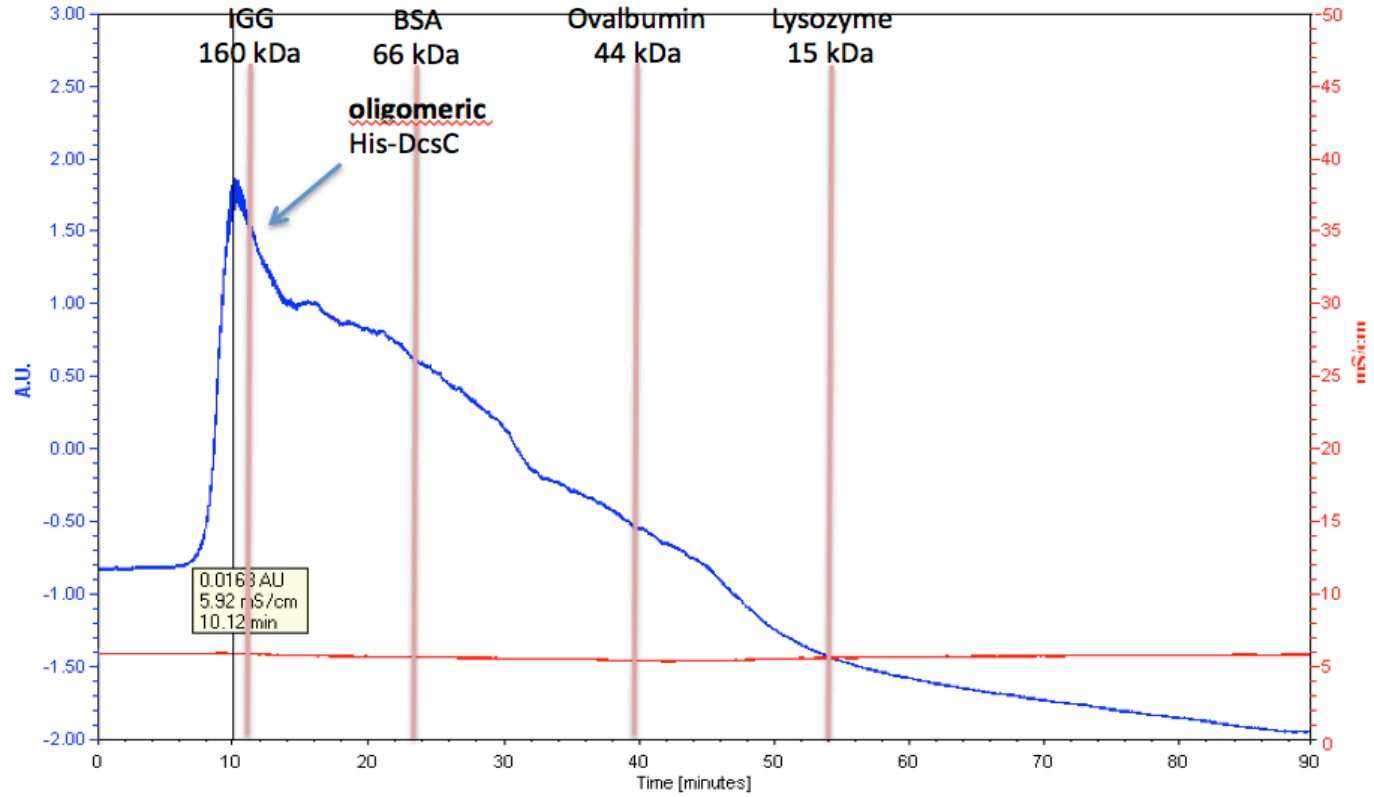
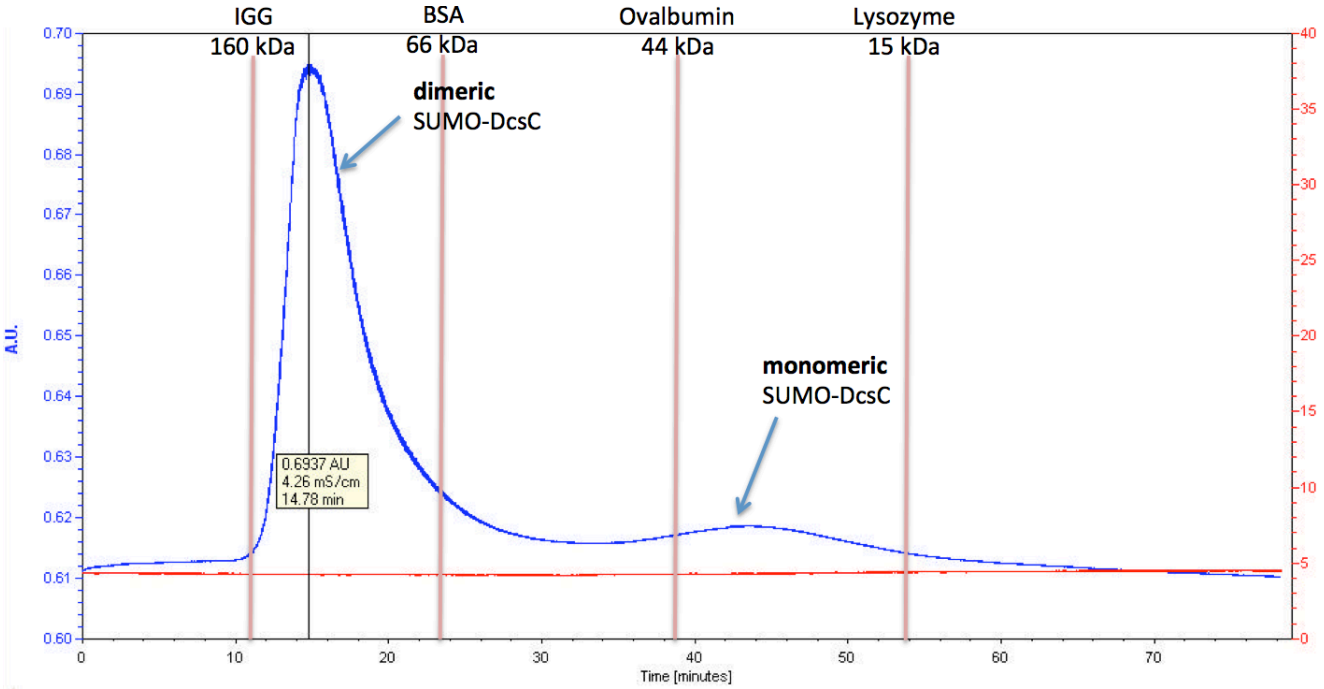


Figure S11. Enzyme activities by ^{1}H-NMR. A. SUMO-DcsC with **1a**. B. SUMO-DcsC with **1b**. C. truncated DcsC with **1a**. D. truncated DcsC with **1b**.

a)


IGG
160 kDa
BSA
66 kDa
Ovalbumin
44 kDa
Lysozyme
15 kDa
oligomeric
His-DcsC
0.0163 AU
5.92 mS/cm
10.12 min
A.U.
mS/cm
Time [minutes]

b)


IGG
160 kDa
BSA
66 kDa
Ovalbumin
44 kDa
Lysozyme
15 kDa
dimeric
SUMO-DcsC
monomeric
SUMO-DcsC
0.6937 AU
4.26 mS/cm
14.78 min
A.U.
Time [minutes]

c)

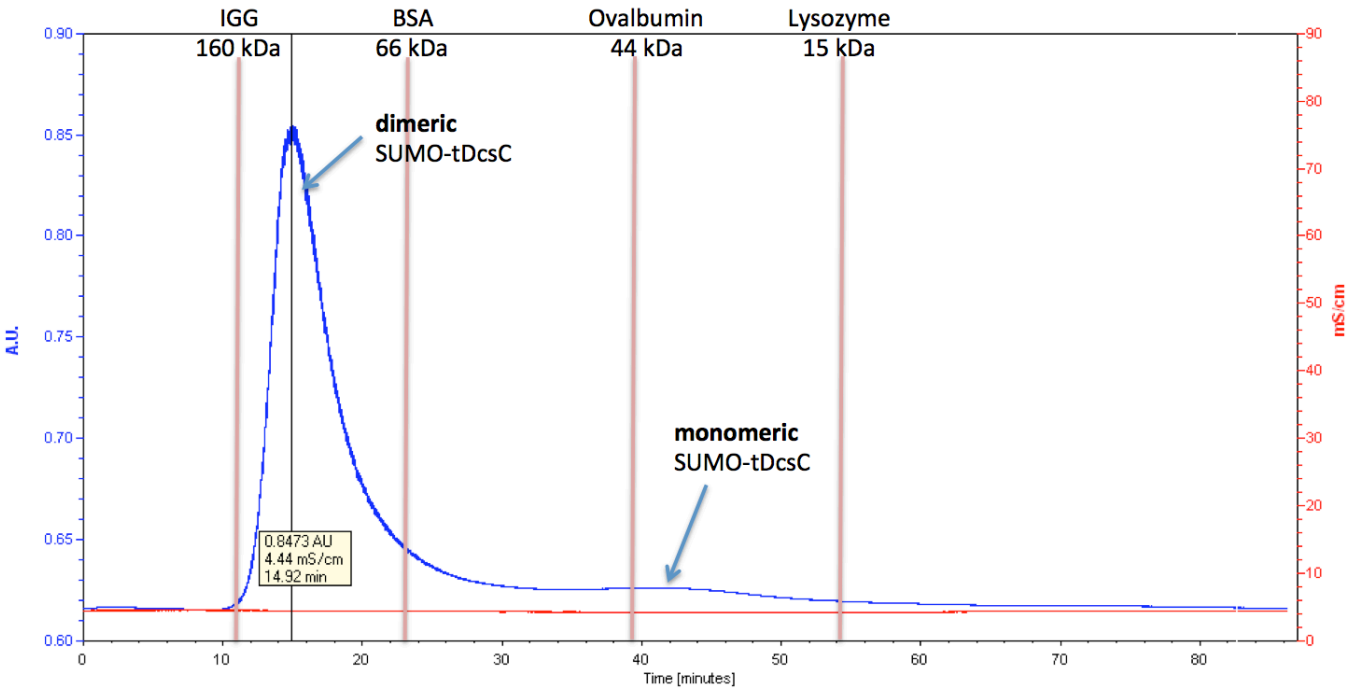
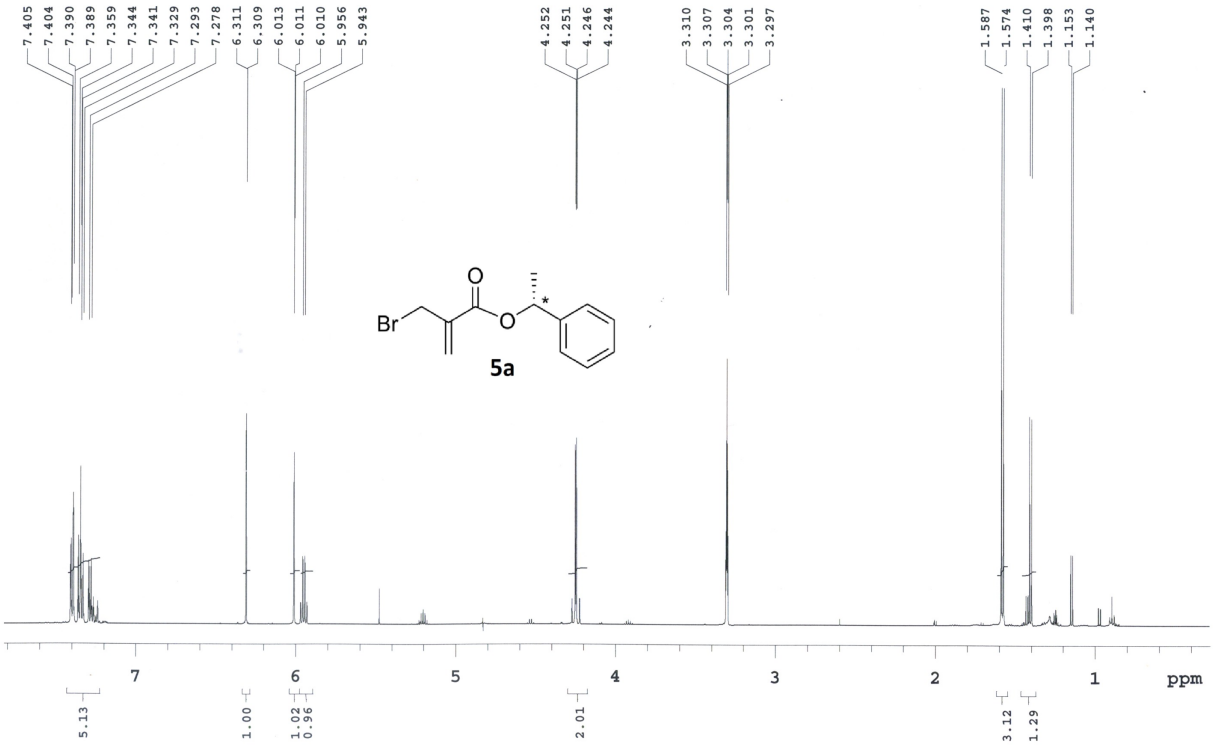
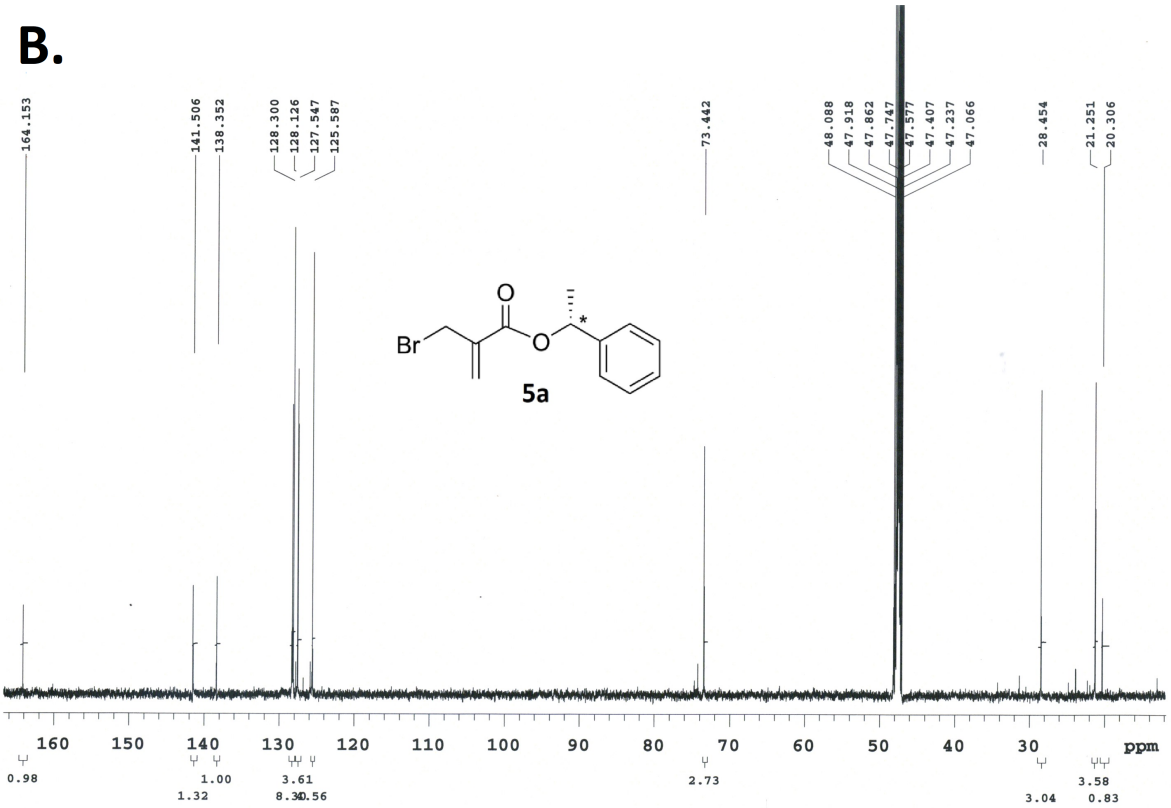


