Supplementary Materials for

Origin of life: protoribosome forms peptide bonds and links RNA and protein dominated worlds

Authors: Tanaya Bose[1], Gil Fridkin[1,2] Chen Davidovich[1]†, Miri Krupkin[1]‡, Nikita Dinger[1§], Alla Falkovich[1], Yoav Peleg[1], Ilana Agmon[3, 4], Anat Bashan[1], Ada Yonath[1*]

Correspondence to: ada.yonath@weizmann.ac.il

This PDF file includes:

Supplementary Text
Figs. S1 to S21
Tables S1

Figure S1: A two-dimensional representation of the RNA constructs designed based on part of *T. thermophilus* 23S rRNA around the PTC. The nucleotides highlighted in yellow were used as the sequences of the RNA constructs tt_A1, tt_P1, tt_A1P1, tt_A2, tt_P2 and tt_A2P2, respectively. Green stretches indicate direct connection between nucleotides and green lines represents positions where the hyper stable CUUCGG hairpin was inserted. rRNA helices and nucleotide numbers (E. coli numbering) are designated in blue and black, respectively. A-loop and P-loop are pointed with red arrows.

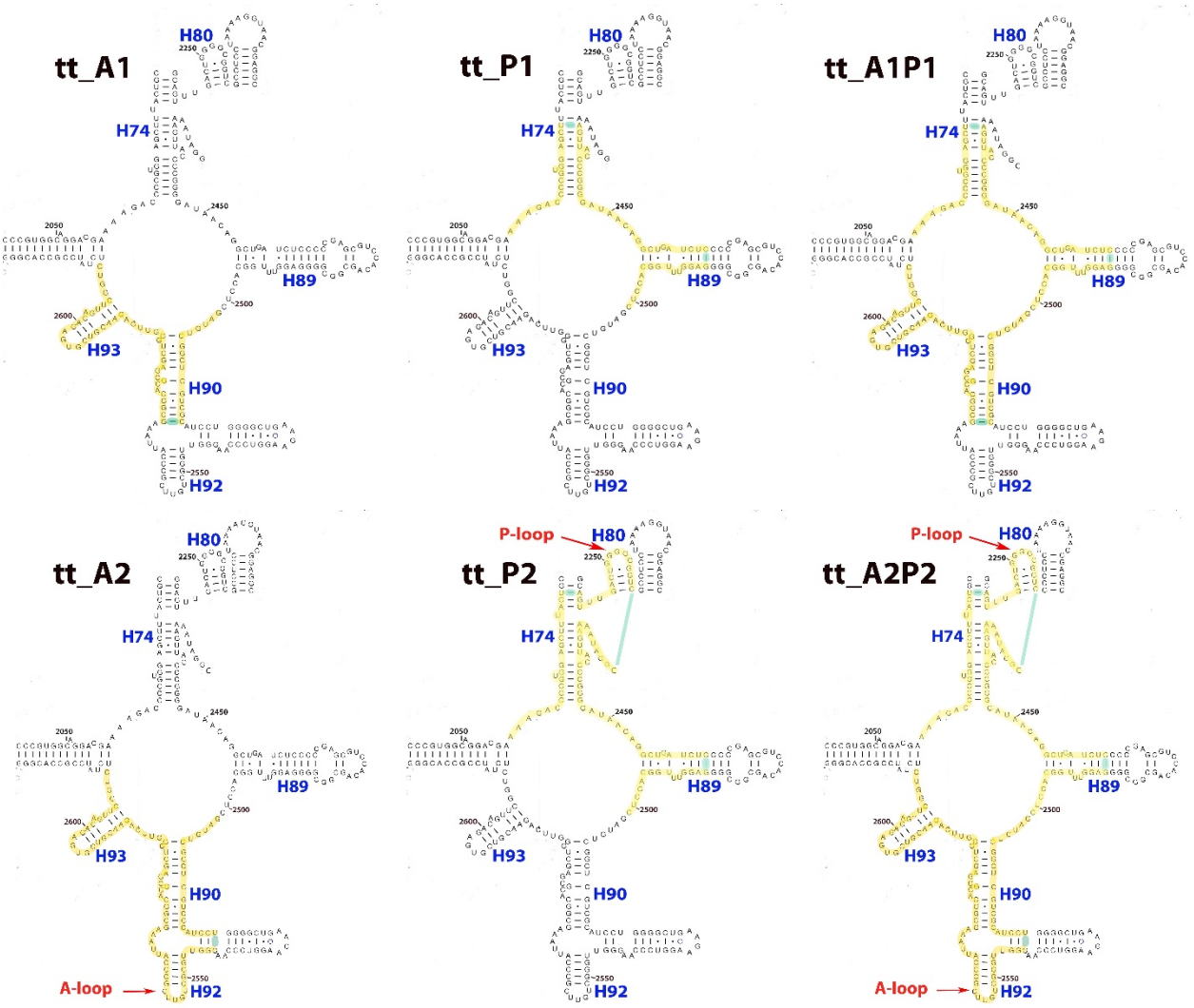


Figure S2: RNA constructs sequence alignment (G- red, A- green, C- orange and U- blue). **A**. The alignment of A-reg short and long sequences. **B.** The alignment of P-reg minimal and extended sequences. GUGA addition to tt_P1c and tt_P2c is marked with a yellow box. **C**. The alignment of long sequences which represent the full proto-ribosome. **D**. The alignment of various P-reg minimal sequences. rRNA sequences were aligned using Clustal omega server (Sievers, Wilm et al. 2011) and imaged using Jalview (Waterhouse, Procter et al. 2009).

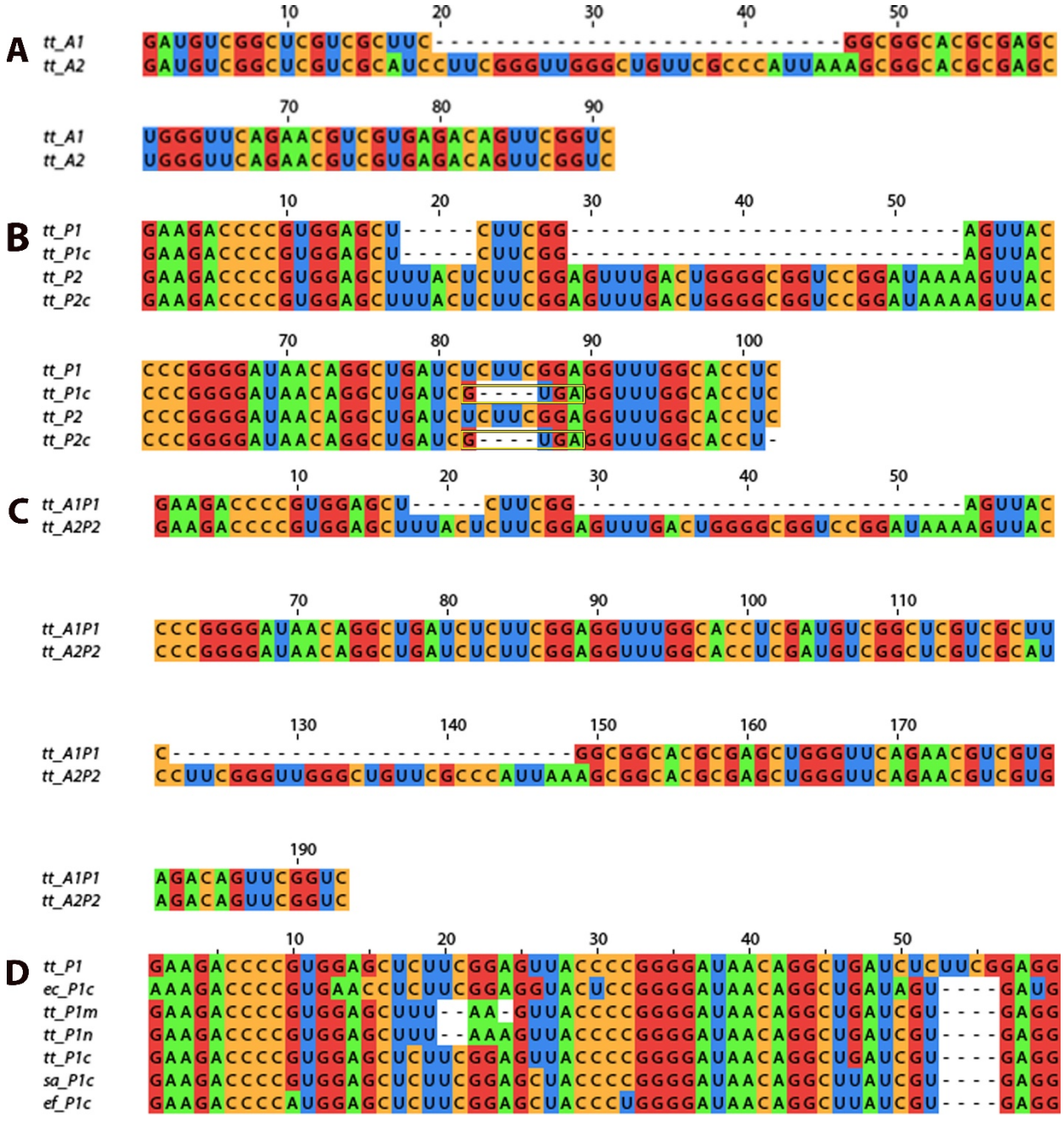


Figure S3: Design schemes of the constructs tt_P1c, tt_P1n and tt_P1m. **A.** Incorporation of a GNRA tetraloop in construct tt_P1 afforded tt_P1c. **B.** Constructs tt_P1n and tt_P1m design. Natural shorter UUAA and UUA loops were added to H74 as shown in the upper image.

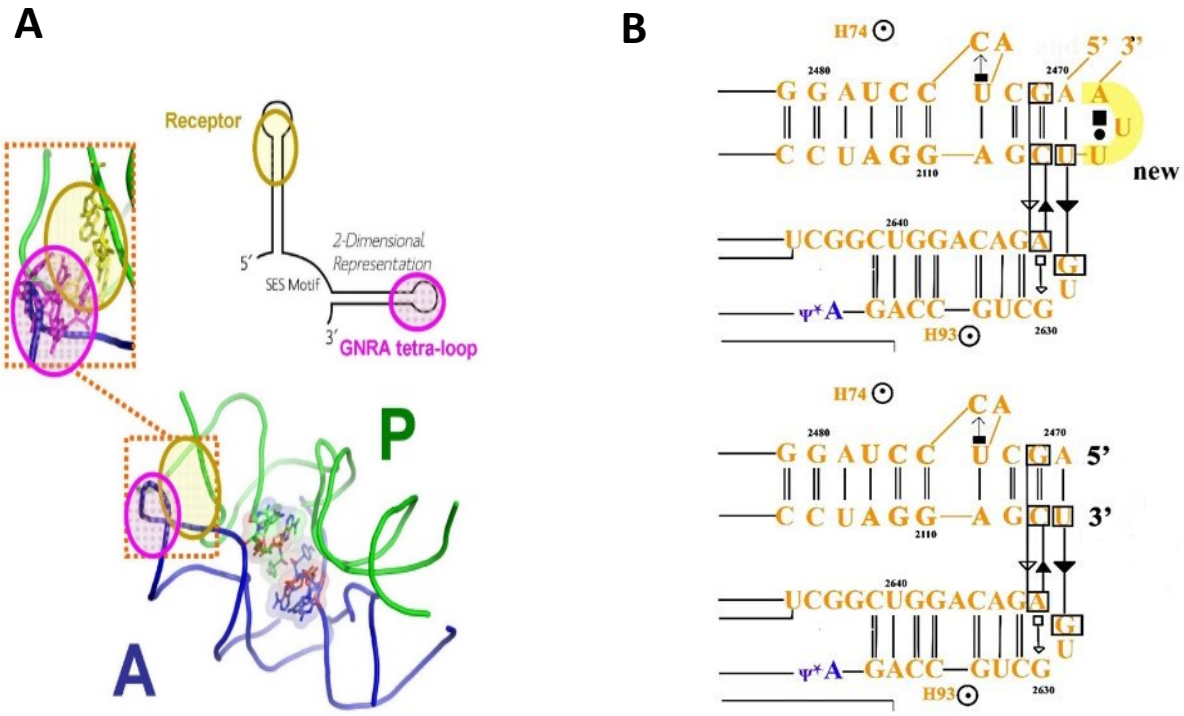


Figure S4: 2D structure predictions of the protoribosome constructs. All predictions were calculated using mfold (*66*) where ΔG values are refined at 37^0C.

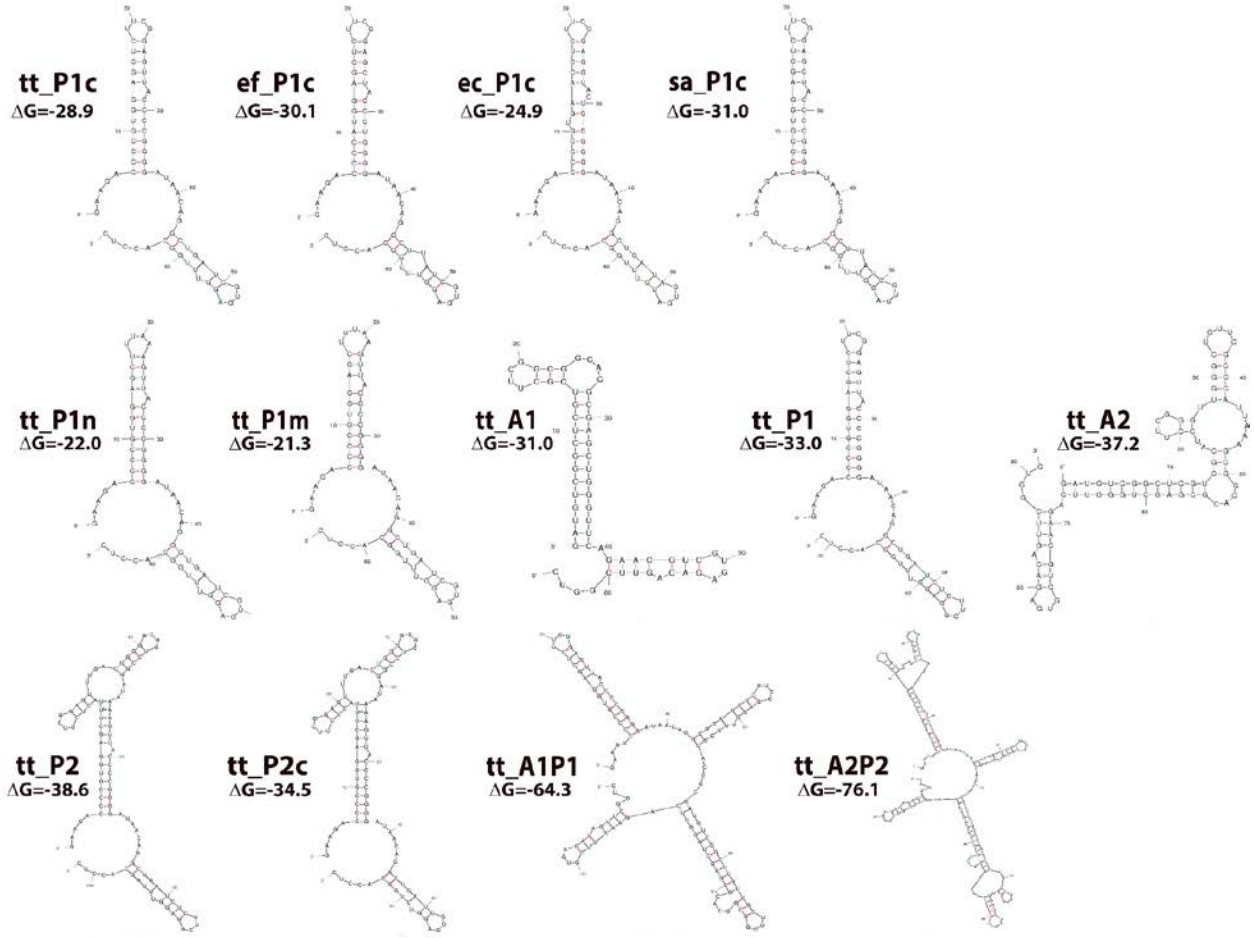


Figure S5: 2D structure predictions of the protoribosome constructs. All predictions were calculated using RNA structure (*67*) where ΔG values are refined at 37^0C.

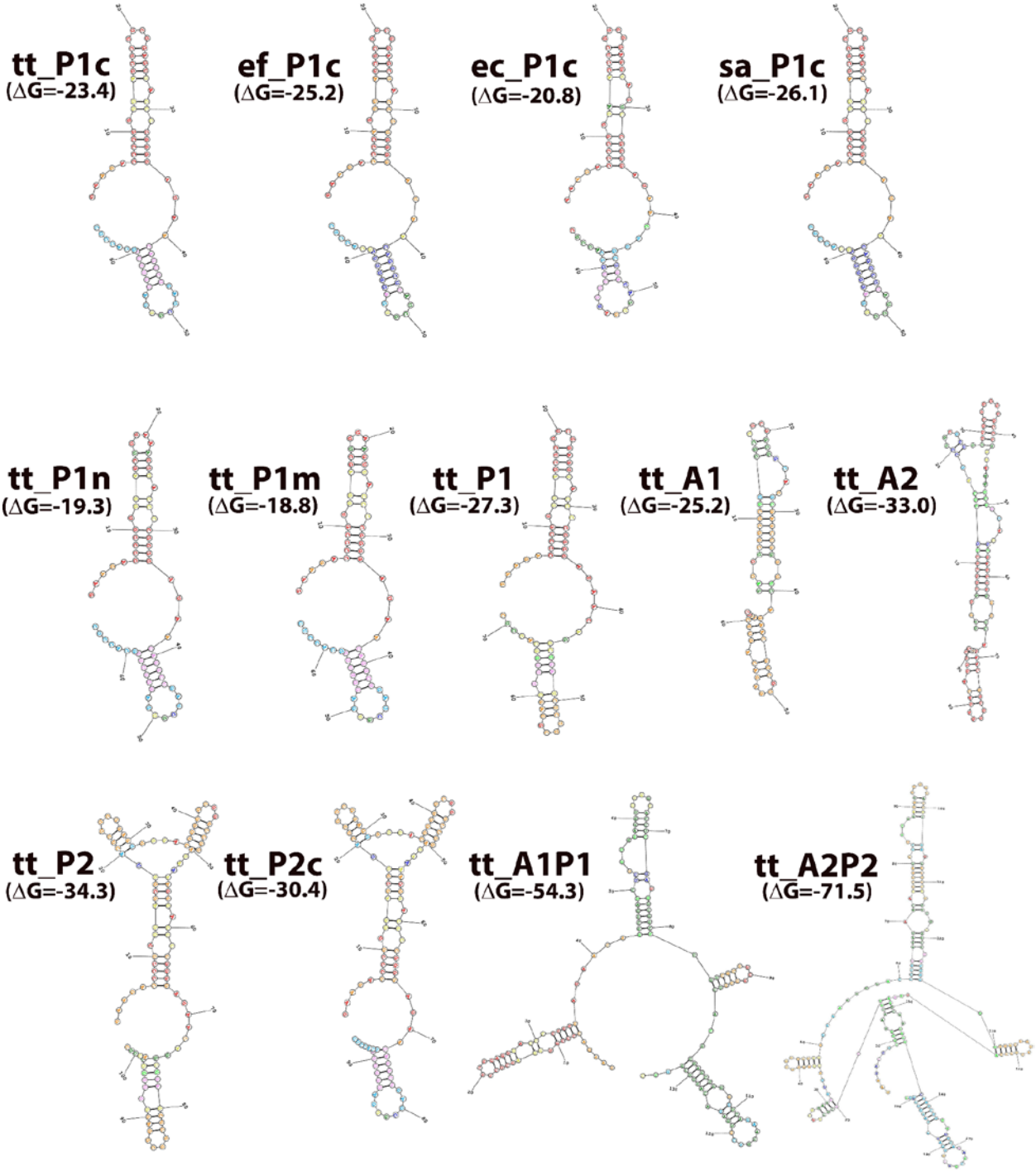


Figure S6. Denaturing gels of the RNA constructs: A. Purified tt_P1, tt_P1c, tt_P2, tt_P2c, tt_A1P1and tt_A2P2 RNA constructs (lanes 4-9). **B.** Purchased ec_p1c, hm_p1c, tt_p1m, tt_p1n, tt_A1 and tt_A2 RNA constructs (lanes 4-9). **C.** Purified sa_p1c and ef_p1c RNA constructs (lanes 4-5). **D.** Denaturing gel of the active constructs after fragment reaction of 24h at 37^0C (lanes 4-8). A single strand RNA (ssRNA) ladder and two DNA ladders (ultralow range (ULR) and 50 base pair (50BP) were used as reference (lanes 1-3). 10% polyacrylamide was used for all gels presented.

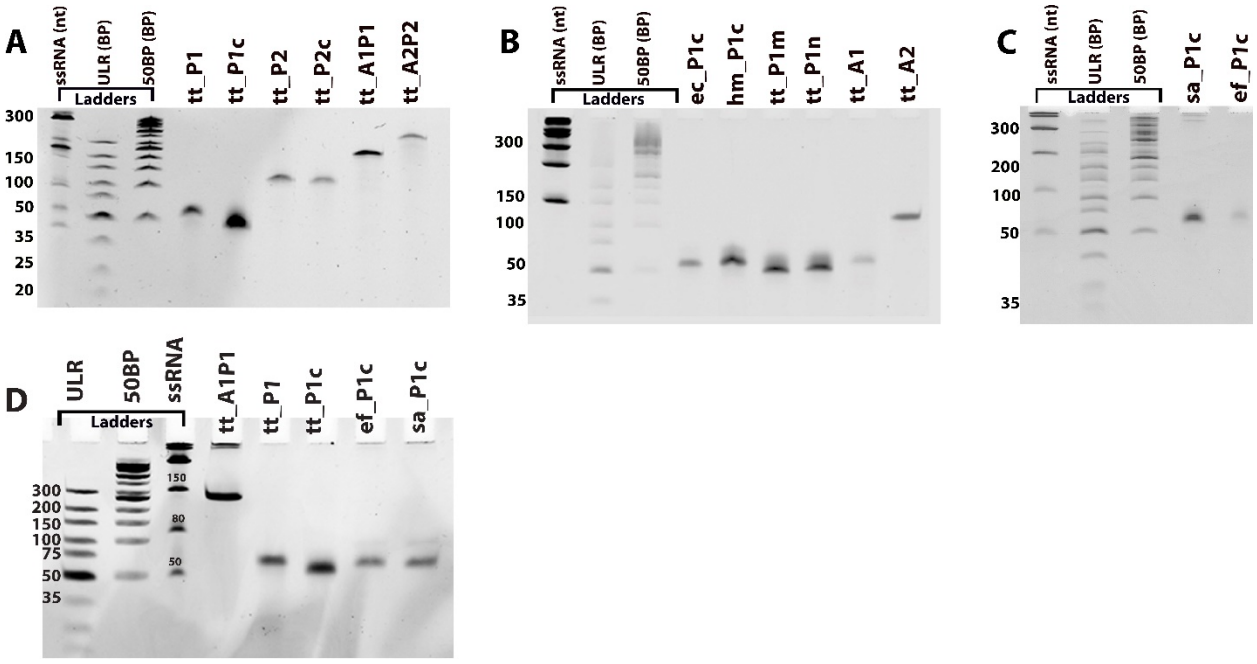


Figure S7: Labeling of RNA oligonucleotides with Fluorecein-5-thiosemicarbazide (FTSC). Sodium periodate was used to cleave the cis diol (3' termini) of RNA into 2',3'-dialdehyde and then, fluorescein-5-thiosemicarbazide, was added to the quenched reaction mixture to label the 3′ terminus of RNA through a condensation reaction between the carbazide and the aldehyde.

