Supplemental Information:

Intein-promoted cyclization of aspartic acid flanking the intein leads to atypical N-terminal cleavage

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Figure S1. LC-ESI-MS of precursor and cleavage fragments from N-terminal cleavage of MIHPab. Mass spectra and the deconvolution results of **A.)** uncleaved MIHPab cleavage mutant precursor, **B.)** N-terminal cleavage fragment, and **C.)** C-terminal cleavage fragment. MIHPab cleavage mutant was reacted for 50°C for 16 h at pH 5 and analyzed LC-ESI-MS as described in the *Materials and Methods.*

Figure S2. pH and temperature dependence of *Pab* PolII intein probed by NMR. A. ^{15}N-^{1}H HSQC at pH 4 (blue), 7 (red) and 10 (purple), showing comparable dispersion at each pH. This shows sample stability over a wide range of pH, which is necessary for NMR pK_a determination. **B.** Temperature dependence of the HSQC from 300 K to 330 K. The HSQC pattern remains essentially the same with increasing temperature, indicating the absence of major conformational change induced by temperature.

Figure S3. LC-ESI-MS of N-terminal fragment from N-terminal cleavage of NIHPab. NIHPab cleavage mutant was reacted at 50°C for 16 h at pH 5, with or without 3 mM aniline or 250 mM hydroxylamine, as indicated. **A.** Samples were separated by a C18 column (total ion chromatograms shown), and analyzed by ESI-MS for NIHPab remaining precursor **(B)** or cleavage product **(C)** after incubation without added nucleophile; NIHPab remaining precursor **(D)** or cleavage product **(E)** after incubation with aniline; and NIHPab remaining precursor **(F)** or cleavage product **(G)** after incubation with hydroxylamine.

Figure S4. LC-ESI-MS of C-terminal fragment from N-terminal cleavage of NIHPab. NIHPab cleavage mutant was reacted at 50°C for 16 h at pH 5, with or without 3 mM aniline or 250 mM hydroxylamine, as indicated. Samples were separated by a C18 column (extracted ion chromatograms shown) and analyzed by ESI-FT-MS for the C-terminal product of cleavage after incubation without added nucleophile **(A)**, with aniline **(B)**, or with hydroxylamine **(C)**.

Figure S5. Time dependence of N-terminal cleavage of NIHPab. NIHPab was incubated at 60°C for the times indicated in buffer C5 as described in the *Materials and Methods*. In B, aniline was added to 3 mM, and in C, hydroxylamine was added to 50 mM, with the pH adjusted as needed to 5.0. We analyzed the cleavage via SDS-PAGE and estimated the relative concentration via densitometry using ImageJ[1]. We fit the data to a first order rate law using Kaleidagraph.

Table S1. Oligonucleotides used in mutagenesis reactions.

Table S2. Observed *m/z* values for the C-terminal cleavage products of the NIHPab cleavage mutant.

Figure S1A

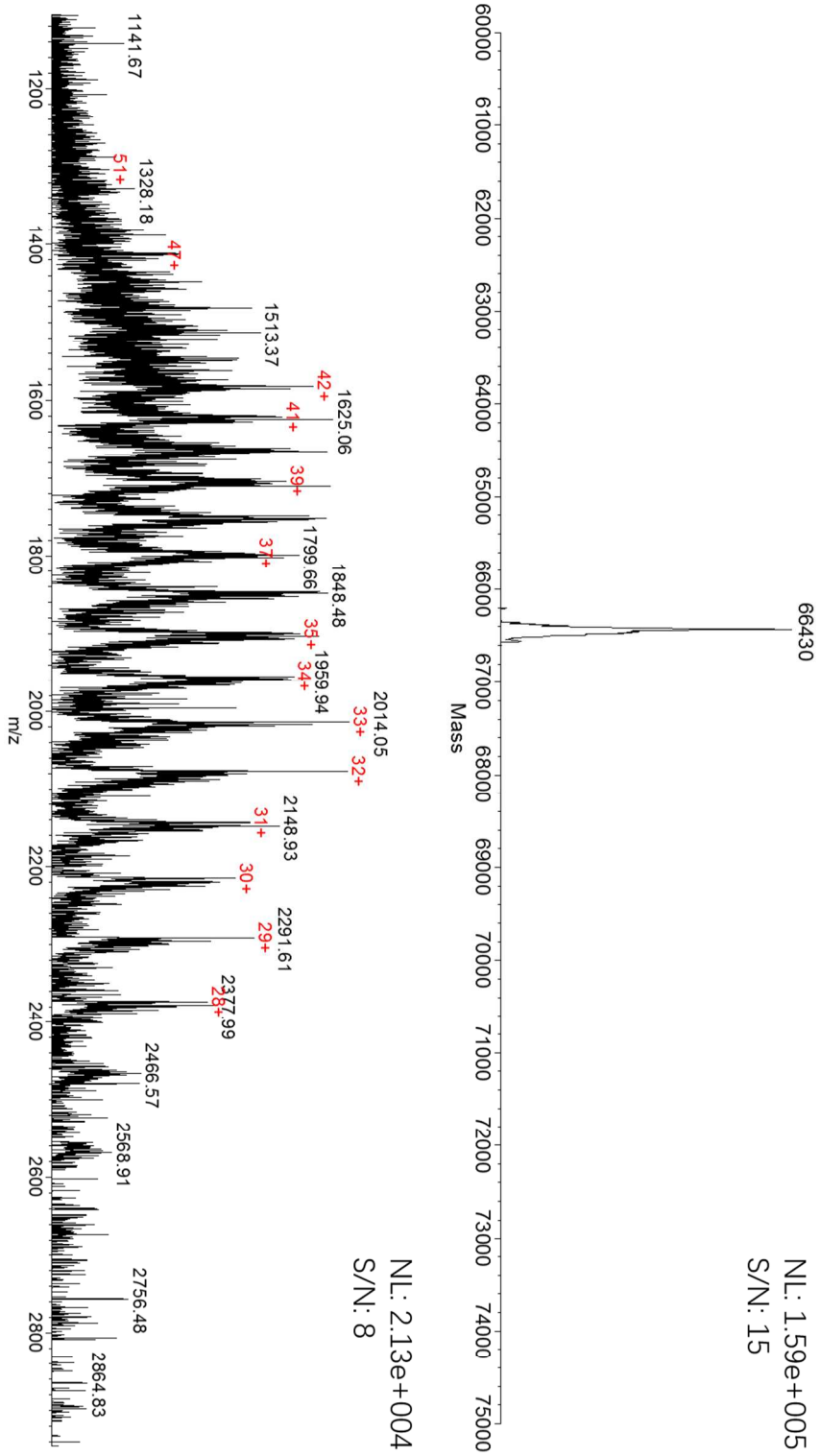
66430
NL: 1.59e+005
S/N: 15
Mass
NL: 2.13e+004
S/N: 8
m/z
1141.67
1328.18
51+
47+
1513.37
1625.06
42+
41+
39+
1799.66
37+
1848.48
35+
1959.94
34+
2014.05
33+
32+
2148.93
31+
30+
2291.61
29+
2377.99
28+
2466.57
2568.91
2756.48
2864.83