Figure S12. Size exclusion chromatogram (Sephadex® G-100, 300 x 15 mm, flow rate 1ml/min, 25mM Tris, 10mM NaCl buffer, pH=7.6) of a) His-DcsC, b) SUMO-DcsC and c) SUMO-tDcsC indicating the presence of dimeric protein aggregates (approx. 90%). The calibration reference standards human immunoglobulin G (IGG, 160 kDa, 11.0 min), bovine serum albumin (BSA, 66 kDa, 23.5 min), chicken ovalbumin (44 kDa, 39.1min) and lysozyme (15 kDa, 54.0 min) are highlighted as red lines.

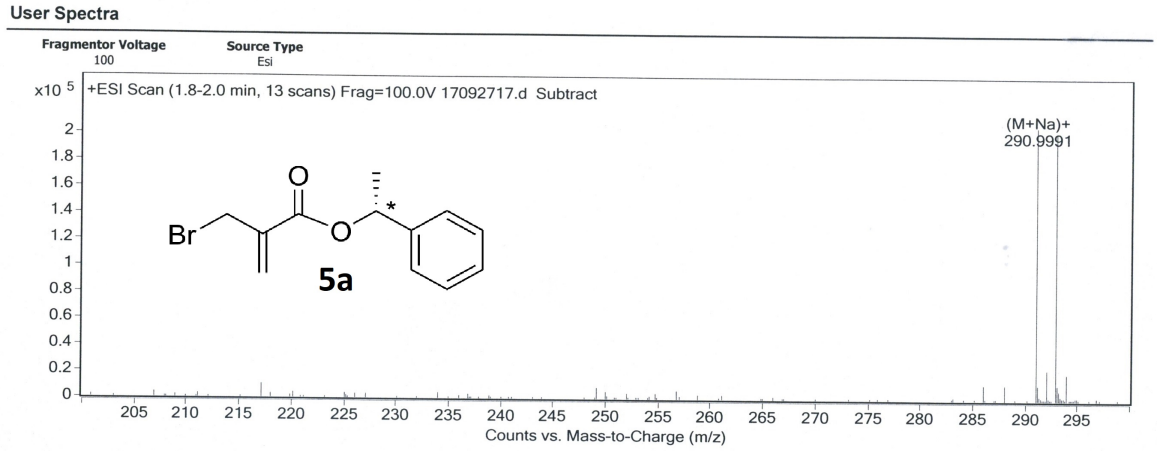
A.


7.405
7.404
7.390
7.389
7.359
7.344
7.341
7.329
7.293
7.278
6.311
6.309
6.013
6.011
6.010
5.956
5.943
4.252
4.251
4.246
4.244
3.310
3.307
3.304
3.301
3.297
1.587
1.574
1.410
1.398
1.153
1.140
Br
O
O
*
5a
7
6
5
4
3
2
1
ppm
5.13
1.00
1.02
0.96
2.01
3.12
1.29

B.


164.153
141.506
138.352
128.300
128.126
127.547
125.587
73.442
48.088
47.918
47.862
47.747
47.577
47.407
47.237
47.066
28.454
21.251
20.306
Br
O
O
*
5a
160
150
140
130
120
110
100
90
80
70
60
50
40
30
ppm
0.98
1.32
1.00
8.30
3.61
0.56
2.73
3.04
3.58
0.83

C.



Formula Calculator Results

Formula	Ion Species	Mass	Calc. Mass	m/z	Calc. m/z	Diff (mDa)	Diff(ppm)	DBE	Ion	Score
C12 H13 Br O2	C12 H13 Br Na O2	268.0099	268.0099	290.9991	290.9991	-0.03	-0.12	6	(M+Na)+	90.96

D.

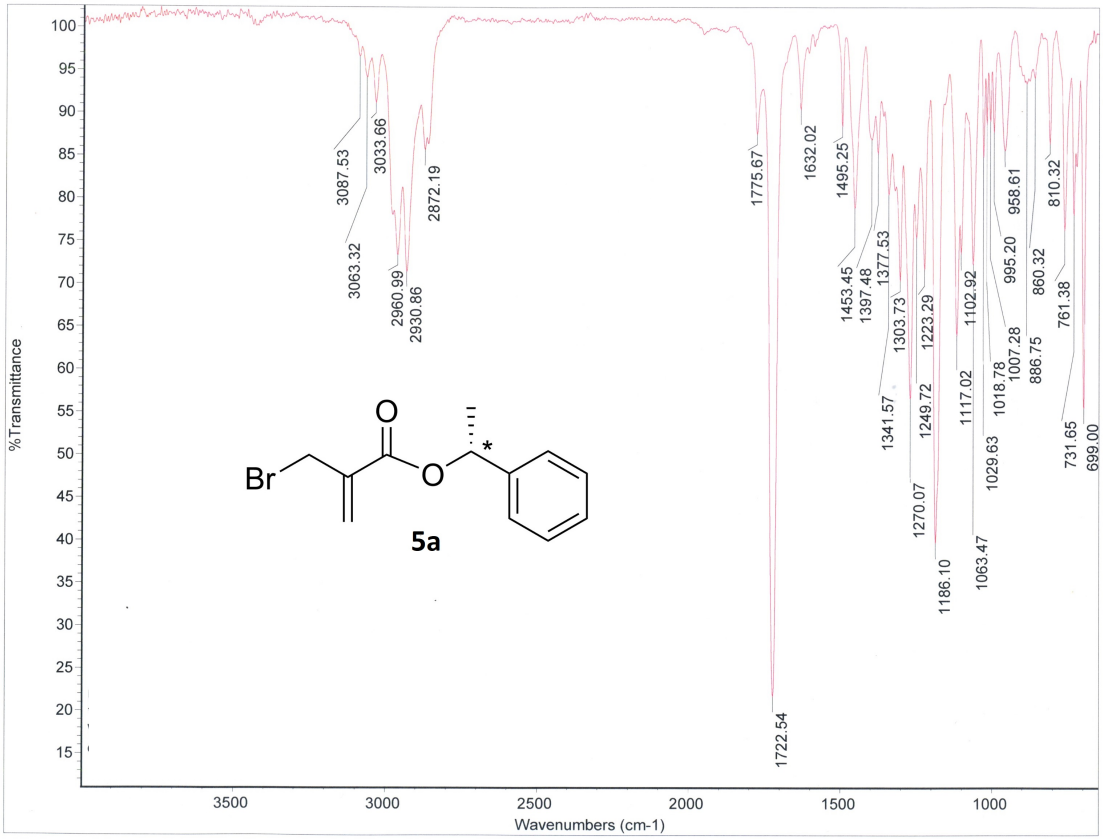
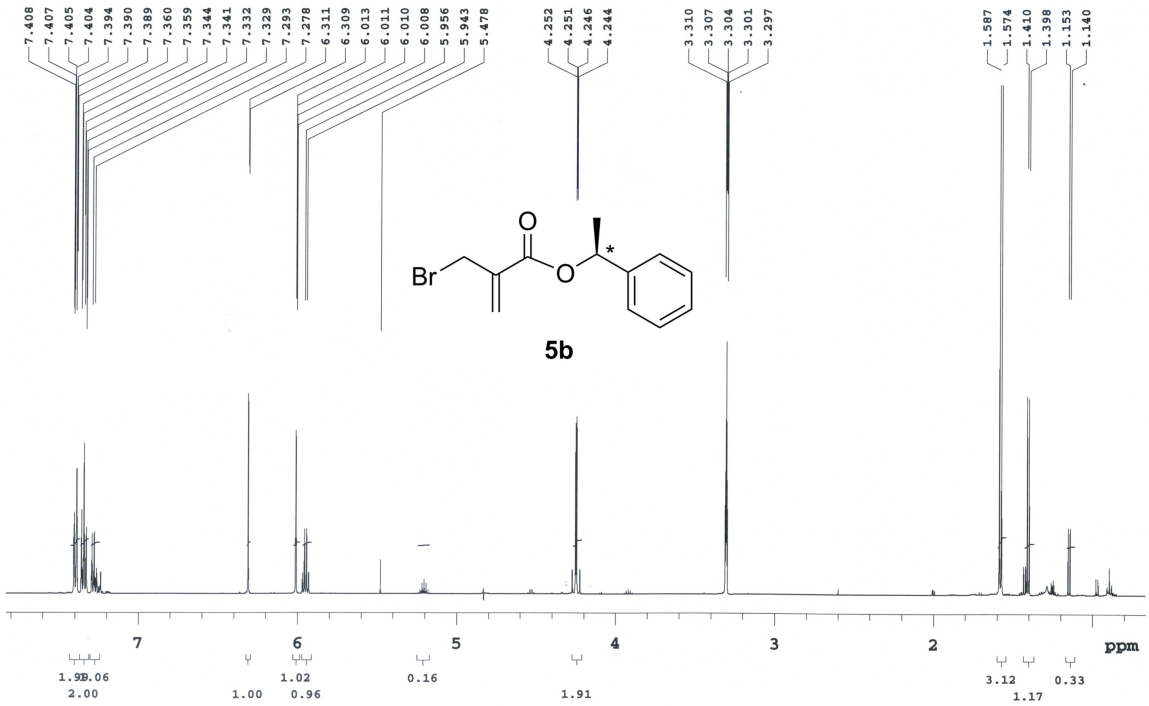
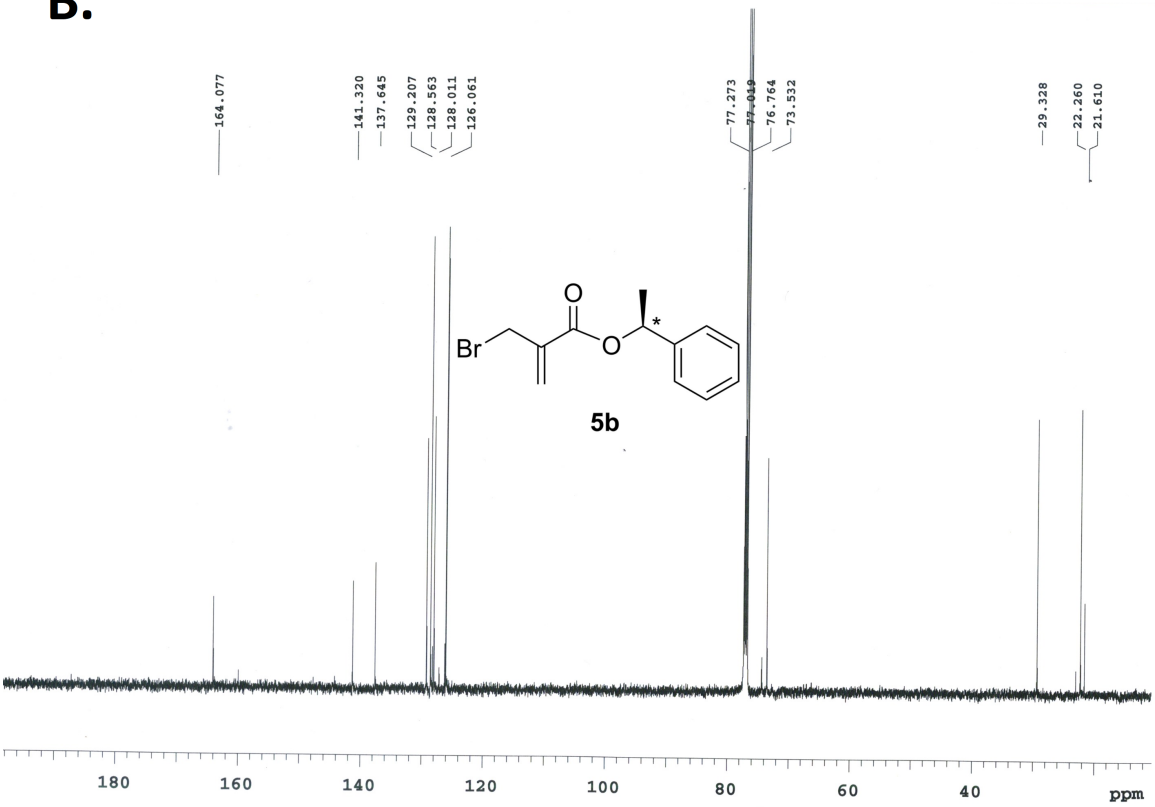