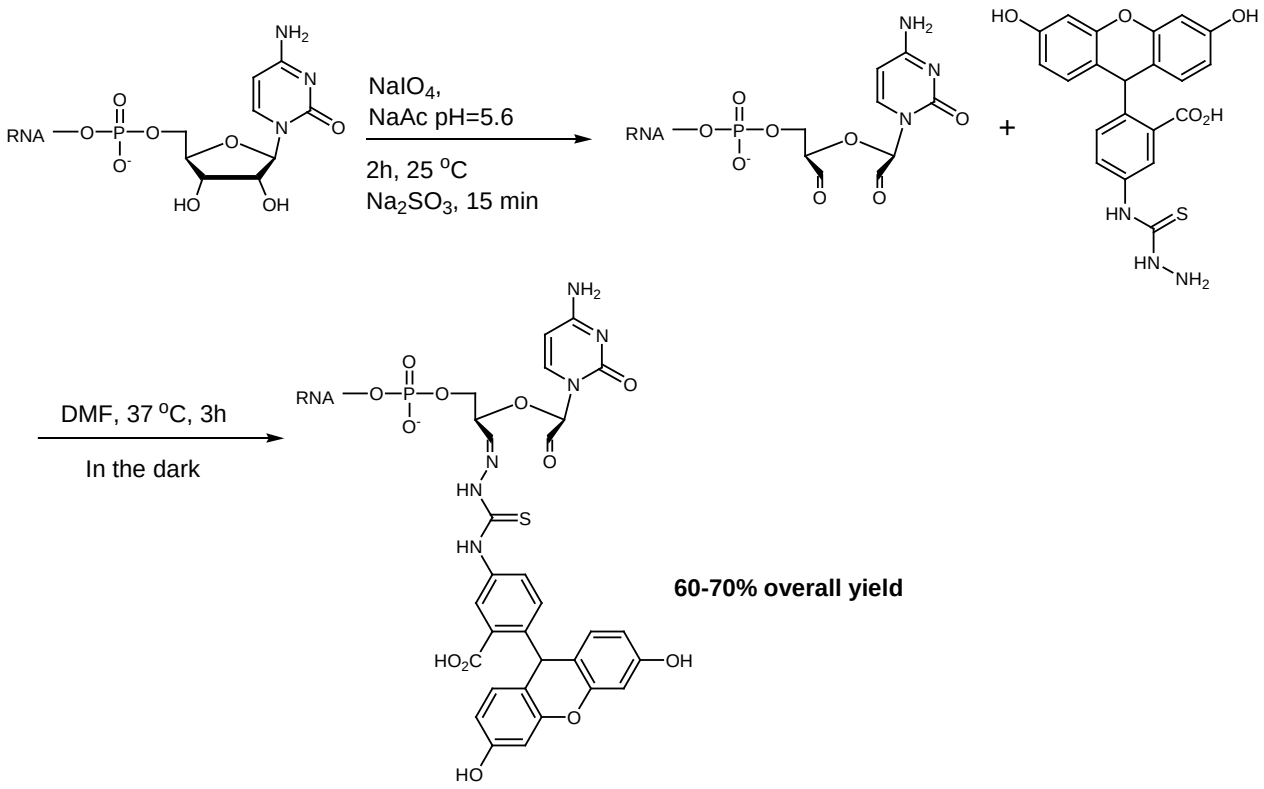
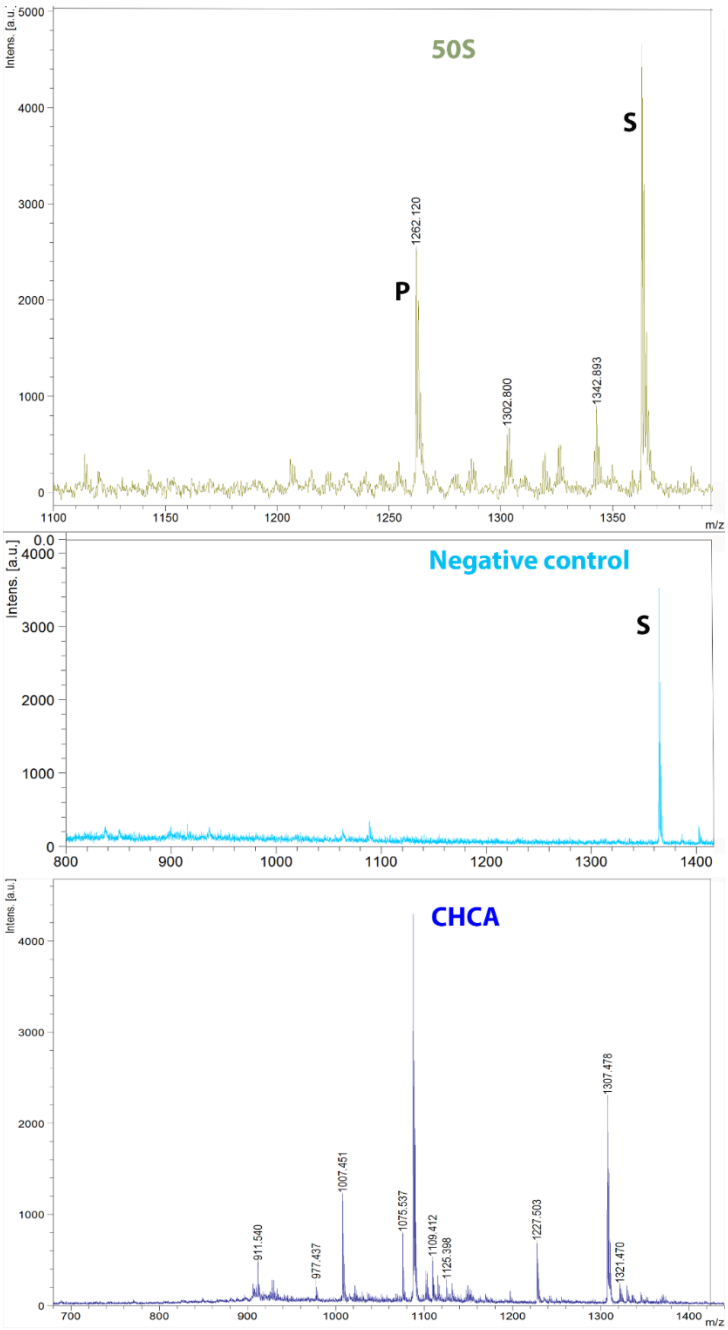
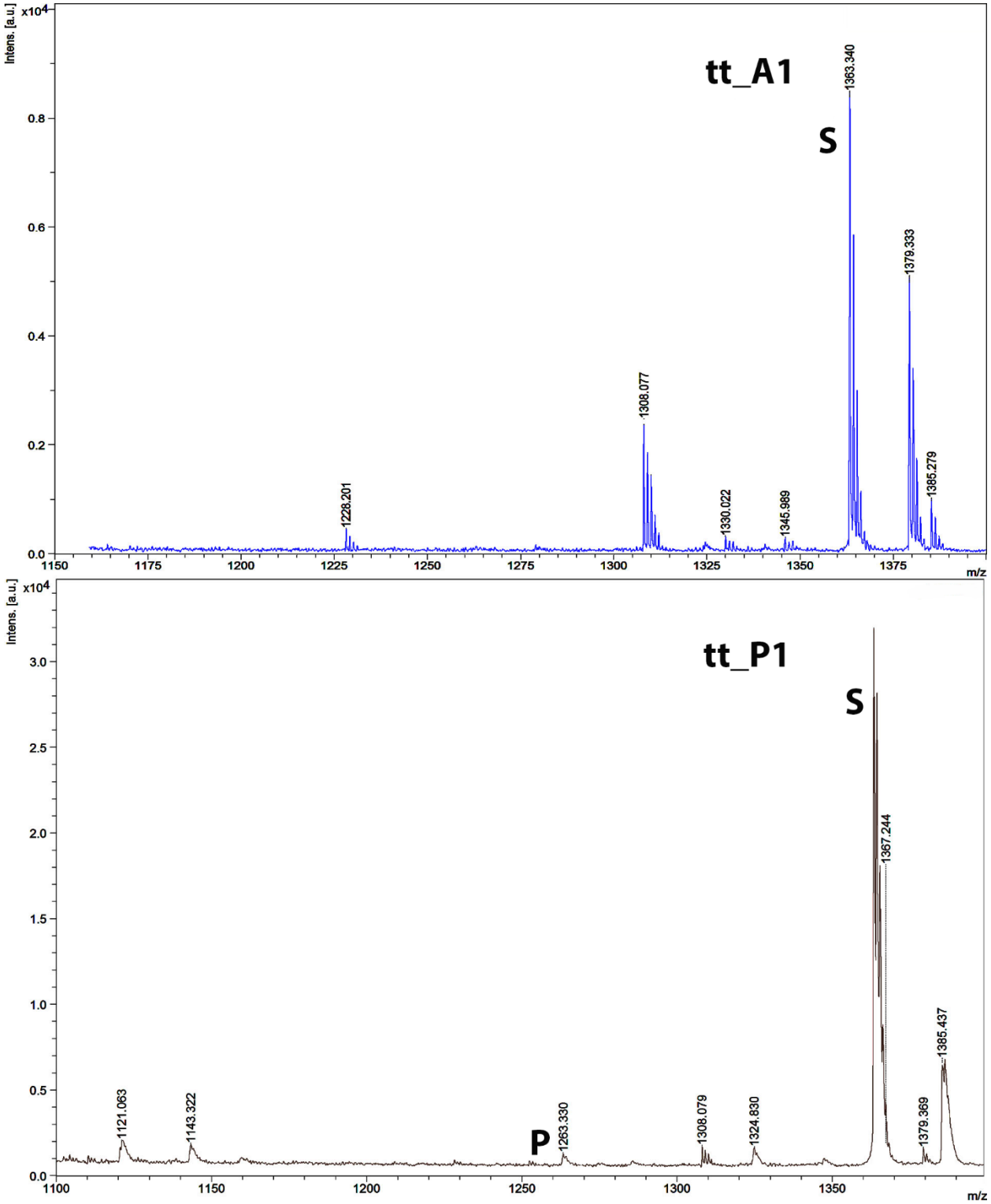


Figure S8: MALDI spectra of the fragment reaction between CCA- pcb and C-Pmn in the presence of the RNA constructs at 37°C. A. Positive (50S) and negative controls (no RNA) and CHCA matrix. **B.** Constructs tt_A1, tt_P1. **C.** Constructs sa_P1c, tt_P1c. **D.** Constructs tt_A1P1, tt_A2P2. **E.** Constructs tt_A2, ec_P1c. **F.** Constructs tt_P1n and tt_P1m. **G.** Constructs tt_P2 and tt_P2c. **H.** Construct cm_tt_P1c. The starting material CCA- pcb and the product C-Pmn-pcb are designated by S and P throughout.

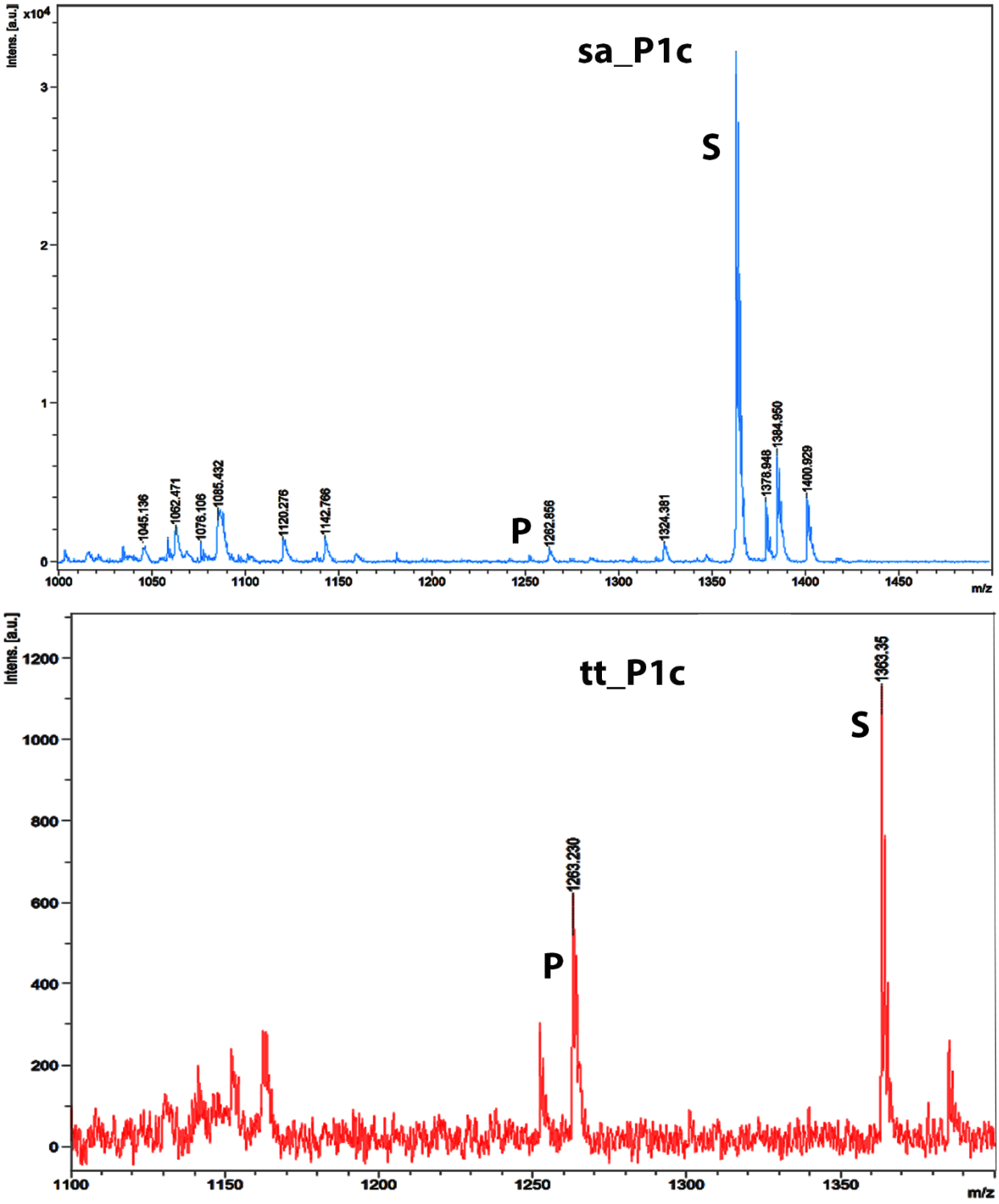
A



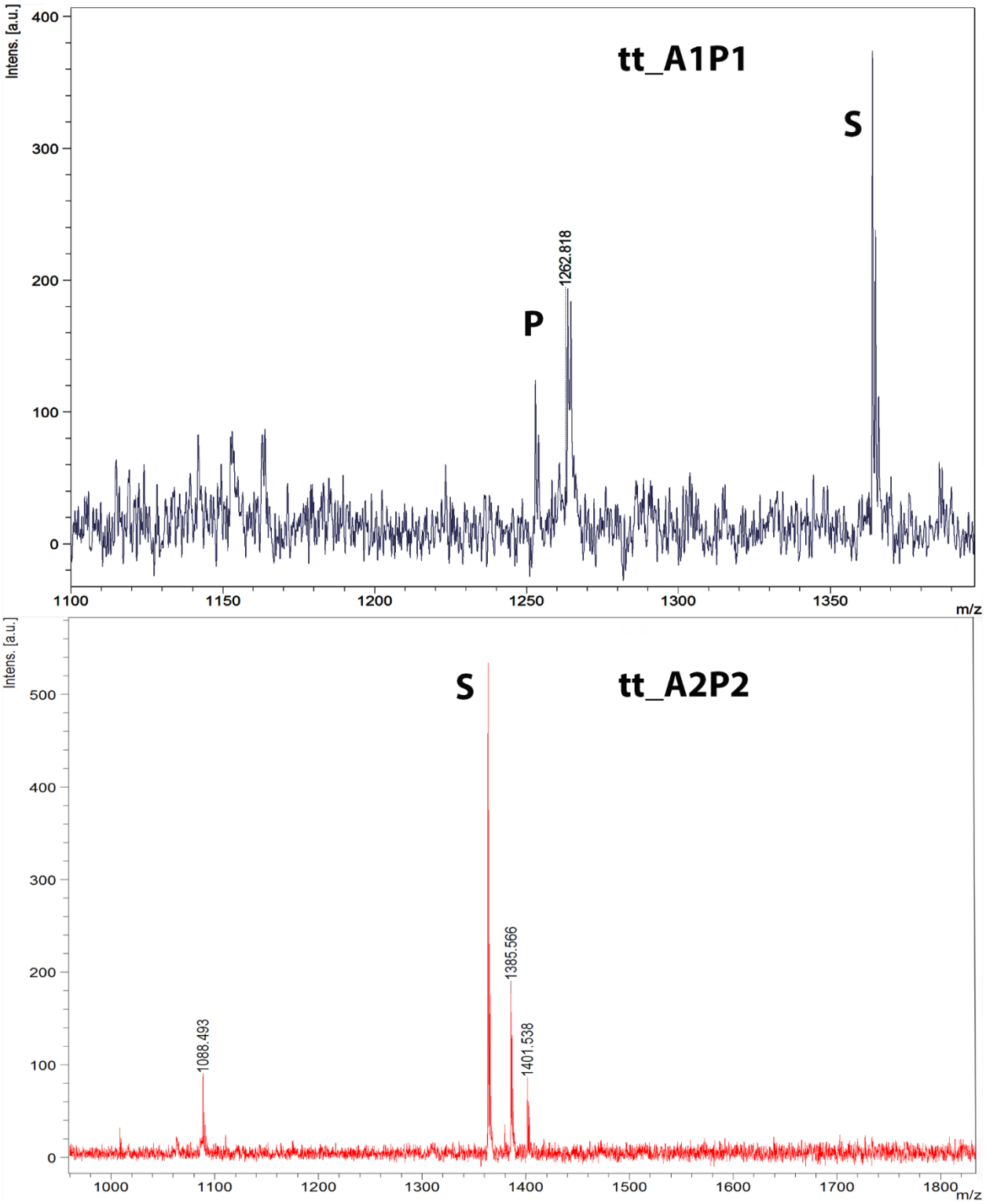
B


tt_A1
S
Intens. [a.u.]
x10^4
1363.340
1379.333
1308.077
1385.279
1228.201
1330.022
1345.989
m/z
tt_P1
S
P
1367.244
1385.437
1121.063
1143.322
1263.330
1308.079
1324.830
1379.369

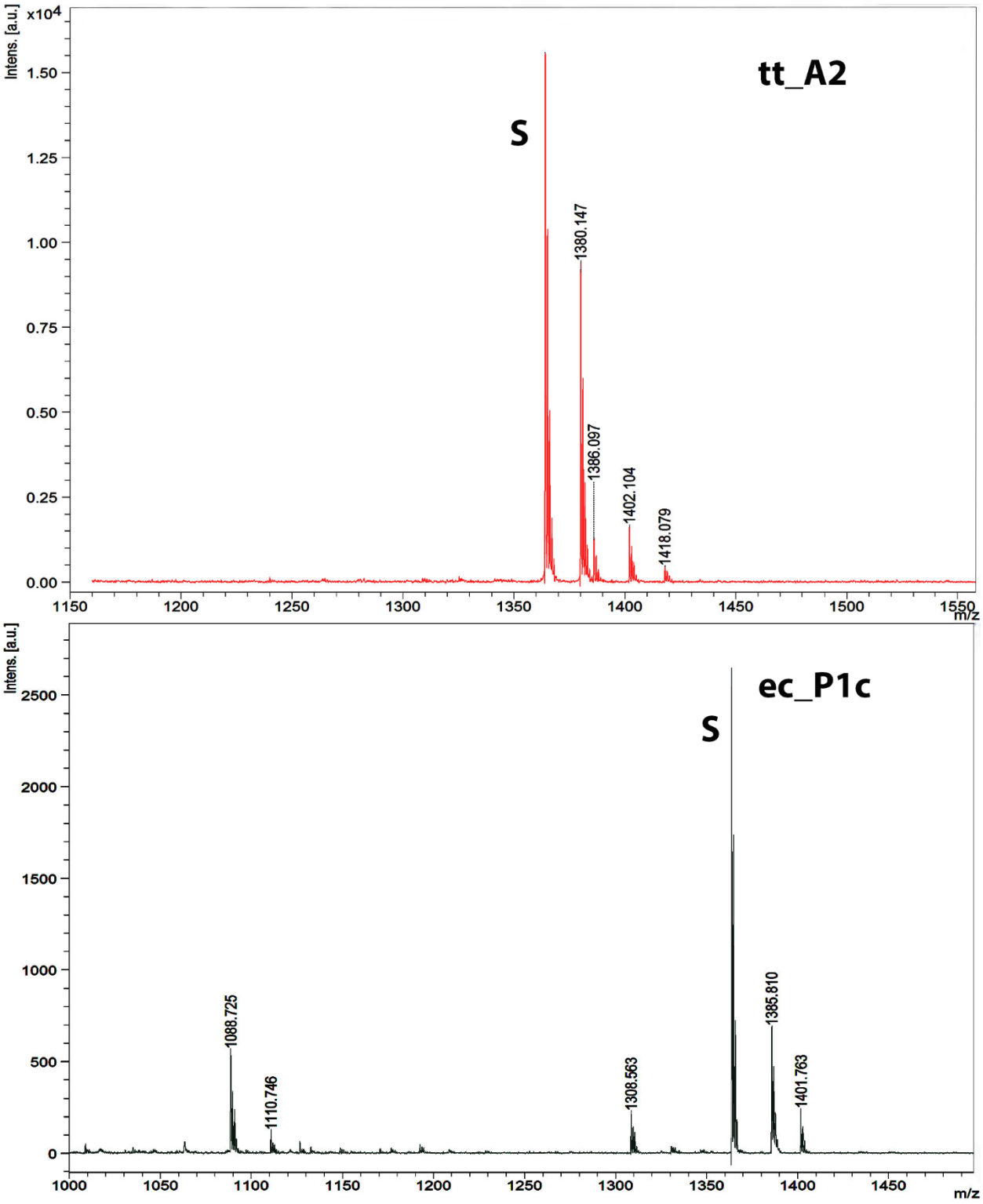
C


sa_P1c
Intens. [a.u.]
x10^4
S
P
1045.136
1062.471
1076.106
1085.432
1120.276
1142.766
1262.856
1324.381
1378.948
1384.950
1400.929
m/z
tt_P1c
Intens. [a.u.]
S
P
1383.35
1263.230
m/z

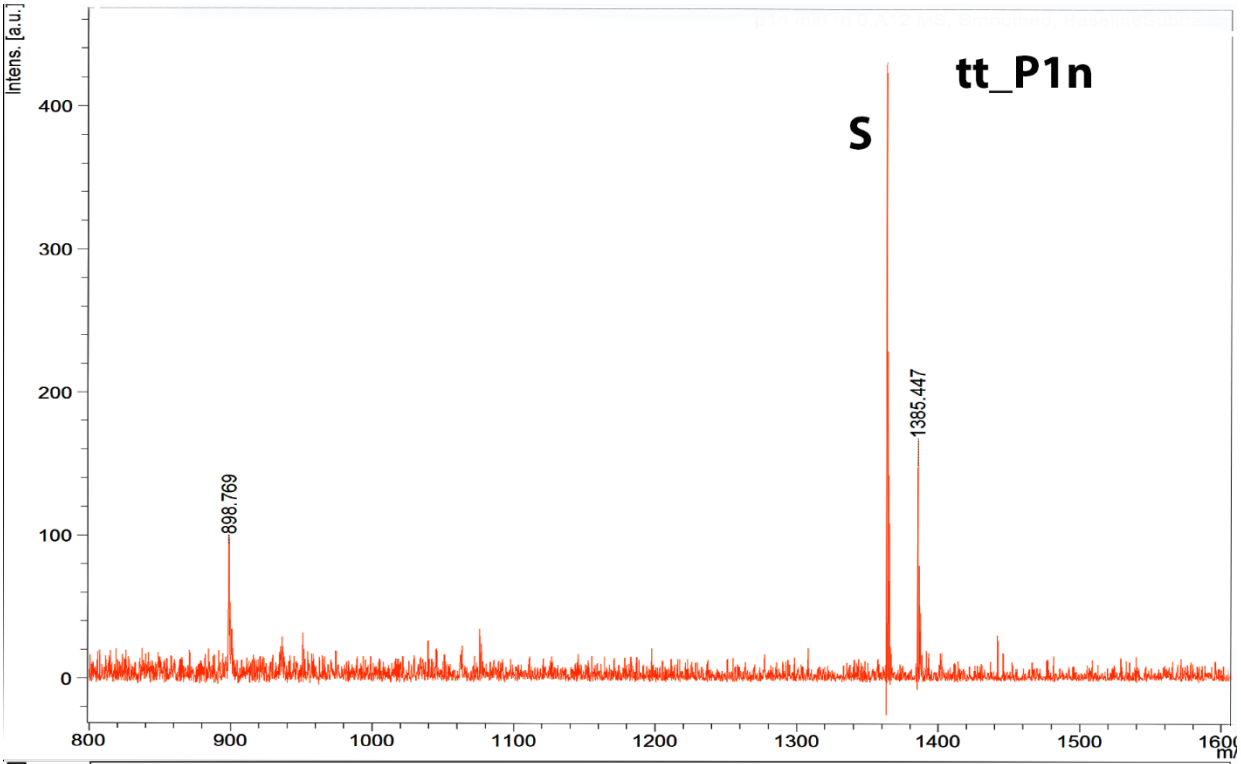
D

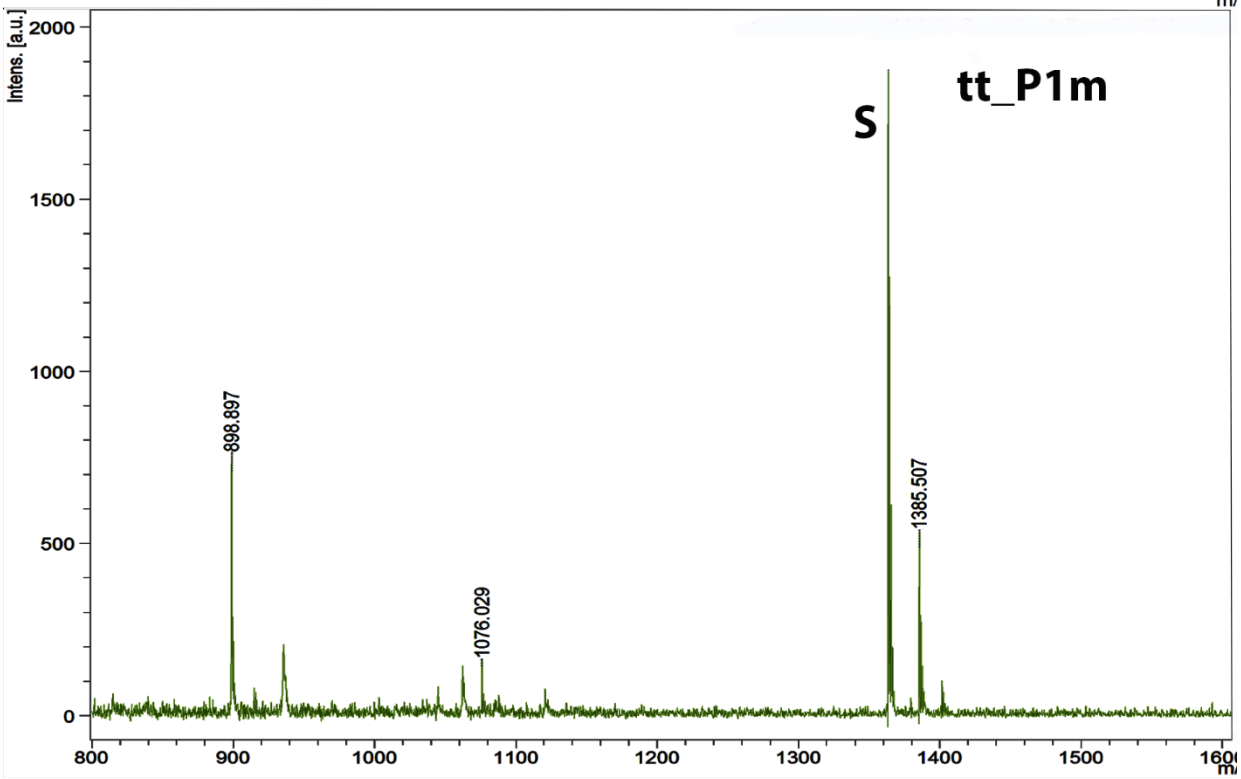

Intens. [a.u.]
tt_A1P1
S
P
1262.818
m/z
Intens. [a.u.]
S
tt_A2P2
1385.566
1088.493
1401.538
m/z