Figure S1B

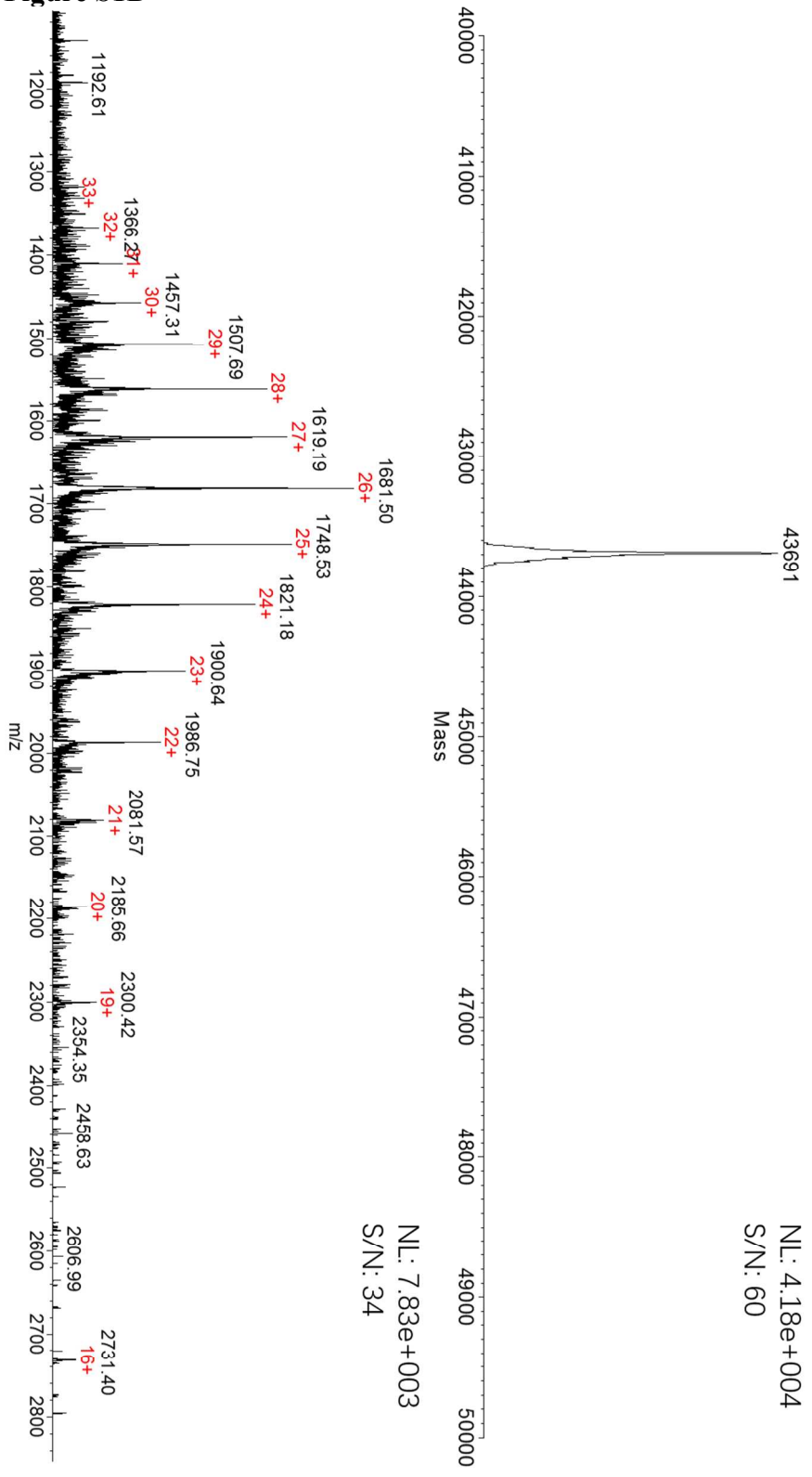
NL: 4.18e+004
S/N: 60
43691
Mass
NL: 7.83e+003
S/N: 34
1192.61
33+
1366.27
32+
31+
1457.31
30+
1507.69
29+
28+
1619.19
27+
1681.50
26+
1748.53
25+
1821.18
24+
1900.64
23+
1986.75
22+
2081.57
21+
2185.66
20+
2300.42
19+
2354.35
2458.63
2606.99
2731.40
16+
m/z

Figure S1C

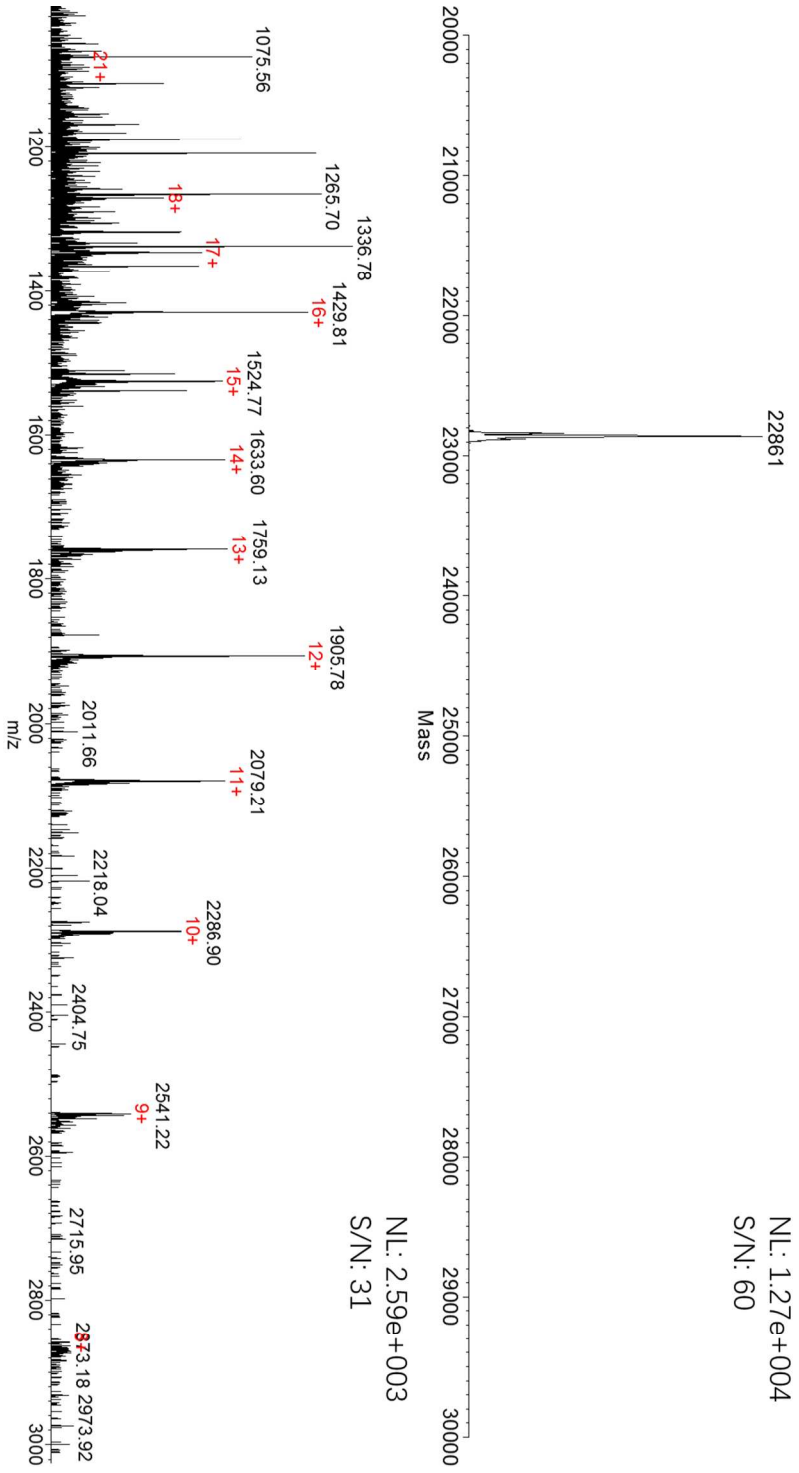
22861
NL: 1.27e+004
S/N: 60
Mass
NL: 2.59e+003
S/N: 31
m/z
1075.56
21+
1265.70
18+
1336.78
17+
1429.81
16+
1524.77
15+
1633.60
14+
1759.13
13+
1905.78
12+
2011.66
2079.21
11+
2218.04
2286.90
10+
2404.75
2541.22
9+
2715.95
2873.18
2973.92

Figure S2A.

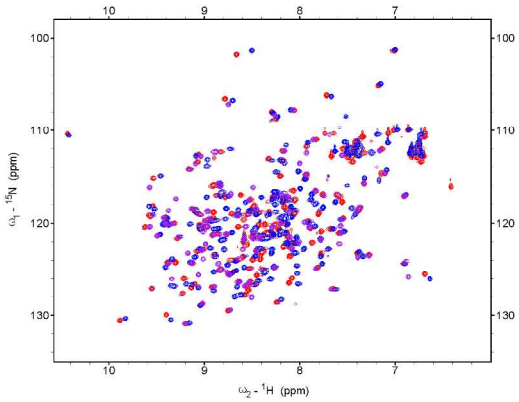

Figure S2B.