Figure S13. Analytical data of **5a**. A. 1H-NMR. B. 13C-NMR. C. ESI. D. FTIR

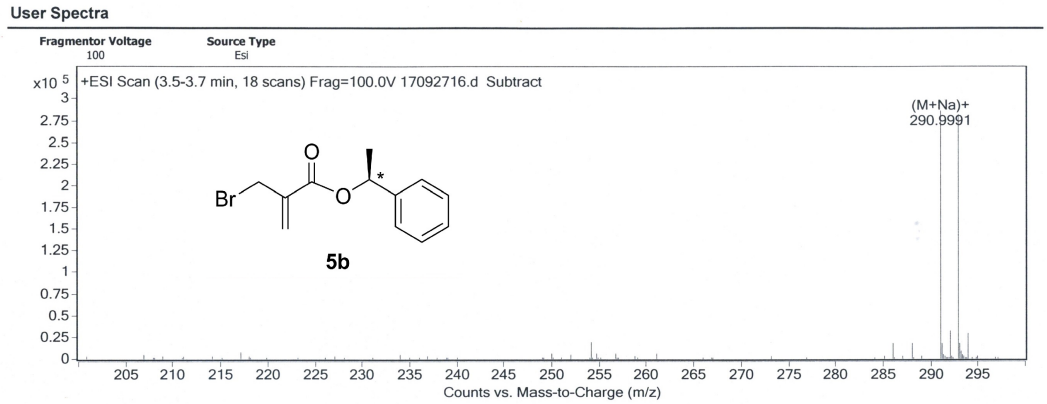
A.


7.408
7.407
7.405
7.404
7.394
7.390
7.389
7.360
7.359
7.344
7.341
7.332
7.329
7.293
7.278
6.311
6.309
6.013
6.011
6.010
6.008
5.956
5.943
5.478
4.252
4.251
4.246
4.244
3.310
3.307
3.304
3.301
3.297
1.587
1.574
1.410
1.398
1.153
1.140
Br
O
O
*
5b
7
6
5
4
3
2
ppm
1.99
1.06
2.00
1.00
1.02
0.96
0.16
1.91
3.12
1.17
0.33

B.


164.077
141.320
137.645
129.207
128.563
128.011
126.061
77.273
77.019
76.764
73.532
29.328
22.260
21.610
Br
O
O
*
5b
180
160
140
120
100
80
60
40
ppm

C.



Formula Calculator Results

Formula	Ion Species	Mass	Calc. Mass	m/z	Calc. m/z	Diff (mDa)	Diff(ppm)	DBE	Ion	Score
C12 H13 Br O2	C12 H13 Br Na O2	268.0099	268.0099	290.9991	290.9991	-0.01	-0.02	6	(M+Na)+	91.49

D.

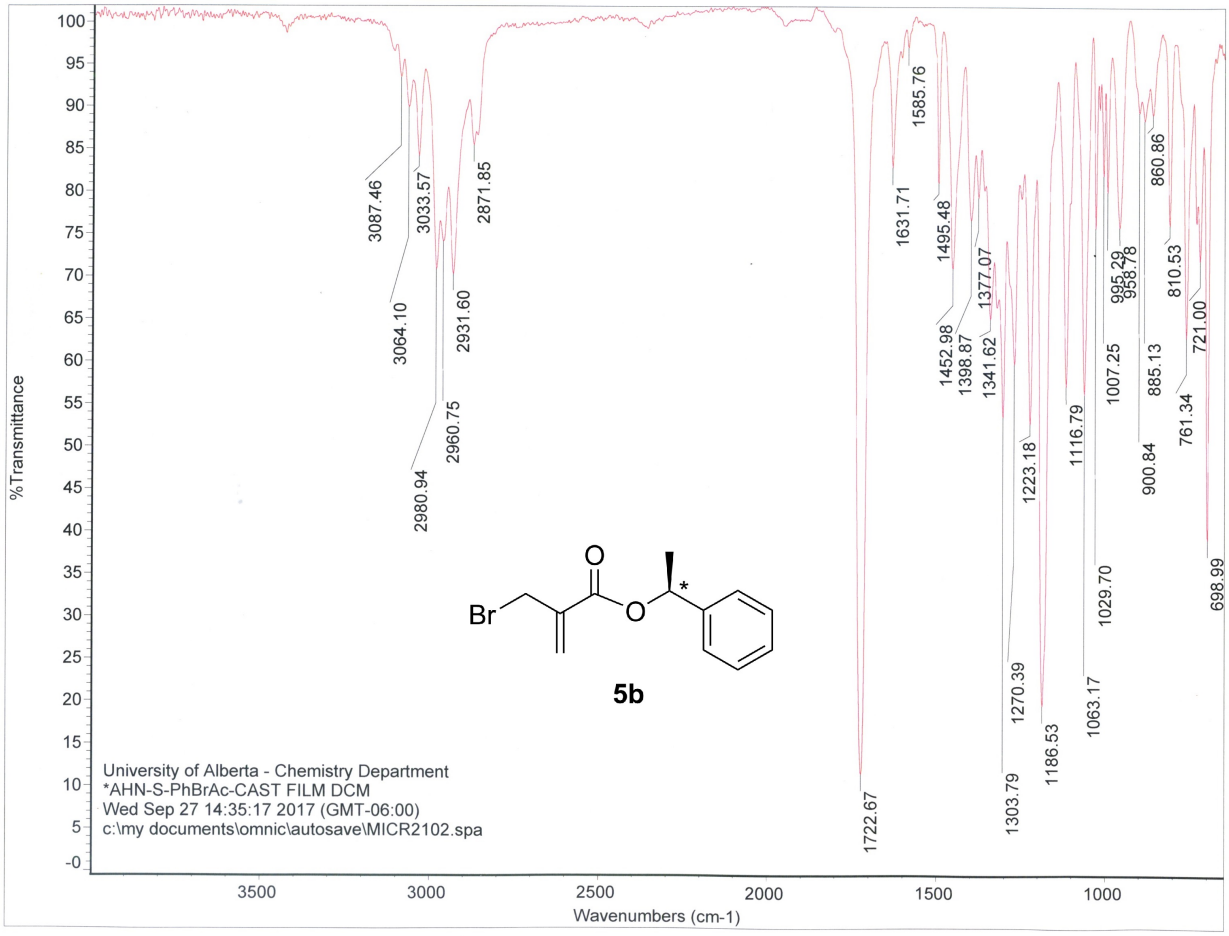
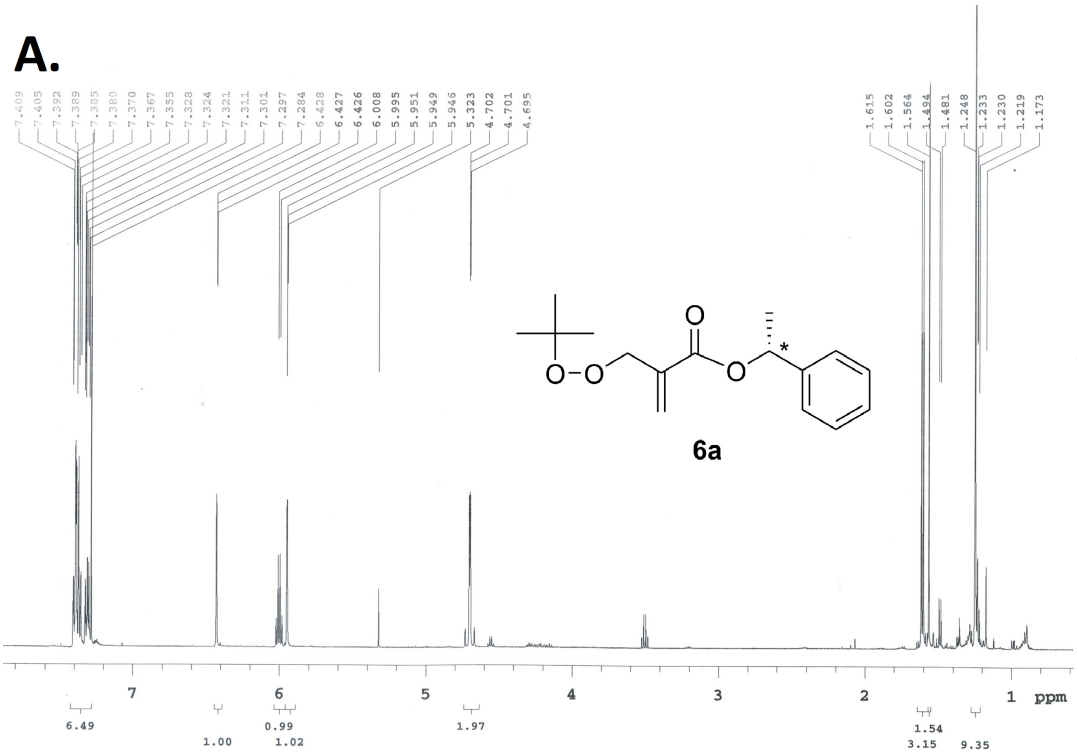
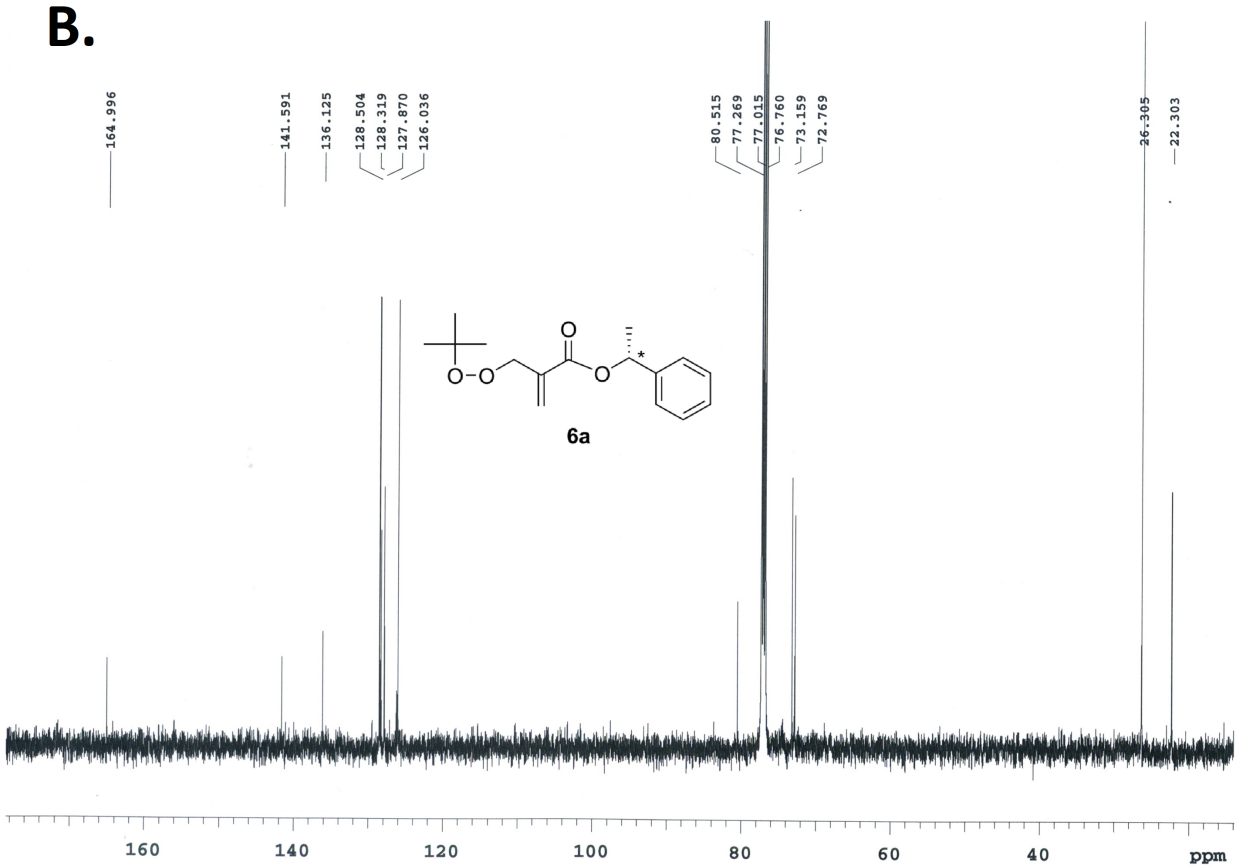


Figure S14. Analytical data of **5b**. A. 1H-NMR. B. 13C-NMR. C. ESI. D. FTIR

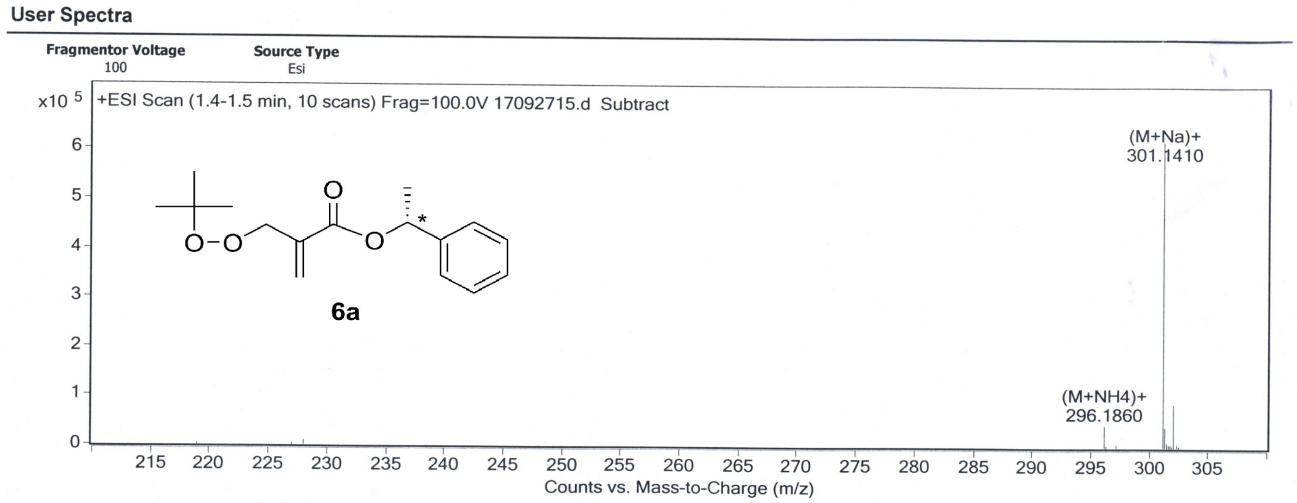
A.