E

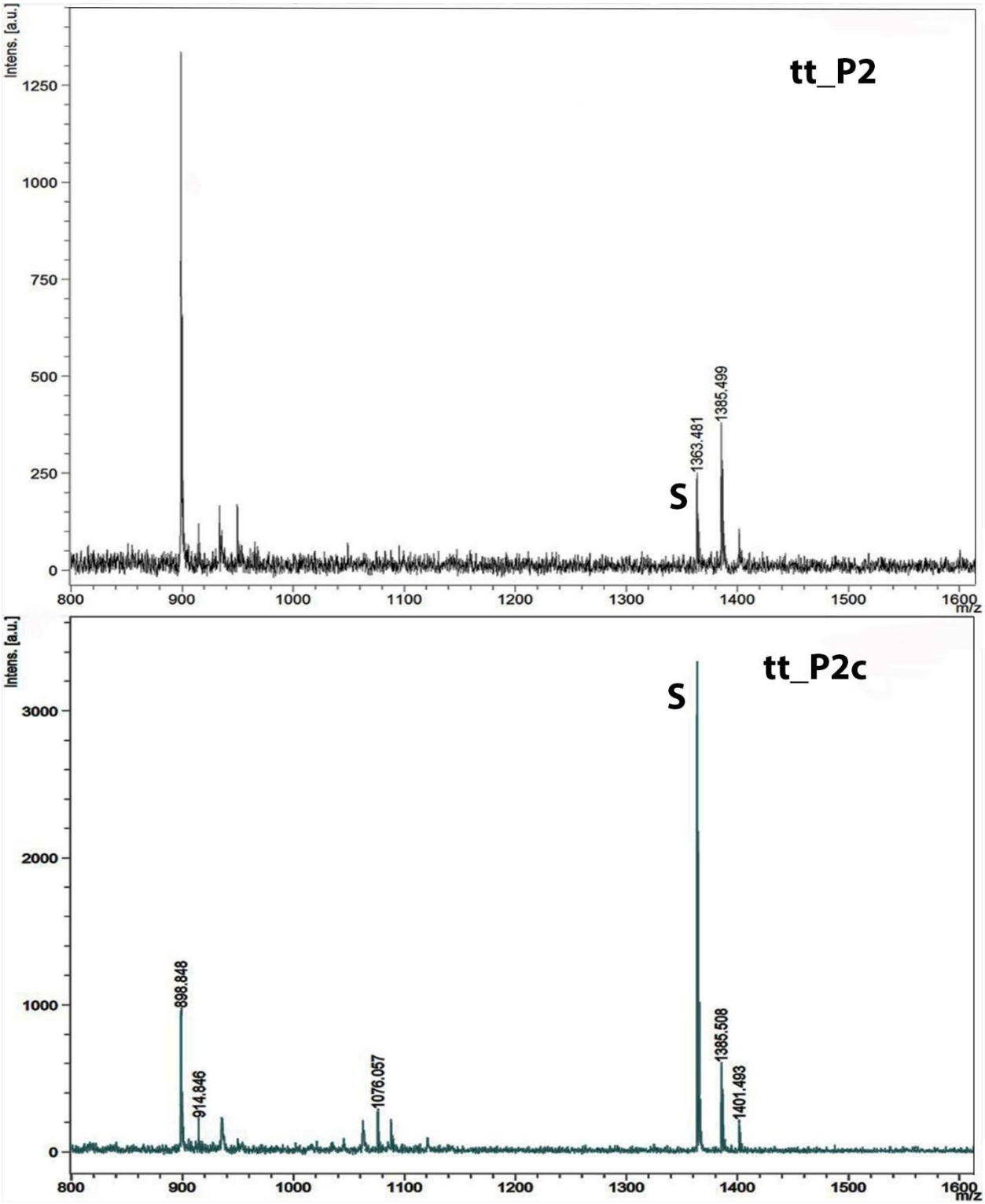

Intens. [a.u.]
x10^4
1.50
1.25
1.00
0.75
0.50
0.25
0.00
1150
1200
1250
1300
1350
1400
1450
1500
1550
m/z
tt_A2
S
1380.147
1386.097
1402.104
1418.079
Intens. [a.u.]
2500
2000
1500
1000
500
0
1000
1050
1100
1150
1200
1250
1300
1350
1400
1450
m/z
ec_P1c
S
1088.725
1110.746
1308.563
1385.810
1401.763

F


tt_P1n
S
Intens. [a.u.]
400
300
200
100
0
898.769
1385.447
800
900
1000
1100
1200
1300
1400
1500


tt_P1m
S
Intens. [a.u.]
2000
1500
1000
500
0
898.897
1076.029
1385.507
800
900
1000
1100
1200
1300
1400
1500

G


tt_P2
Intens. [a.u.]
1250
1000
750
500
250
0
S
1363.481
1385.499
800
900
1000
1100
1200
1300
1400
1500
1600
m/z
tt_P2c
Intens. [a.u.]
3000
2000
1000
0
S
898.848
914.846
1076.057
1385.508
1401.493
800
900
1000
1100
1200
1300
1400
1500
1600
m/z

H

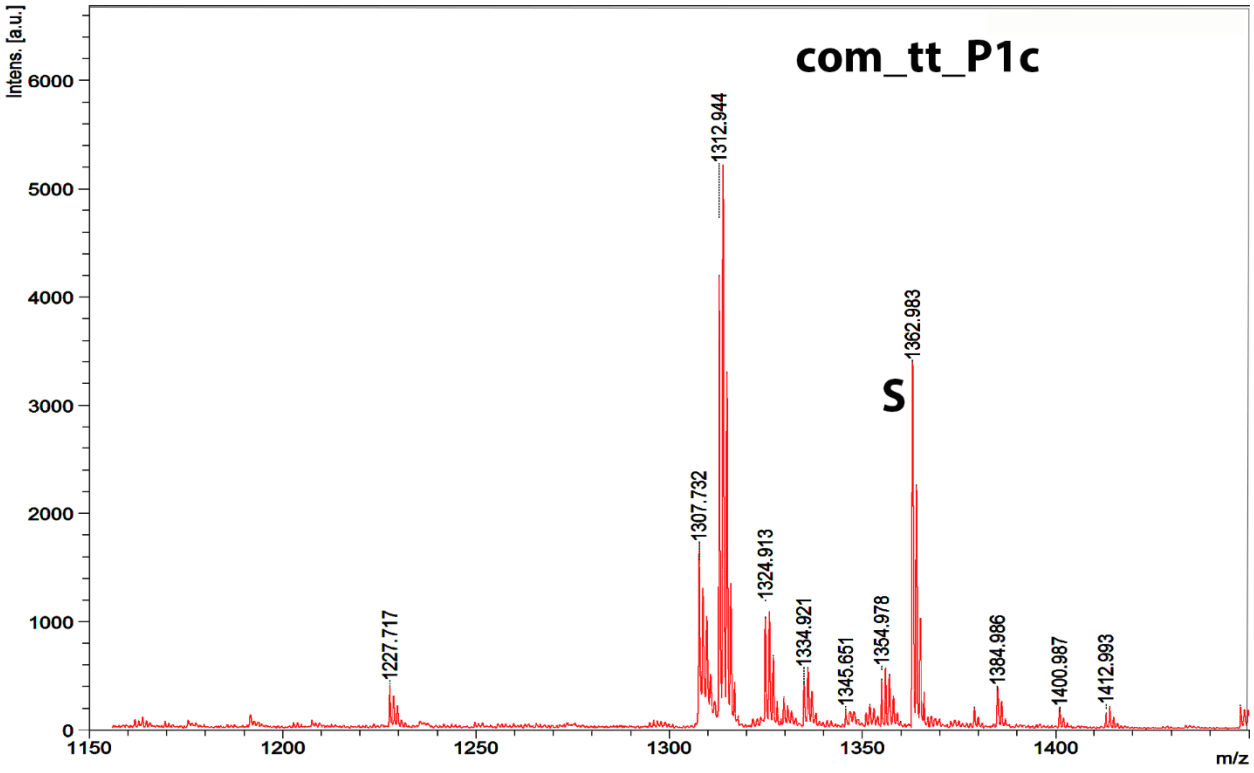
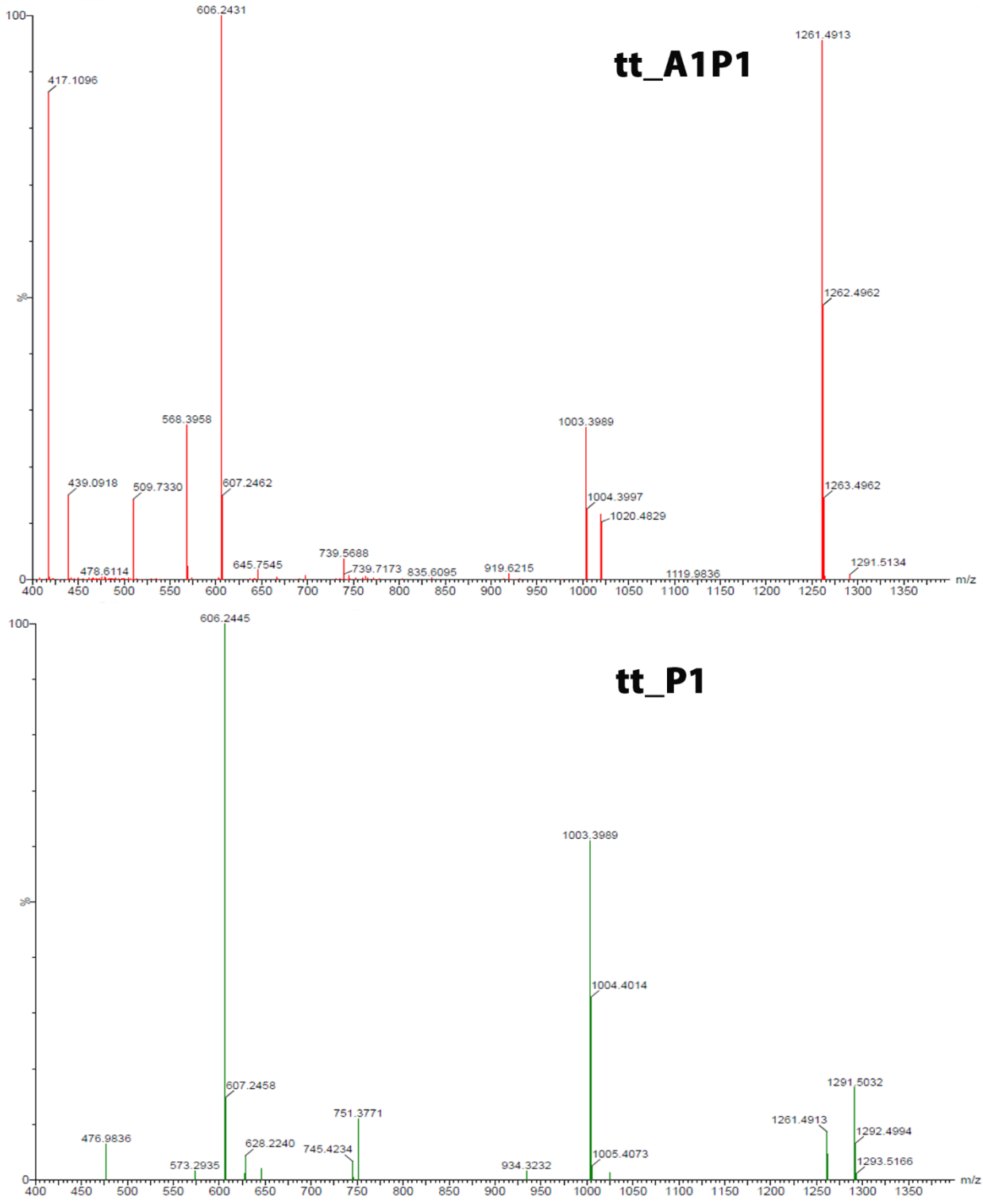

com_tt_P1c
Intens. [a.u.]
6000
5000
4000
3000
2000
1000
0
1150
1200
1250
1300
1350
1400
m/z
1312.944
1362.983
S
1307.732
1324.913
1334.921
1227.717
1345.651
1354.978
1384.986
1400.987
1412.993

Figure S9: LC-MS spectra of A. tt_A1P1 and tt_P1 B. sa_P1c and tt_P1c

A



B

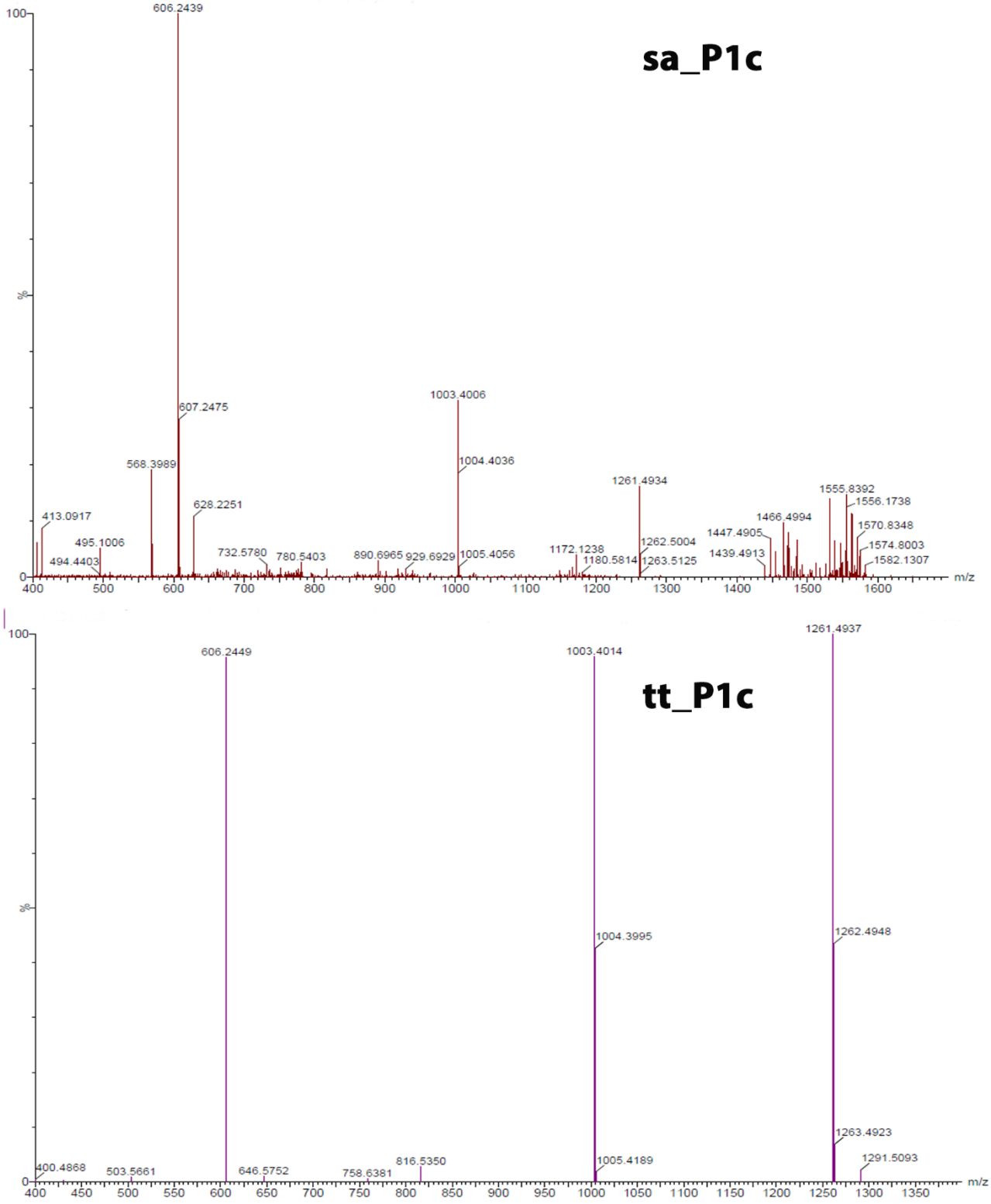
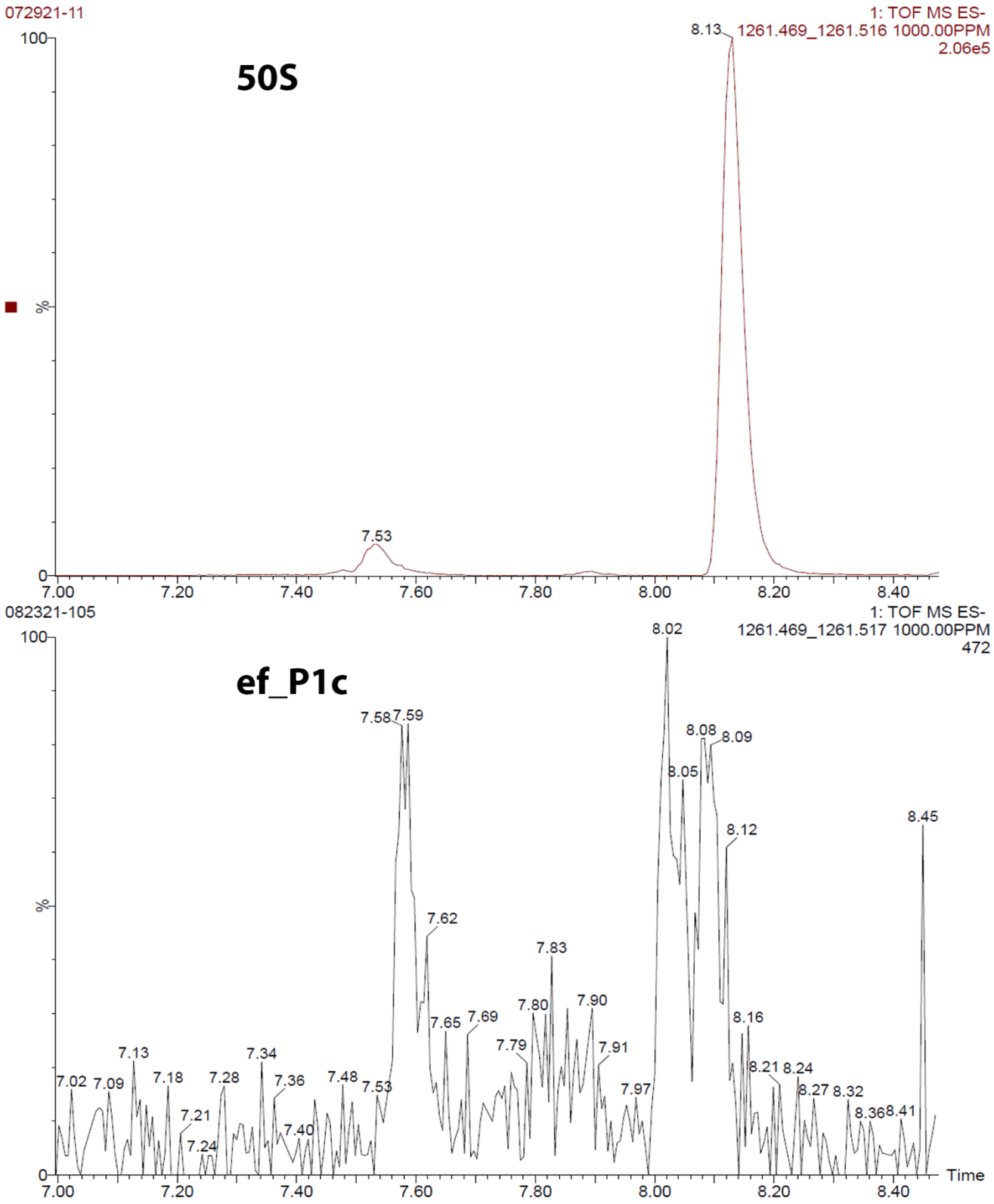
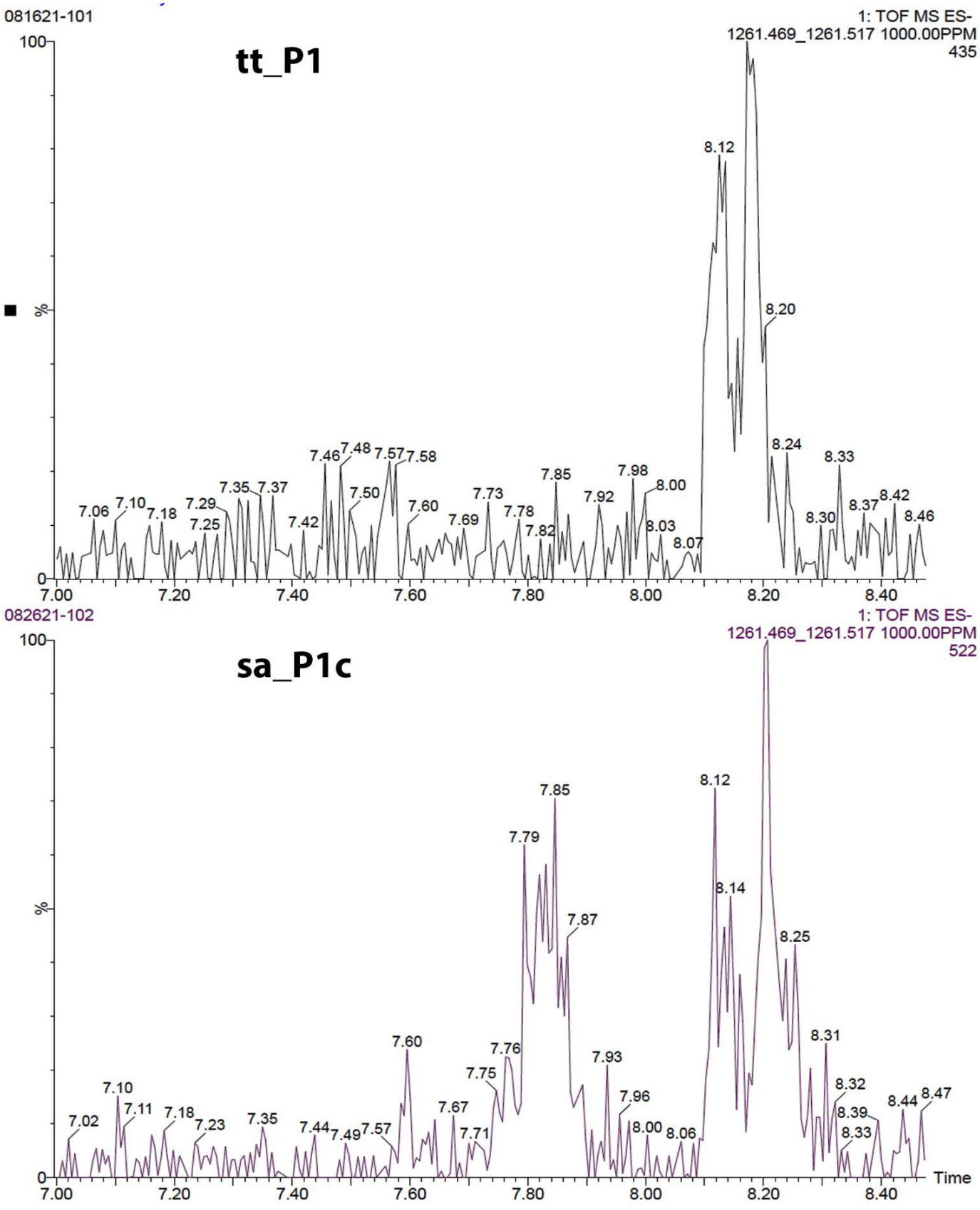

sa_P1c
606.2439
607.2475
1003.4006
1004.4036
568.3989
628.2251
1261.4934
413.0917
495.1006
494.4403
732.5780
780.5403
890.6965
929.6929
1005.4056
1172.1238
1180.5814
1262.5004
1263.5125
1439.4913
1447.4905
1466.4994
1555.8392
1556.1738
1570.8348
1574.8003
1582.1307
m/z
tt_P1c
606.2449
1003.4014
1261.4937
1004.3995
1262.4948
1263.4923
1005.4189
816.5350
400.4868
503.5661
646.5752
758.6381
1291.5093
m/z

