Figure S1, related to Figure 1 and Introduction.
The final natural product is encoded as a core sequence within a linear precursor peptide. Two proteases cleave off the core peptide from the precursor. They are the A-protease (PatA homolog) and the G-protease (PatG homolog) that carry out N- and C-terminal proteolysis, respectively. The G-protease is also a macrocyclase that carries out N-C cyclization of the core in tandem with proteolysis. Additional posttranslational enzymes may append further modification to the core, such as heterocyclization (conversion of cysteine, serine or threonine residues to azolines) or prenylation (to add isoprene units to serine, threonine or tyrosine residues).

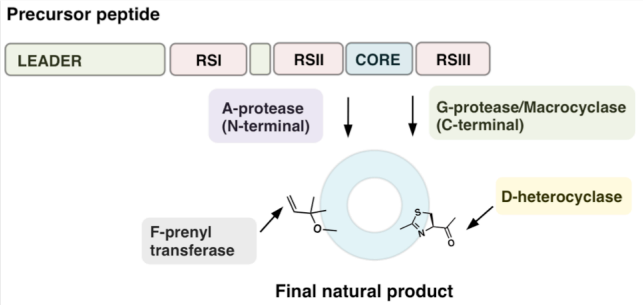


Figure S2 (A-F), related to Figure 2A and Results (Sequential enzymatic reactions reconstitute full-pathway activity)

A. Chemical structures and mass spectra of reaction intermediates formed during sequential addition of enzymes to 1 to yield the monoprenylated trunkamide derivative 6. (i) Structures of intermediates **1**-**6**. (ii) Mass spectra of each of the species **1**, **2**, **4** and **5** in reaction mixtures. (iii) LC trace (top) and mass spectrum (bottom) of TruF1 prenylation of reaction mixture containing the cyclic peptide **5**, to yield the monoprenylated product **6**. As judged from integration of chromatogram, only about 30% of **5** was singly prenylated to yield **6** and the doubly prenylated product was not detectable. (iv) FTMS of product **6** with a mass error of less than 3.5 ppm and (v) the corresponding MS/MS fragmentation spectrum showing loss of the prenyl group.

B. Mass spectrum of the products 5 and 6 resulting from full-length TruG. The activity of TruG on substrate **1** after reaction with ThcD and PatA, in comparison to the same reaction (shown in Figure S2-A) catalyzed by the PatG protease/macrocyclase domain. This shows that the full-length TruG and the PatG protease domain are functionally interchangeable.

C. NMR characterization of product 5. (i) Proton NMR, (ii) HSQC spectra, (iii) COSY spectra, (iv) ROESY and tabular interpretation of data in (v).

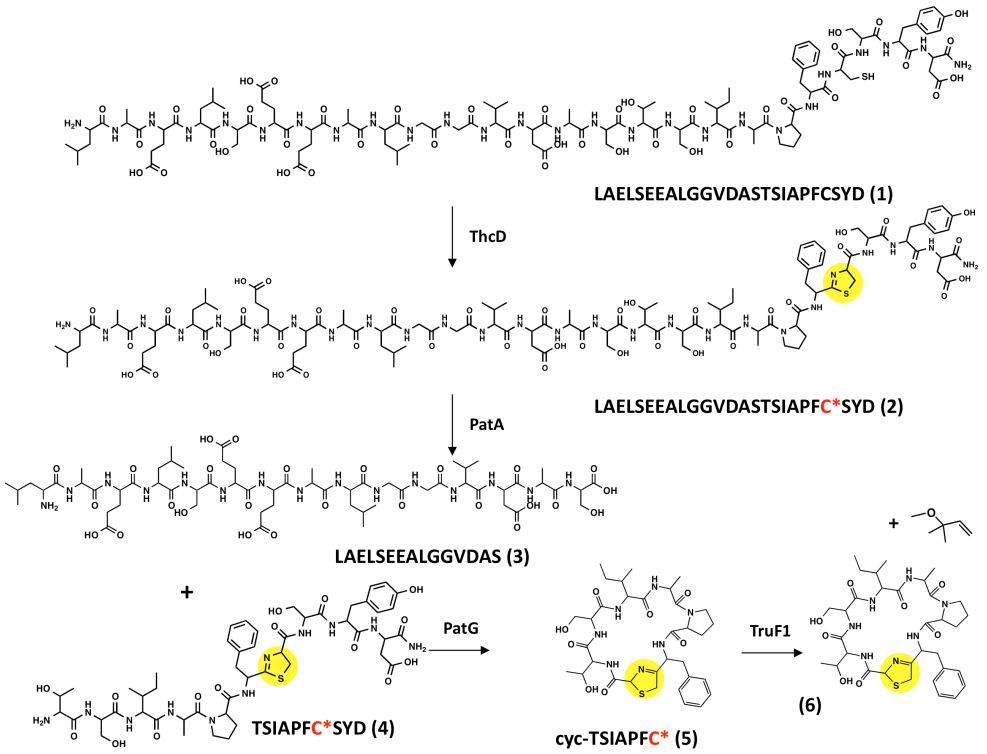
D. Stepwise addition of enzymes results in nearly complete conversion of 1 to products 5 and 6. Shown is a combined mass spectrum of the entire LC-MS chromatogram, in which only **5** and **6** are readily observed as major products. All other possible starting materials, side products, or intermediates resulting from these peptides were present in a combined total that was <5% of the peak height of **5**, indicating that the reactions are highly selective and efficient.

E. Purification of reaction intermediates 2 and 4 and subsequent conversion to their PatA and PatG products. (i) On the left is the HPLC trace of reaction **1** + ThcD (top) and **1** + ThcD + PatA (bottom). The fractions marked by arrows were collected and their identity was confirmed to be **2** and **4** respectively by LC-MS. The LC traces are shown on the right. (ii) Mass spectra of the purified intermediates **2** and **4**. (iii) Mass spectra of purified **2** and **4** after reaction with PatA and PatG, showing formation of products **4** and **5** respectively.

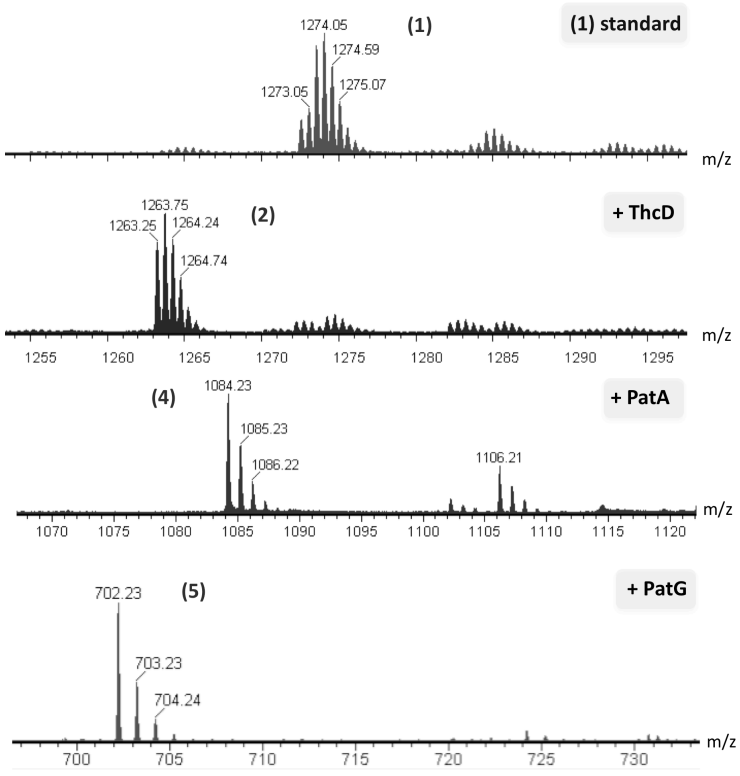
F. Formation of product 5a from substrate 1a. (i) Mass spectrum of product **5a** with the corresponding structure is shown. The reaction was carried out for 24 h using substrate **5a** (75 μM) and PatG (10 μM) in standard assay conditions as detailed in Methods. (ii) The graph shows the relative percentage of reaction completion to products **5** and **5a** after a 24 h reaction, showing that formation of the proline containing macrocycle **5a** was 4-folds slower.