7.409
7.405
7.392
7.389
7.385
7.380
7.370
7.367
7.355
7.328
7.324
7.321
7.311
7.301
7.297
7.284
6.428
6.427
6.426
6.008
5.995
5.951
5.949
5.946
5.323
4.702
4.701
4.695
1.615
1.602
1.564
1.494
1.481
1.248
1.233
1.230
1.219
1.173
O
O-O
O
*
6a
7
6
5
4
3
2
1 ppm
6.49
1.00
0.99
1.02
1.97
1.54
3.15
9.35

B.


164.996
141.591
136.125
128.504
128.319
127.870
126.036
80.515
77.269
77.015
76.760
73.159
72.769
26.305
22.303
O
O-O
O
*
6a
160
140
120
100
80
60
40
ppm

C.



Formula Calculator Results

Formula	Ion Species	Mass	Calc. Mass	m/z	Calc. m/z	Diff (mDa)	Diff(ppm)	DBE	Ion	Score
C16 H22 O4	C16 H26 N O4	278.1522	278.1518	296.186	296.1856	-0.34	-1.23	6	(M+NH4)+	83.28
C16 H22 O4	C16 H22 Na O4	278.1518	278.1518	301.141	301.141	0.06	0.21	6	(M+Na)+	78.59

D.

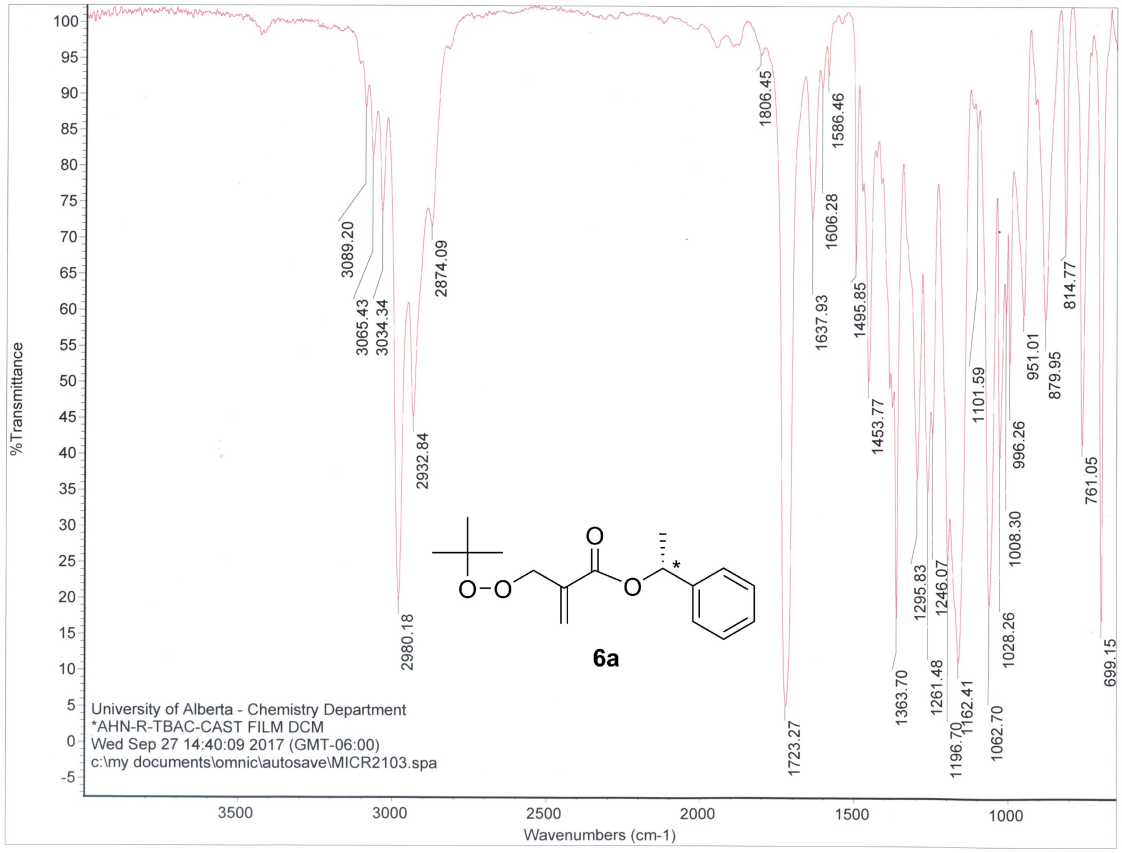
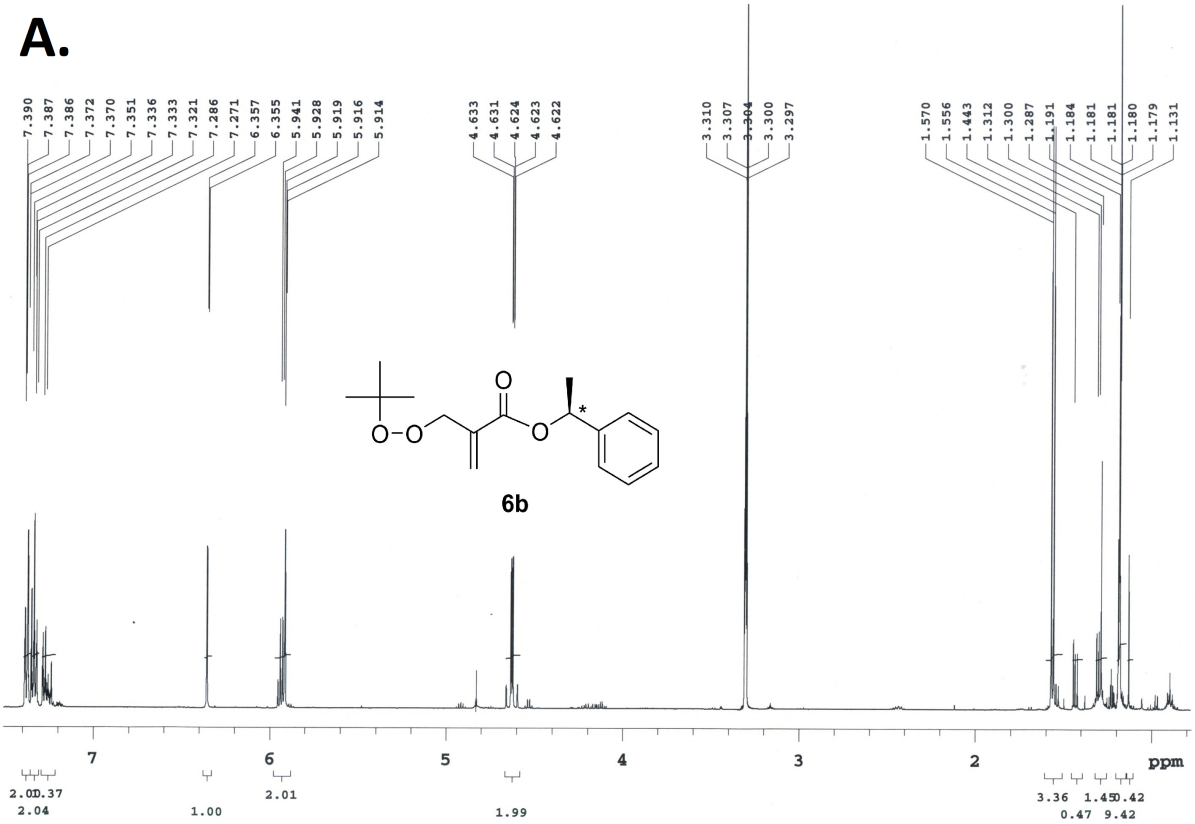
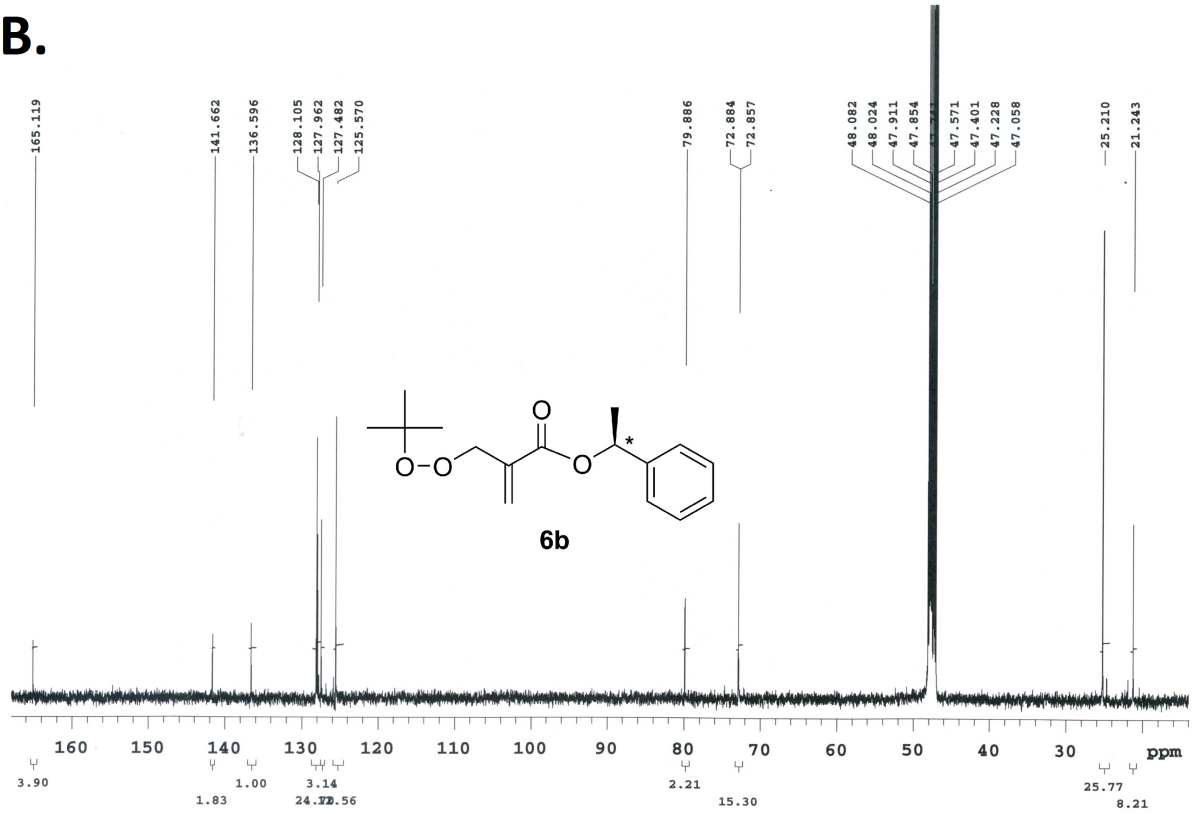


Figure S15. Analytical data of **6a**. A. 1H-NMR. B. 13C-NMR. C. ESI. D. FTIR

A.


7.390
7.387
7.386
7.372
7.370
7.351
7.336
7.333
7.321
7.286
7.271
6.357
6.355
5.941
5.928
5.919
5.916
5.914
4.633
4.631
4.624
4.623
4.622
3.310
3.307
3.304
3.300
3.297
1.570
1.556
1.443
1.312
1.300
1.287
1.191
1.184
1.181
1.181
1.180
1.179
1.131
O
O-O
O
*
6b
7
6
5
4
3
2
ppm
2.04
1.00
2.01
1.99
3.36
0.47
9.42

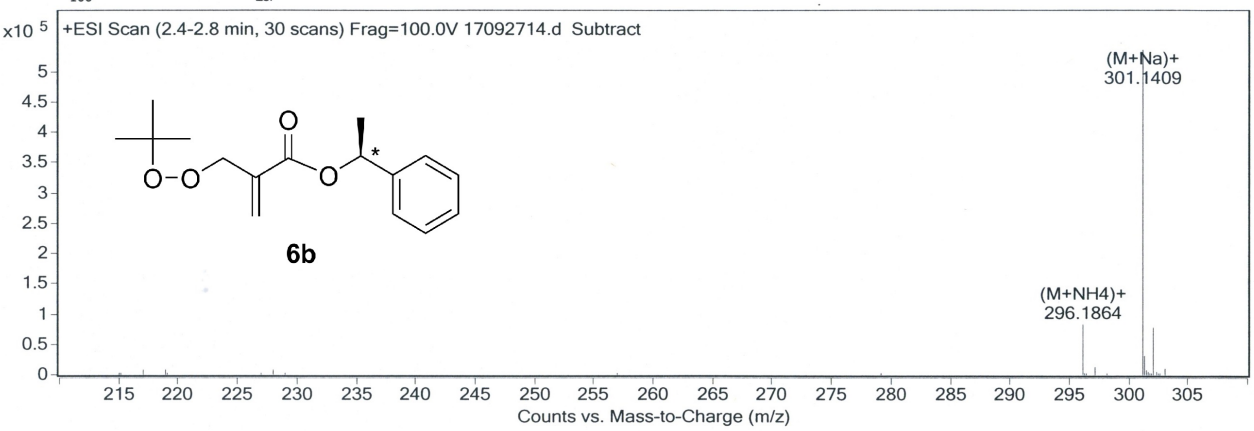
B.