Figure S10: UPLC chromatograms of 50S, ef_P1c, tt_P1, sa_P1c, tt_A1P1, tt_P1c




081621-101
1: TOF MS ES-
1261.469_1261.517 1000.00PPM
435
tt_P1
100
%
0
7.00
7.20
7.40
7.60
7.80
8.00
8.20
8.40
8.12
8.20
8.24
8.33
8.37
8.42
8.46
8.30
8.03
8.07
7.46
7.48
7.57
7.58
7.50
7.60
7.85
7.98
8.00
7.73
7.78
7.82
7.92
7.35
7.37
7.29
7.25
7.06
7.10
7.18
7.42
7.69
082621-102
1: TOF MS ES-
1261.469_1261.517 1000.00PPM
522
sa_P1c
100
%
0
Time
7.85
7.79
8.12
8.14
7.87
8.25
8.31
7.60
7.76
7.93
7.75
8.32
8.44
8.47
7.10
7.11
7.18
7.96
8.39
7.02
7.23
7.35
7.44
7.49
7.57
7.67
7.71
8.00
8.06
8.33

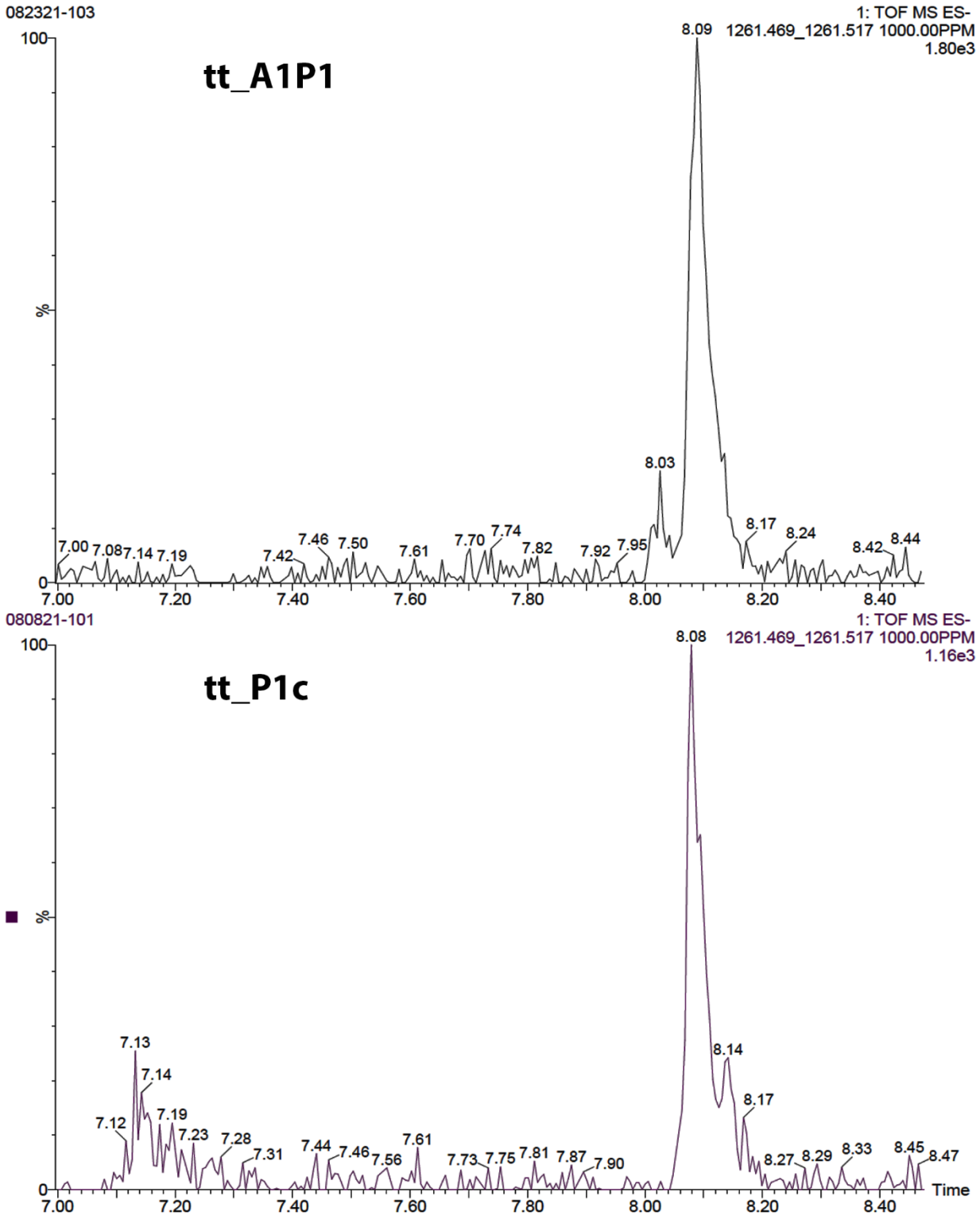
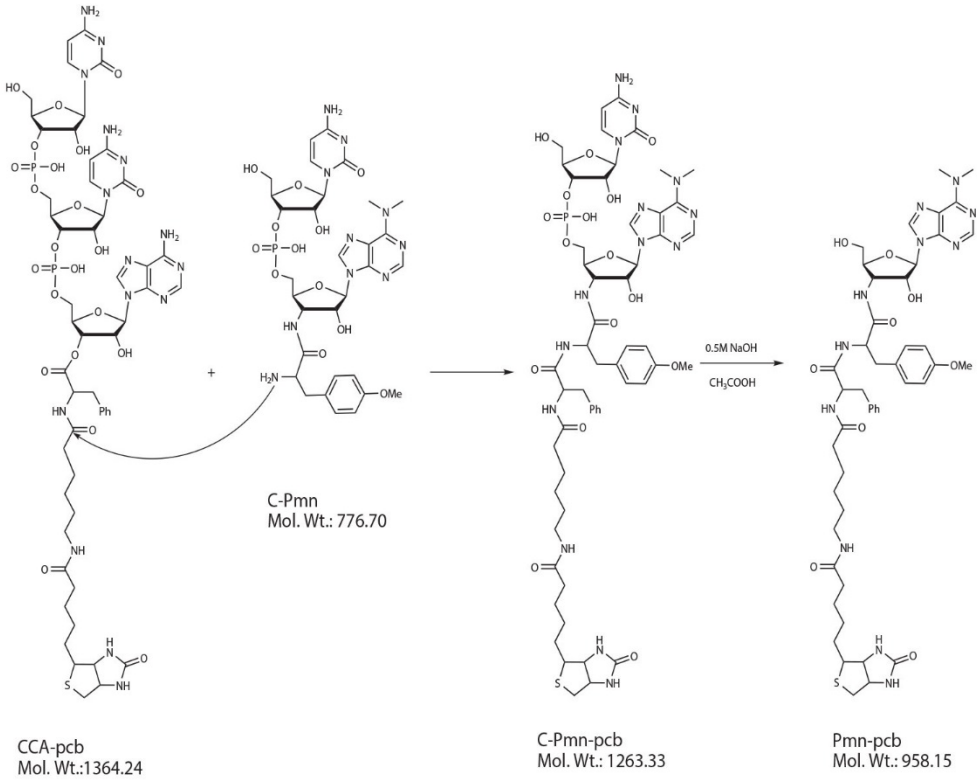
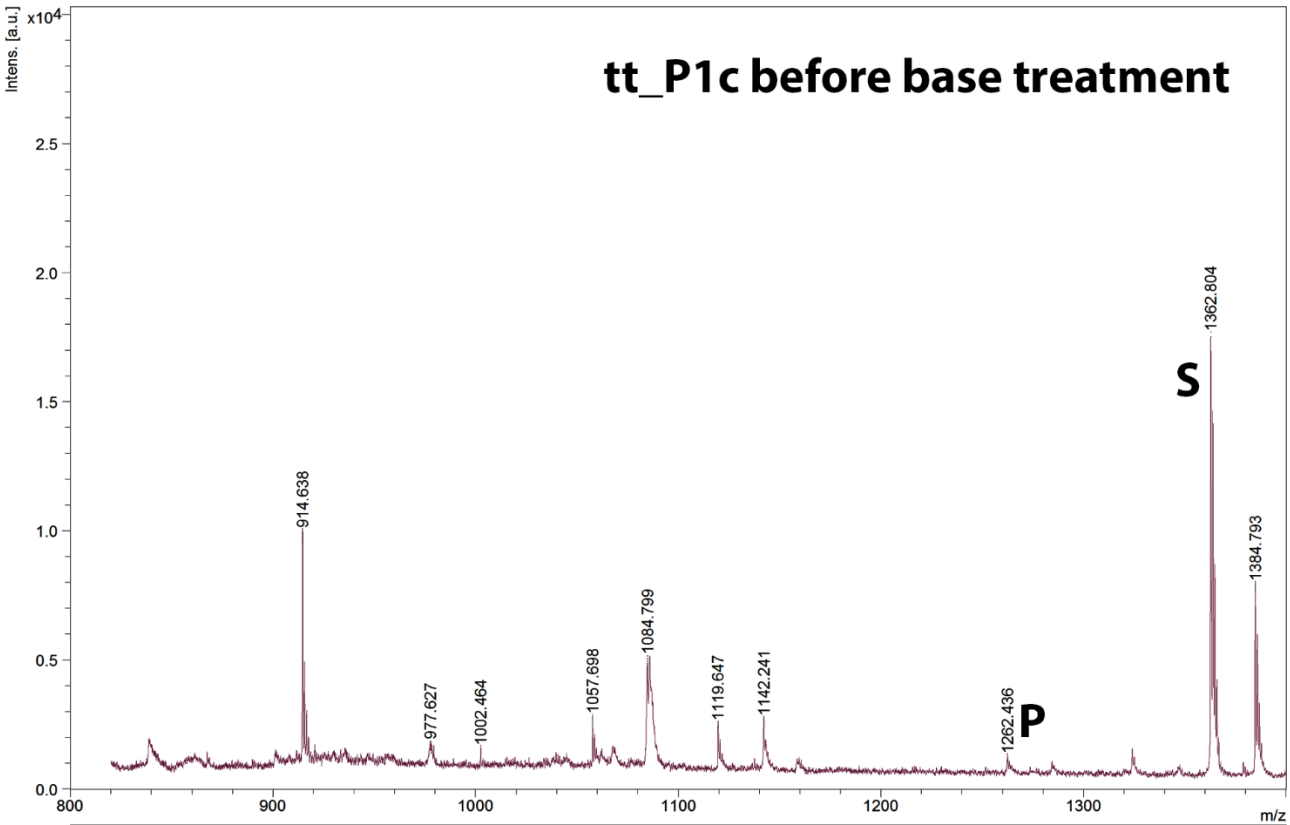

082321-103
1: TOF MS ES-
1261.469_1261.517 1000.00PPM
1.80e3
tt_A1P1
100
%
0
8.09
8.03
8.17
8.24
8.42
8.44
7.00
7.08
7.14
7.19
7.42
7.46
7.50
7.61
7.70
7.74
7.82
7.92
7.95
7.00
7.20
7.40
7.60
7.80
8.00
8.20
8.40
080821-101
1: TOF MS ES-
1261.469_1261.517 1000.00PPM
1.16e3
tt_P1c
100
%
0
8.08
8.14
8.17
7.13
7.14
7.12
7.19
7.23
7.28
7.31
7.44
7.46
7.56
7.61
7.73
7.75
7.81
7.87
7.90
8.27
8.29
8.33
8.45
8.47
Time
7.00
7.20
7.40
7.60
7.80
8.00
8.20
8.40

Figure S11: Verification of the product identity; presence of a peptide bond. A. Schematic representation of the product basic hydrolysis. B. MALDI spectra of the reaction mixture of construct tt_P1c before and after base treatment.

A



B


tt_P1c before base treatment
Intens. [a.u.]
x10^4
2.5
2.0
1.5
1.0
0.5
0.0
914.638
977.627
1002.464
1057.698
1084.799
1119.647
1142.241
1262.436
P
1362.804
S
1384.793
800
900
1000
1100
1200
1300
m/z

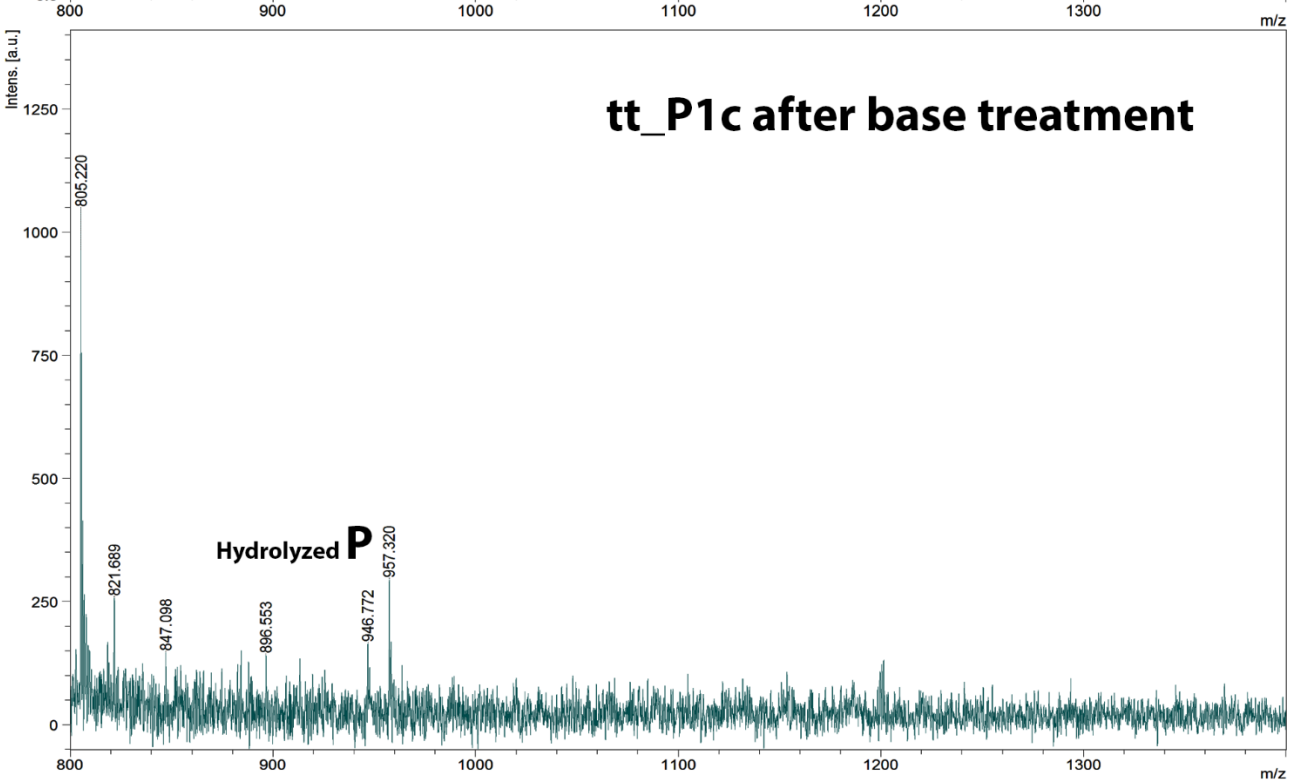
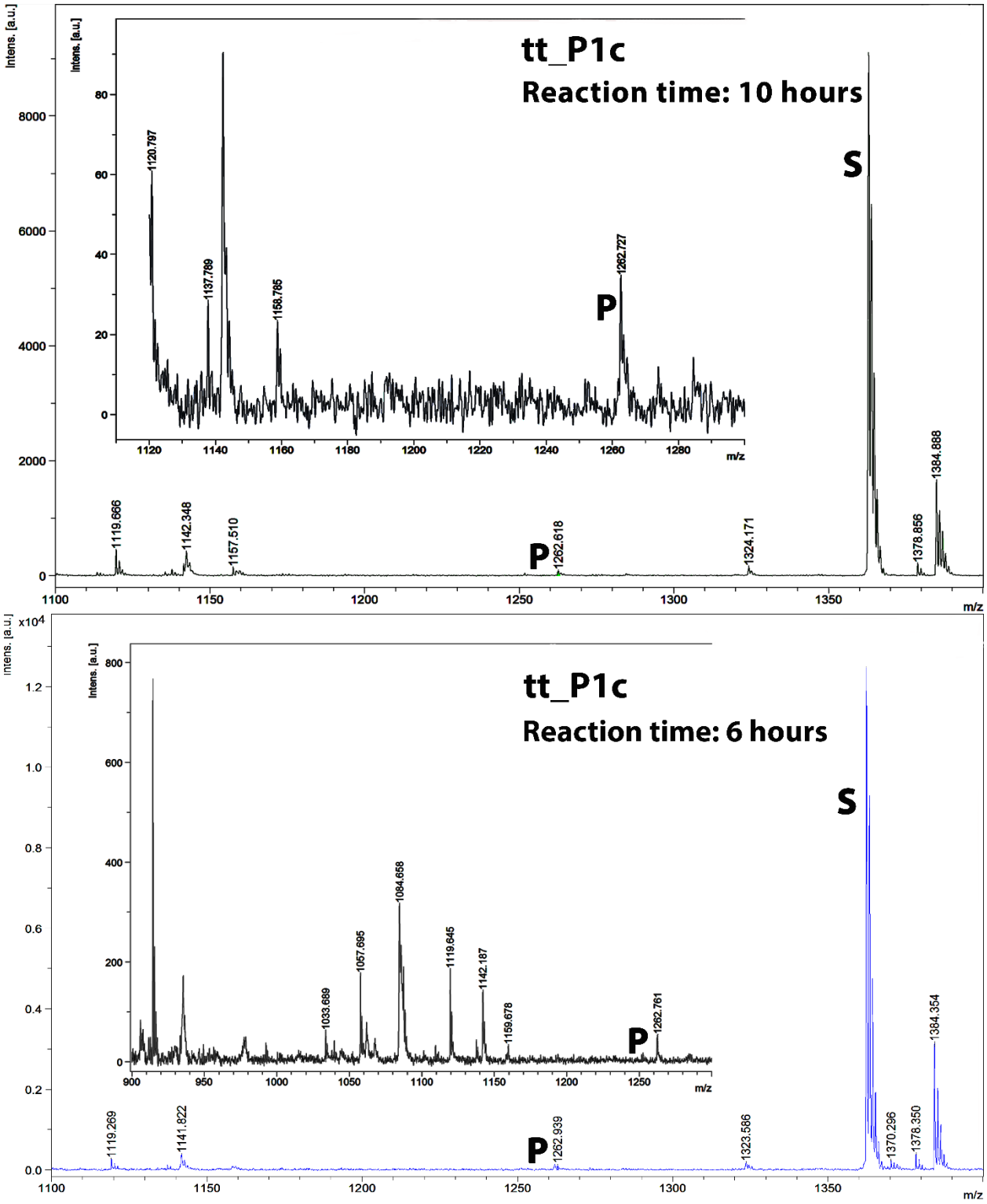
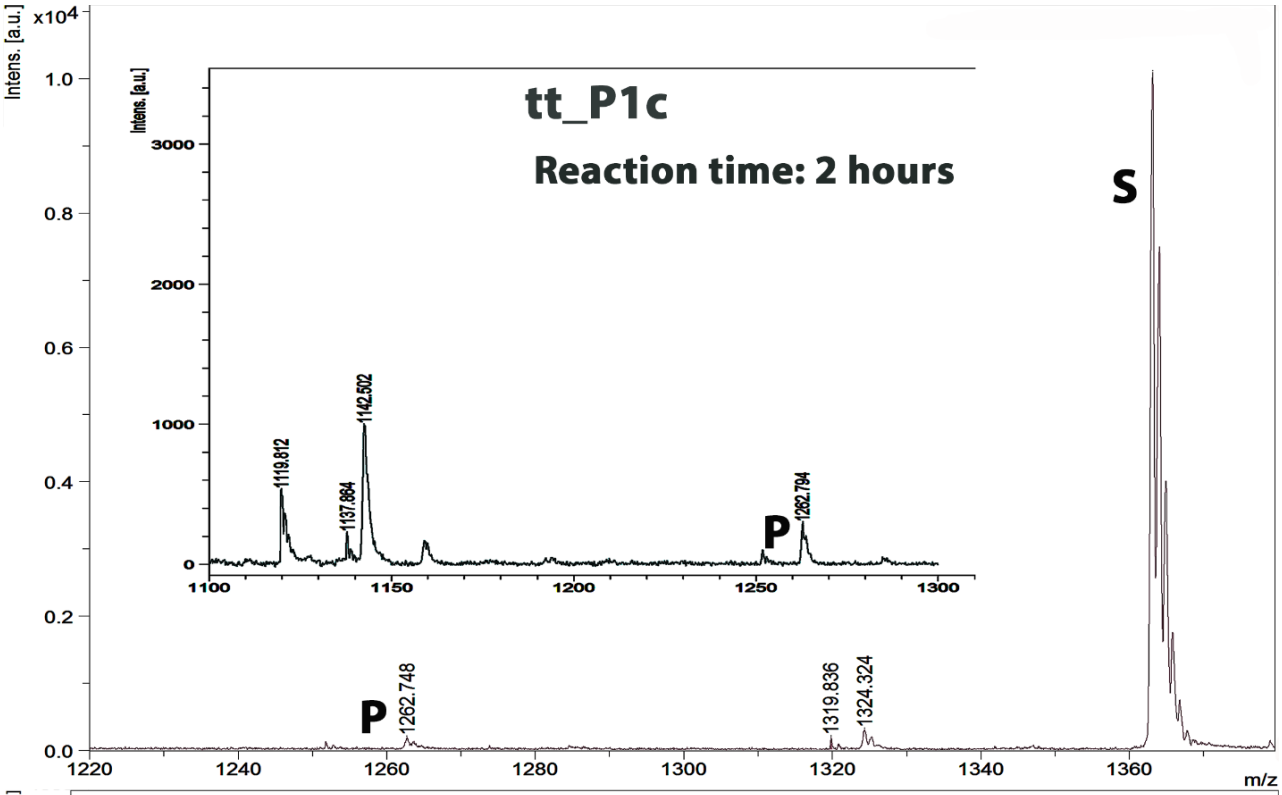
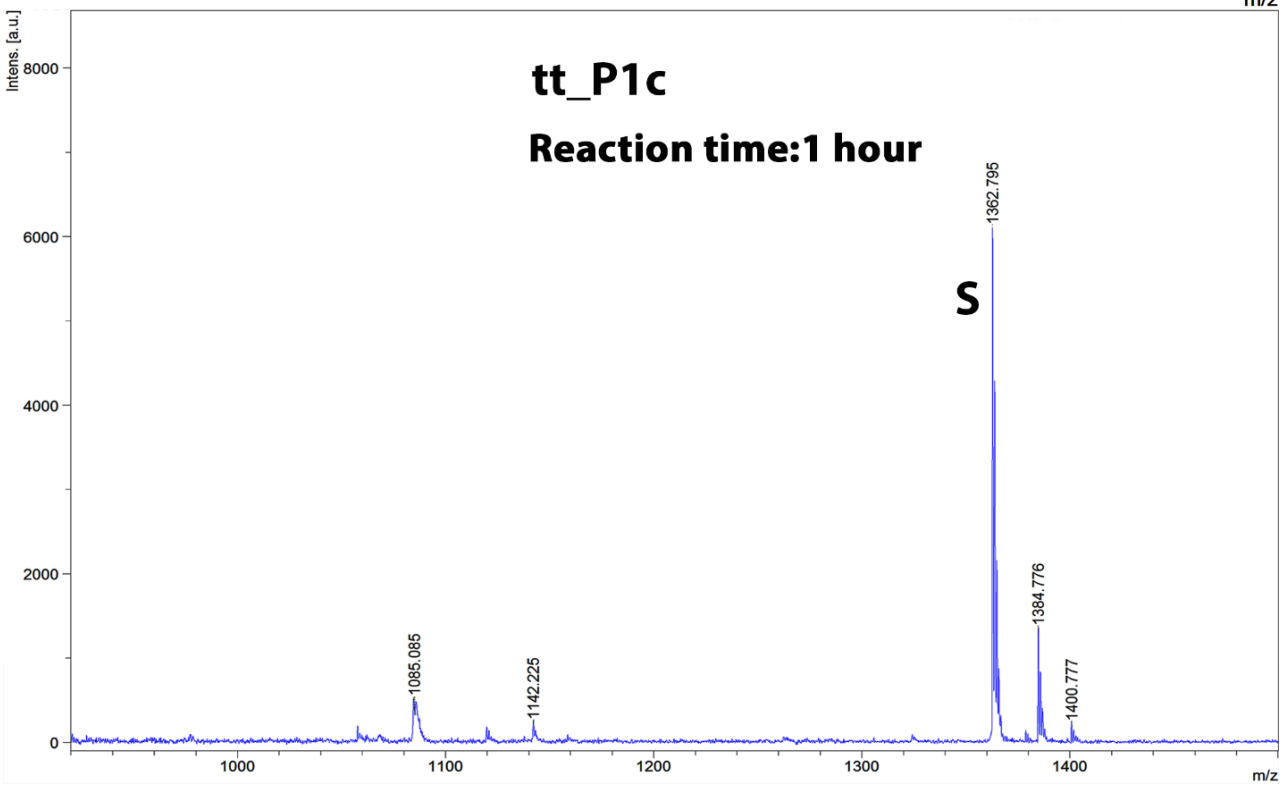

tt_P1c after base treatment
Intens. [a.u.]
1250
1000
750
500
250
0
805.220
821.689
847.098
896.553
946.772
957.320
Hydrolyzed P
800
900
1000
1100
1200
1300
m/z

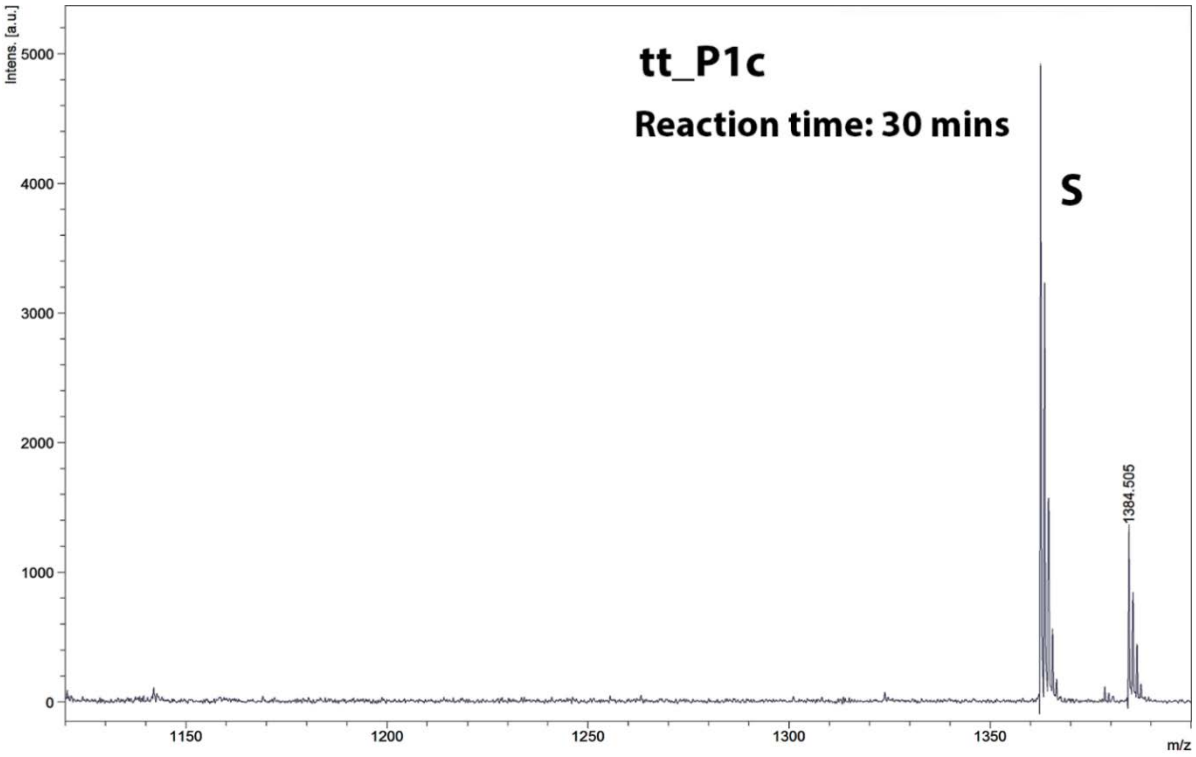
Figure S12: MALDI spectra of the fragment reaction for various time durations A. tt_P1c B. 50S.