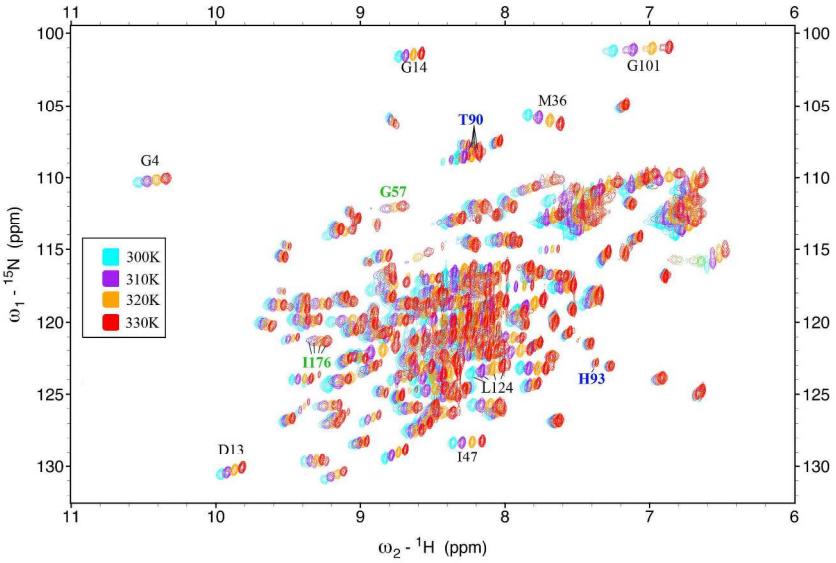

Figure S3A.

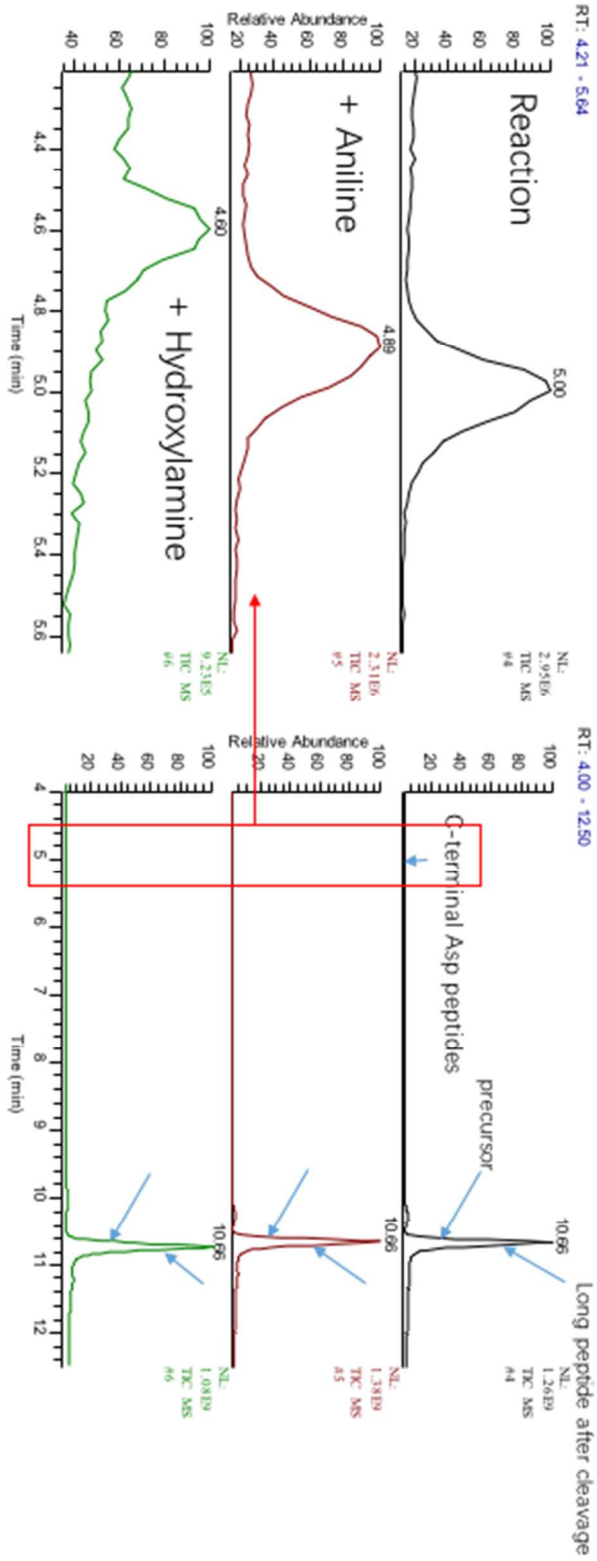
RT: 4.21 - 5.64
Relative Abundance
Reaction
+ Aniline
+ Hydroxylamine
5.00
4.89
4.60
NL: 2.95E6 TIC MS #4
NL: 2.31E6 TIC MS #5
NL: 9.23E5 TIC MS #6
Time (min)
RT: 4.00 - 12.50
C-terminal Asp peptides
precursor
Long peptide after cleavage
10.66
NL: 1.26E9 TIC MS #4
NL: 1.38E9 TIC MS #5
NL: 1.08E9 TIC MS #6

Figure S3B.

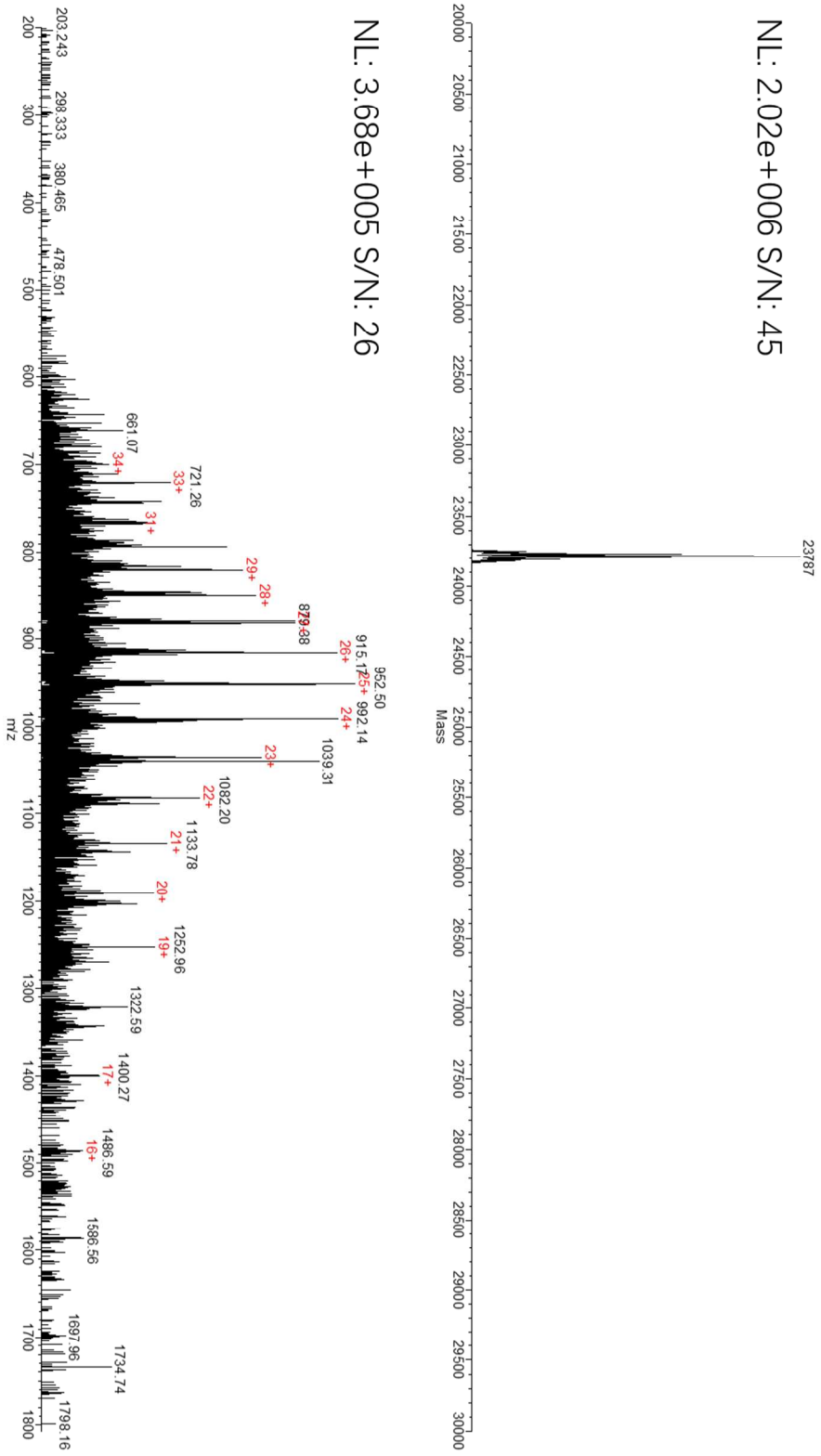
NL: 2.02e+006 S/N: 45
23787
Mass
NL: 3.68e+005 S/N: 26
203.243
298.333
380.465
478.501
661.07
34+
721.26
33+
31+
29+
28+
879.88
26+
915.12
952.50
25+
992.14
24+
1039.31
23+
1082.20
22+
1133.78
21+
20+
1252.96
19+
1322.59
1400.27
17+
1486.59
16+
1586.56
1697.96
1734.74
1798.16
m/z

Figure S3C.