165.119
141.662
136.596
128.105
127.962
127.482
125.570
79.886
72.884
72.857
48.082
48.024
47.911
47.854
47.571
47.401
47.228
47.058
25.210
21.243
O
O-O
O
*
6b
160
150
140
130
120
110
100
90
80
70
60
50
40
30
ppm
3.90
1.83
1.00
3.14
2.21
15.30
25.77
8.21

C.

User Spectra

Fragmentor Voltage 100 **Source Type** Esi



Formula Calculator Results

Formula	Ion Species	Mass	Calc. Mass	m/z	Calc. m/z	Diff (mDa)	Diff(ppm)	DBE	Ion	Score
C16 H22 O4	C16 H26 N O4	278.1526	278.1518	296.1864	296.1856	-0.79	-2.82	6	(M+NH4)+	88.89
C16 H22 O4	C16 H22 Na O4	278.1517	278.1518	301.1409	301.141	0.12	0.44	6	(M+Na)+	92.13

D.

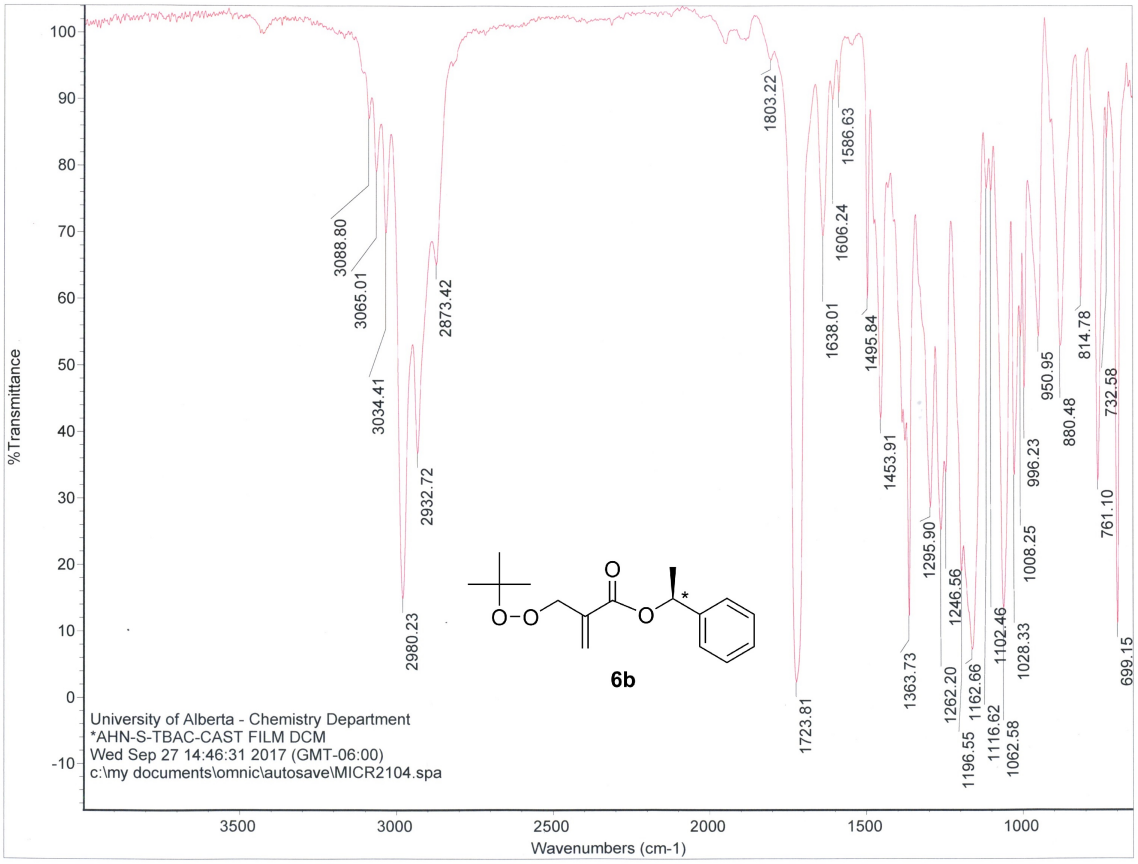
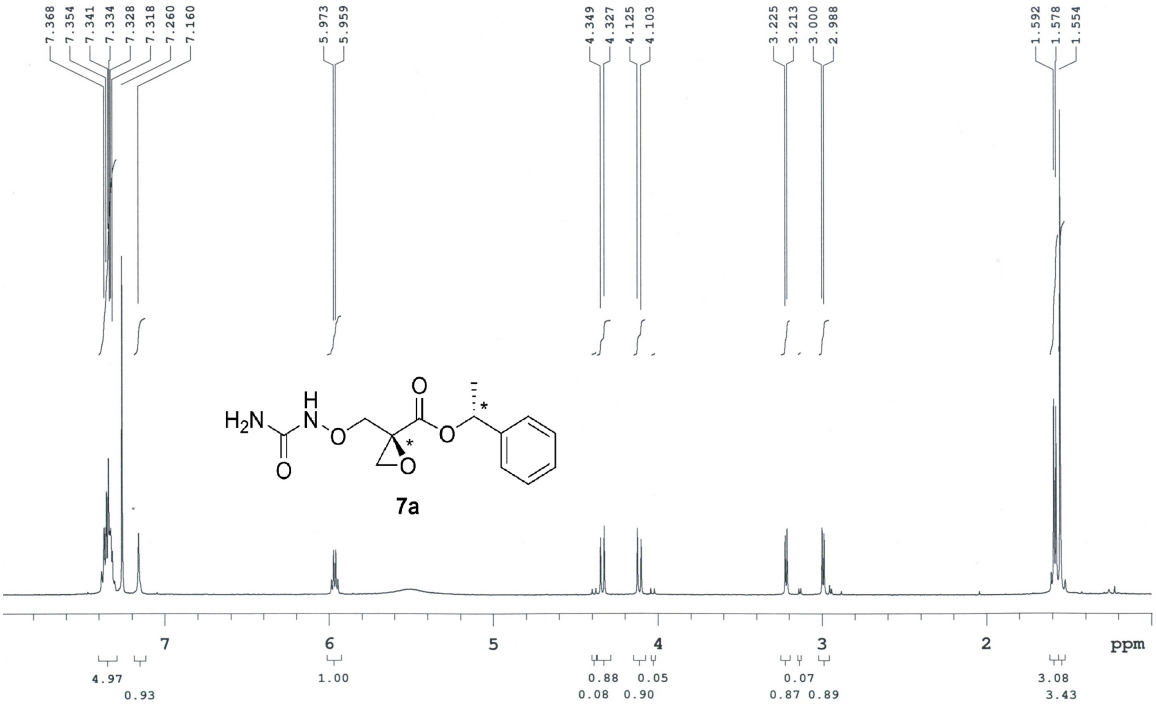
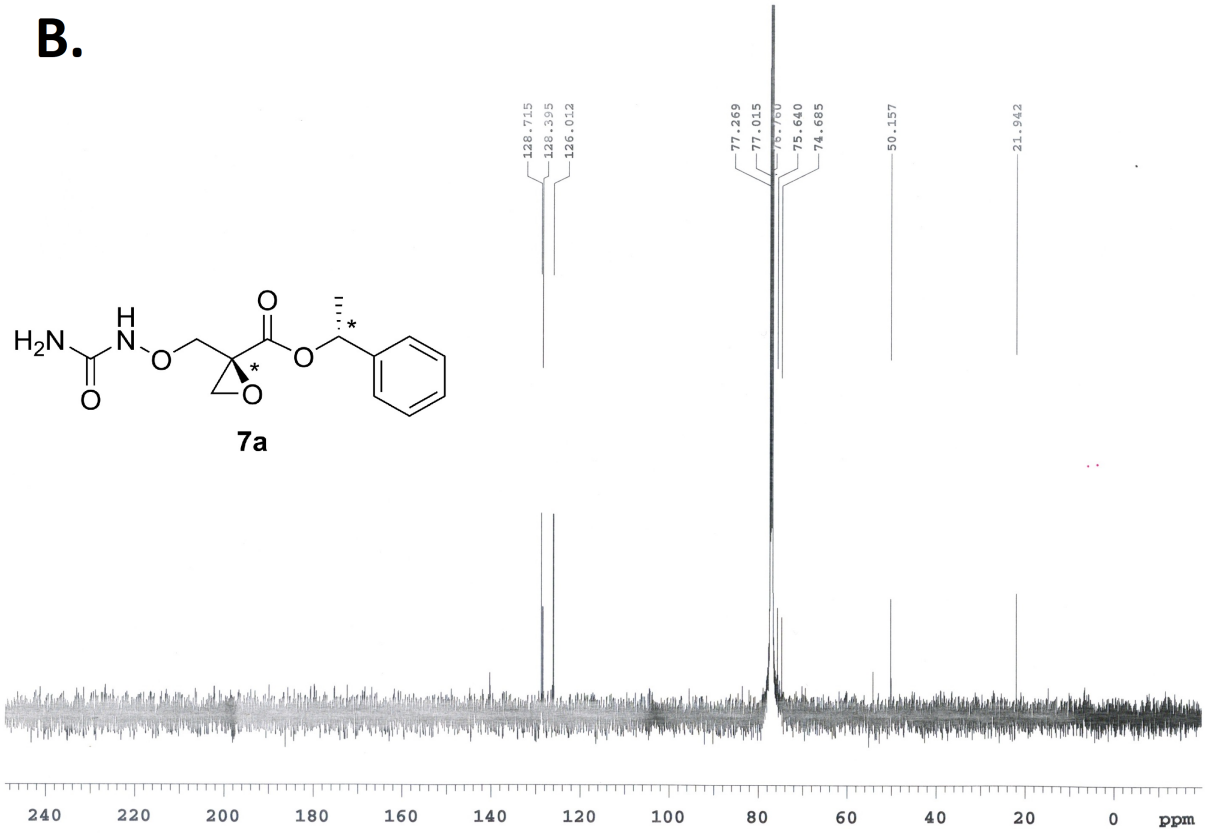


Figure S16. Analytical data of **6b**. A. 1H-NMR. B. 13C-NMR. C. ESI. D. FTIR

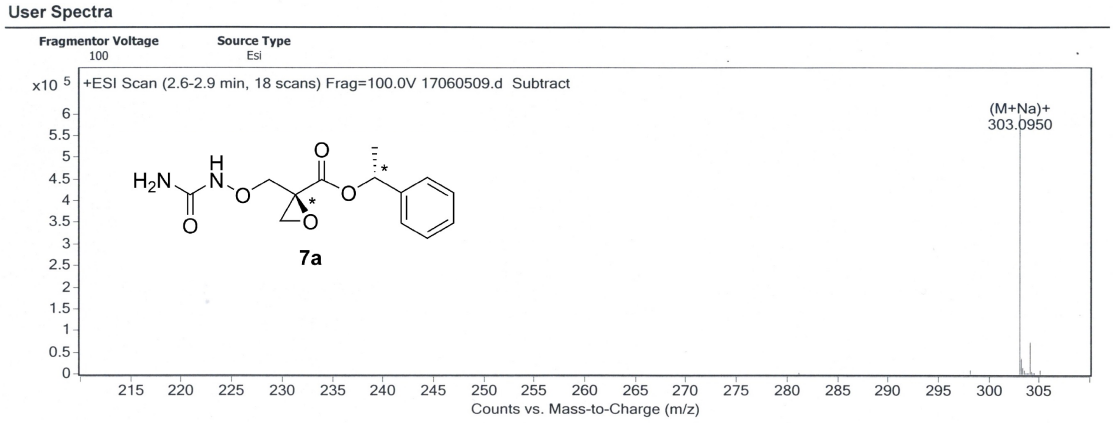
A.


7.368
7.354
7.341
7.334
7.328
7.318
7.260
7.160
5.973
5.959
4.349
4.327
4.125
4.103
3.225
3.213
3.000
2.988
1.592
1.578
1.554
7a
7
6
5
4
3
2
ppm
4.97
0.93
1.00
0.88
0.08
0.05
0.90
0.07
0.87
0.89
3.08
3.43

B.


128.715
128.395
126.012
77.269
77.015
75.640
74.685
50.157
21.942
7a
240
220
200
180
160
140
120
100
80
60
40
20
0
ppm

C.



Formula Calculator Results

Formula	Ion Species	Mass	Calc. Mass	m/z	Calc. m/z	Diff (mDa)	Diff(ppm)	DBE	Ion	Score
C13 H16 N2 O5	C13 H16 N2 Na O5	280.1058	280.1059	303.095	303.0951	0.17	0.59	7	(M+Na)+	89.87

D.