A

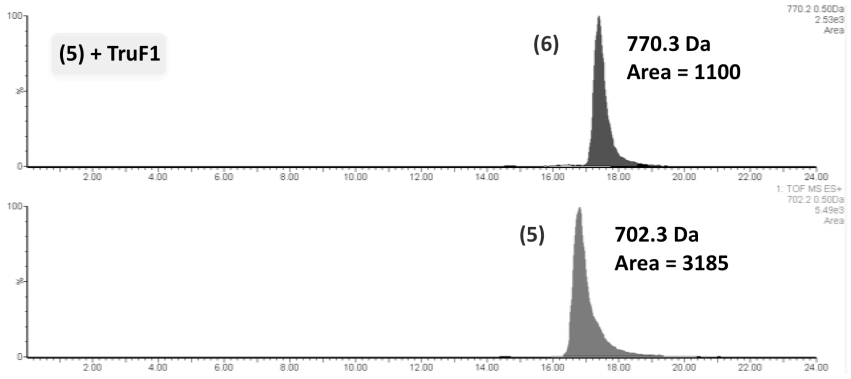
(i)

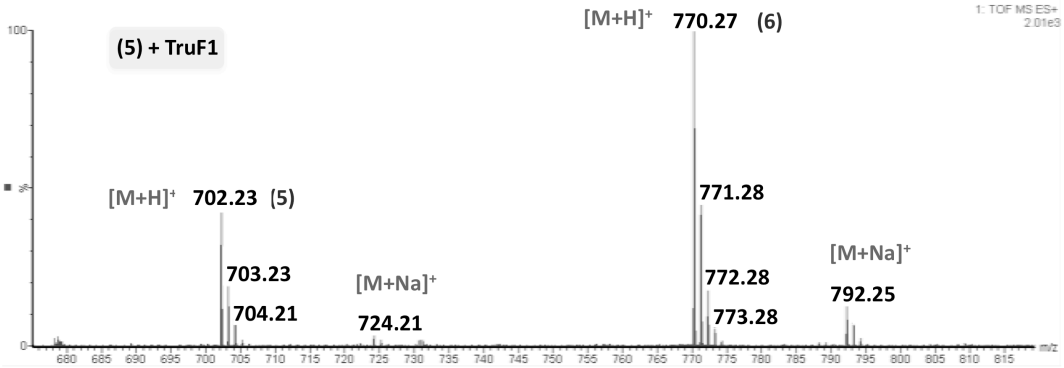

LAELSEEALGGVDASTSIAPFCSYD (1)
ThcD
LAELSEEALGGVDASTSIAPFC*SYD (2)
PatA
LAELSEEALGGVDAS (3)
+
PatG
TSIAPFC*SYD (4)
cyc-TSIAPFC* (5)
TruF1
(6)

(ii)

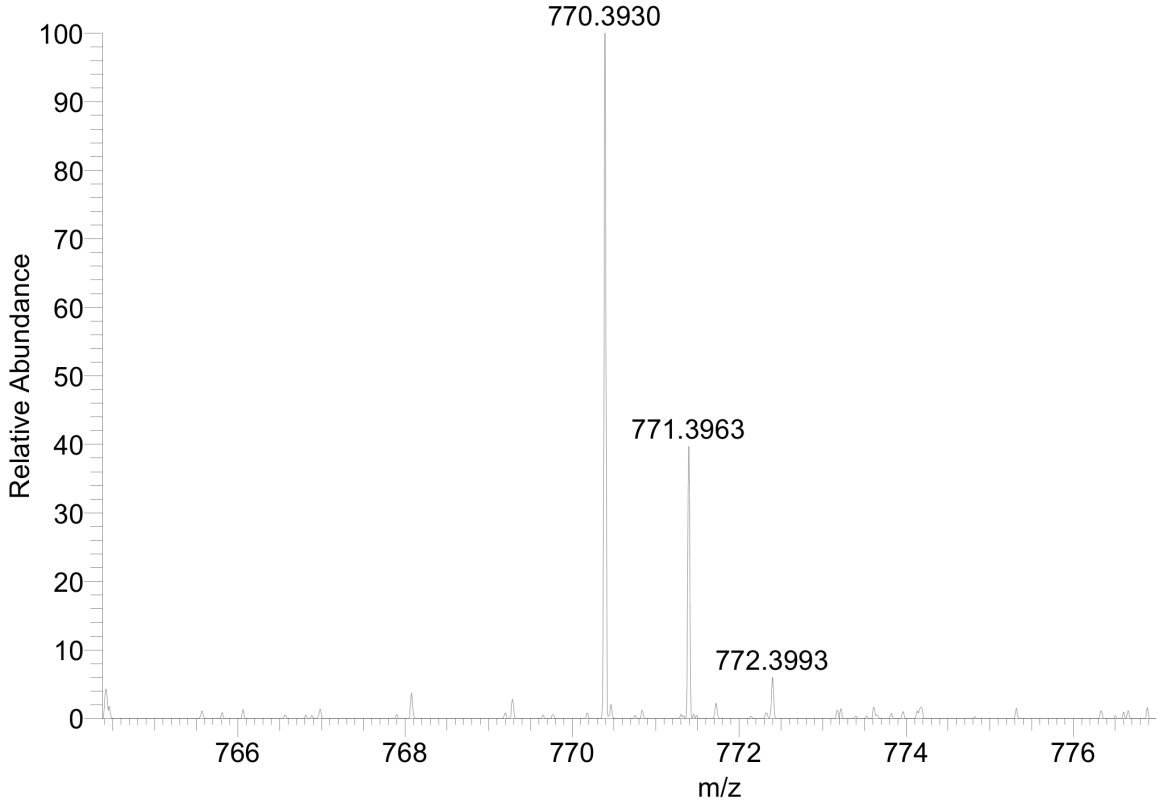

(1)
(1) standard
1274.05
1274.59
1273.05
1275.07
m/z
(2)
+ ThcD
1263.75
1263.25
1264.24
1264.74
1255
1260
1265
1270
1275
1280
1285
1290
1295
(4)
+ PatA
1084.23
1085.23
1086.22
1106.21
1070
1075
1080
1085
1090
1095
1100
1105
1110
1115
1120
(5)
+ PatG
702.23
703.23
704.24
700
705
710
715
720
725
730

(iii)