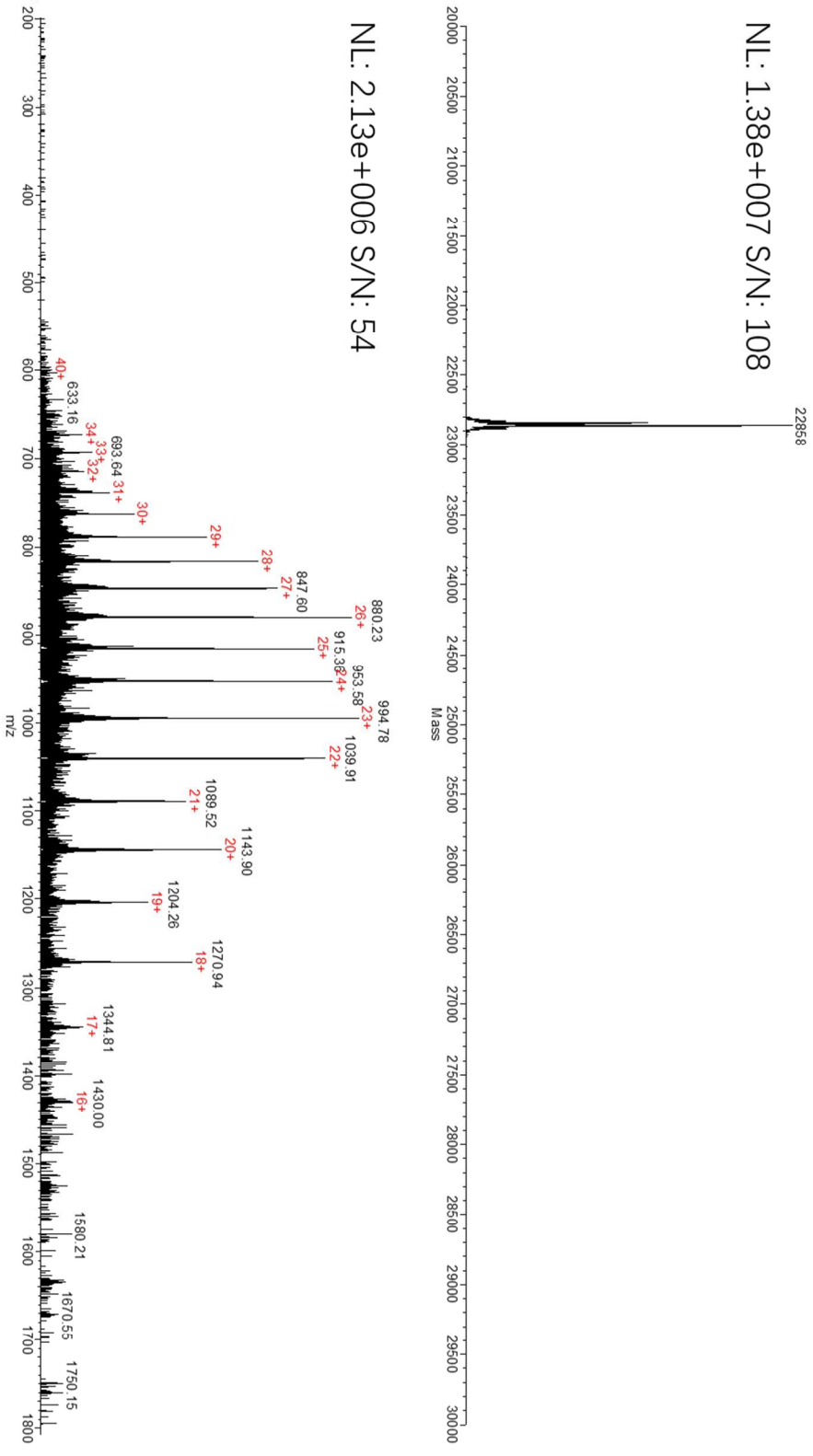
NL: 1.38e+007 S/N: 108
22858
Mass
NL: 2.13e+006 S/N: 54
633.16
683.64
847.60
880.23
915.38
953.58
994.78
1039.91
1089.52
1143.90
1204.26
1270.94
1344.81
1430.00
1580.21
1670.55
1750.15
m/z

Figure S3D.

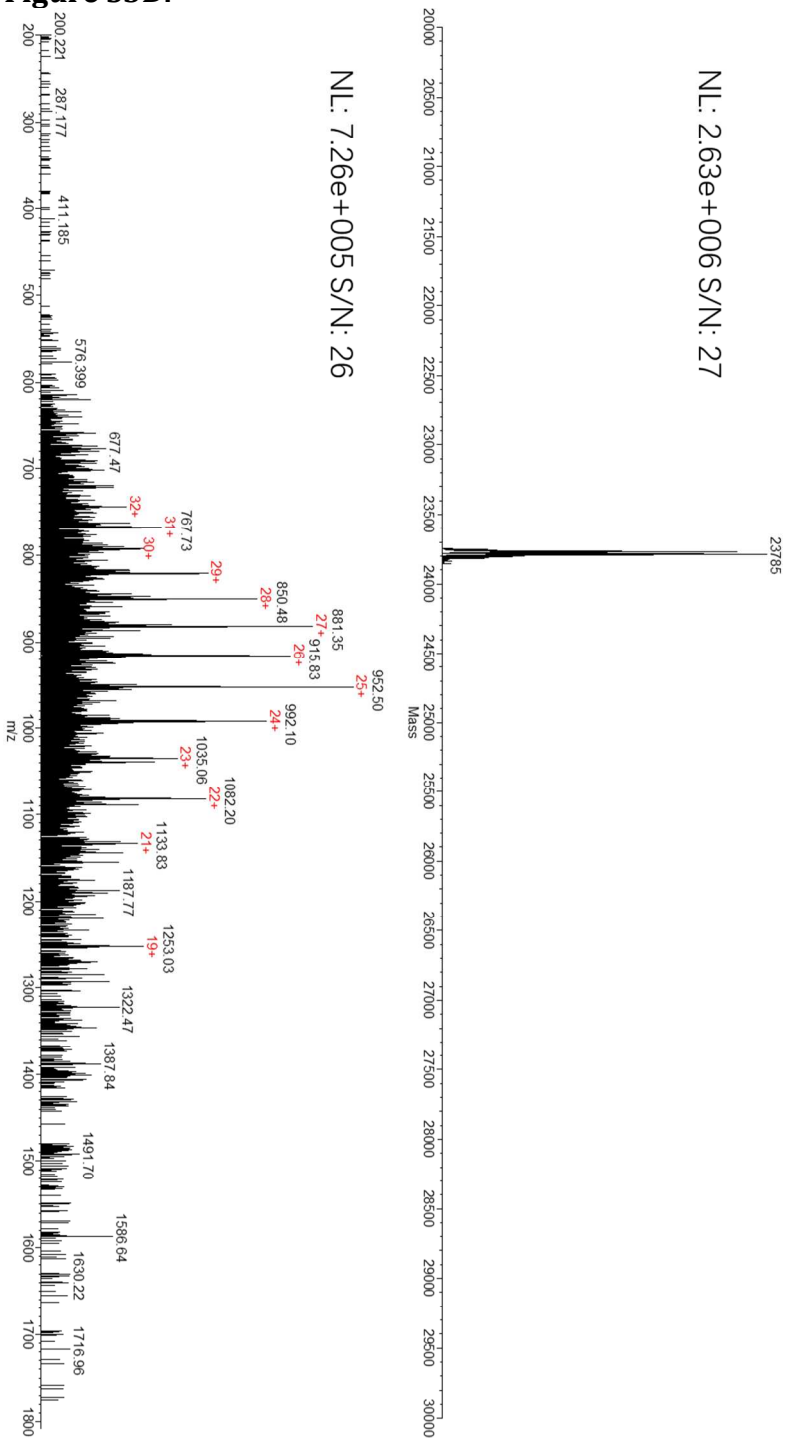
NL: 2.63e+006 S/N: 27
23785
Mass
NL: 7.26e+005 S/N: 26
200.221
287.177
411.185
576.399
677.47
767.73
32+
31+
30+
29+
850.48
28+
881.35
27+
915.83
26+
952.50
25+
992.10
24+
1035.06
23+
1082.20
22+
1133.83
21+
1187.77
1253.03
19+
1322.47
1387.84
1491.70
1586.64
1630.22
1716.96
m/z

Figure S3E.