A

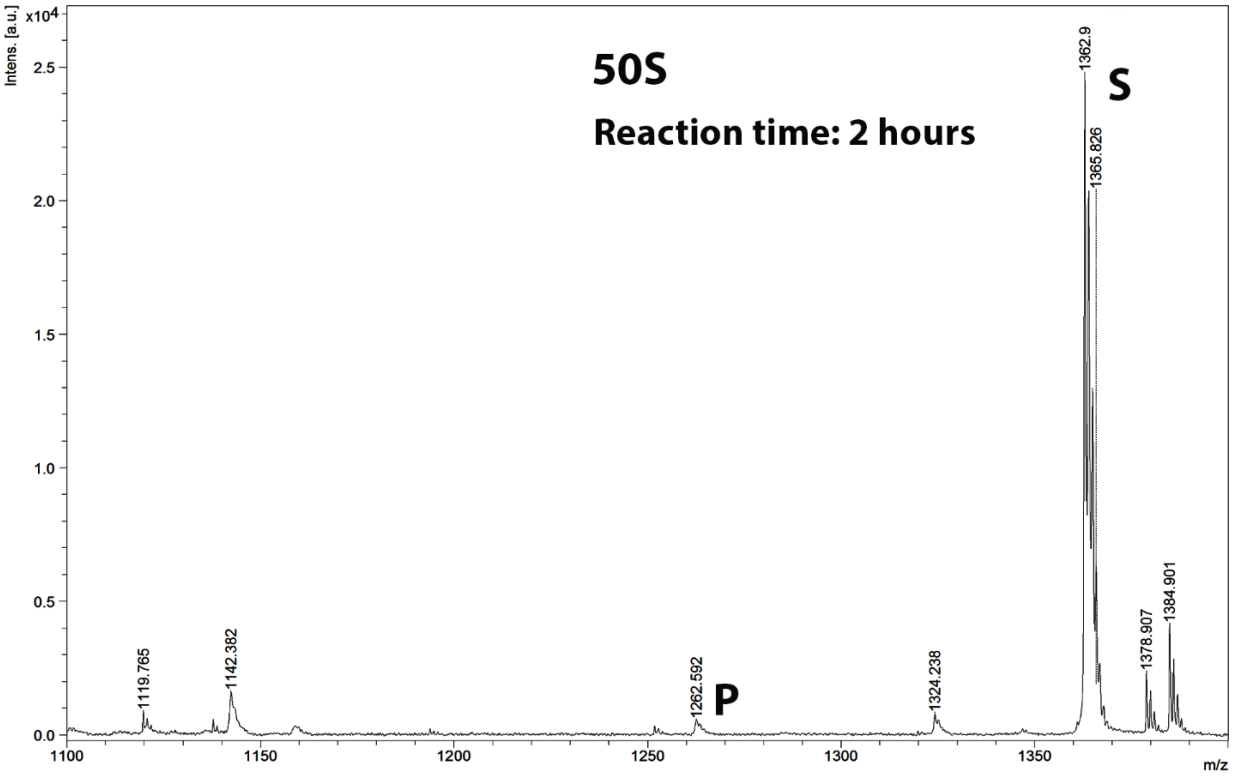



Intens. [a.u.]
x10^4
1.0
0.8
0.6
0.4
0.2
0.0
1220
1240
1260
1280
1300
1320
1340
1360
m/z
P
1262.748
1319.836
1324.324
S
tt_P1c
Reaction time: 2 hours
Intens. [a.u.]
3000
2000
1000
0
1100
1150
1200
1250
1300
1119.812
1137.864
1142.502
P
1282.794


Intens. [a.u.]
8000
6000
4000
2000
0
1000
1100
1200
1300
1400
m/z
tt_P1c
Reaction time:1 hour
S
1362.795
1384.776
1400.777
1085.085
1142.225


tt_P1c
Reaction time: 30 mins
S
1384.505
Intens. [a.u.]
5000
4000
3000
2000
1000
0
1150
1200
1250
1300
1350
m/z

B


50S
Reaction time: 2 hours
S
P
1362.9
1365.826
1384.901
1378.907
1324.238
1262.592
1142.382
1119.765
Intens. [a.u.]
x10^4
2.5
2.0
1.5
1.0
0.5
0.0
1100
1150
1200
1250
1300
1350
m/z

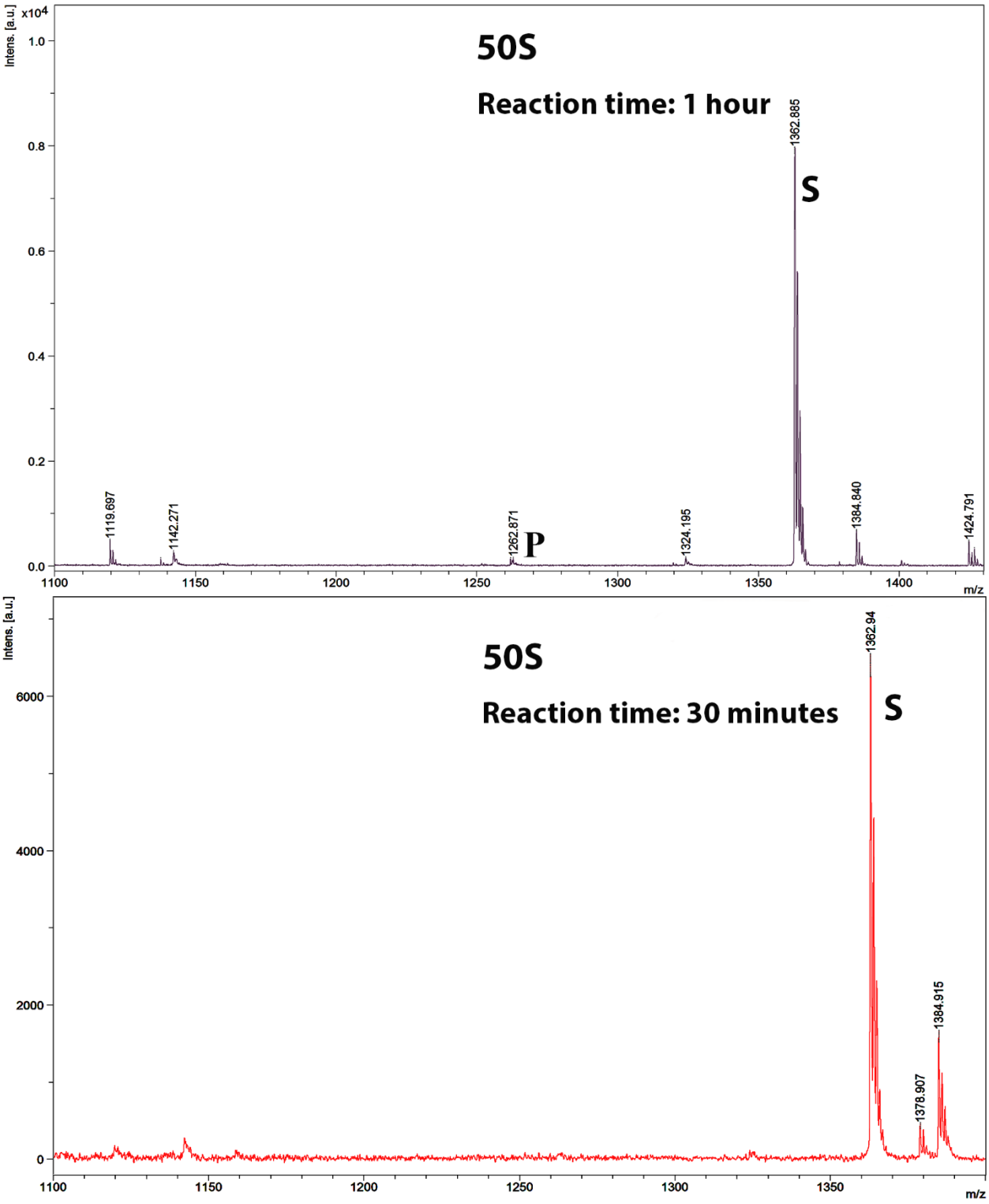
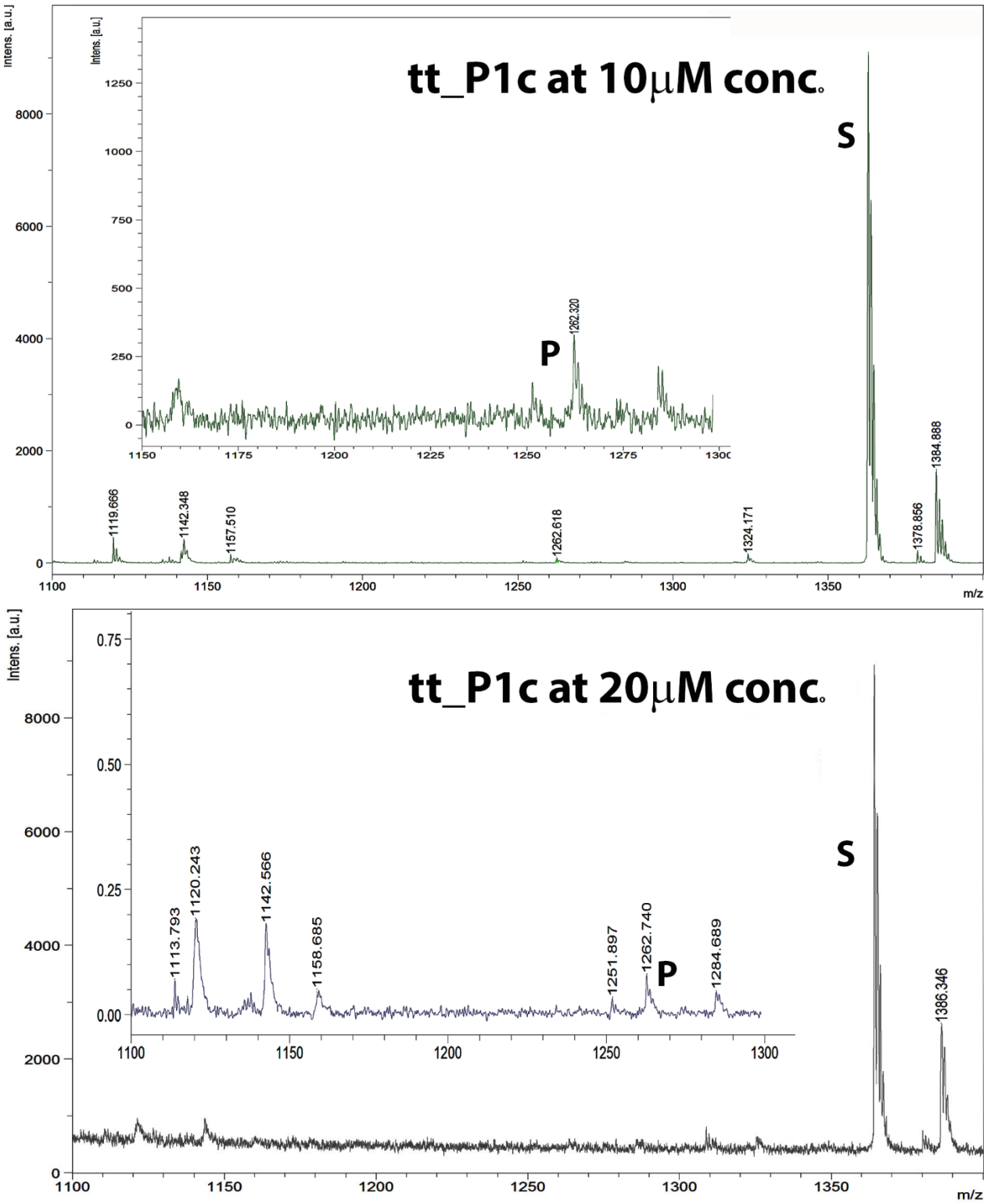
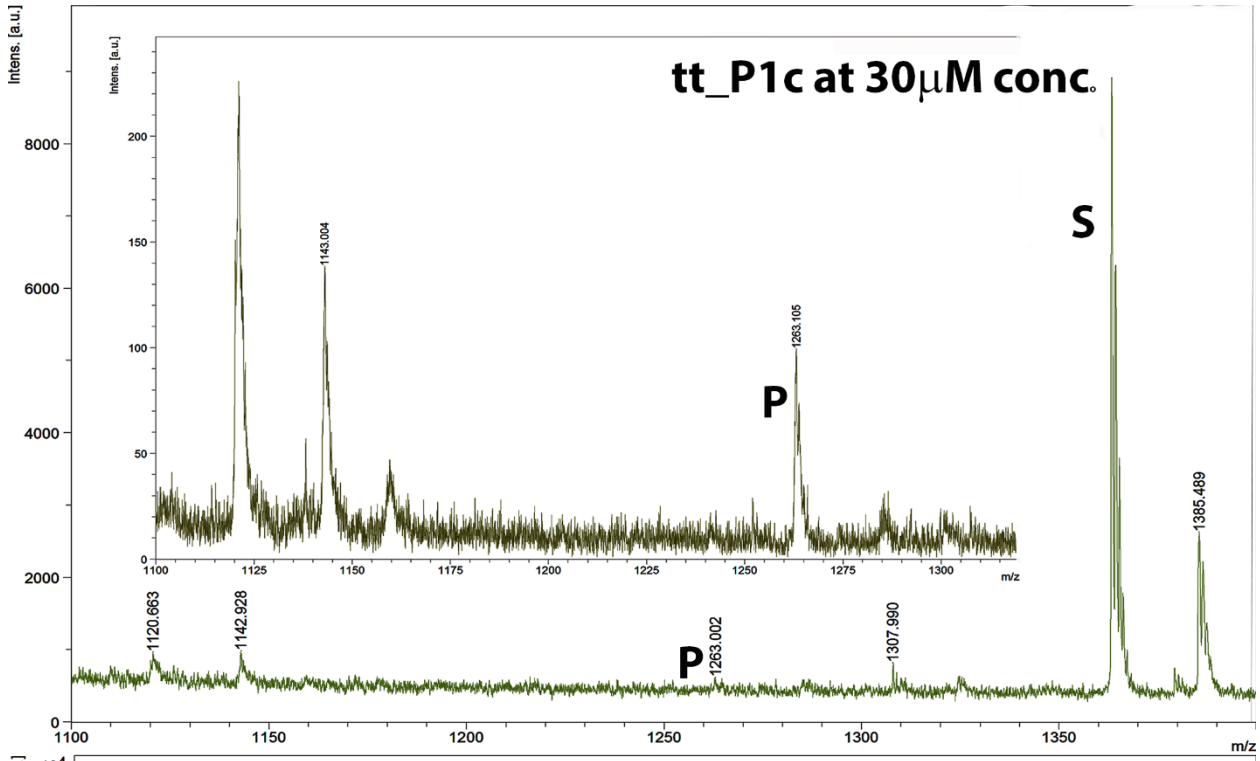
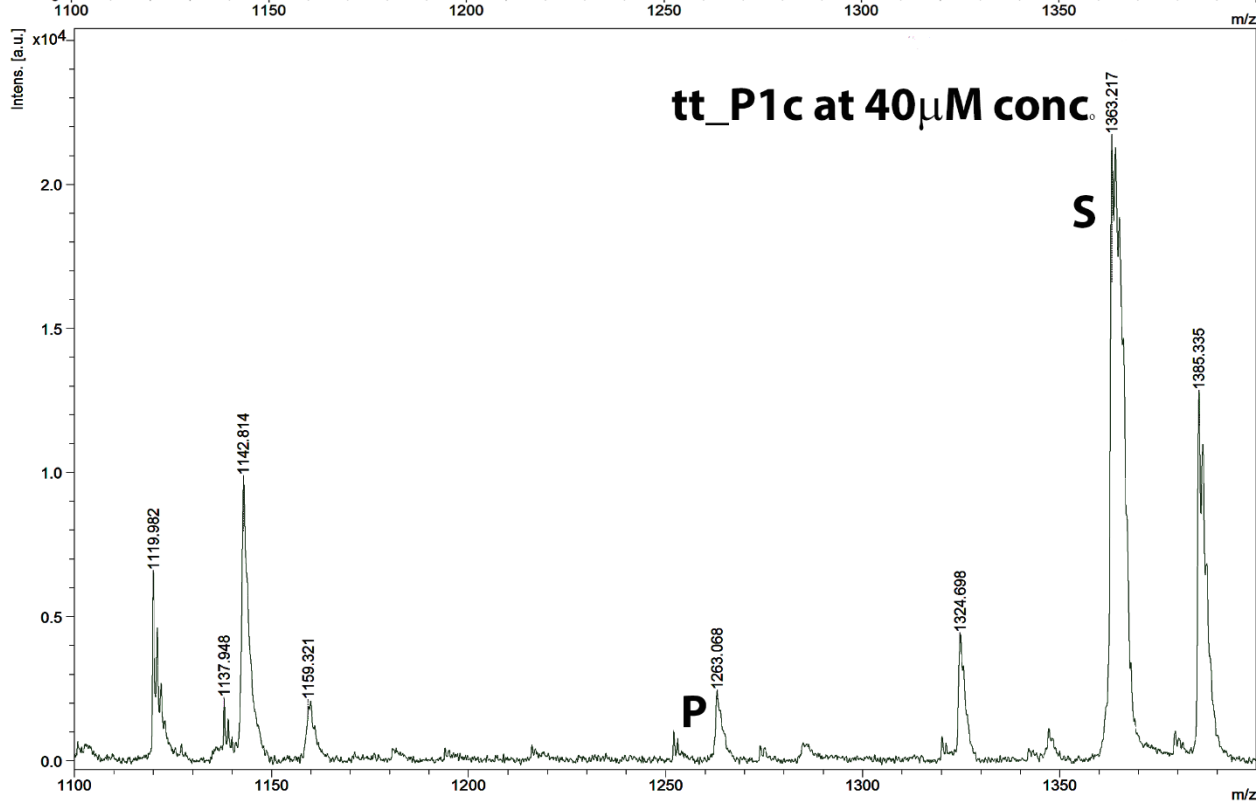
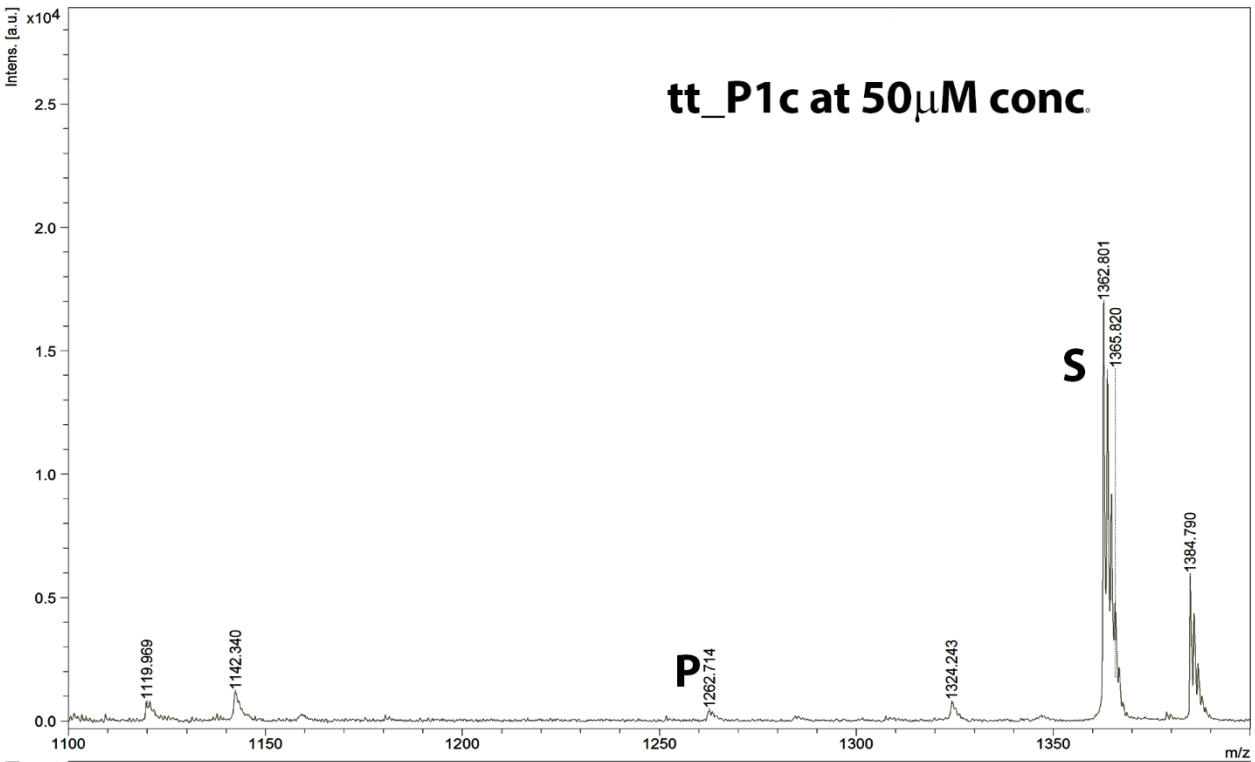

50S
Reaction time: 1 hour
S
P
1362.885
1119.697
1142.271
1262.871
1324.195
1384.840
1424.791
Intens. [a.u.]
x10^4
m/z
50S
Reaction time: 30 minutes
S
1362.94
1384.915
1378.907
Intens. [a.u.]
m/z

Figure S13: MALDI spectra of fragment reactions using 10, 20, 30, 40, 50 and 100 µM concentration of construct tt_P1c.




tt_P1c at 30μM conc.
Intens. [a.u.]
S
P
1385.489
1263.105
1143.004
1120.663
1142.928
1263.002
1307.990
m/z


tt_P1c at 40μM conc.
Intens. [a.u.]
x10^4
S
P
1363.217
1385.335
1142.814
1119.982
1137.948
1159.321
1263.068
1324.698
m/z


tt_P1c at 50μM conc.
Intens. [a.u.]
x10^4
2.5
2.0
1.5
1.0
0.5
0.0
1119.969
1142.340
P
1262.714
1324.243
S
1362.801
1365.820
1384.790
1100
1150
1200
1250
1300
1350
m/z

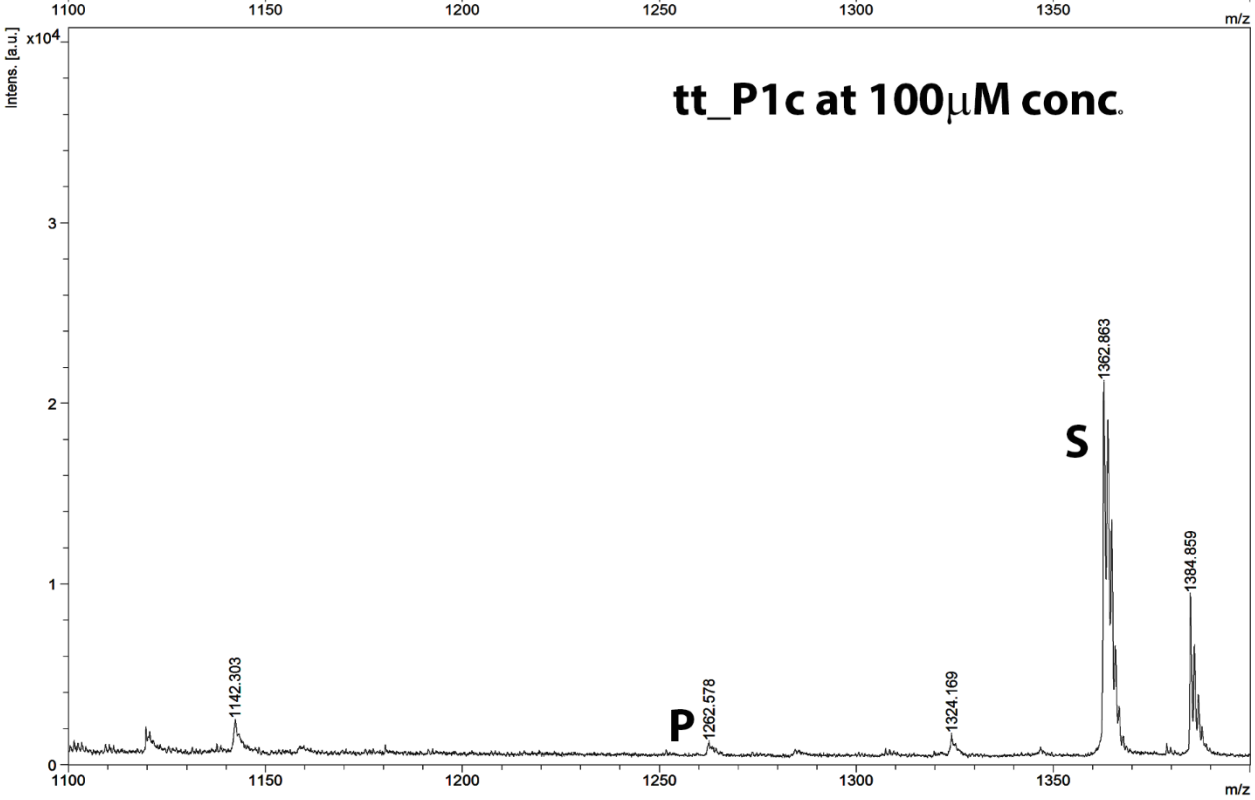

tt_P1c at 100μM conc.
Intens. [a.u.]
x10^4
3
2
1
0
1142.303
P
1262.578
1324.169
S
1362.863
1384.859
1100
1150
1200
1250
1300
1350
m/z

Figure S14: MALDI spectra of the reactions at 2.8 µM concentration of tt_P1c and different substrate concentrations.