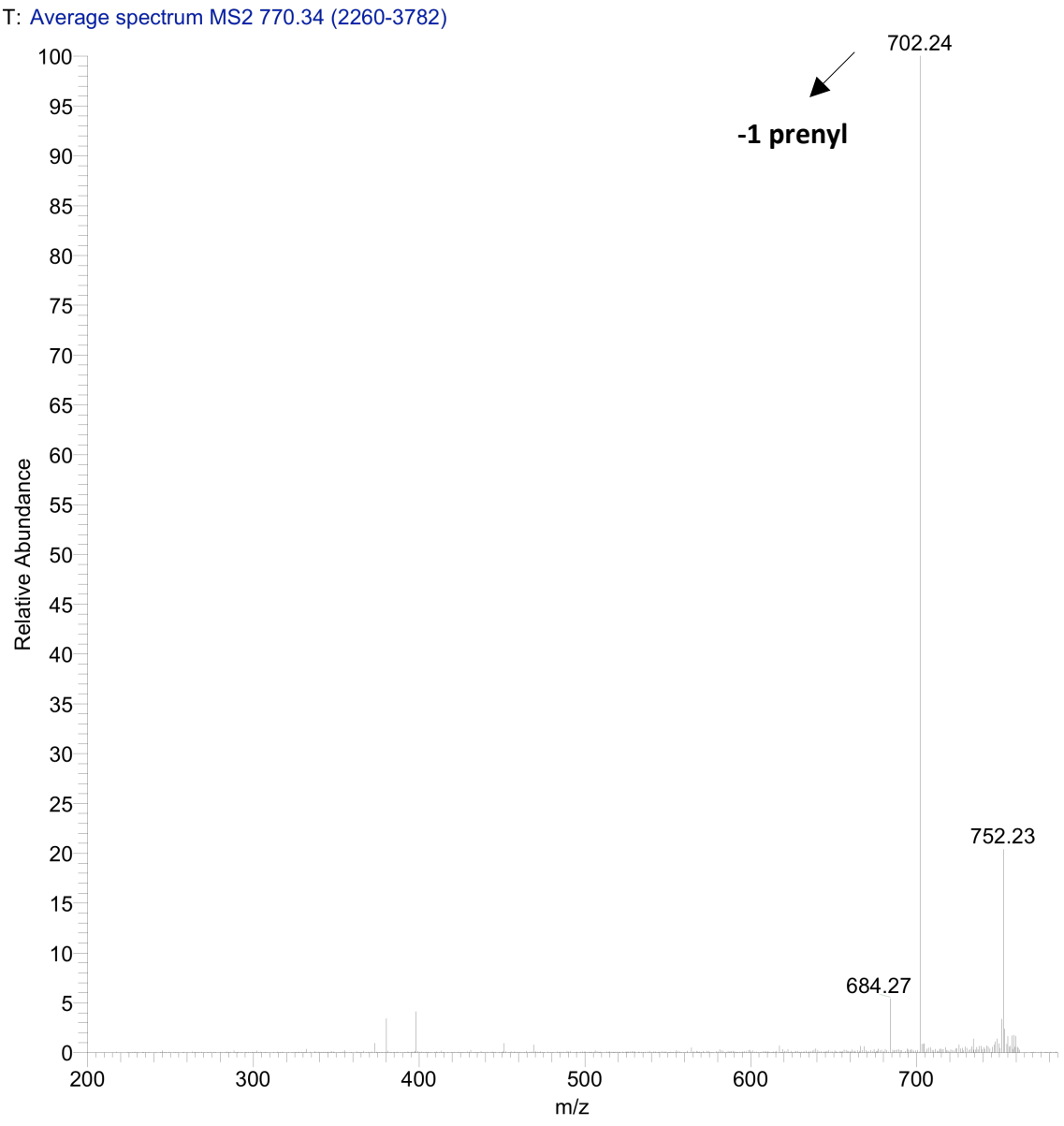

(5) + TruF1
(6)
770.3 Da
Area = 1100
(5)
702.3 Da
Area = 3185


(5) + TruF1
[M+H]+ 702.23 (5)
703.23
704.21
[M+Na]+
724.21
[M+H]+ 770.27 (6)
771.28
772.28
773.28
[M+Na]+
792.25

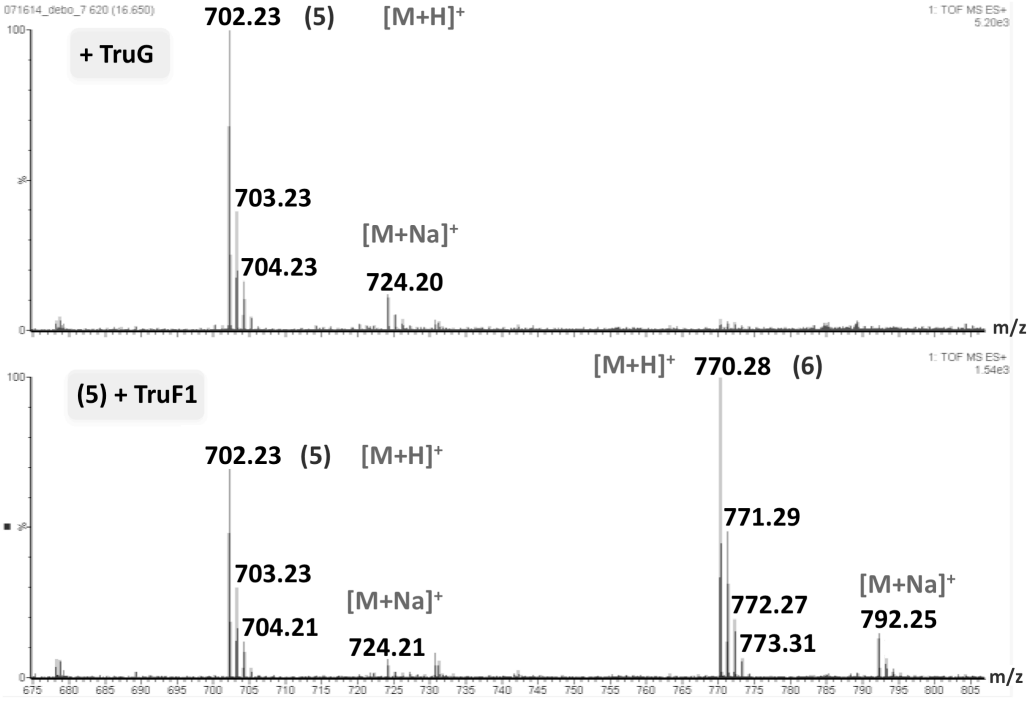
(iv)


770.3930
771.3963
772.3993
100
90
80
70
60
50
40
30
20
10
0
Relative Abundance
766
768
770
772
774
776
m/z

(v)

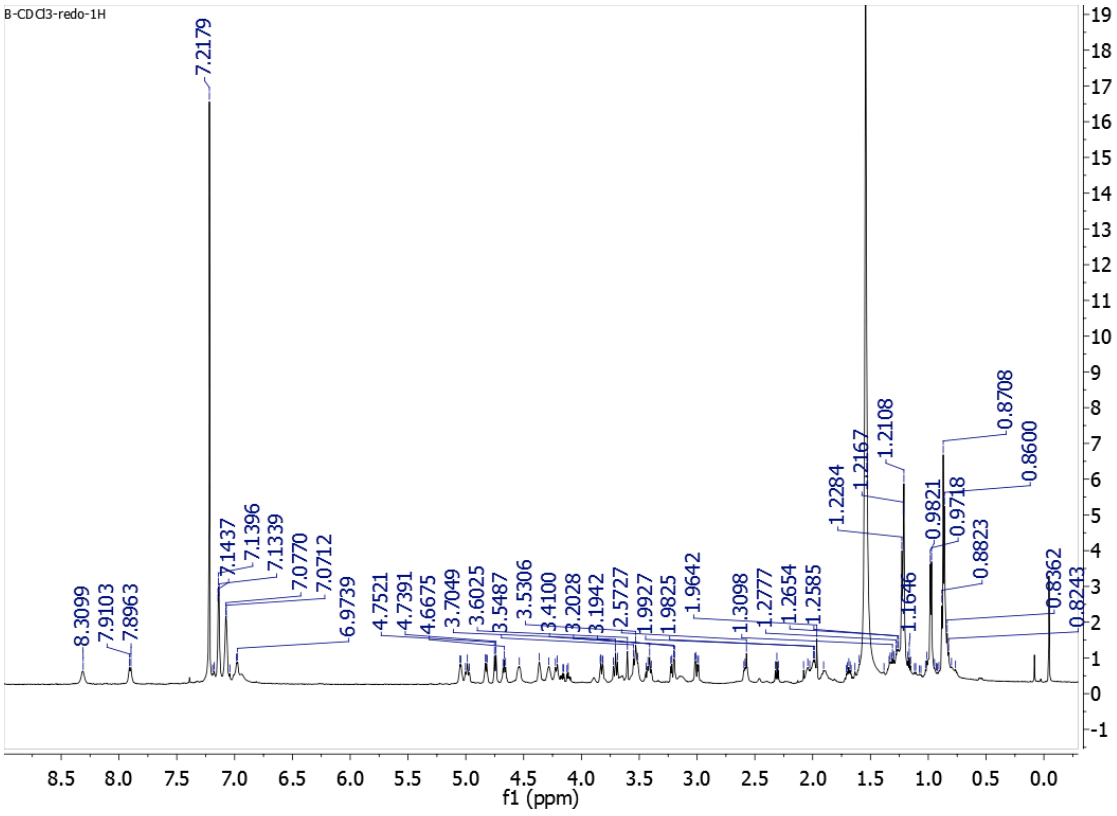

T: Average spectrum MS2 770.34 (2260-3782)
702.24
-1 prenyl
752.23
684.27
Relative Abundance
m/z
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
200
300
400
500
600
700