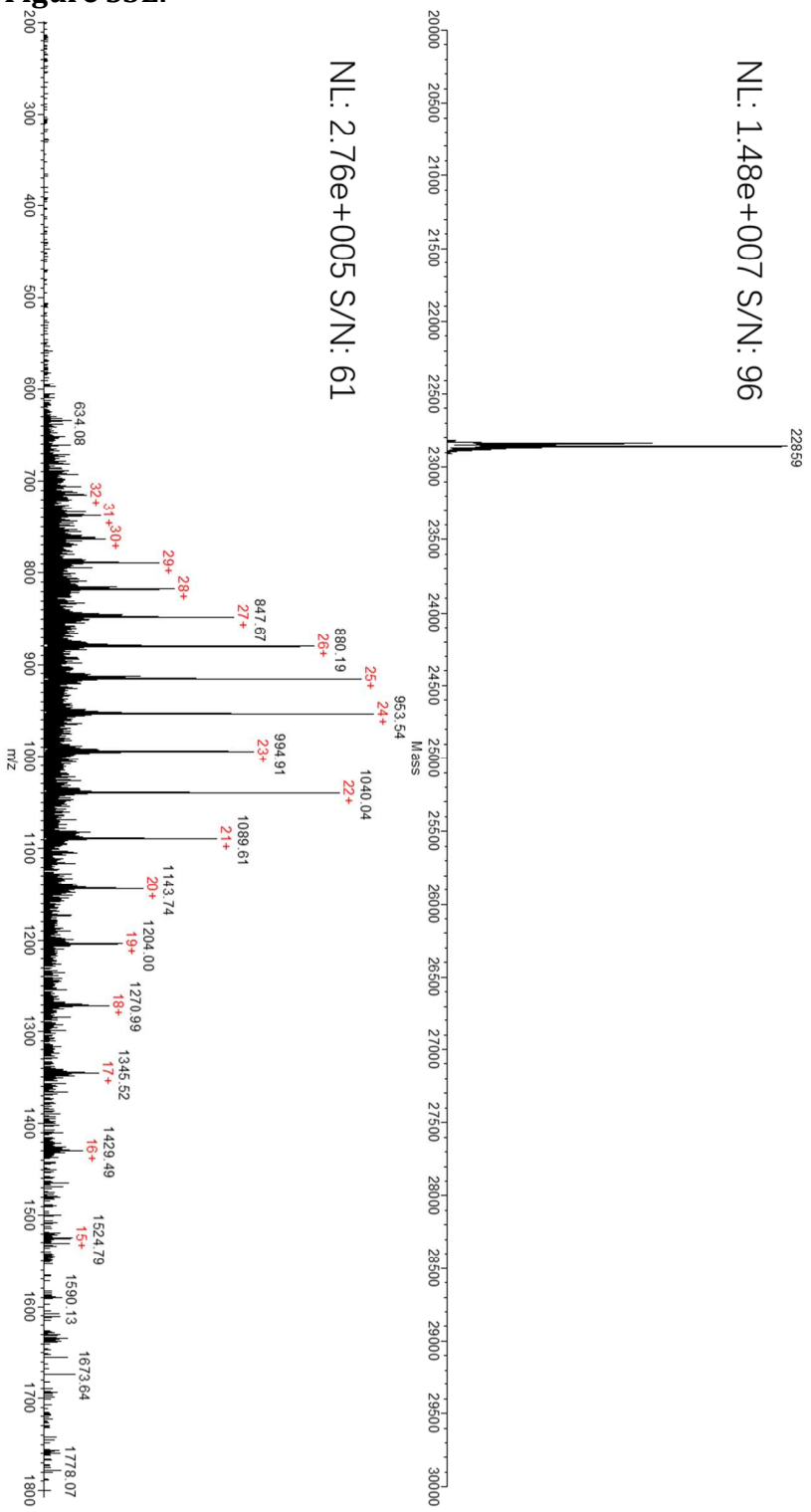
NL: 1.48e+007 S/N: 96
22859
Mass
NL: 2.76e+005 S/N: 61
634.08
32+
31+
30+
29+
28+
847.67
27+
680.19
26+
25+
953.54
24+
994.91
23+
1040.04
22+
1089.61
21+
1143.74
20+
1204.00
19+
1270.99
18+
1345.52
17+
1429.49
16+
1524.79
15+
1590.13
1673.64
1778.07
m/z

Figure S3F.

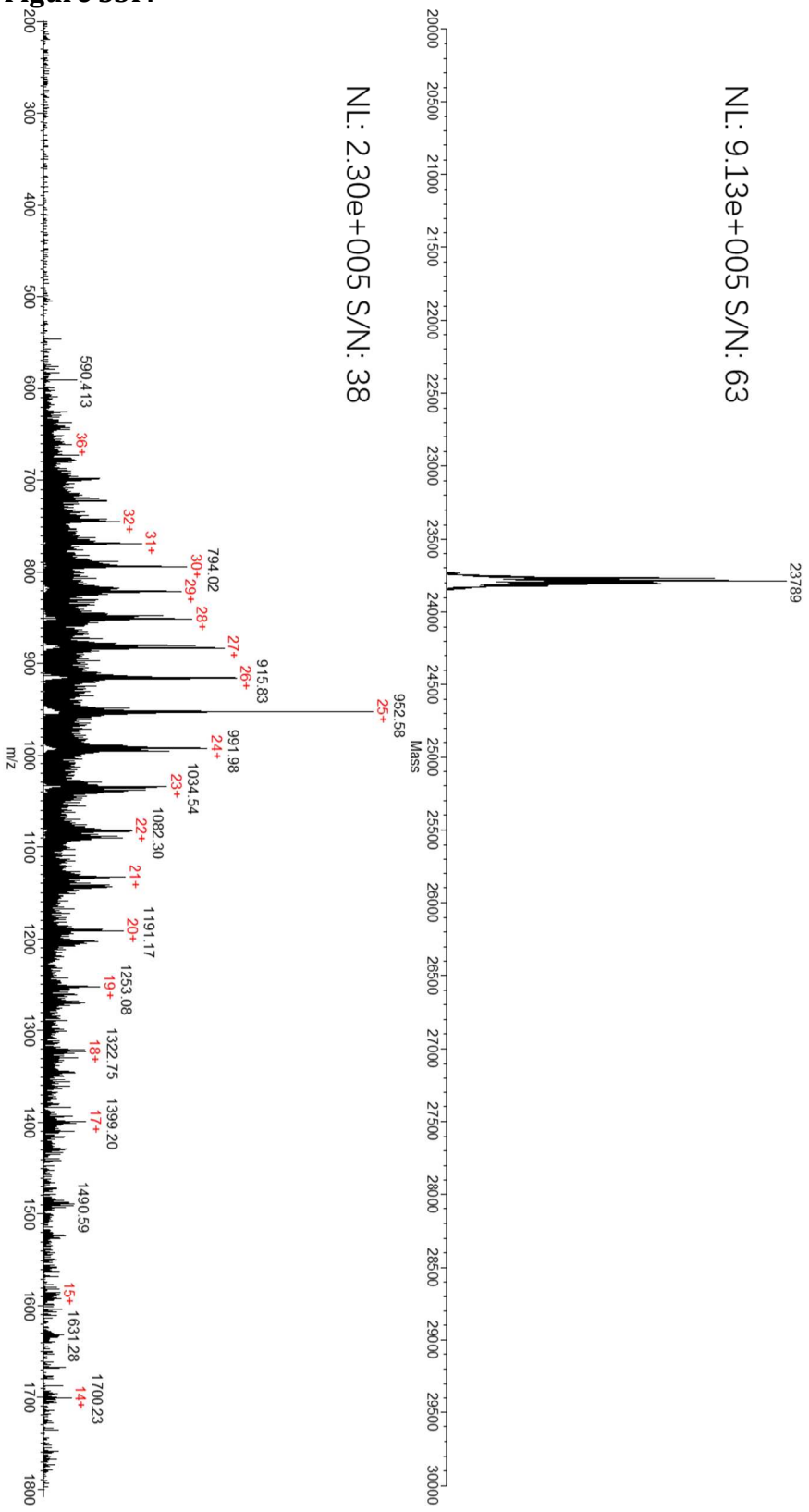
NL: 9.13e+005 S/N: 63
23789
Mass
NL: 2.30e+005 S/N: 38
590.413
36+
32+
31+
794.02
30+
29+
28+
27+
26+
915.83
952.58
25+
991.98
24+
1034.54
23+
1082.30
22+
21+
1191.17
20+
1253.08
19+
1322.75
18+
1399.20
17+
1490.59
15+
1631.28
1700.23
14+
m/z