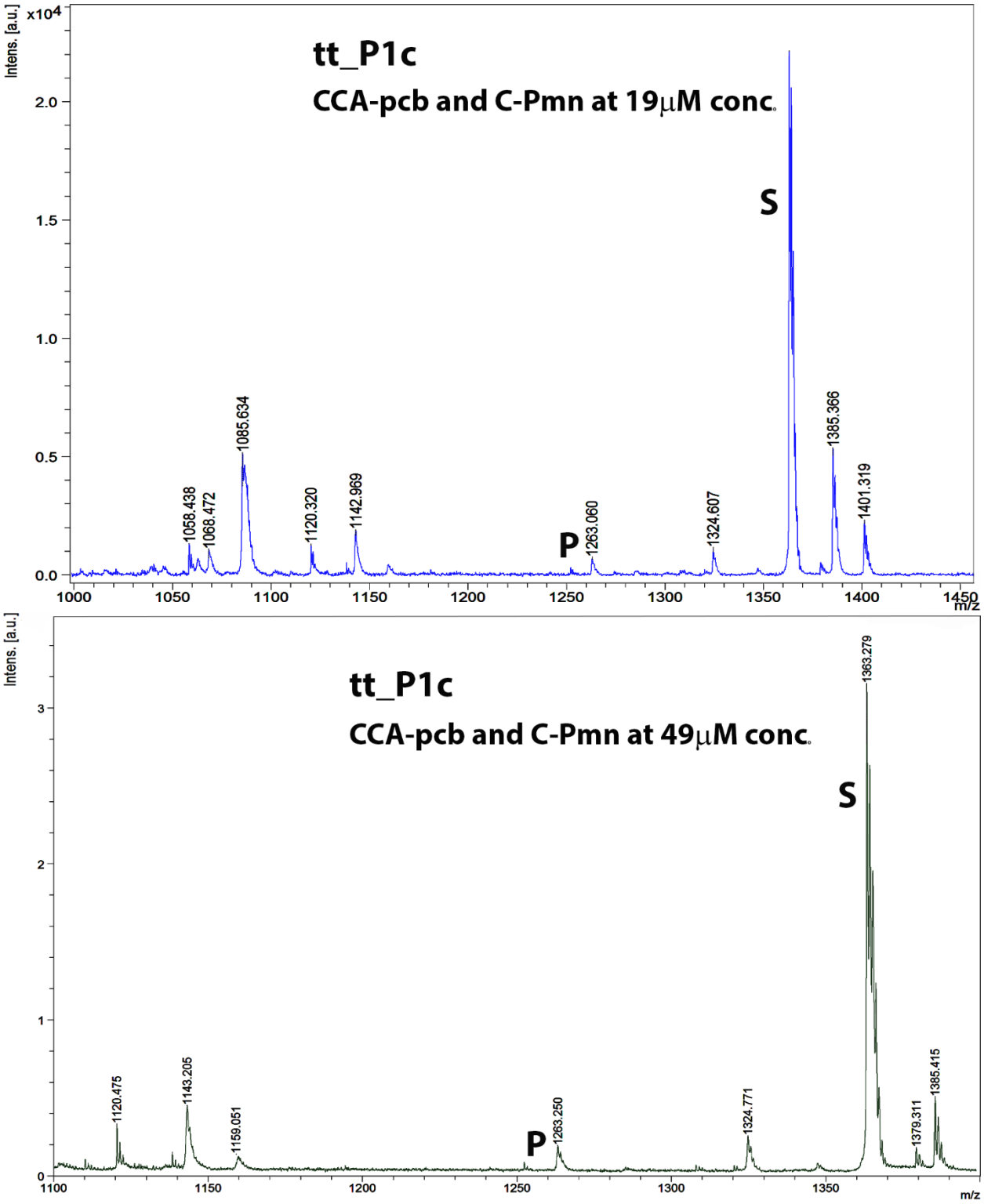
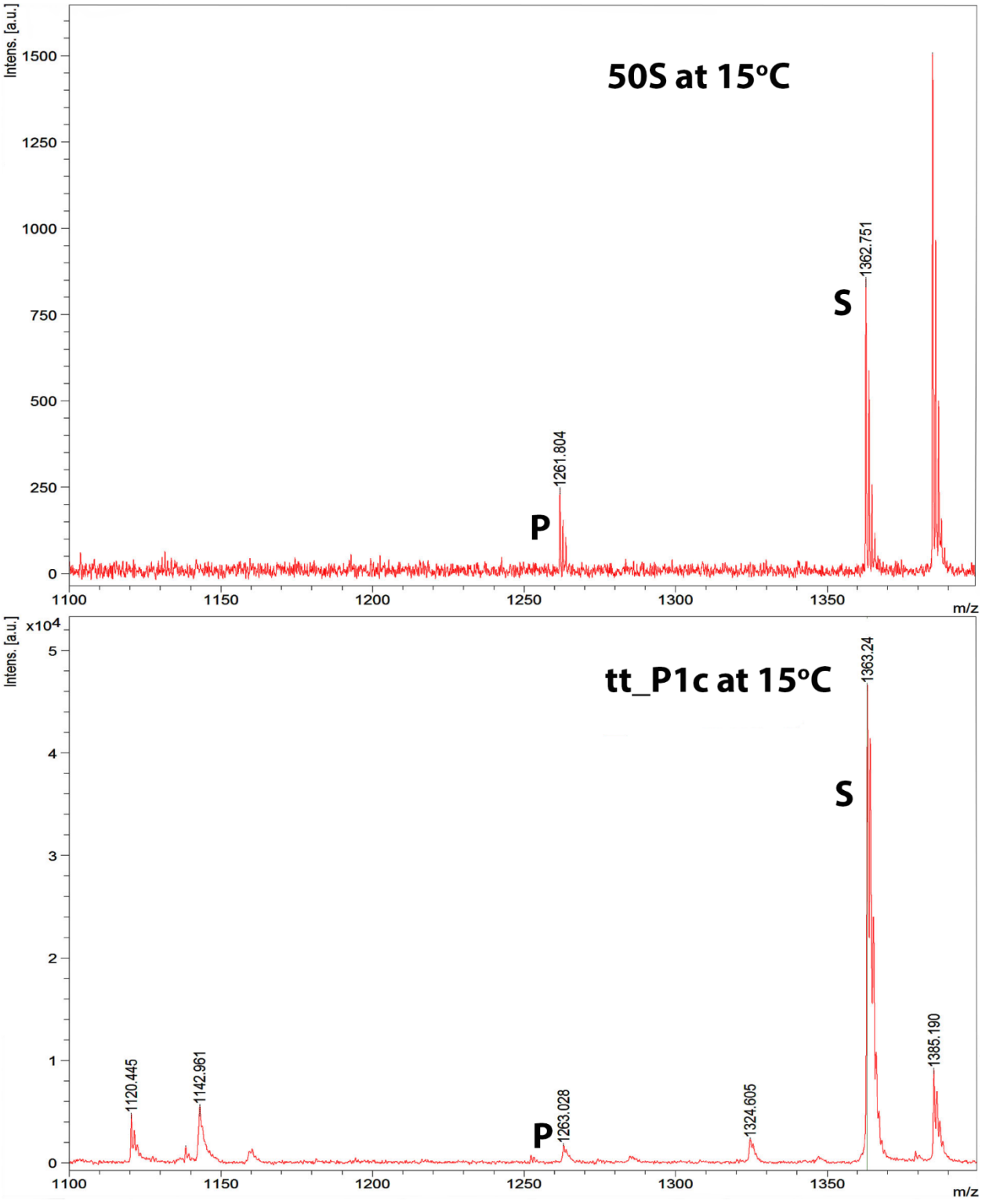
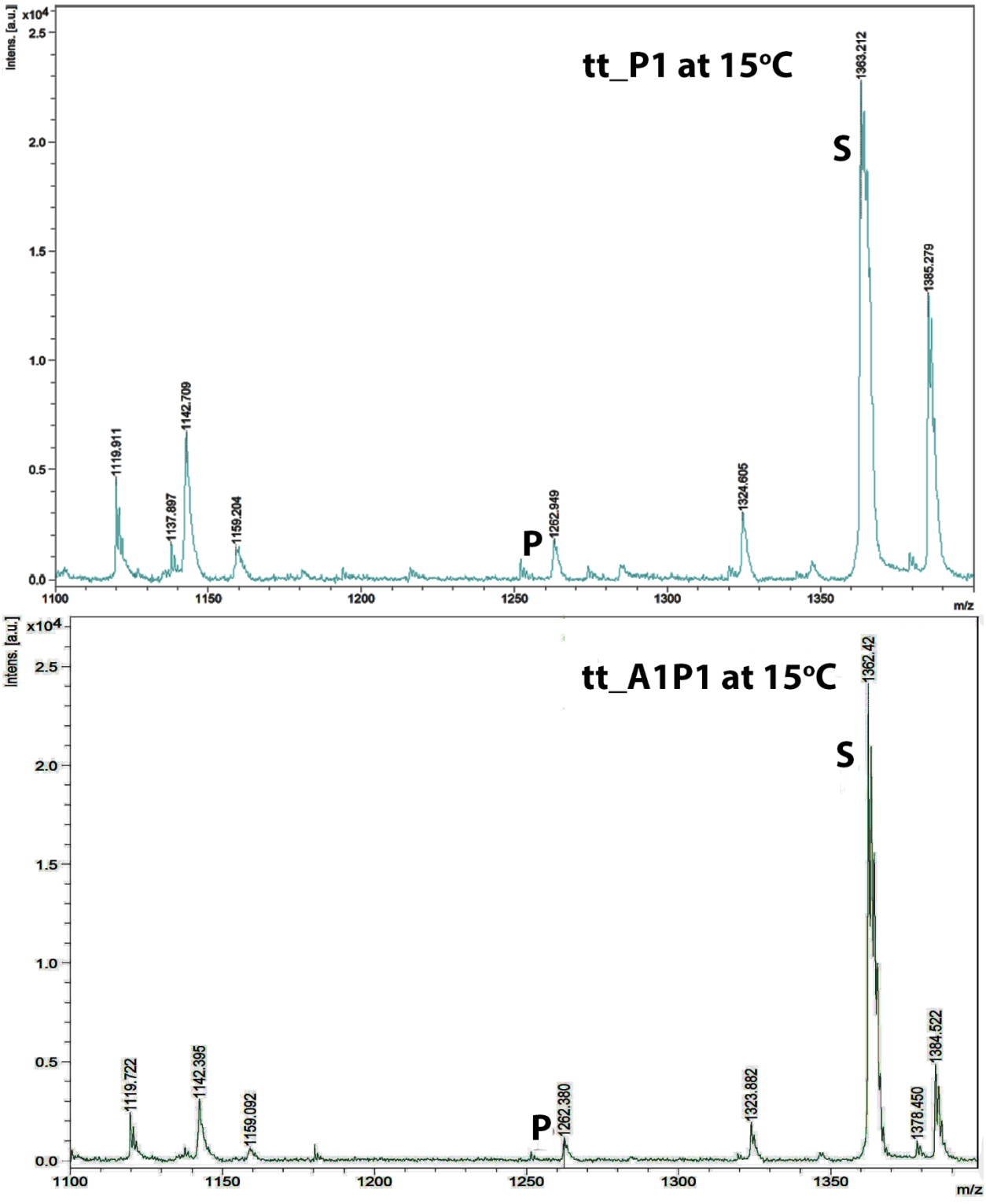


Figure S15: MALDI spectra of the reaction mixtures at 15°C. A. Positive control (50S) and the construct tt_P1c. **B.** The constructs tt_P1, tt_A1P1. **C**. The constructs ef_P1c and sa_P1c.

A



B


tt_P1 at 15°C
Intens. [a.u.]
x10^4
2.5
2.0
1.5
1.0
0.5
0.0
1100
1150
1200
1250
1300
1350
m/z
1119.911
1137.897
1142.709
1159.204
1262.949
1324.605
1383.212
1385.279
S
P
tt_A1P1 at 15°C
Intens. [a.u.]
x10^4
2.5
2.0
1.5
1.0
0.5
0.0
1100
1150
1200
1250
1300
1350
m/z
1119.722
1142.395
1159.092
1262.380
1323.882
1362.42
1378.450
1384.522
S
P

C

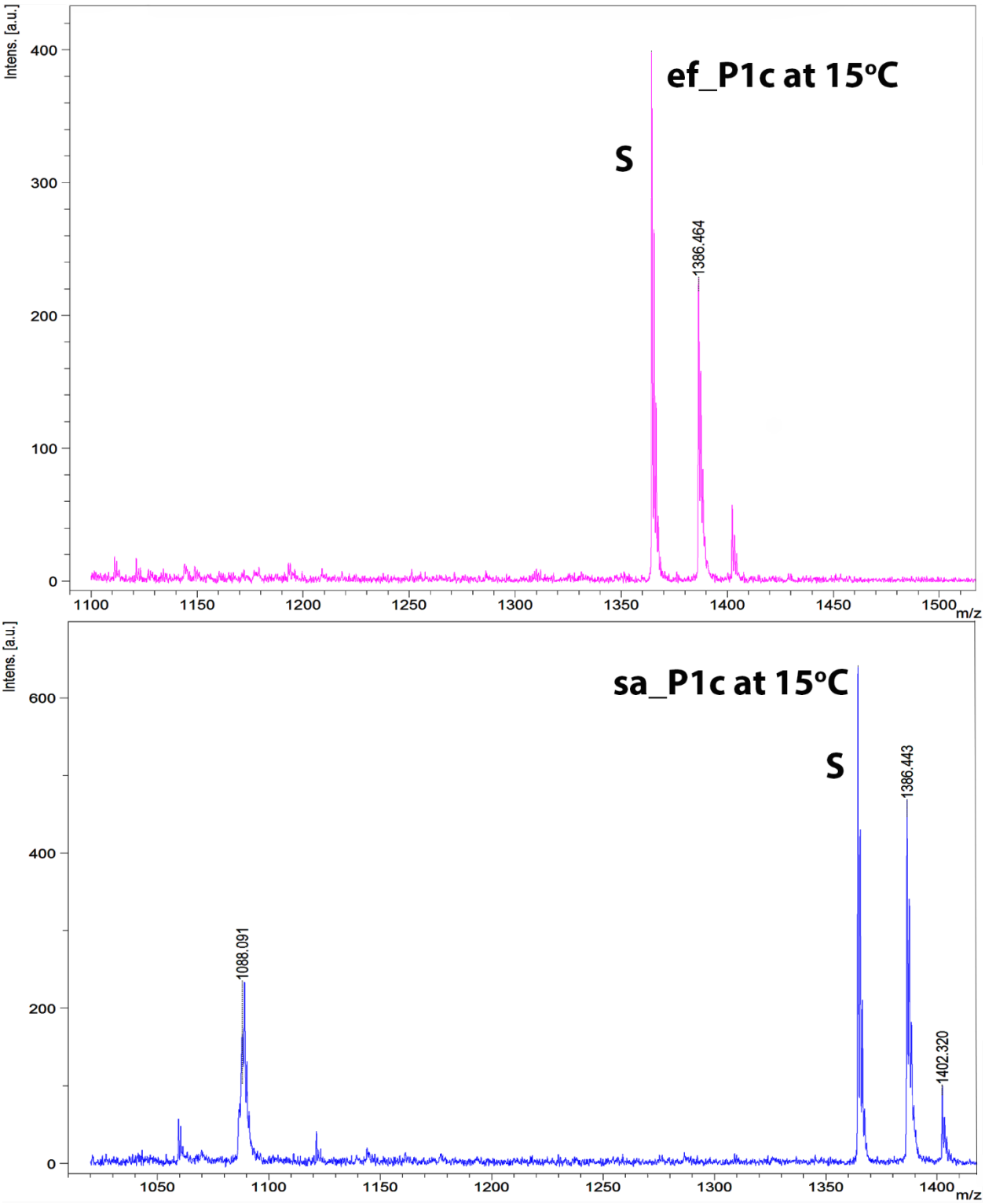
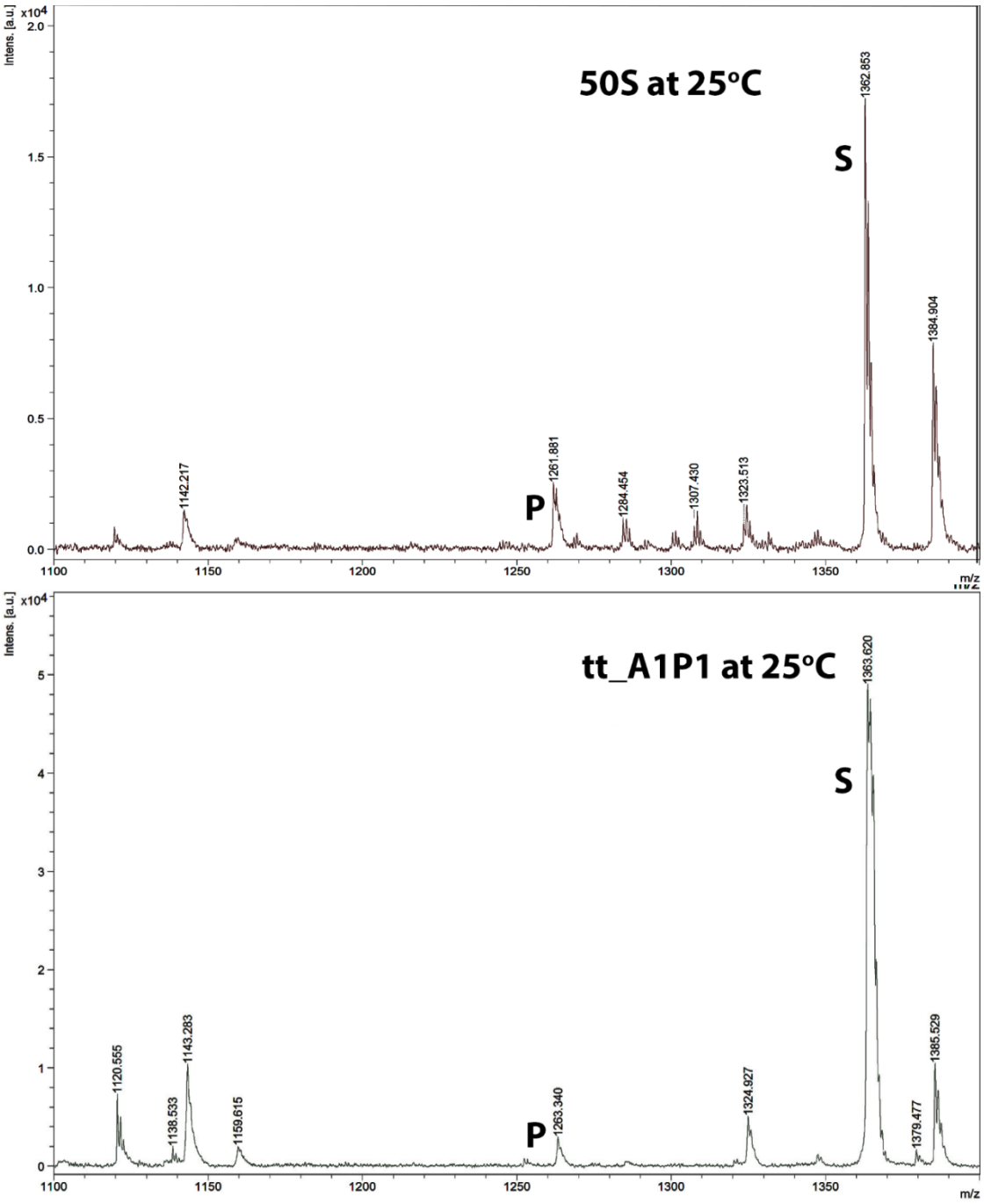
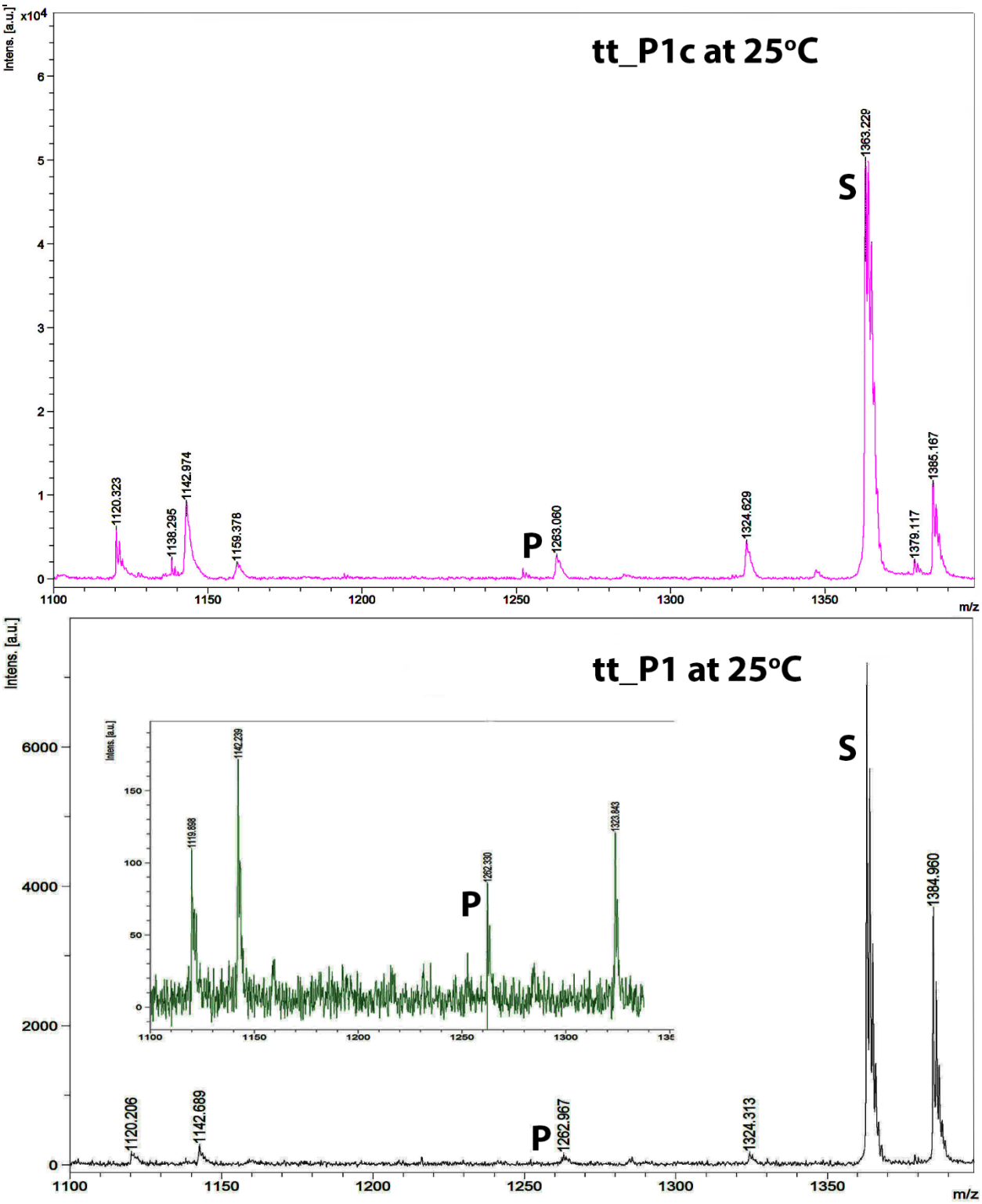

Intens. [a.u.]
ef_P1c at 15°C
S
1386.464
m/z
Intens. [a.u.]
sa_P1c at 15°C
S
1386.443
1088.091
1402.320
m/z

Figure S16: MALDI spectra of the reaction mixtures at 25°C. A. Positive control (50S) and the construct tt_A1P1. **B.** The constructs tt_P1c and tt_P1. **C**. The constructs ef_P1c and sa_P1c.