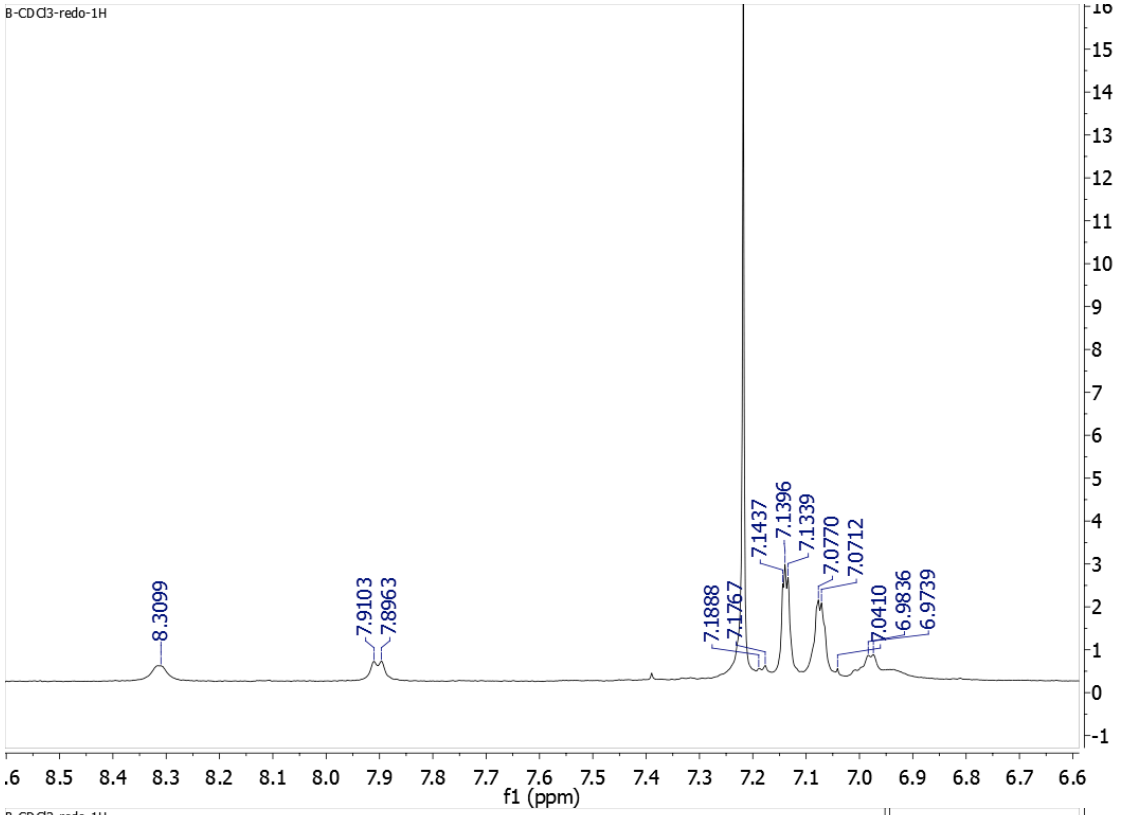
B


071614_debo_7 620 (16.650)
1: TOF MS ES+
5.20e3
100
%
0
+ TruG
702.23 (5) [M+H]+
703.23
704.23
[M+Na]+
724.20
m/z
1: TOF MS ES+
1.54e3
100
%
0
(5) + TruF1
[M+H]+ 770.28 (6)
702.23 (5) [M+H]+
703.23
704.21
[M+Na]+
724.21
771.29
772.27
773.31
[M+Na]+
792.25
m/z
675 680 685 690 695 700 705 710 715 720 725 730 735 740 745 750 755 760 765 770 775 780 785 790 795 800 805

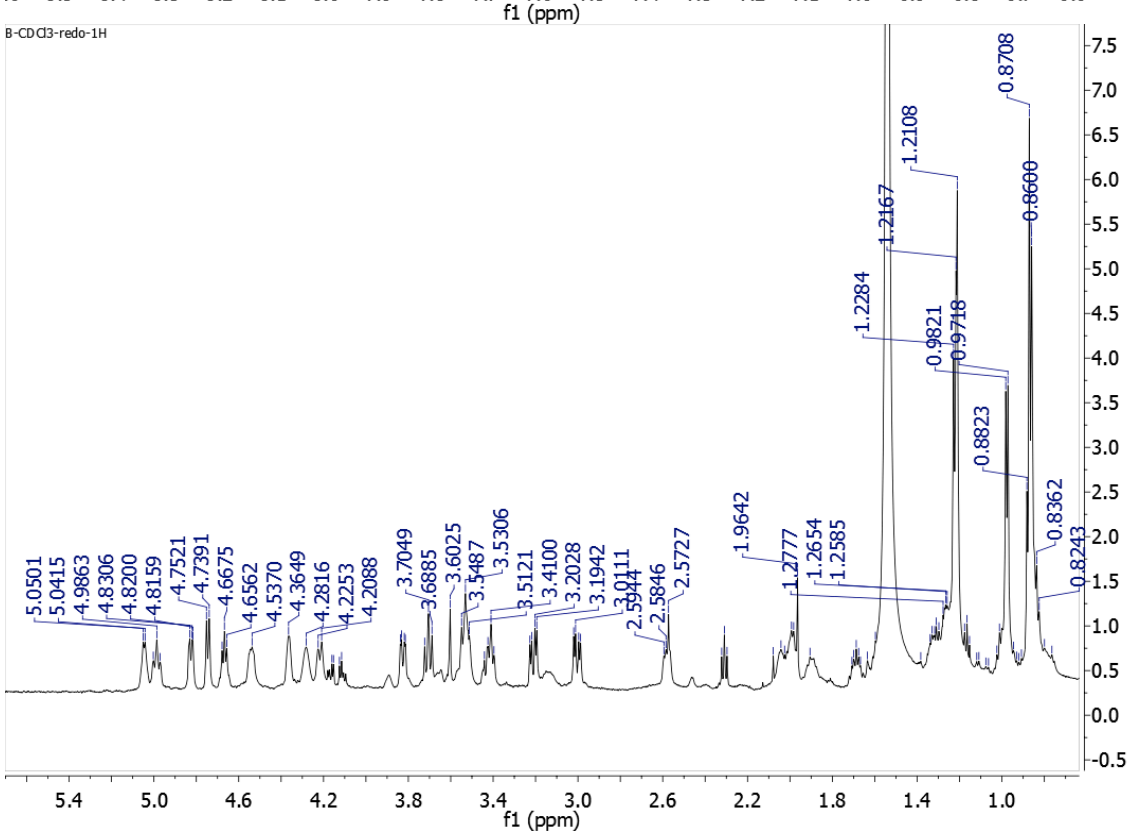
C

(i)