Figure S3G.

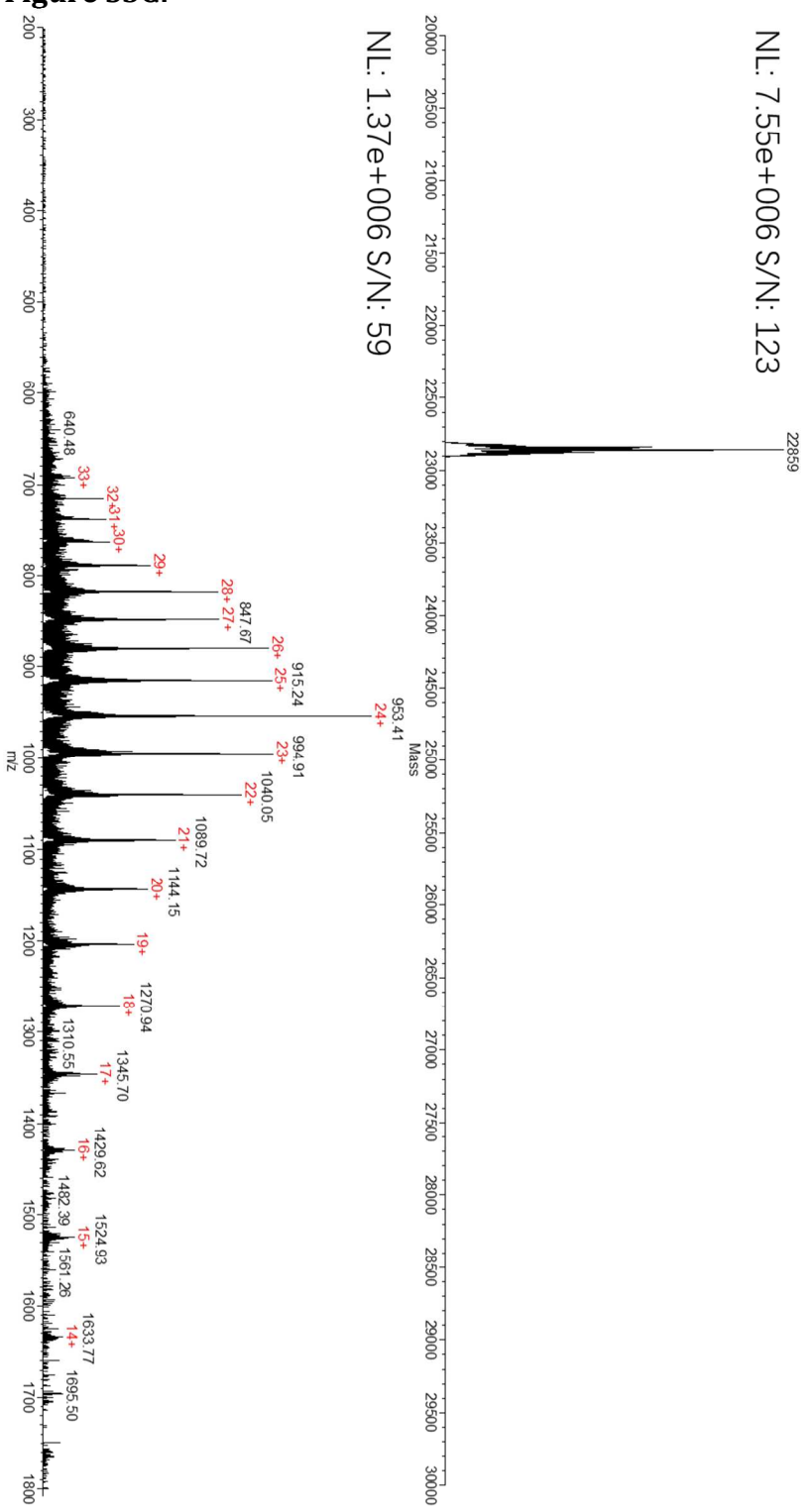
NL: 7.55e+006 S/N: 123
22859
Mass
NL: 1.37e+006 S/N: 59
953.41
24+
915.24
25+
26+
847.67
28+
27+
29+
32+
31+
30+
33+
640.48
994.91
23+
1040.05
22+
1089.72
21+
1144.15
20+
19+
1270.94
18+
1310.55
1345.70
17+
1429.62
16+
1482.39
1524.93
15+
1561.26
1633.77
14+
1695.50
m/z

Figure S4A.

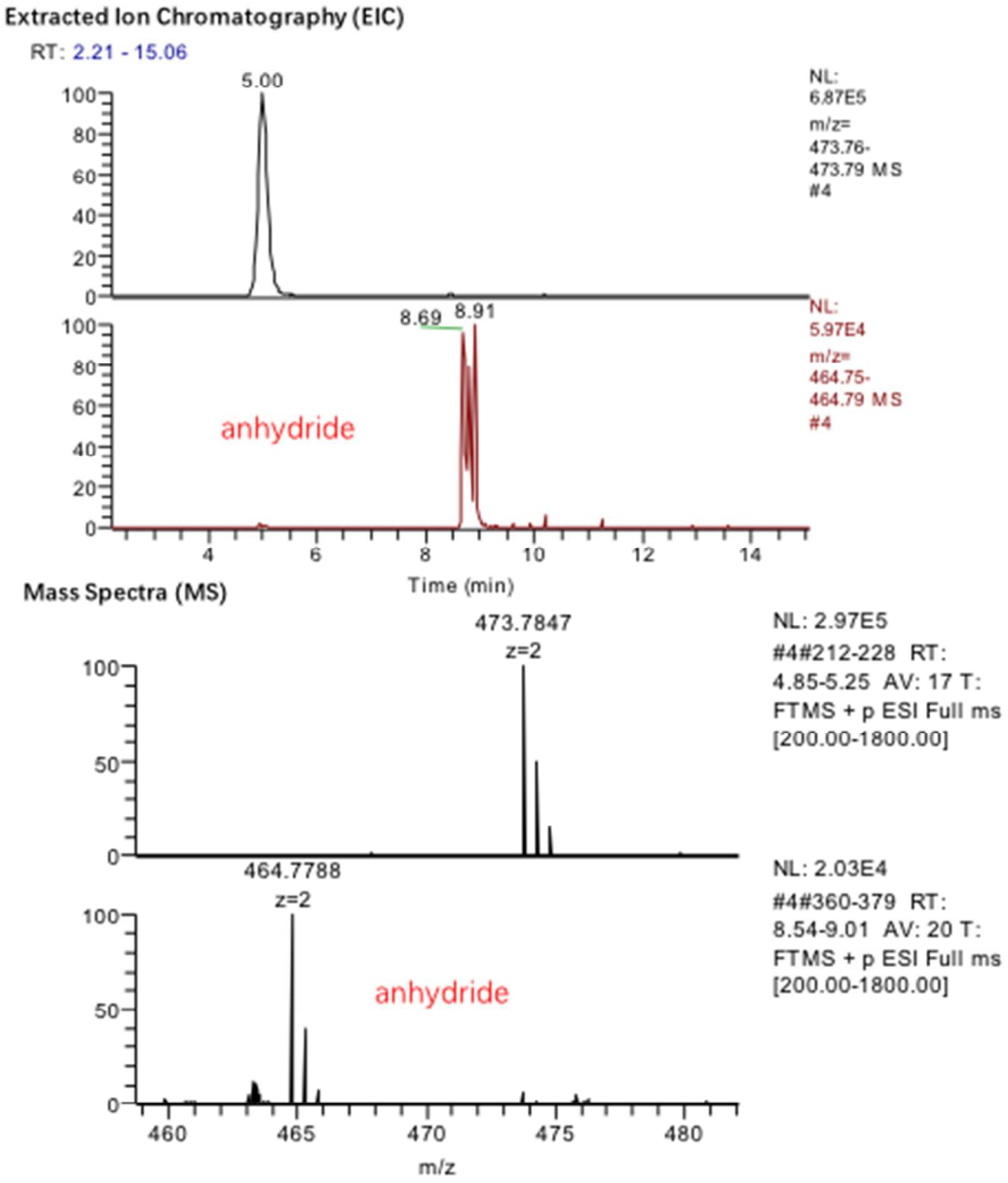
Extracted Ion Chromatography (EIC)
RT: 2.21 - 15.06
5.00
NL:
6.87E5
m/z=
473.76-
473.79 MS
#4
8.69
8.91
NL:
5.97E4
m/z=
464.75-
464.79 MS
#4
anhydride
Time (min)
Mass Spectra (MS)
473.7847
z=2
NL: 2.97E5
#4#212-228 RT:
4.85-5.25 AV: 17 T:
FTMS + p ESI Full ms
[200.00-1800.00]
464.7788
z=2
NL: 2.03E4
#4#360-379 RT:
8.54-9.01 AV: 20 T:
FTMS + p ESI Full ms
[200.00-1800.00]
anhydride
m/z