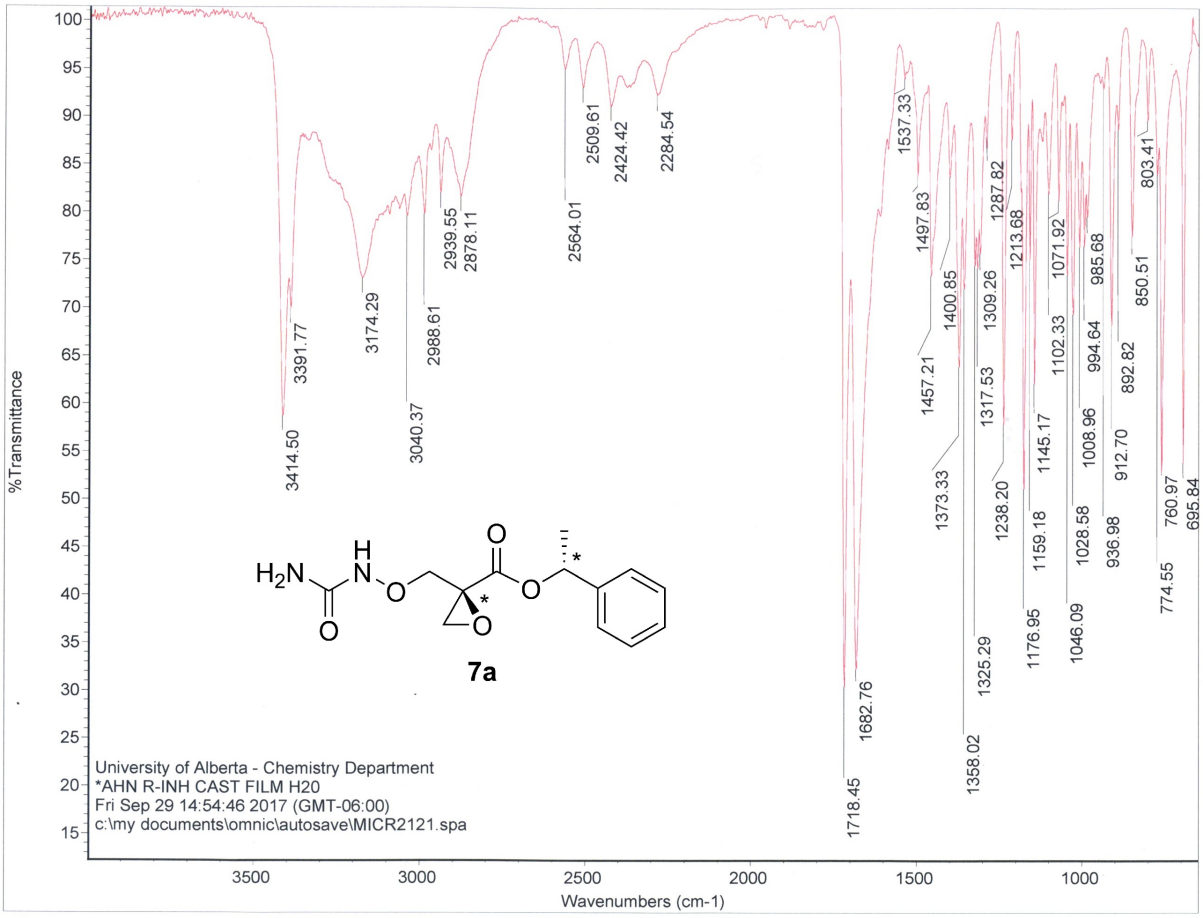
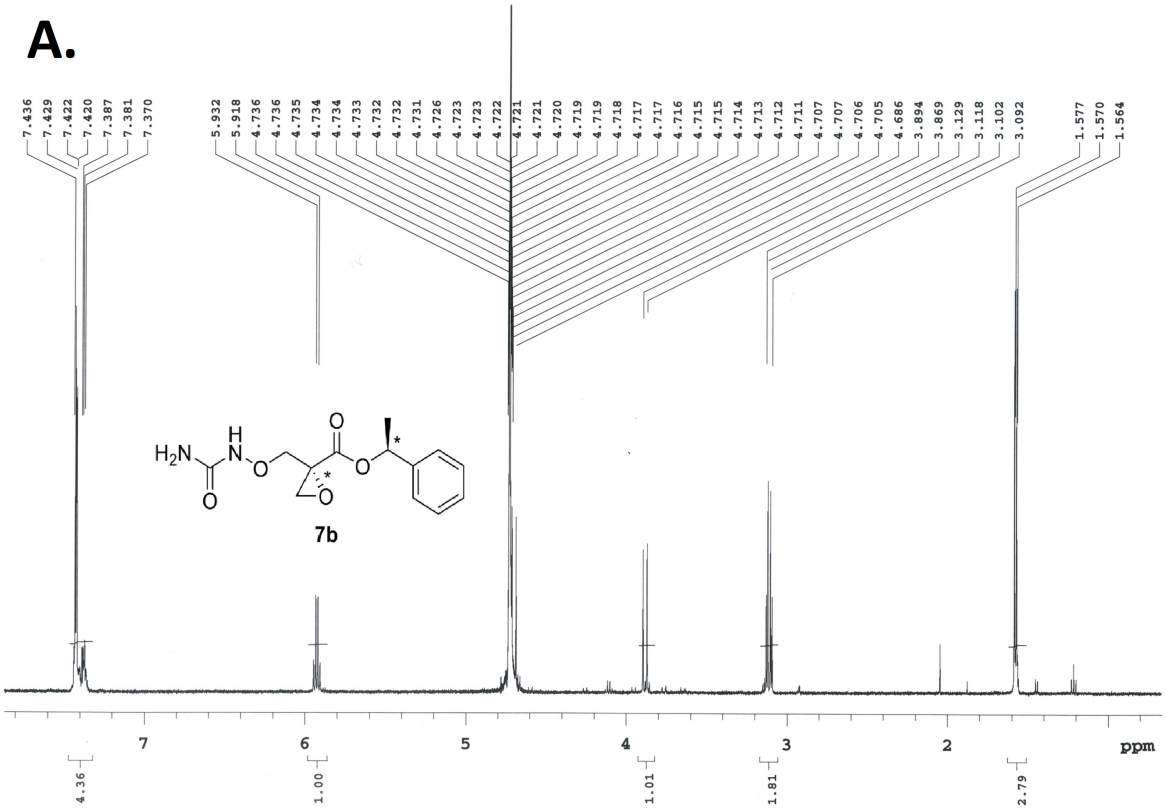


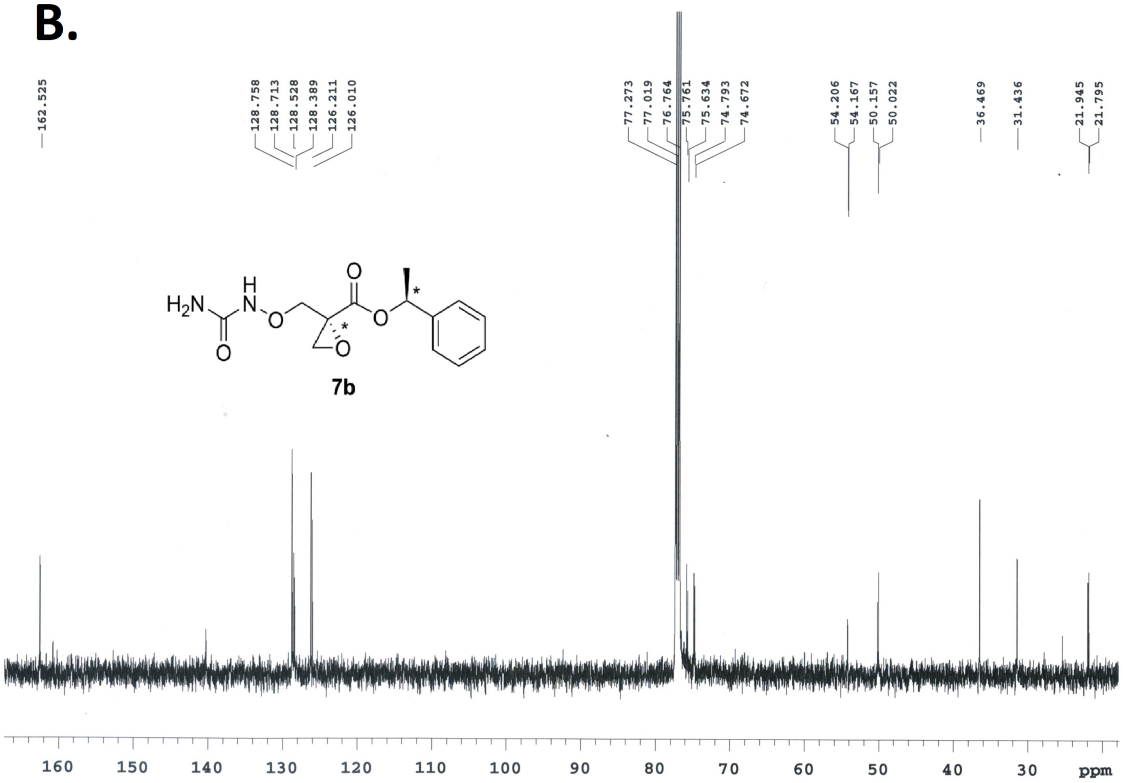
Figure S17. Analytical data of **7a**. A. 1H-NMR. B. 13C-NMR. C. ESI. D. FTIR

A.


7.436
7.429
7.422
7.420
7.387
7.381
7.370
5.932
5.918
4.736
4.736
4.735
4.734
4.734
4.733
4.732
4.732
4.731
4.726
4.723
4.723
4.722
4.721
4.721
4.720
4.719
4.719
4.718
4.717
4.717
4.716
4.715
4.715
4.714
4.713
4.712
4.711
4.707
4.707
4.706
4.705
4.686
3.894
3.869
3.129
3.118
3.102
3.092
1.577
1.570
1.564
7
6
5
4
3
2
ppm
4.36
1.00
1.01
1.81
2.79

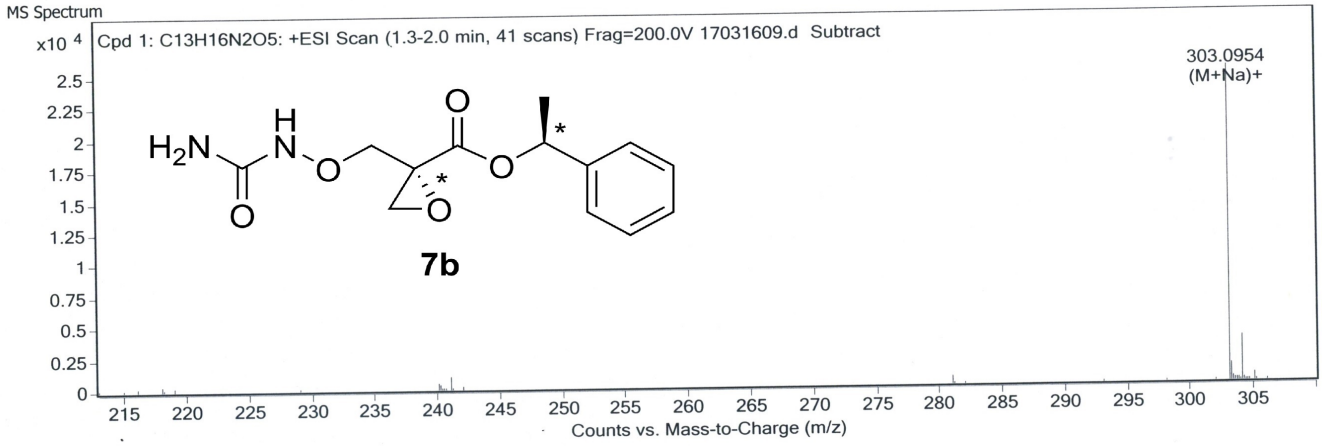
7b

B.


162.525
128.758
128.713
128.528
128.389
126.211
126.010
77.273
77.019
76.764
75.761
75.634
74.793
74.672
54.206
54.167
50.157
50.022
36.469
31.436
21.945
21.795
160
150
140
130
120
110
100
90
80
70
60
50
40
30
ppm

7b

C.



MS Spectrum Peak List

Ion Formula	Ion type	*Measured m/z*	*Calc m/z*	Diff(ppm)	z	Abund
C13 H16 N2 Na O5	(M+Na)+	303.0954	303.0951	0.71	1	25272

D.