A



B


tt_P1c at 25°C
Intens. [a.u.]
x10^4
S
P
1120.323
1138.295
1142.974
1159.378
1263.060
1324.629
1363.229
1379.117
1385.167
m/z
tt_P1 at 25°C
Intens. [a.u.]
S
P
1120.206
1142.689
1262.967
1324.313
1384.960
1119.698
1142.239
1262.330
1323.843
m/z

C

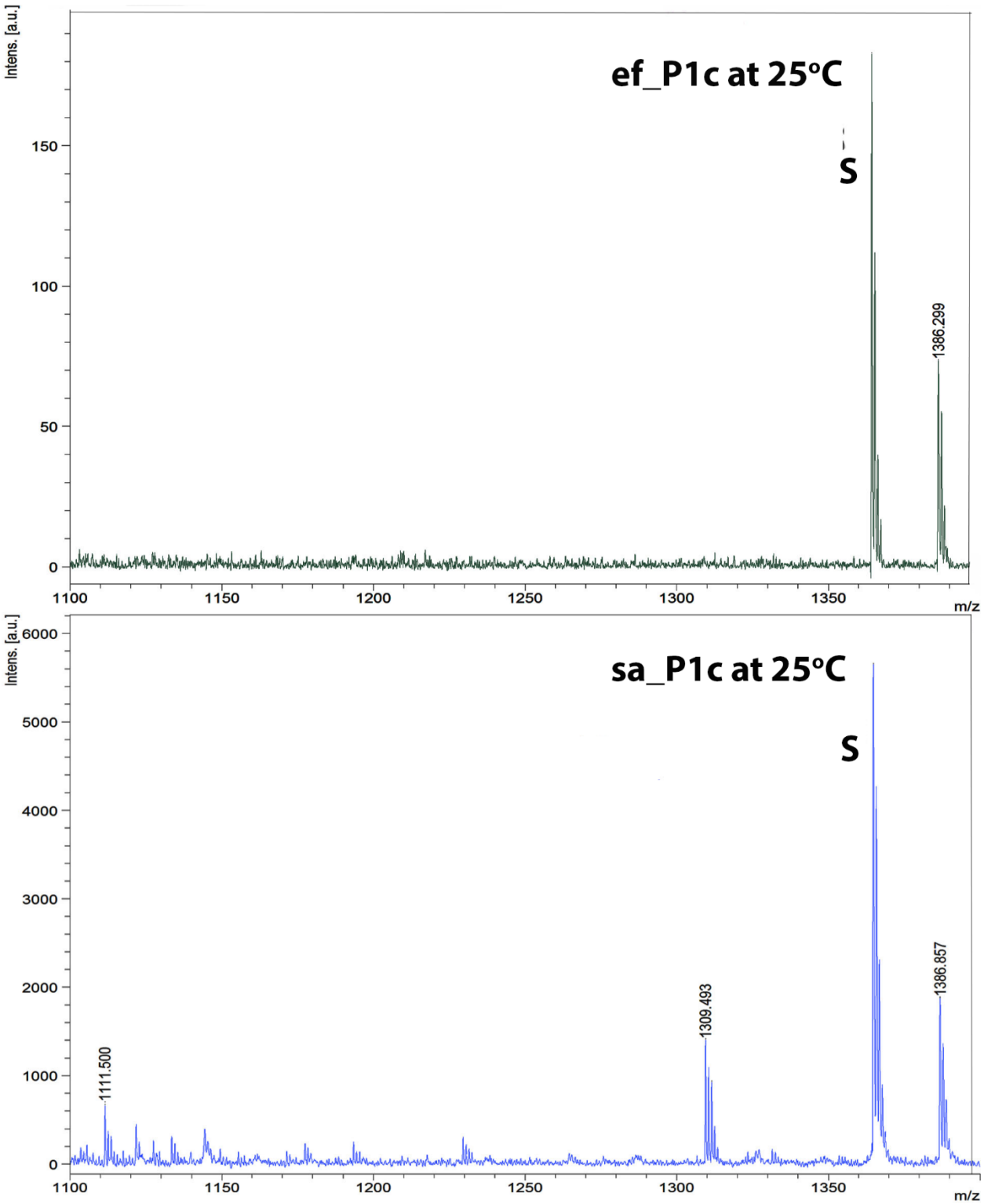
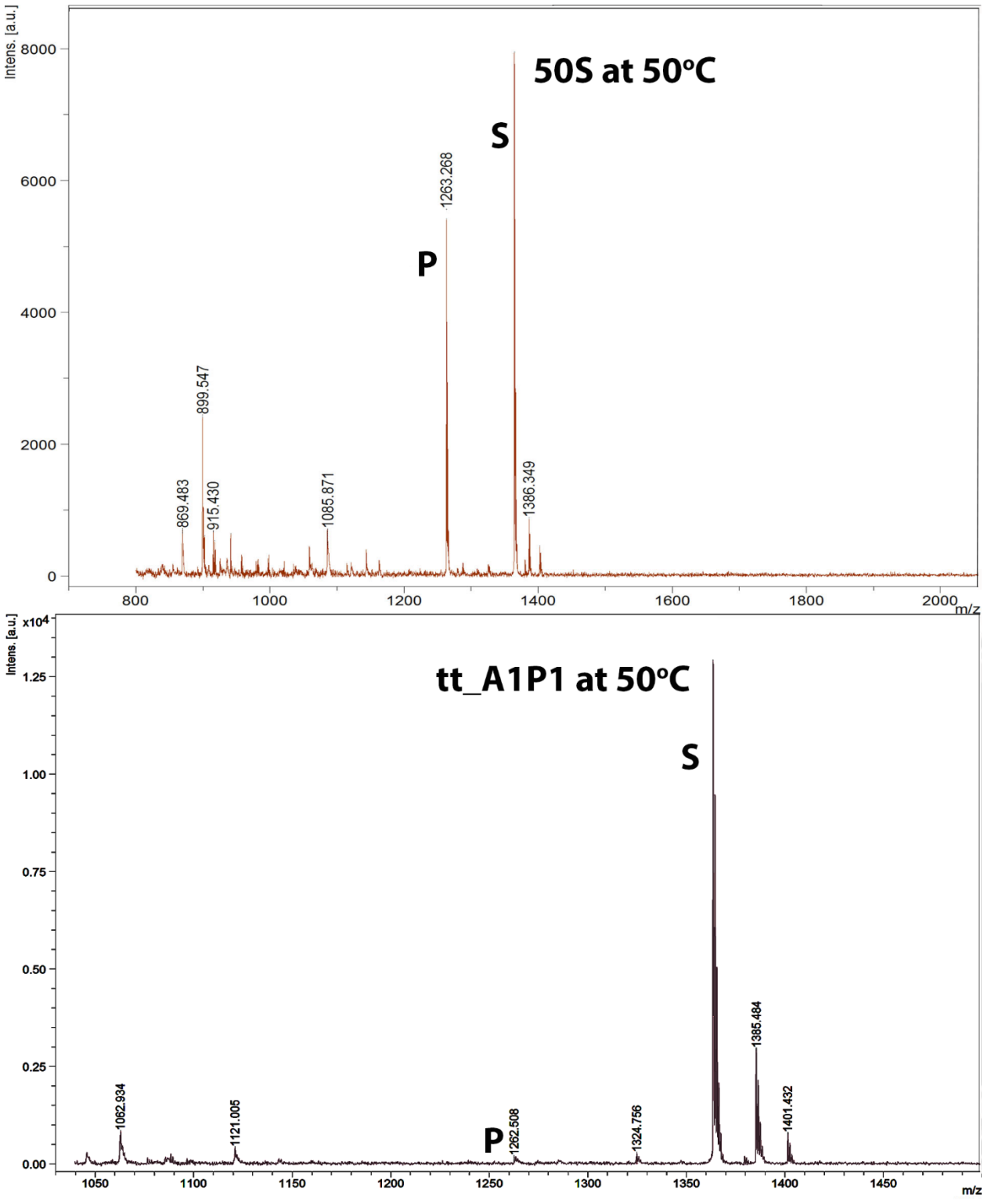
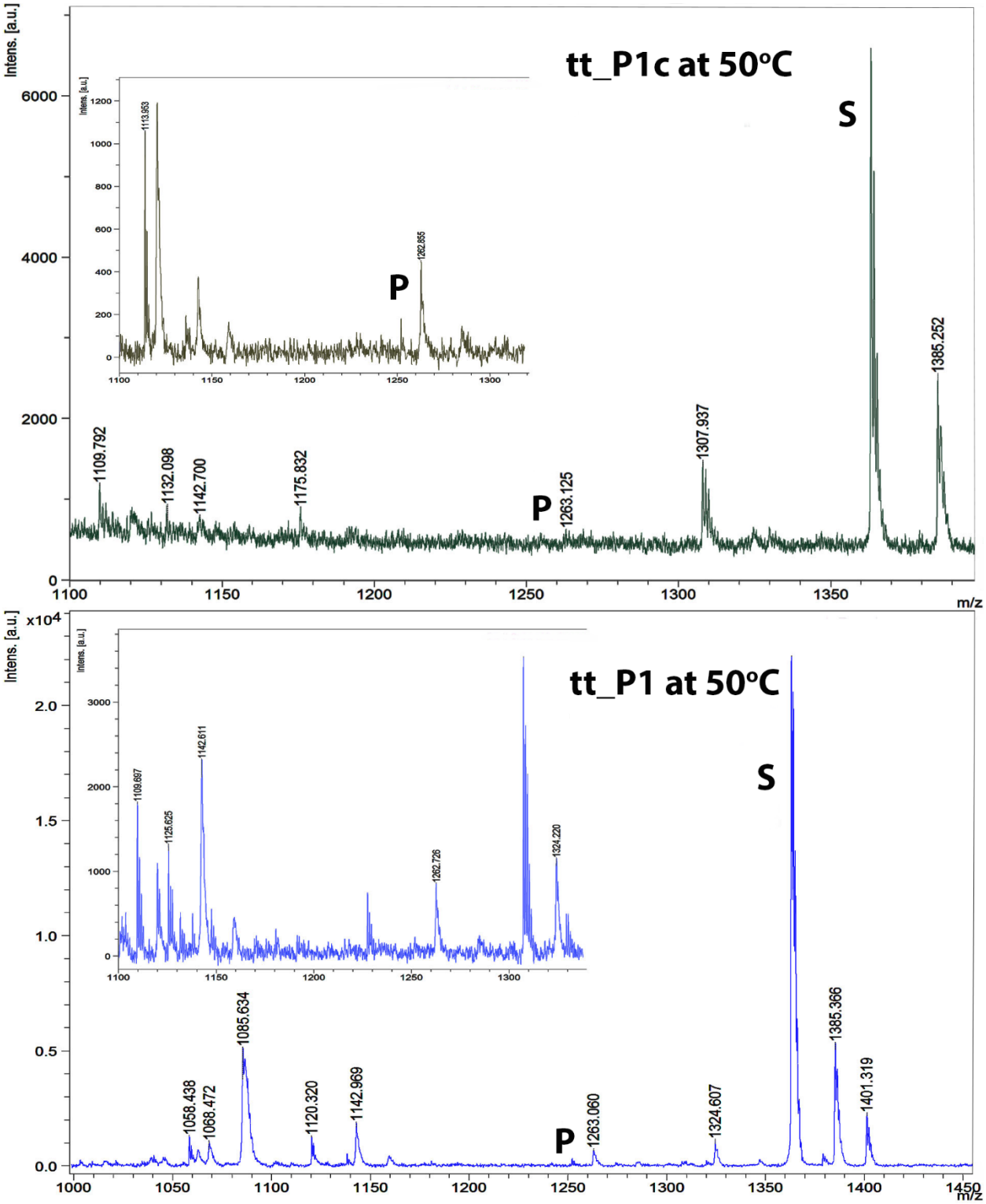

ef_P1c at 25°C
S
1386.299
Intens. [a.u.]
150
100
50
0
1100
1150
1200
1250
1300
1350
m/z
sa_P1c at 25°C
S
1386.857
1309.493
1111.500
Intens. [a.u.]
6000
5000
4000
3000
2000
1000
0
1100
1150
1200
1250
1300
1350
m/z

Figure S17: MALDI spectra of the reaction mixtures at 50°C. A. positive control (50S) and the construct tt_A1P1 **B.** The constructs tt_P1c and tt_P1. **C.** The constructs sa_P1c and ef_P1c.

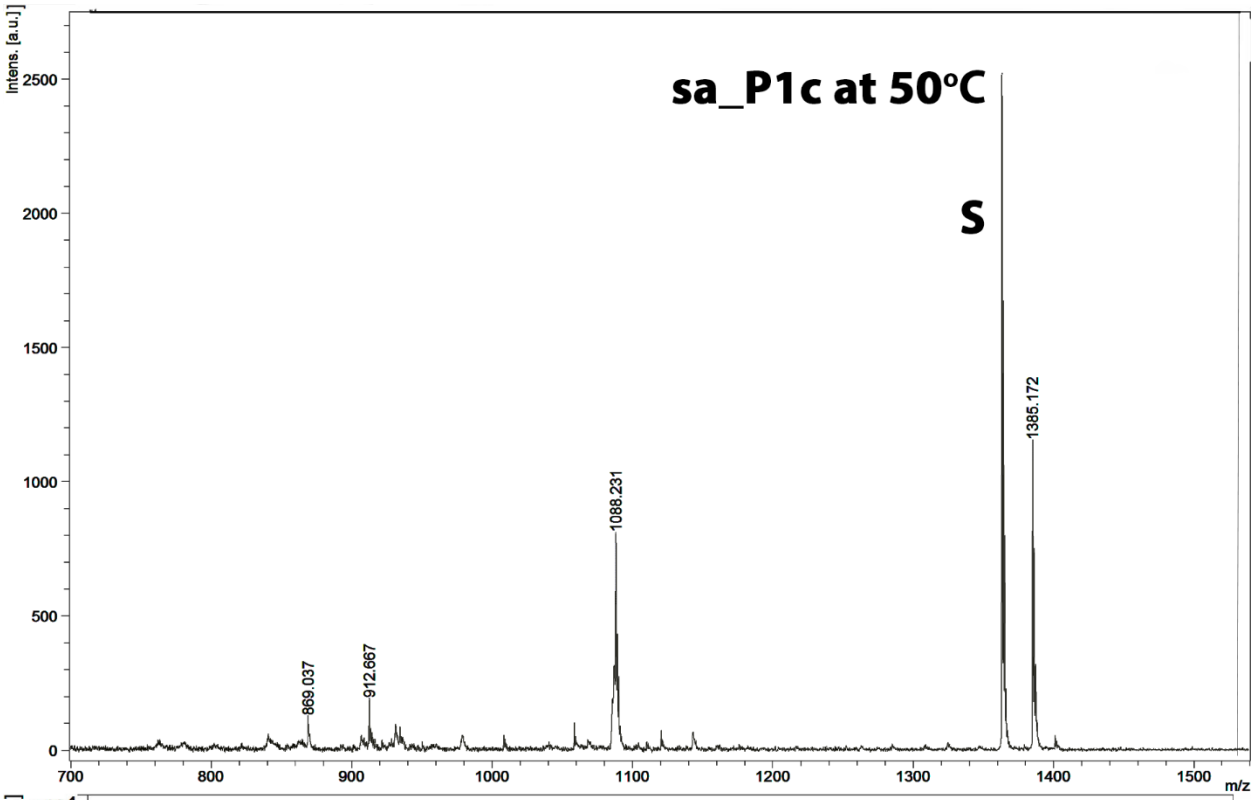
A



B


tt_P1c at 50°C
Intens. [a.u.]
S
P
1109.792
1132.098
1142.700
1175.832
1263.125
1307.937
1385.252
m/z
tt_P1 at 50°C
x10^4
1109.697
1125.625
1142.611
1262.726
1324.220
1058.438
1068.472
1085.634
1120.320
1142.969
1263.060
1324.607
1385.366
1401.319

C


sa_P1c at 50°C
S
Intens. [a.u.]
2500
2000
1500
1000
500
0
869.037
912.667
1088.231
1385.172
700
800
900
1000
1100
1200
1300
1400
1500
m/z

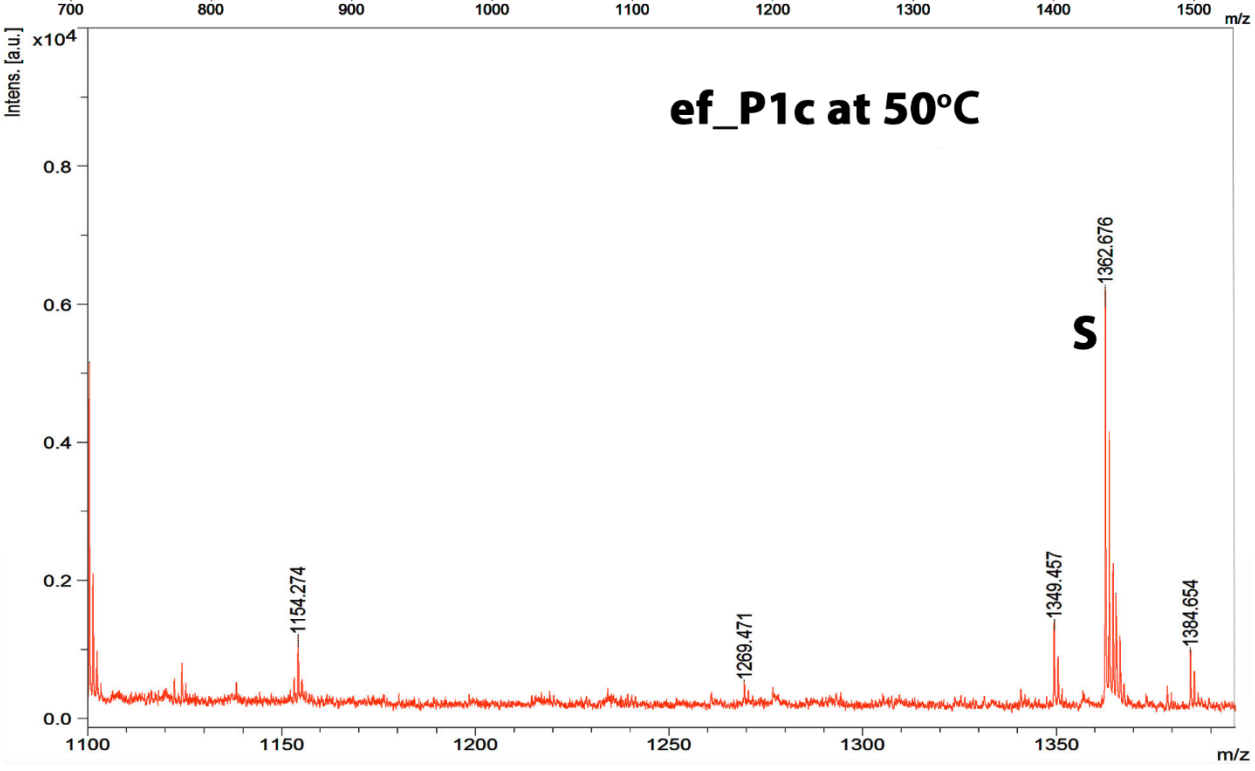

ef_P1c at 50°C
S
Intens. [a.u.]
x10^4
0.8
0.6
0.4
0.2
0.0
1154.274
1269.471
1349.457
1362.676
1384.654
1100
1150
1200
1250
1300
1350
m/z

Figure S18: EtBr stained native gel of RNA constructs under various controlled conditions of dimerization. **A-C.** tt_P1c, tt_P1, and tt_A1P1, respectively in the absence of salts. **D.** tt_P1, tt_P1c, and tt_A1P1 with 30 mM KCl and no Mg^{2+} **E.** tt_P1c, tt_P1, and tt_A1P1 with no salts but in the presence of the substrates CCA-pcb and C-Pmn. **F.** tt_A1, tt_A2 and ec_P1c at 20 µM RNA concentration. Gels were imaged at the wavelength of 254nm.