Figure S4B.

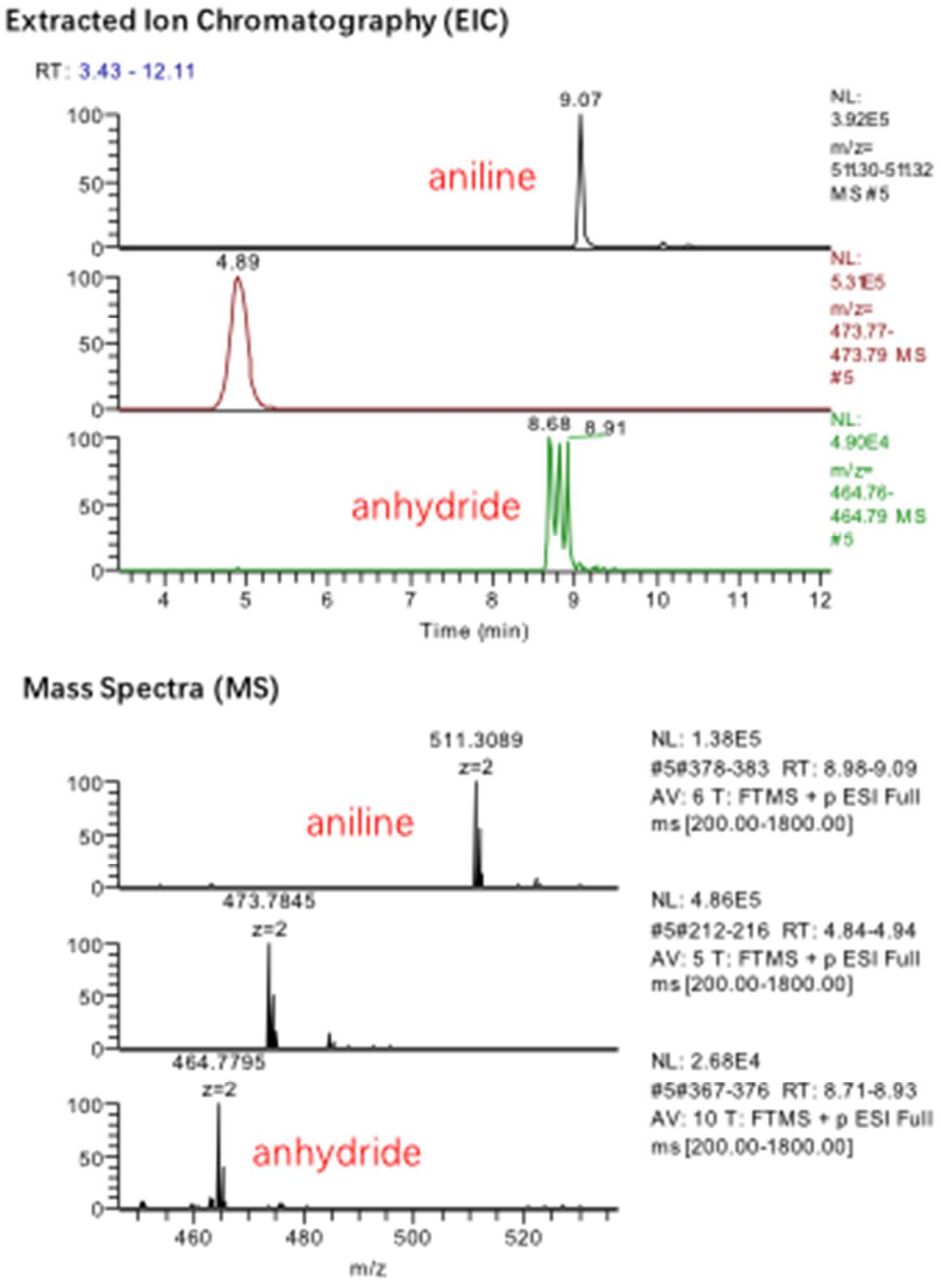
Extracted Ion Chromatography (EIC)
RT: 3.43 - 12.11
9.07
aniline
NL: 3.92E5 m/z= 511.30-511.32 MS #5
4.89
NL: 5.31E5 m/z= 473.77-473.79 MS #5
8.68 8.91
anhydride
NL: 4.90E4 m/z= 464.76-464.79 MS #5
Time (min)
Mass Spectra (MS)
511.3089
z=2
aniline
NL: 1.38E5
#5#378-383 RT: 8.98-9.09
AV: 6 T: FTMS + p ESI Full ms [200.00-1800.00]
473.7845
z=2
NL: 4.86E5
#5#212-216 RT: 4.84-4.94
AV: 5 T: FTMS + p ESI Full ms [200.00-1800.00]
464.7795
z=2
anhydride
NL: 2.68E4
#5#367-376 RT: 8.71-8.93
AV: 10 T: FTMS + p ESI Full ms [200.00-1800.00]
m/z

Figure S4C.