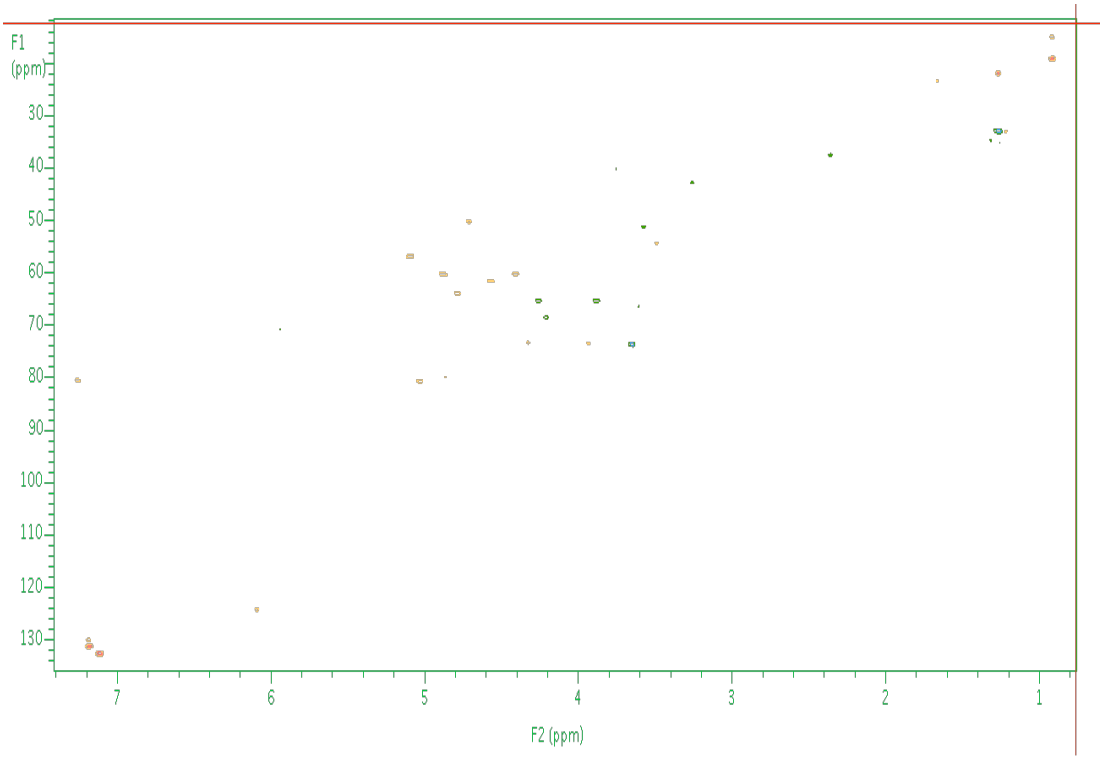

B-CDCl3-redo-1H
7.2179
7.1437
7.1396
7.1339
7.0770
7.0712
6.9739
8.3099
7.9103
7.8963
4.7521
4.7391
4.6675
3.7049
3.6025
3.5487
3.5306
3.4100
3.2028
3.1942
2.5727
1.9927
1.9825
1.9642
1.3098
1.2777
1.2654
1.2585
1.2284
1.2167
1.2108
1.1646
0.9821
0.9718
0.8823
0.8708
0.8600
0.8362
0.8243
f1 (ppm)


B-CDCl3-redo-1H
8.3099
7.9103
7.8963
7.1888
7.1767
7.1437
7.1396
7.1339
7.0770
7.0712
7.0410
6.9836
6.9739
f1 (ppm)

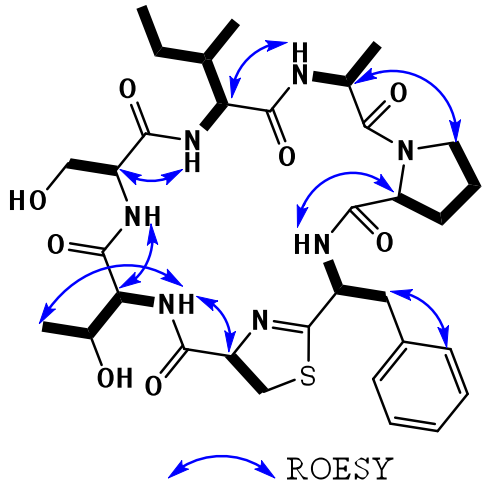
(ii)


B-CDCl3-redo-1H
5.0501
5.0415
4.9863
4.8306
4.8200
4.8159
4.7521
4.7391
4.6675
4.6562
4.5370
4.3649
4.2816
4.2253
4.2088
3.7049
3.6885
3.6025
3.5487
3.5306
3.5121
3.4100
3.2028
3.1942
3.0111
2.5944
2.5846
2.5727
1.9642
1.2777
1.2654
1.2585
1.2284
1.2167
1.2108
0.9821
0.9718
0.8823
0.8708
0.8600
0.8362
0.8243
f1 (ppm)

(iii)


F1
(ppm)
30
40
50
60
70
80
90
100
110
120
130
7
6
5
4
3
2
1
F2 (ppm)

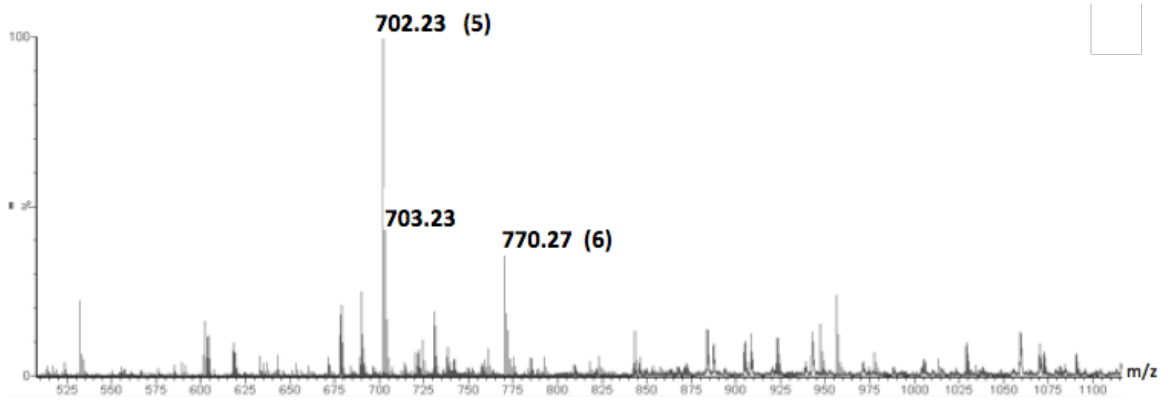
(iv)