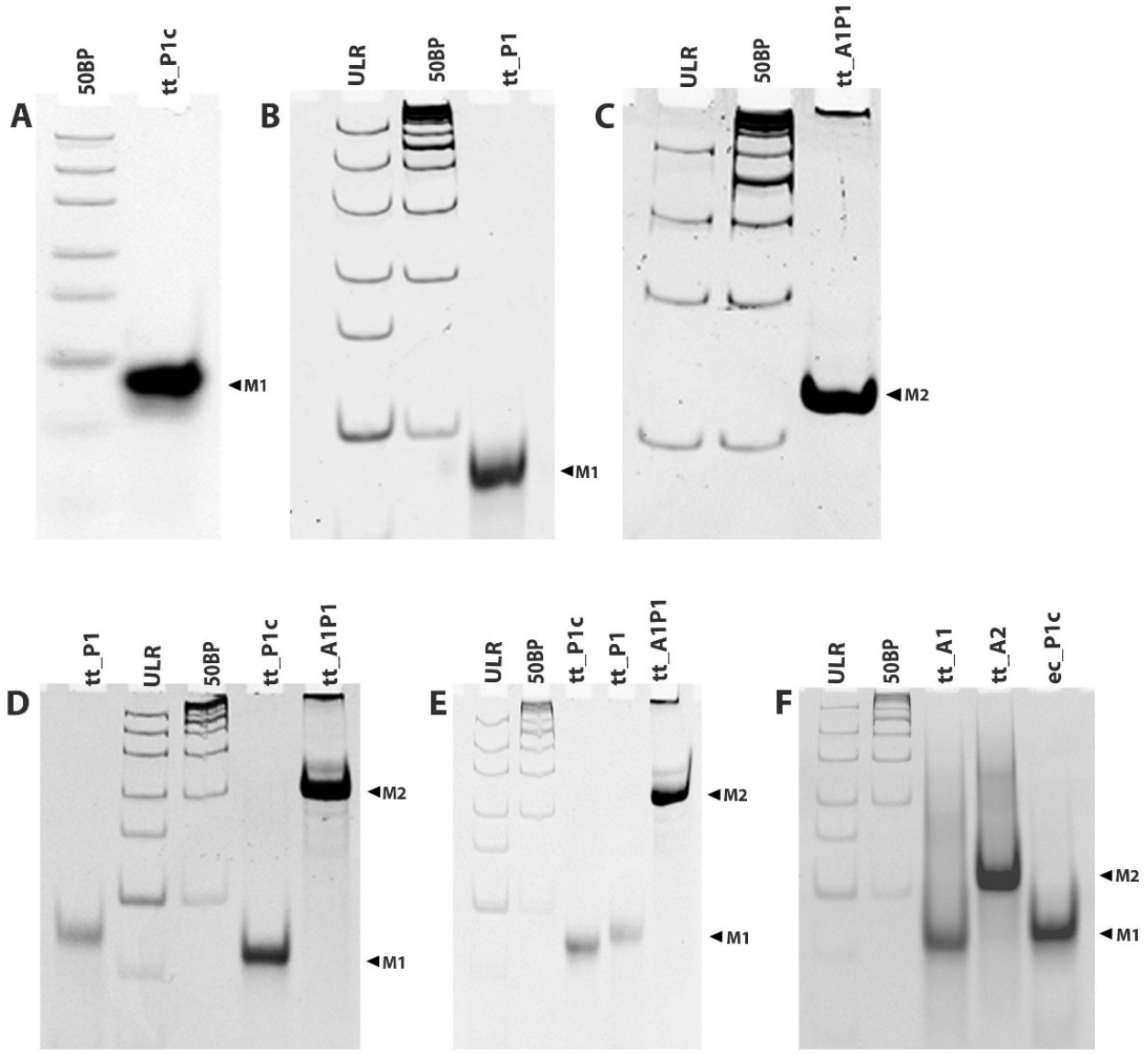
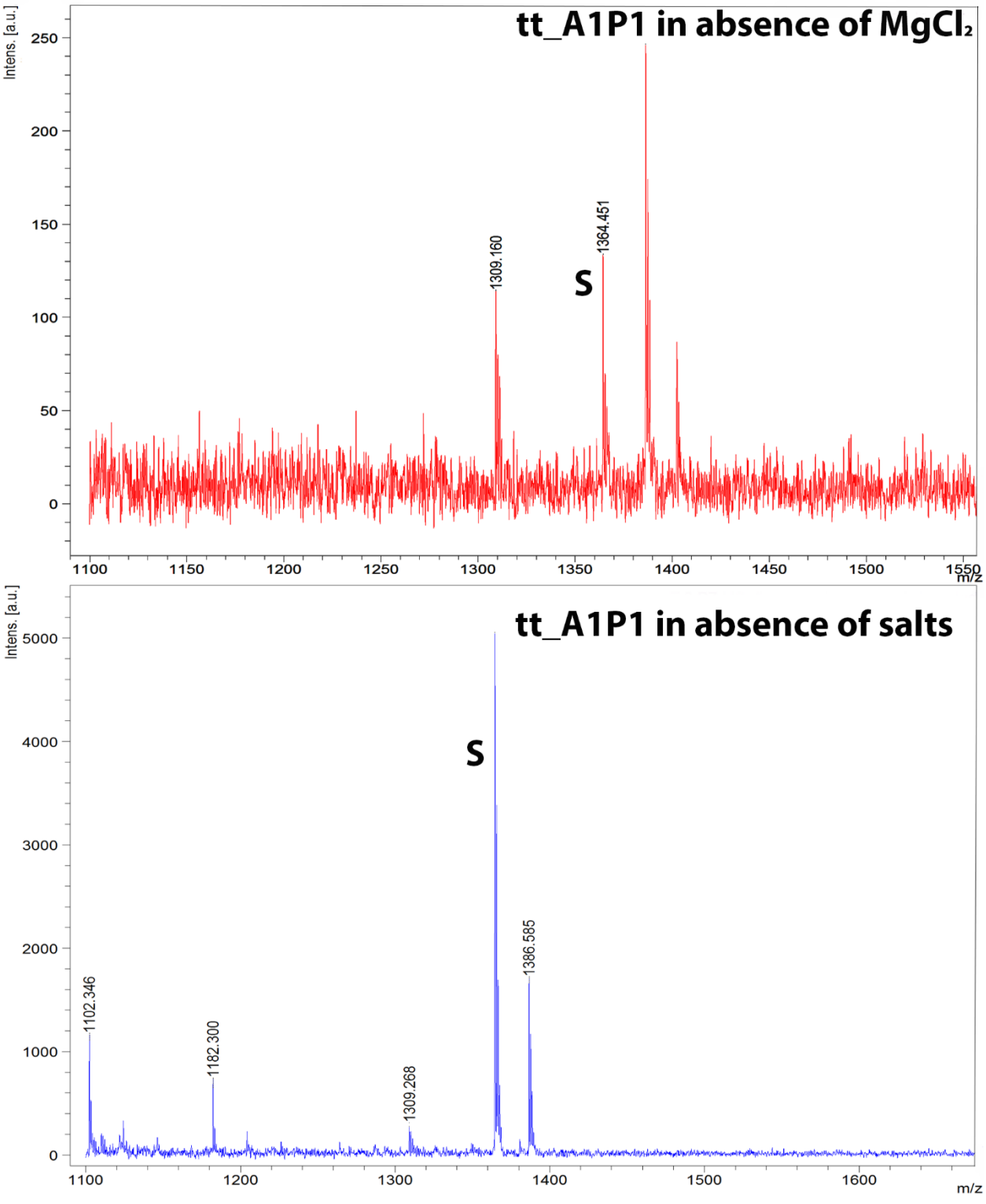
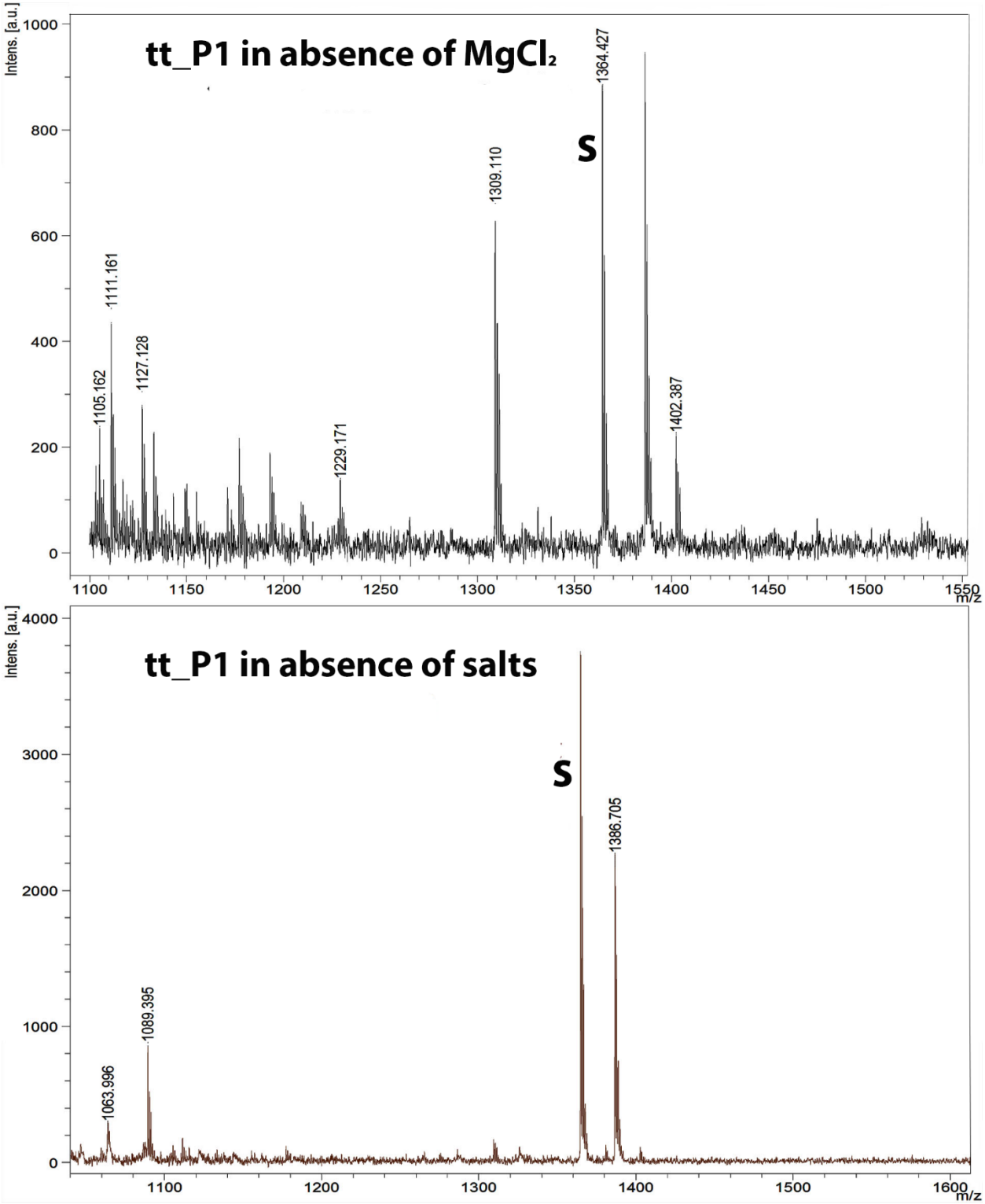


Figure S19: MALDI spectra of tt_A1P1, tt_P1 and tt_P1c reactions performed in the absence of KCl and $MgCl_2$ (salts) or in the absence of $MgCl_2$.

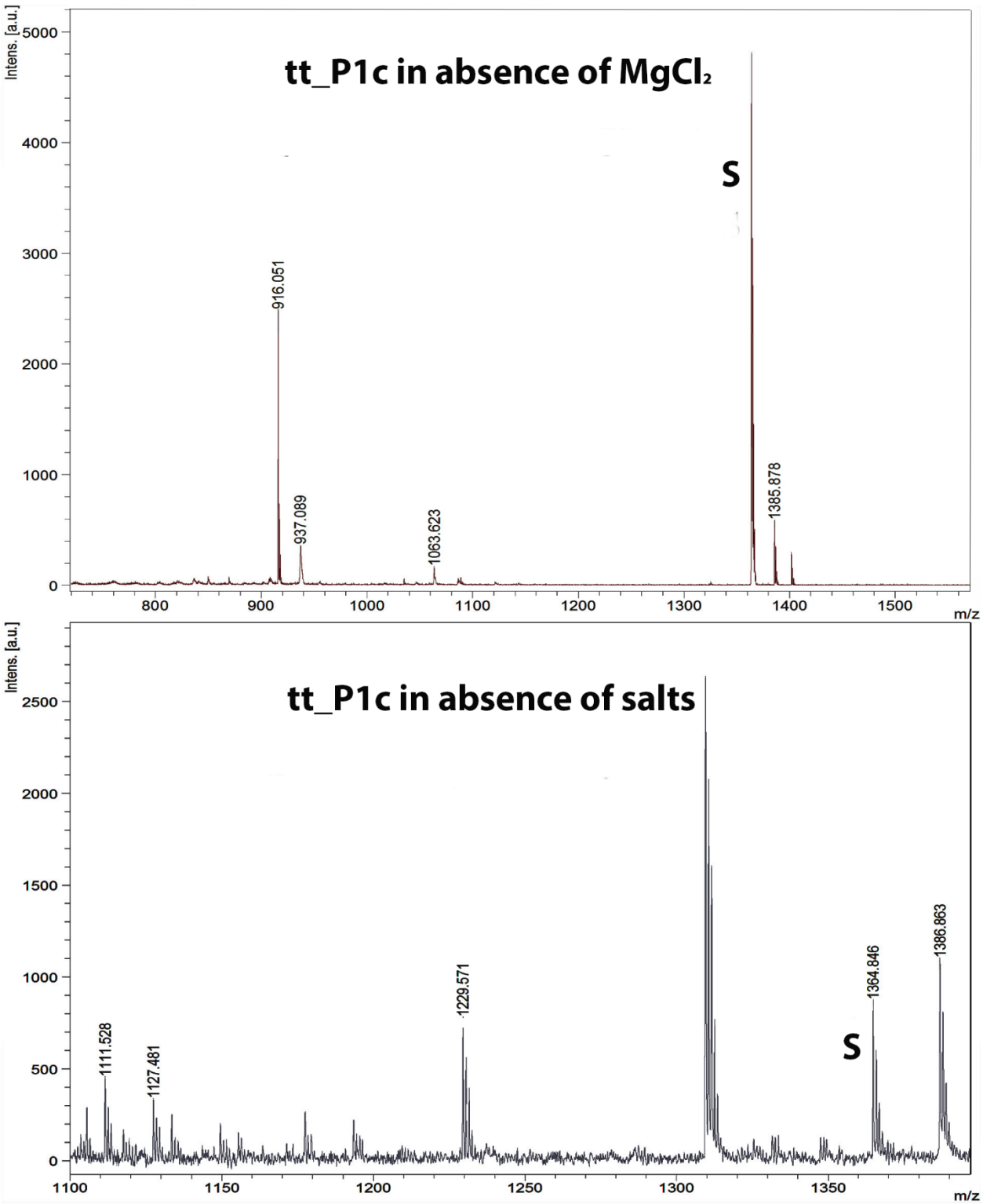
A



B


tt_P1 in absence of MgCl₂
Intens. [a.u.]
1000
800
600
400
200
0
1105.162
1111.161
1127.128
1229.171
1309.110
S
1364.427
1402.387
1100
1150
1200
1250
1300
1350
1400
1450
1500
1550
m/z
tt_P1 in absence of salts
Intens. [a.u.]
4000
3000
2000
1000
0
1063.996
1089.395
S
1386.705
1100
1200
1300
1400
1500
1600
m/z

C


tt_P1c in absence of MgCl₂
Intens. [a.u.]
5000
4000
3000
2000
1000
0
916.051
937.089
1063.623
S
1385.878
800
900
1000
1100
1200
1300
1400
1500
m/z
tt_P1c in absence of salts
Intens. [a.u.]
2500
2000
1500
1000
500
0
1111.528
1127.481
1229.571
S
1364.846
1386.863
1100
1150
1200
1250
1300
1350
m/z

Derivation of Equation used to calculate the dimerization constant (K_d)

The reaction between a labelled and unlabelled oligomer can be represented as follows:

$$M + M^* \underset{}{\overset{Kd}{\rightleftharpoons}} MM^*$$

$$M + M \underset{}{\overset{Kd}{\rightleftharpoons}} MM$$

[M = Unlabelled oligomer, M* = Labelled oligomer]

Since, the labelled and unlabelled oligomers are same RNA sequences, we assume that they have same K_d

$$\mathbf{K_d = \frac{[M][M^*]}{[MM^*]} = \frac{[M][M]}{[MM]}}$$

$\mathbf{M_0^* = [M^*] + [MM^*]}$ M_0^* = Total Concentration of labelled oligomer

$\mathbf{M_0 = [M] + 2[MM] + [MM^*]}$ M_0= Total concentration of unlabelled oligomer

$\mathbf{Y = \frac{[MM^*]}{[MM^*]+[M^*]} *100}$ where Y= Dimer Fraction %

Substituting the values in the above equation,

$$\mathbf{Dimer\ \% = \left\{\frac{\sqrt{K_d(K_d+8x)}-K_d-2x}{2(K_d-X)}\right\} * 100} \qquad \mathbf{(1)}$$

Where X = M_0 or concentration of the unlabelled oligomers
Y = Dimer %

$$\mathbf{where,\ Dimer\% = \frac{[Dimer]}{[Dimer+Monomer]} * 100} \qquad \mathbf{(2)}$$

Figure S20: Determination of the dimerization constant (K_d) of the RNA constructs using fluorescently imaged EMSA native gels. **A-F.** Dimerization was assayed at increasing RNA concentrations from 0.0μM (left) to 42μM (right) (lanes 1-12 RNA concentrations were 0, 0.08, 0.16, 0.24, 0.4, 0.72, 1.36, 2.46, 5.2, 10.4, 21 and 42μM respectively), with constant (0.15 μM) Fluorecein-5-thiosemicarbazide labelled RNA concentration in all lanes. EMSA was performed for tt_P1c, tt_A1P1, tt_P1, tt_P1m, sa_P1c and ef_P1c constructs, respectively.

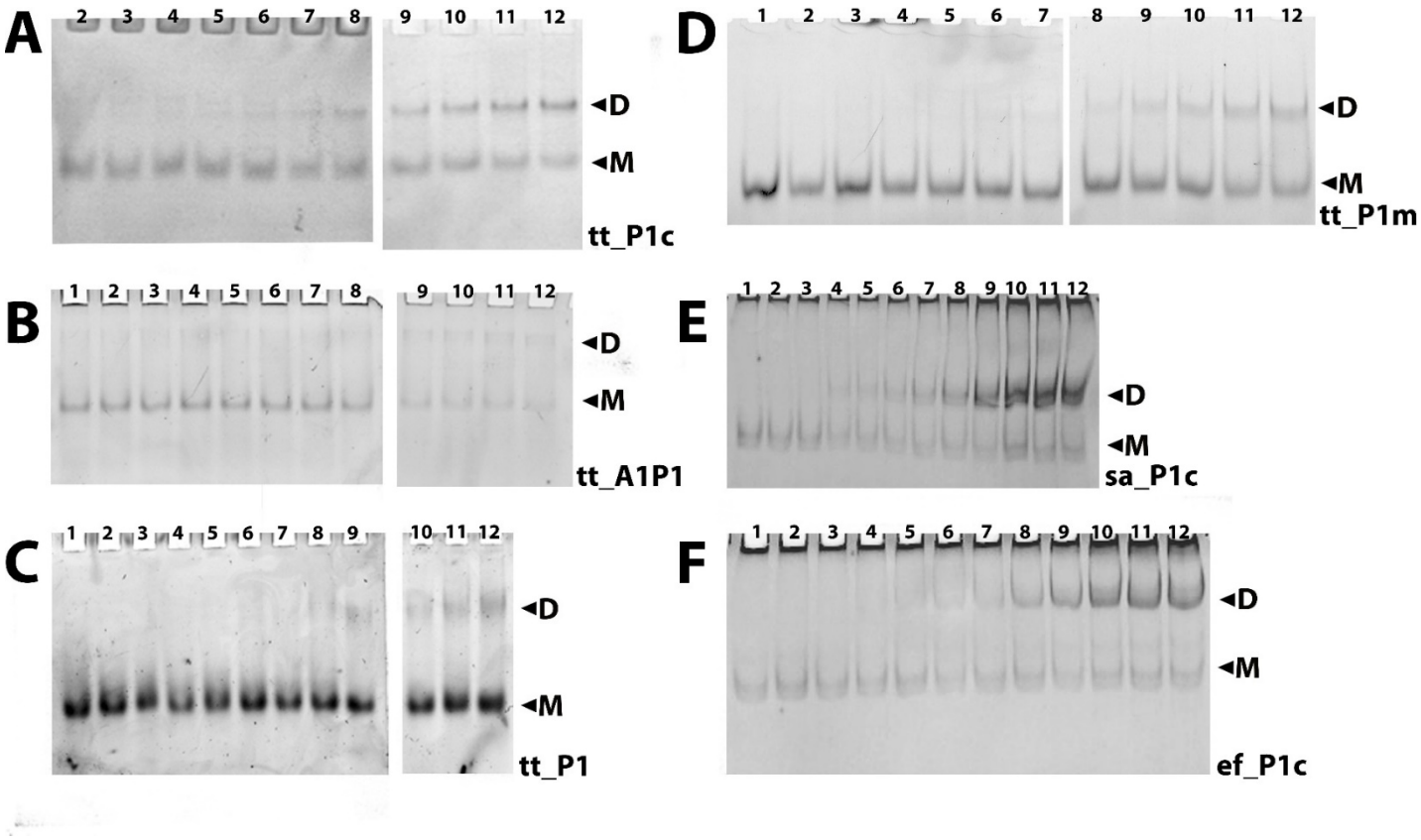


Figure S21: EtBr staining of the native gels used for the determination of the K_d of the RNA constructs. A-G. RNA construct dimerization is shown for tt_P1c, tt_A1P1, tt_P1, tt_P1m, tt_P1n, sa_P1c and ef_P1c, respectively. Dimerization was assayed at increasing RNA concentrations from 0.0μM (left) to 42μM (right), with constant (0.15μM) labelled RNA concentration in all lanes (lanes 1-12 RNA concentrations are: 0, 0.08, 0.16, 0.24, 0.4, 0.72, 1.36, 2.46, 5.2, 10.4, 21 and 42 μM, respectively). Gels were imaged at a wavelength of 254nm.

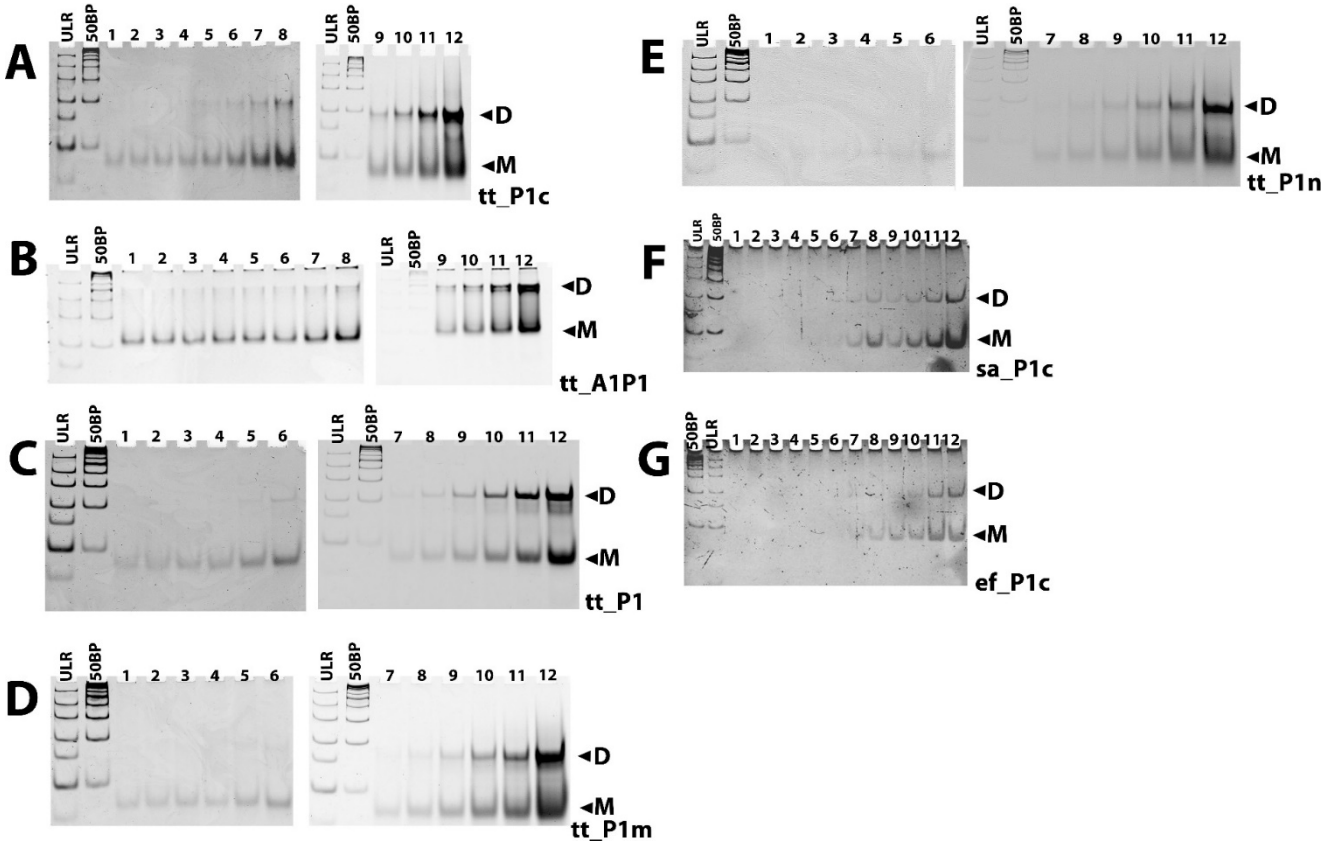


Table S1: Sequences of the RNA constructs

RNA Construct	Sequence*
tt_A1	GAUGUCGGCUCGUCGCUUCGGCGGCACGCGAGCUGGGUUCAGAACG UCGUGAGACAGUUCGGUC
tt_P1	GAAGACCCCGUGGAGCUCUUCGGAGUUACCCCGGGGAUAACAGGCU GAUCUCUUCGGAGGUUUGGCACCUC
tt_A1P1	GAAGACCCCGUGGAGCUCUUCGGAGUUACCCCGGGGAUAACAGGCU GAUCUCUUCGGAGGUUUGGCACCUCGAUGUCGGCUCGUCGCUUCGG CGGCACGCGAGCUGGGUUCAGAACGUCGUGAGACAGUUCGGUC
tt_A2	GAUGUCGGCUCGUCGCAUCCUUCGGGUUGGGCUGUUCGCCCAUUAA AGCGGCACGCGAGCUGGGUUCAGAACGUCGUGAGACAGUUCGGUC
tt_P2	GAAGACCCCGUGGAGCUUUACUCUUCGGAGUUUGACUGGGGCGGUC CGGAUAAAAGUUACCCCGGGGAUAACAGGCUGAUCUCUUCGGAGGU UUGGCACCUC
tt_A2P2	GAAGACCCCGUGGAGCUUUACUCUUCGGAGUUUGACUGGGGCGGUC CGGAUAAAAGUUACCCCGGGGAUAACAGGCUGAUCUCUUCGGAGGU UUGGCACCUCGAUGUCGGCUCGUCGCAUCCUUCGGGUUGGGCUGUU CGCCCAUUAAAGCGGCACGCGAGCUGGGUUCAGAACGUCGUGAGAC AGUUCGGUC
tt_P2c	GAAGACCCCGUGGAGCUUUACUCUUCGGAGUUUGACUGGGGCGGUC CGGAUAAAAGUUACCCCGGGGAUAACAGGCUGAUCGUGAGGUUUGG CACCUC
tt_P1c	GAAGACCCCGUGGAGCUCUUCGGAGUUACCCCGGGGAUAACAGGCU GAUCGUGAGGUUUGGCACCUC
ec-P1c	AAAGACCCCGUGAACCUCUUCGGAGGUACUCCGGGGAUAACAGGCU GAUAGUGAUGUUUGGCACCUC
sa_P1c	GAAGACCCCGUGGAGCUCUUCGGAGCUACCCCGGGGAUAACAGGCU UAUCGUGAGGUUUGGCACCUC
ef_P1c	GAAGACCCCAUGGAGCUCUUCGGAGCUACCCUGGGGAUAACAGGCU UAUCGUGAGGUUUGGCACCUC
tt_P1m	GAAGACCCCGUGGAGCUUUAAGUUACCCCGGGGAUAACAGGCUGAU CGUGAGGUUUGGCACCUC
tt_P1n	GAAGACCCCGUGGAGCUUUAAAGUUACCCCGGGGAUAACAGGCUGA UCGUGAGGUUUGGCACCUC
com_tt_P1c	GAGGUGCCAAACCUCACGAUCAGCCUGUUAUCCCCGGGGUAACUCC GAAGAGCUCCACGGGGUCUUC

***** *Closing inserted loops are colored; hyper stable CUUCGG loop is colored red, GNRA (GUGA) tetraloop is colored cyan and H74 natural loop is colored green.*