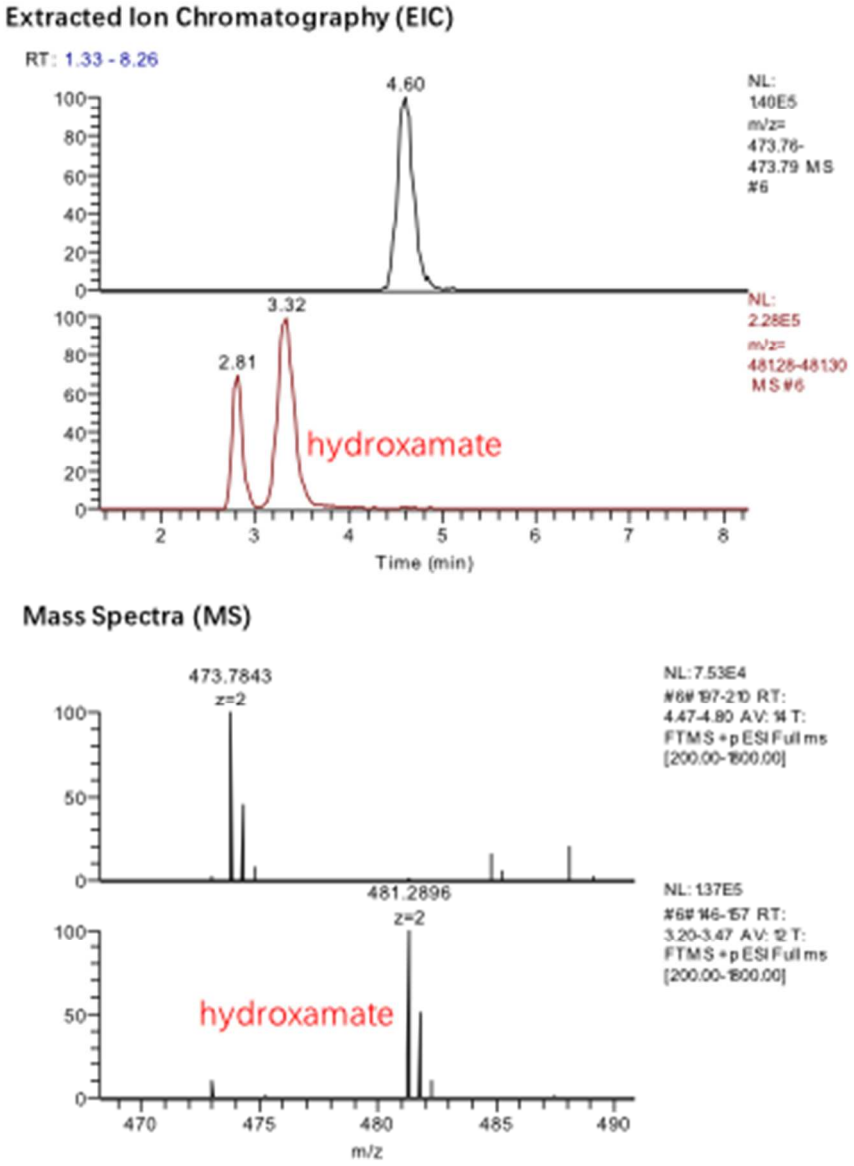
Extracted Ion Chromatography (EIC)
RT: 1.33 - 8.26
4.60
NL:
140E5
m/z=
473.76-
473.79 MS
#6
3.32
2.81
hydroxamate
NL:
2.28E5
m/z=
48128-48130
MS #6
Time (min)
Mass Spectra (MS)
473.7843
z=2
NL: 7.53E4
#6# 197-210 RT:
4.47-4.80 AV: 14 T:
FTMS + p ESI Full ms
[200.00-1800.00]
481.2896
z=2
NL: 137E5
#6# 146-157 RT:
3.20-3.47 AV: 12 T:
FTMS + p ESI Full ms
[200.00-1800.00]
hydroxamate
m/z

Figure S5.

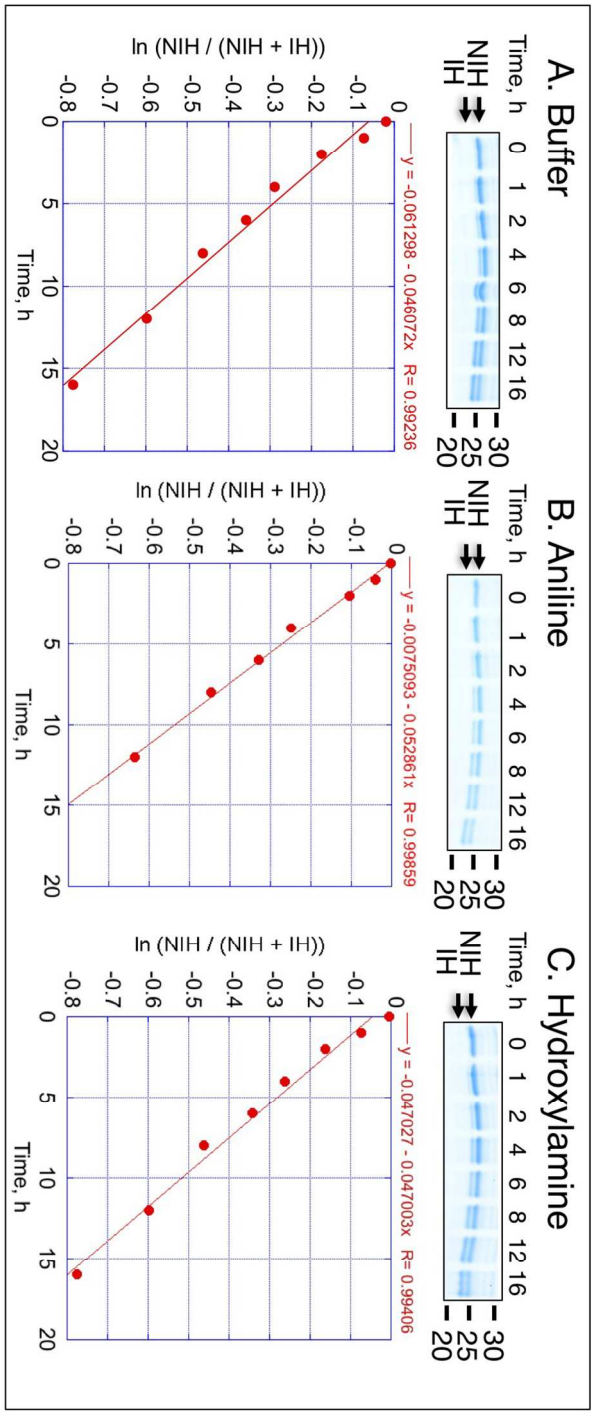
A. Buffer
B. Aniline
C. Hydroxylamine
Time, h
0 1 2 4 6 8 12 16
NIH
IH
30
25
20
ln (NIH / (NIH + IH))
Time, h
y = -0.061298 - 0.046072x R= 0.99236
y = -0.0075093 - 0.052861x R= 0.99859
y = -0.047027 - 0.047003x R= 0.99406

Table S1. Oligonucleotides used in mutagenesis for this publication (others previously described, including C1A mutation.)

Mutation	Oligonucleotides used
T90A	Upper: 5'-GATGGAAGAAGTTTTGAAACCGCCGTAGATCATCCAGTTTTAG Lower: 5'-CTAAAACTGGATGATCTACGGCGGTTTCAAAACTTCTTCCATC
H93A	Upper: 5'-ATGGAAGAAGTTTTGAAACCACCGTAGATGCTCCAGTTTTAGTTTATGAAA Lower: 5'-TTTCATAAACTAAAACTGGAGCATCTACGGTGGTTTCAAAACTTCTTCCAT
T90A/H93A	Upper: 5'-GGAAGAAGTTTTGAAACCGCCGTAGATGCTCCAGTTTTAG Lower: 5'-CTAAAACTGGAGCATCTACGGCGGTTTCAAAACTTCTTCC
C1A/N-1D	Upper: 5'-CATGCTGCGAAGAGGAGAGACGCCTTCCCGGGTGATACTAG Lower: 5'-CTAGTATCACCCGGGAAGGCGTCTCTCCTCTTCGCAGCATG
C1A/N-1E	Upper: 5'-TGAAGCTTAAGAGGAGAGAGGCCTTCCCGG Lower: 5'-CCGGGAAGGCCTCTCTCCTCTTAAGCTTCA
C1A/N-1Q	Upper: 5'-ATAGCATATGAAGCTTAAGAGGAGACAAGCCTTCCCGGGTG Lower: 5'-CACCCGGGAAGGCTTGTCTCCTCTTAAGCTTCATATGCTAT

Table S2. Observed *m/z* values for the C-terminal cleavage products of the NIHPab cleavage mutant.

	Components	*m/z* observed	charge state	Experimental Mw_{mono}	Theoretical Mw_{mono}	Error (ppm)
A	Asp	473.7847	2	945.5537	945.5542	-0.49
	Anhydride	464.7788	2	927.5419	927.5436	-1.79
B	Asp	473.7845	2	945.5533	945.5542	-0.91
	Anhydride	464.7795	2	927.5433	927.5436	-0.28
	Aniline	511.3089	2	1020.6021	1020.6015	0.63
C	Asp	473.7843	2	945.5529	945.5542	-1.33
	Hydroxamate	481.2896	2	960.5635	960.5651	-1.62

References

[1] Abramoff, M. D., Magalhaes, P. J., and Ram, S. J. (2004) Image Processing with ImageJ, *Biophotonics International 11*, 36-42.