H
N
O
O
N
N
H
O
HO
NH
O
HN
O
NH
N
OH
O
S
ROESY

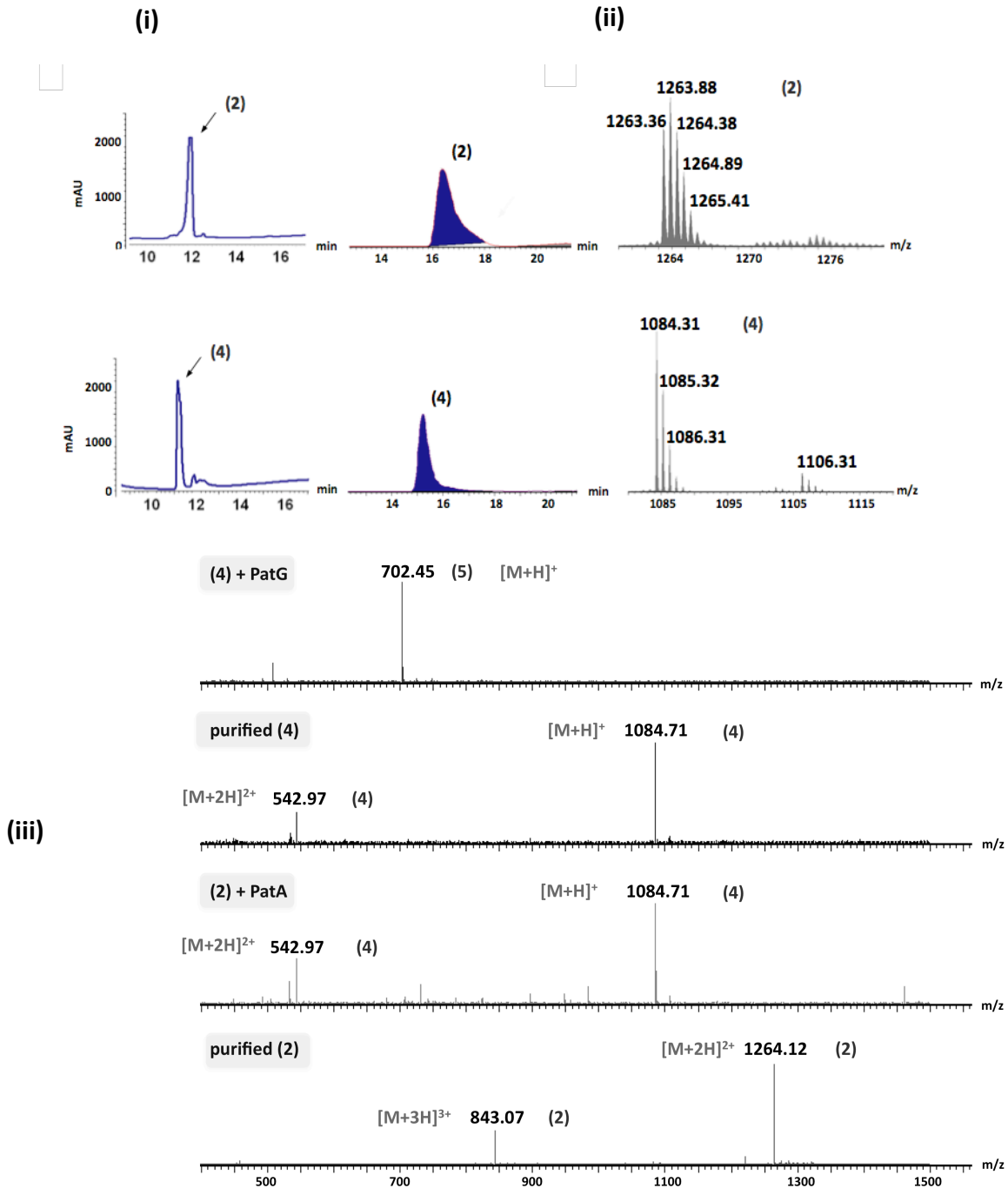
(v)

		^{1}H NMR	HSQC
Ile	NH	7.15 m	
	α	4.54 dd (5.8, 8.0)	61.1
	β	2.07 m	ND
	γ	0.90 m; 1.28 m	18.9; 32.7
	δ	0.88 m	14.8
Ser	NH	7.01 m	
	α	4.40 m	59.8
	β	3.87 m; 4.25 m	65.0
Thr	NH	7.92 d (9.0)	
	α	4.86 dd (9.0, 2.9)	59.5
	β	4.31 m	72.6
	γ	1.00 m	ND
Thiazoline	α	5.01 dd (9.0, 8.0)	80.0
	β	3.72m; 3.56m	39.5
Phe	NH	8.31 h	
	α	5.08 m	55.9
	β	3.24 dd (14.0, 5.4); 3.04 dd (14.0, 5.3)	42.3
	Benzene ring	7.14~7.18 m	131.0~132.0
Pro	α	4.79 dd (9.3, 2.6)	63.5
	β	2.61 m; 1.73 m	ND
	γ	2.34 m; 1.64 m	37.2
	δ	3.57 m; 3.45 m	50.8
Ala	NH	7.03 m	
	α	4.69 dd (6.9, 6.8)	49.8
	β	1.24 m	ND

D

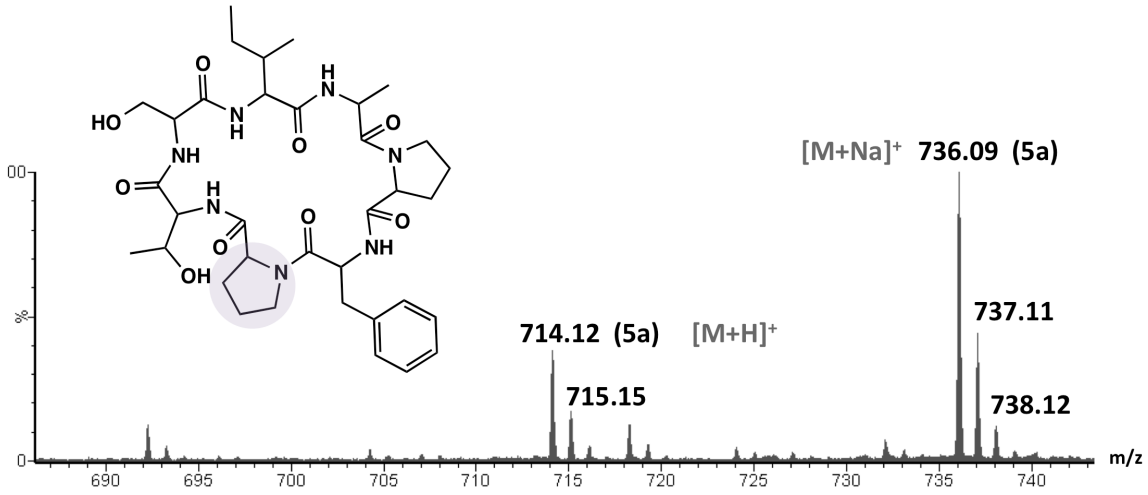

702.23 (5)
703.23
770.27 (6)
100
%
0
525 550 575 600 625 650 675 700 725 750 775 800 825 850 875 900 925 950 975 1000 1025 1050 1075 1100
m/z

E


(i)
(ii)
(2)
mAU
2000
1000
0
10 12 14 16
min
(2)
14 16 18 20
min
1263.88
(2)
1263.36
1264.38
1264.89
1265.41
1264 1270 1276
m/z
(4)
2000
1000
0
10 12 14 16
min
(4)
14 16 18 20
min
1084.31
(4)
1085.32
1086.31
1106.31
1085 1095 1105 1115
m/z
(iii)
(4) + PatG
702.45 (5) [M+H]+
m/z
purified (4)
[M+H]+ 1084.71 (4)
[M+2H]2+ 542.97 (4)
m/z
(2) + PatA
[M+H]+ 1084.71 (4)
[M+2H]2+ 542.97 (4)
m/z
purified (2)
[M+2H]2+ 1264.12 (2)
[M+3H]3+ 843.07 (2)
500 700 900 1100 1300 1500
m/z

F

(i)


[M+Na]+ 736.09 (5a)
737.11
714.12 (5a)
[M+H]+
715.15
738.12
m/z
100
%
0
690
695
700
705
710
715
720
725
730
735
740

(ii)