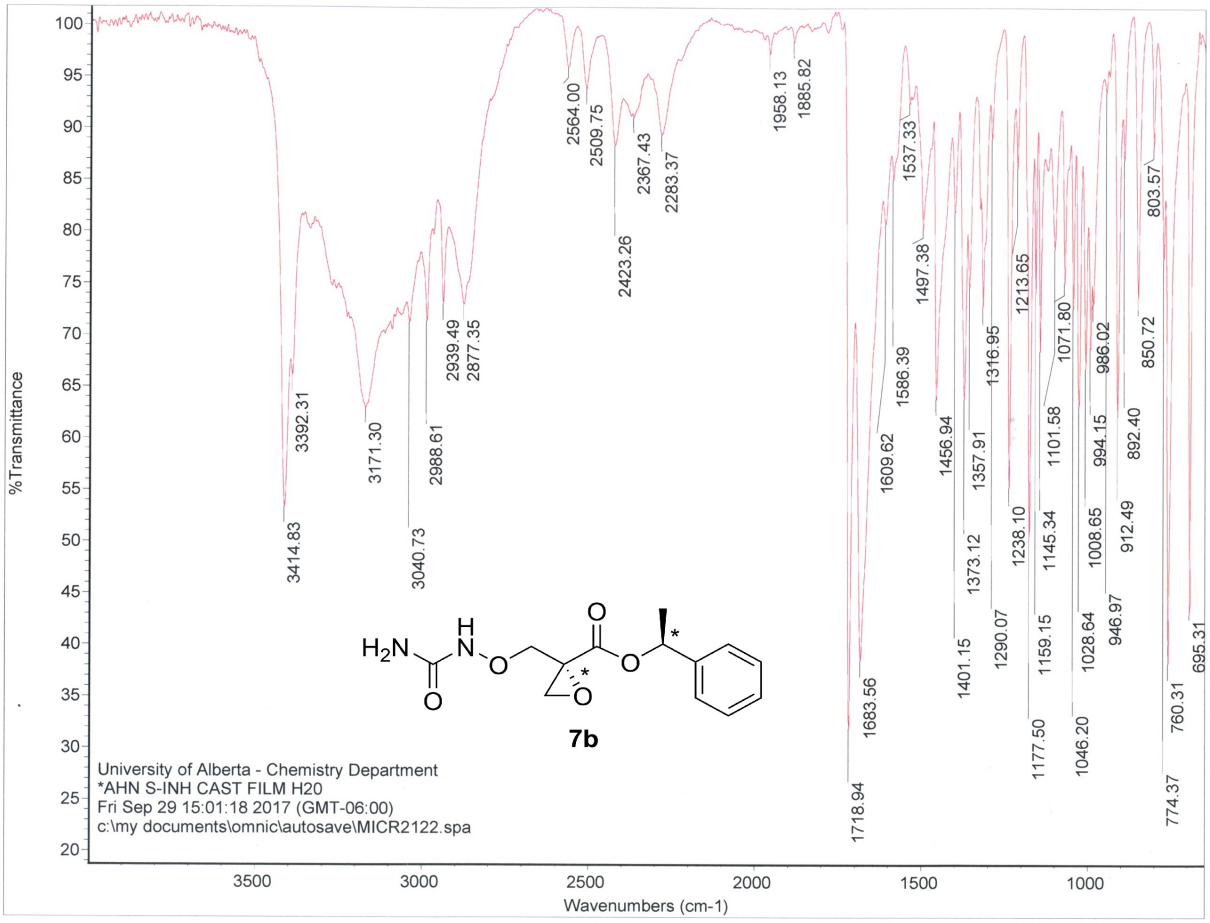
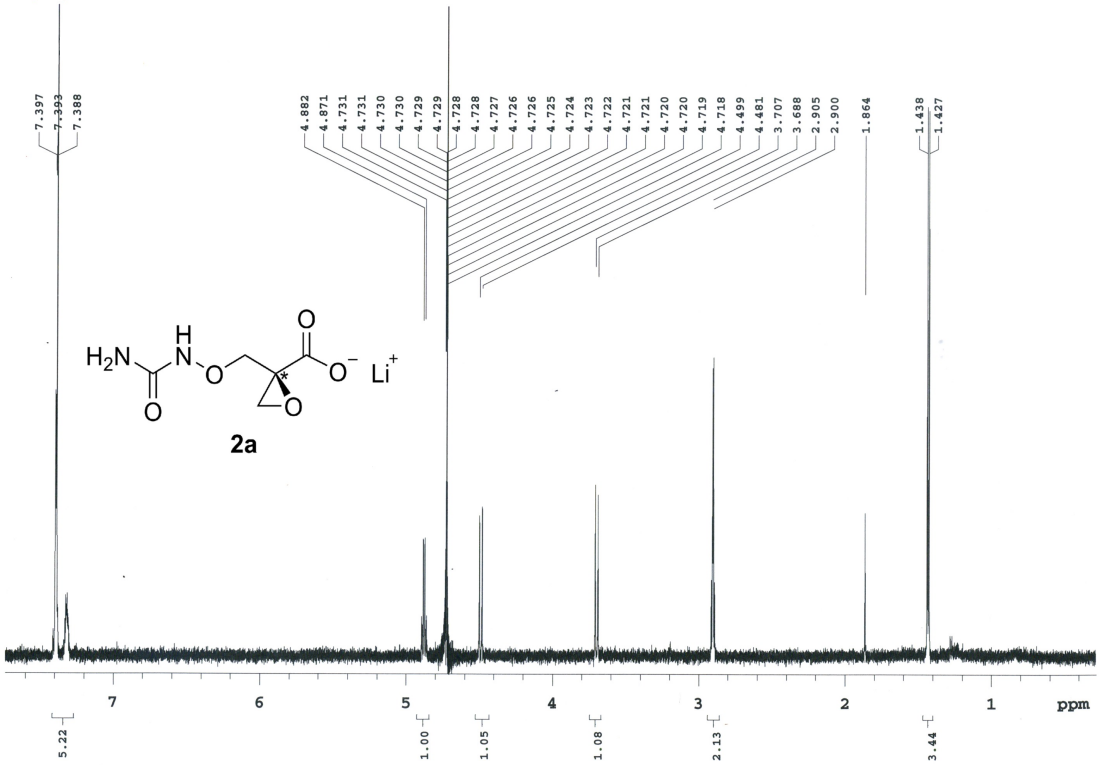
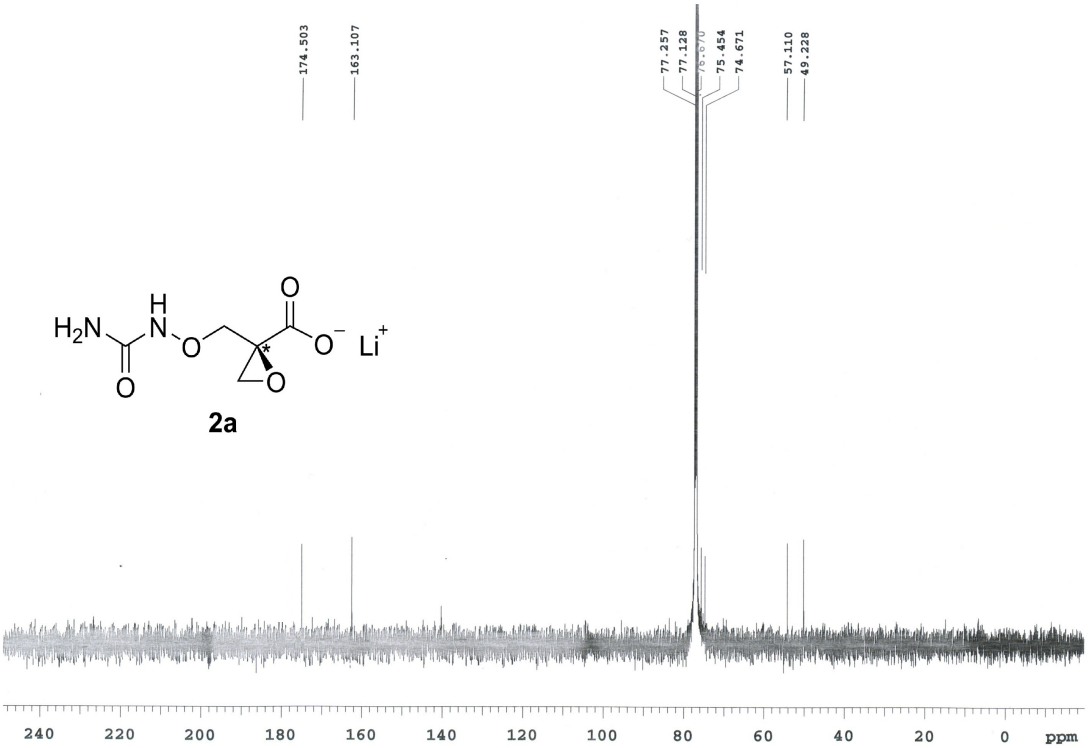


Figure S18. Analytical data of **7b**. A. 1H-NMR. B. 13C-NMR. C. ESI. D. FTIR

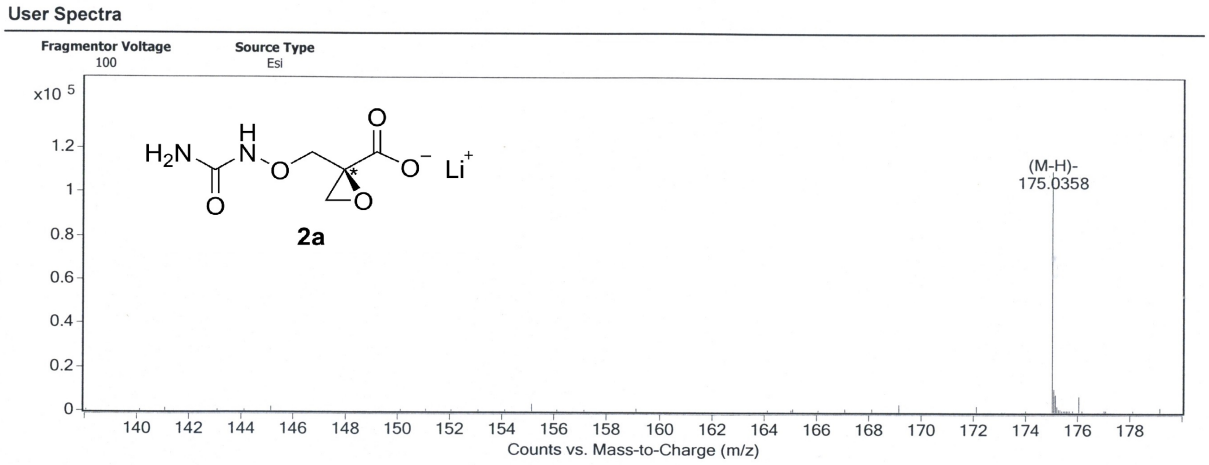
A.


7.397
7.393
7.388
4.882
4.871
4.731
4.731
4.730
4.730
4.729
4.729
4.728
4.728
4.727
4.726
4.726
4.725
4.724
4.723
4.722
4.721
4.721
4.720
4.720
4.719
4.718
4.499
4.481
3.707
3.688
2.905
2.900
1.864
1.438
1.427
H2N
H
N
O
O
O
O
O−
Li+
2a
7
6
5
4
3
2
1
ppm
5.22
1.00
1.05
1.08
2.13
3.44

B.


174.503
163.107
77.257
77.128
75.454
74.671
57.110
49.228
H2N
H
N
O
O
O
O
O−
Li+
2a
240
220
200
180
160
140
120
100
80
60
40
20
0
ppm

C.



Formula Calculator Results

Formula	Ion Species	Mass	Calc. Mass	m/z	Calc. m/z
C5 H8 N2 O5	C5 H7 N2 O5	176.0429	176.0433	175.0358	175.036

D.