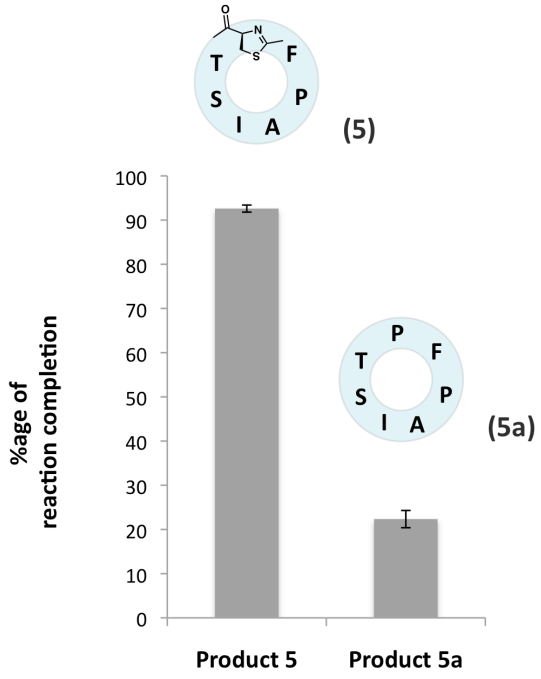
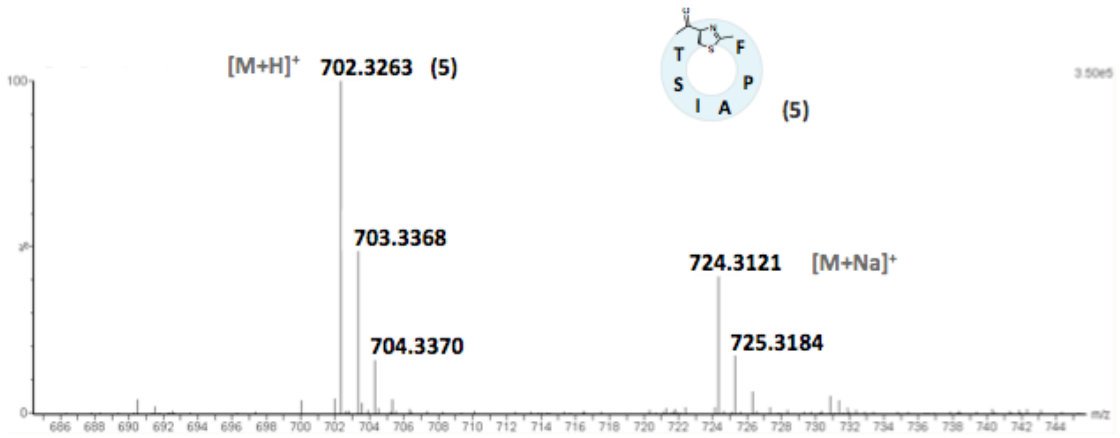
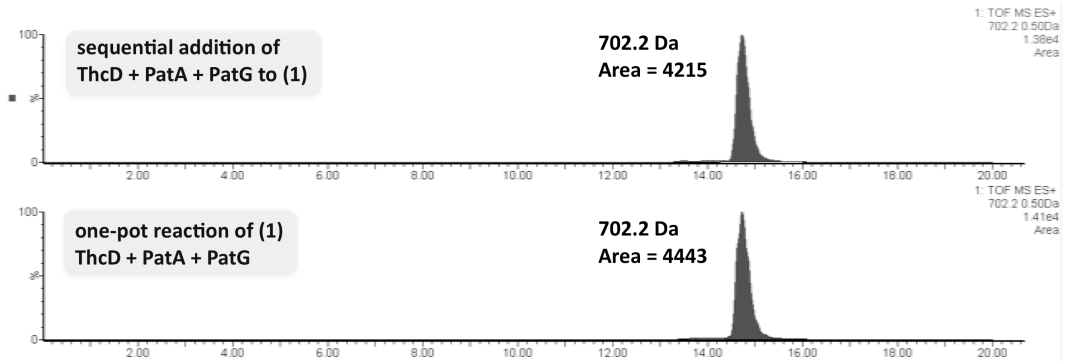

T
F
S
P
I
A
(5)
%age of reaction completion
100
90
80
70
60
50
40
30
20
10
0
P
F
T
P
S
I
A
(5a)
Product 5
Product 5a

Figure S2 (G-K), related to Figure 2B and Results (One-pot synthesis of cyanobactins)
G. Accurate mass measurement of product 5 from a one-pot reaction. Accurate mass measurement of cyclic peptide **5** (2.3 ppm error) from a one-pot reaction of starting substrate **1** with enzymes ThcD, PatA, PatG and TruF1 for 18 h.
H. A comparison of the amount of cyclic product 5 formed in one-pot reaction versus sequential addition of each enzyme. As based on integration of the LC area peak, roughly the same amounts of product **5** were formed in both cases. The one-pot reaction was carried out for 7.5 h, whereas in case of the sequential reaction, PatG was added for 7.5 h to a reaction mixture of **1** treated with ThcD first and then PatA for 1 h each. The same starting concentration of substrate **1** (100 μM) with ThcD (2 μM), PatA (2 μM) and PatG (10 μM) in the reaction mixture was maintained in both cases.
I. One-pot reaction to 5 with higher amount of PatA. The LC trace of an 18 h one-pot reaction with **1** (200 μM), ThcD (2 μM), PatA (7 μM) and PatG (10 μM). (i) The higher amounts of PatA in comparison to ThcD led to the non-heterocyclized proteolysed side-product **7** (bottom) along with the cyclic peptide **5** (top). (ii) Mass spectrum of the species **7** that also exists as a disulphide linked dimer.
J. Mass spectrum of the monoprenylated product 6 from one-pot reaction. Mass spectrum of the monoprenylated product **6** from one-pot reaction of **1** (100 μM) with ThcD (2 μM), PatA (2 μM), PatG (10 μM) and TruF1 (10 μM) in reaction mixtures for 18 h. of **1** (100 μM) with ThcD (2 μM), PatA (2 μM), PatG (10 μM) and TruF1 (10 μM) in reaction mixtures for 18 h.
K. Comparison of time-course of formation of the PatG product 5 in comparison to the ThcD product 2 and the PatA product 4. (i) In a one-pot reaction with **1** (50 μM), ThcD (2 μM), PatA (2 μM) and PatG (10 μM), the time-course for the formation of the PatG product **5** was followed by LC-MS, showing that the reaction is not complete by 7.5 h. (ii) Under the same conditions of **1** (50 μM) and ThcD (2 μM), the reaction for formation of **2** reaches completion by 1 h (as deduced from the disappearance of **1** from the mass spectrum) (iii) Similarly the PatA reaction (2 μM) with purified intermediate **2** (50 μM) is also complete within 1 h (as deduced from the disappearance of **2** from the mass spectrum) to form the product **4** under the same reaction conditions. This shows that formation of the PatG product **5** is the limiting step in the one-pot reaction, since both ThcD and PatA activity reach completion much faster. Each data point is an average of duplicate reactions. The area is an absolute value from the integration of the displayed mass peak from the total ion chromatogram of the mass spectrum, representing relative abundance of the product species.

G

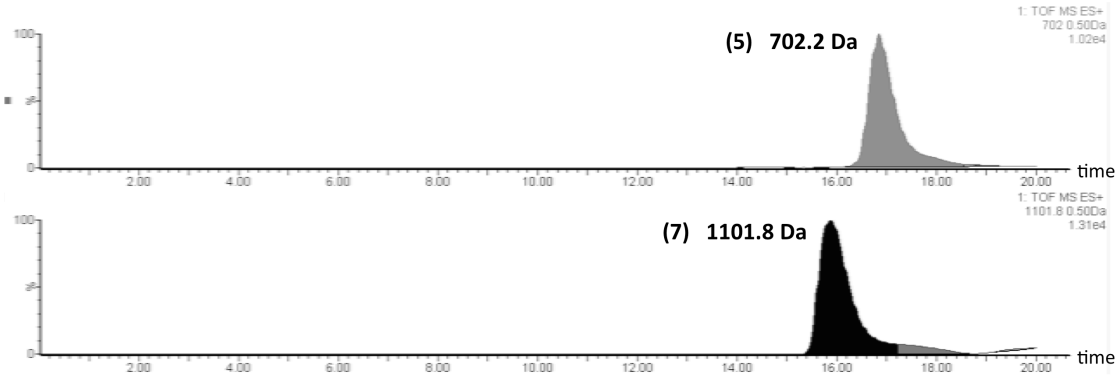

[M+H]+ 702.3263 (5)
703.3368
704.3370
724.3121 [M+Na]+
725.3184
T F
S P
I A
(5)
m/z