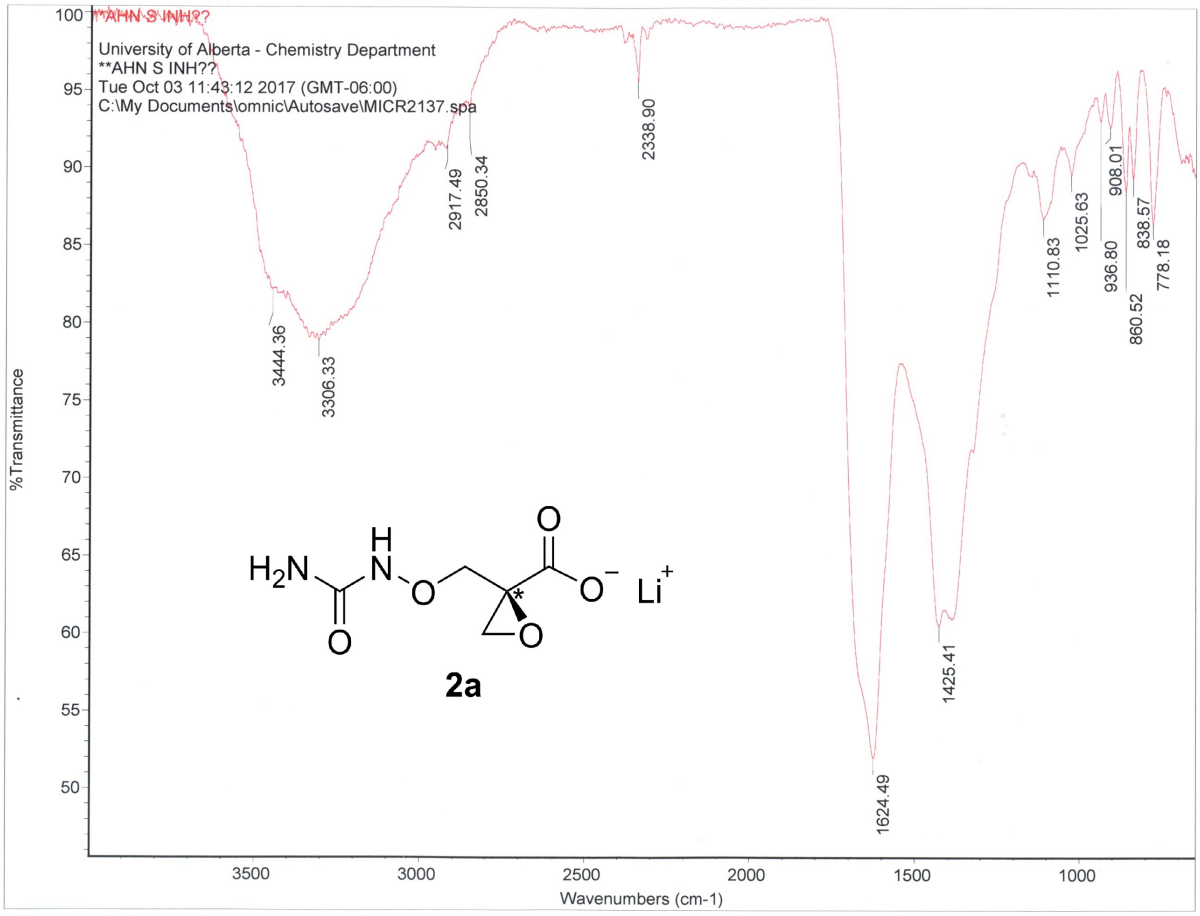
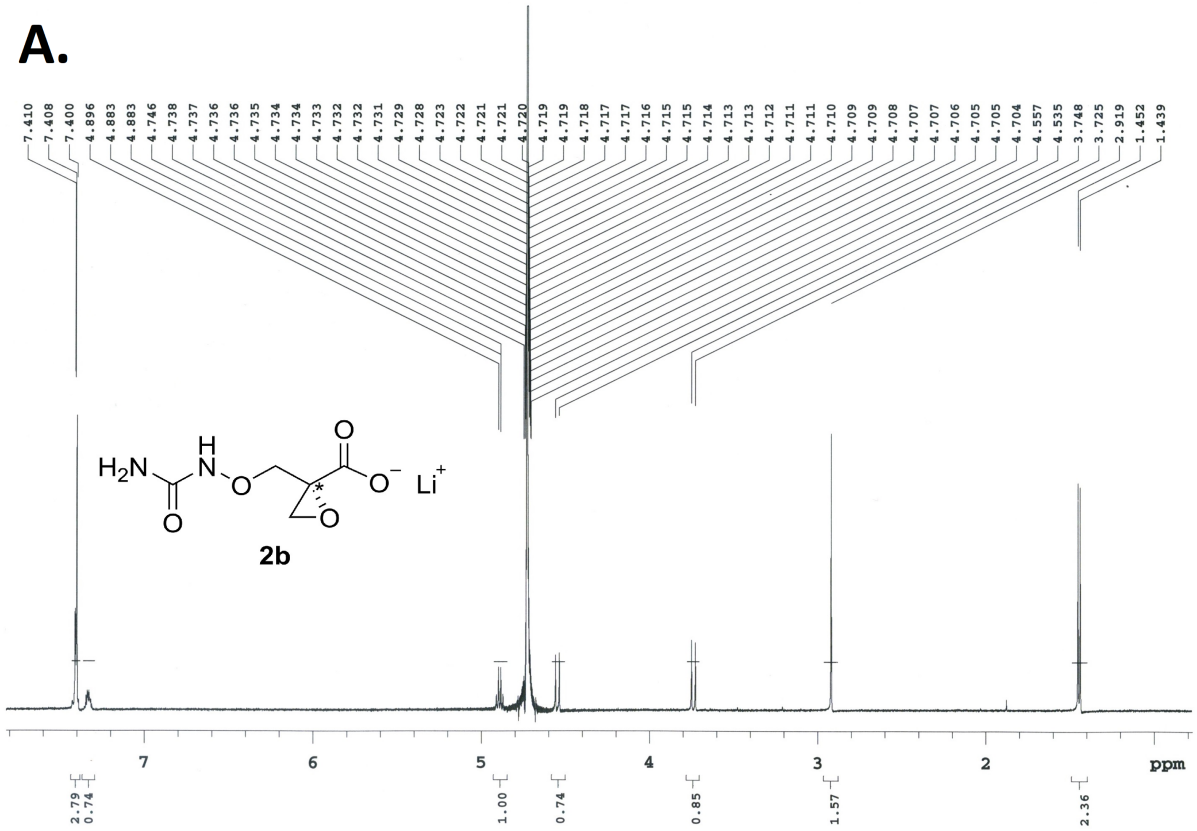
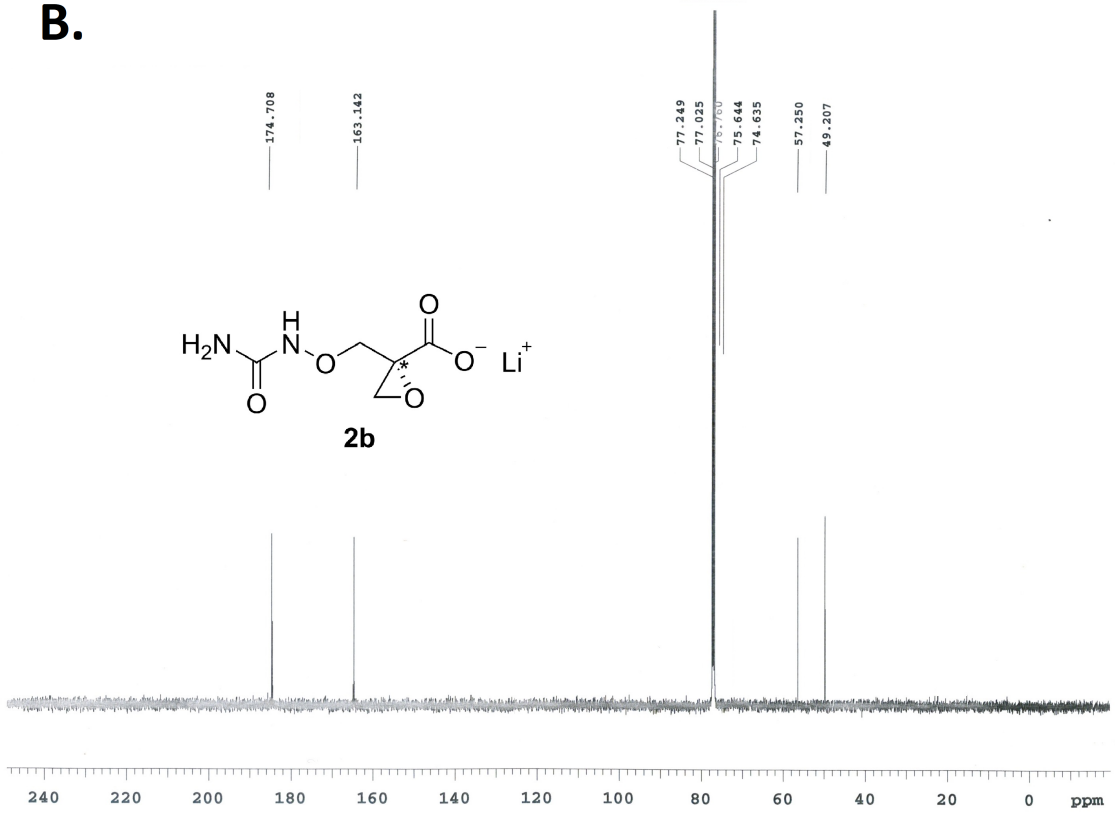


Figure S19. Analytical data of **2a**. A. 1H-NMR. B. 13C-NMR. C. ESI. D. FTIR

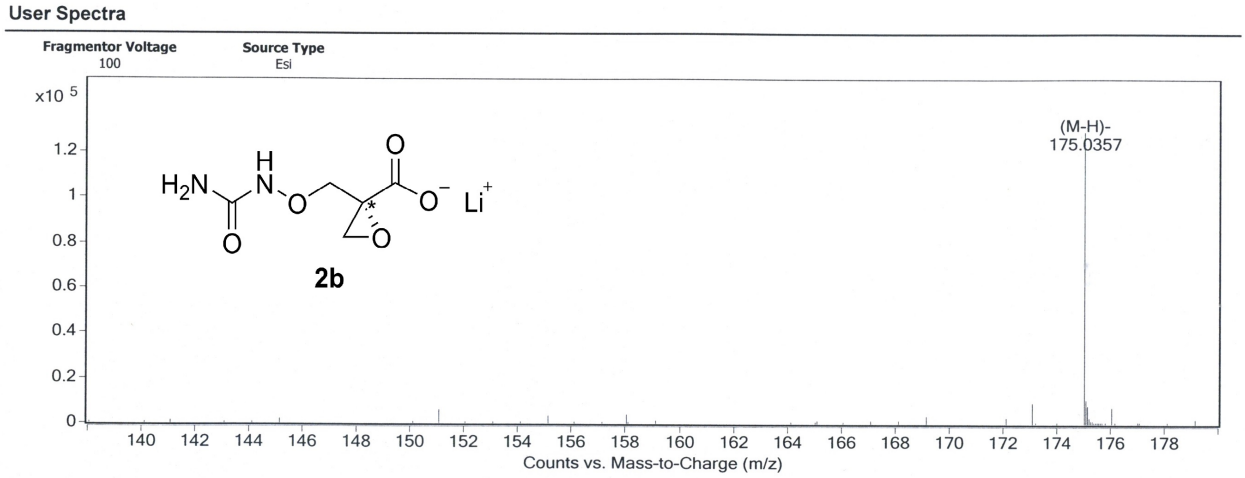
A.


7.410
7.408
7.400
4.896
4.883
4.883
4.746
4.738
4.737
4.736
4.736
4.735
4.734
4.734
4.733
4.732
4.732
4.731
4.729
4.728
4.723
4.722
4.721
4.721
4.720
4.719
4.719
4.718
4.717
4.717
4.716
4.715
4.715
4.714
4.713
4.713
4.712
4.711
4.711
4.710
4.709
4.709
4.708
4.707
4.707
4.706
4.705
4.705
4.704
4.557
4.535
3.748
3.725
2.919
1.452
1.439
H2N
H
N
O
O
O
O
O−
Li+
*
2b
7
6
5
4
3
2
ppm
2.79
0.74
1.00
0.74
0.85
1.57
2.36

B.


174.708
163.142
77.249
77.025
75.644
74.635
57.250
49.207
H2N
H
N
O
O
O
O
O−
Li+
*
2b
240
220
200
180
160
140
120
100
80
60
40
20
0
ppm

C.



Formula Calculator Results

Formula	Ion Species	Mass	Calc. Mass	m/z	Calc. m/z
C5 H8 N2 O5	C5 H7 N2 O5	176.0429	176.0433	175.0357	175.036

D.

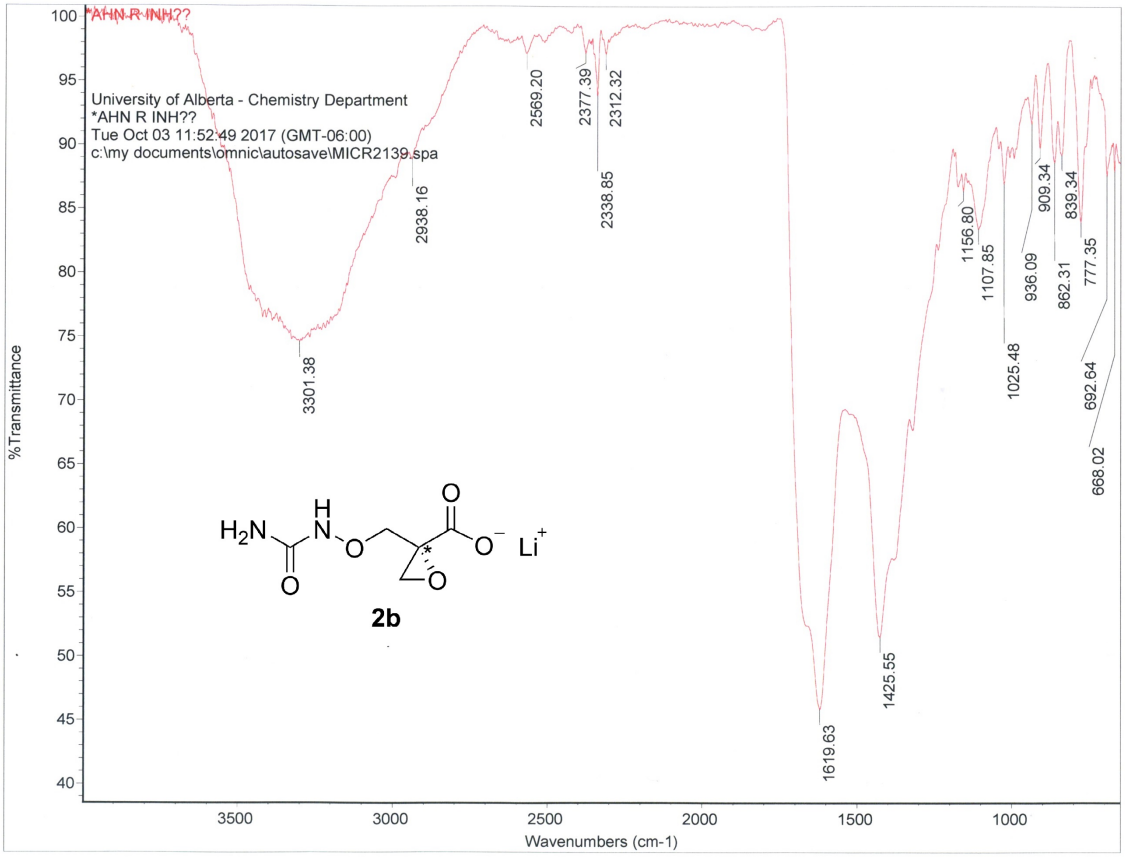


Figure S20. Analytical data of **2b**. A. 1H-NMR. B. 13C-NMR. C. ESI. D. FTIR

Literature:

[1] Programs for diffractometer operation, data collection, data reduction and absorption correction were those supplied by Bruker.

[2] Sheldrick, G. M. *Acta Crystallogr.* **2015**, *A71*, 3–8. (*SHELXT-2014*)

[3] Sheldrick, G. M. *Acta Crystallogr.* **2015**, *C71*, 3–8. (*SHELXL-2014*)

[4] $F_C^* = kF_C[1 + x\{0.001F_C^2\lambda^3/\sin(2\theta)\}]^{-1/4}$ where k is the overall scale factor.

[5] Flack, H. D. *Acta Crystallogr.* **1983**, *A39*, 876–881; Flack, H. D.; Bernardinelli, G. *Acta Crystallogr.* **1999**, *A55*, 908–915; Flack, H. D.; Bernardinelli, G. *J. Appl. Cryst.* **2000**, *33*, 1143–1148. The Flack parameter will refine to a value near zero if the structure is in the correct configuration and will refine to a value near one for the inverted configuration. The low anomalous scattering power of the atoms in this structure (none heavier than oxygen) implies that the data cannot be used for absolute structure assignment, thus the Flack parameter is provided for informational purposes only. The stereochemistry was assigned on the basis of the established stereochemistry of the precursor compound.

[6] $S = [\Sigma w(F_O^2 - F_C^2)^2/(n - p)]^{1/2}$ (n = number of data; p = number of parameters varied; $w = [\sigma^2(F_O^2) + (0.0429P)^2 + 0.0614P]^{-1}$ where $P = [\mathrm{Max}(F_O^2, 0) + 2F_C^2]/3$).

[7] $R_1 = \Sigma||F_O| - |F_C||/\Sigma|F_O|$; $wR_2 = [\Sigma w(F_O^2 - F_C^2)^2/\Sigma w(F_O^4)]^{1/2}$.