H

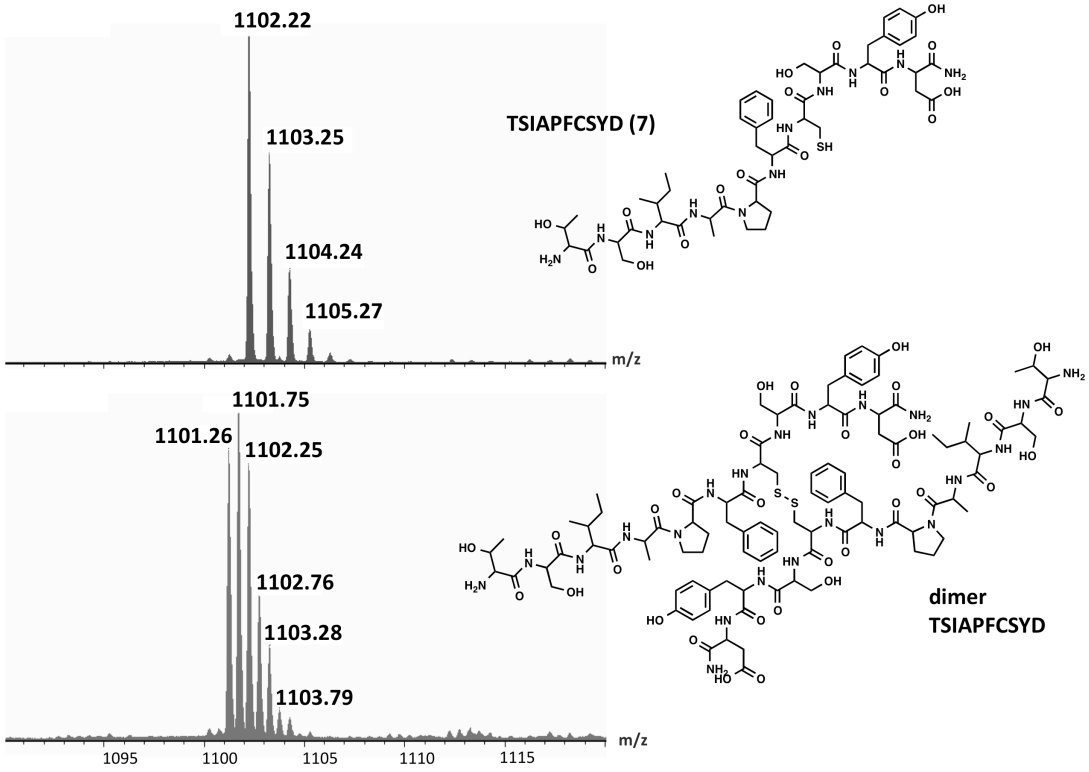

sequential addition of
ThcD + PatA + PatG to (1)
702.2 Da
Area = 4215
one-pot reaction of (1)
ThcD + PatA + PatG
702.2 Da
Area = 4443

I

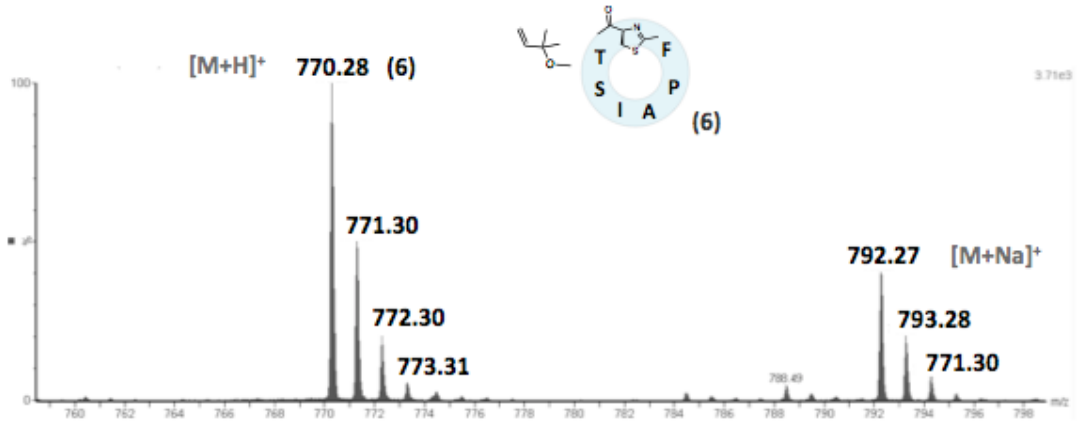
(i)


1: TOF MS ES+
702 0.50Da
1.02e4
(5) 702.2 Da
time
1: TOF MS ES+
1101.8 0.50Da
1.31e4
(7) 1101.8 Da
time

(ii)

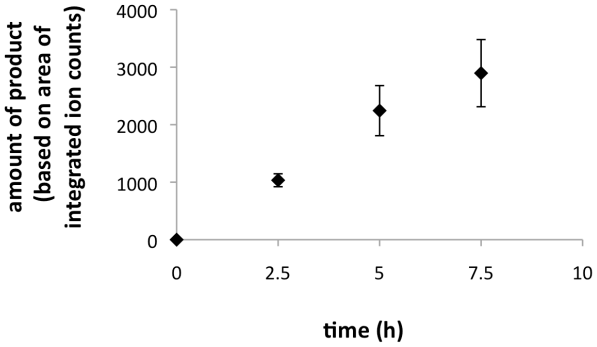

1102.22
1103.25
1104.24
1105.27
m/z
TSIAPFCSYD (7)
1101.75
1101.26
1102.25
1102.76
1103.28
1103.79
m/z
1095
1100
1105
1110
1115
dimer
TSIAPFCSYD

J

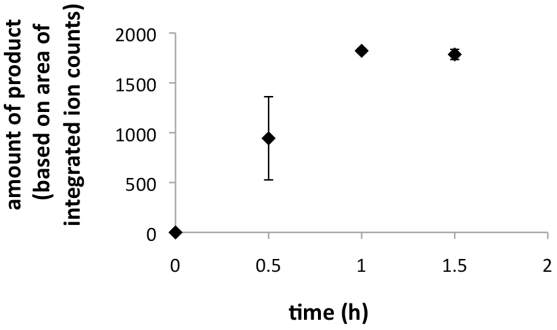

[M+H]+
770.28
(6)
T
F
S
P
I
A
(6)
771.30
772.30
773.31
792.27
[M+Na]+
793.28
771.30
100
0
760
762
764
766
768
770
772
774
776
778
780
782
784
786
788
790
792
794
796
798
m/z

K

(i) **Product formation of (5) in one-pot reaction starting from (1)**



(ii) **Product formation of (4) by PatA from precursor (2)**



(iii) **Product formation of (2) by ThcD from starting material (1)**

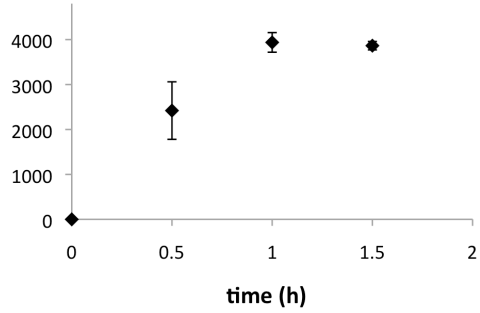


Figure S3 (A-I), related to Figure 3 and Results (Unprecedented macrocycle size plasticity)

A. Dependence of cyanobactin enzymes on DTT. In presence of DTT, PatA activity is inhibited such that PatG cyclizes the ThcD product **2** to yield the large macrocycle **8** (top). In absence of DTT, PatA activity is uninhibited, such that the ThcD product **2** is cleaved to form **4**, which in turn is processed to **5** by PatG (bottom). Reactions were carried out in one pot with **1** (100 μM), ThcD (2 μM), PatA (2 μM) and PatG (10 μM) in optimised mixtures containing Tris pH 7.5 (50 mM), $MgCl_2$ (5 mM), $CaCl_2$ (10 mM), ATP (1 mM) and DTT (6 mM, if present) for 18 h.

B. Mass spectrum of the product 8 resulting from full-length TruG activity. Mass spectrum of the product **8** resulting from full-length TruG activity instead of the PatG protease domain.

C. Mass spectrum of 8 from a one-pot reaction. Mass spectrum of an 18 h one-pot reaction starting from substrate **1** (100 μM) along with ThcD (2 μM) and PatG (10 μM) to lead to the macrocyclic product **8**.

D. MS data showing that compound 8 is macrocyclic. (i) FT-ICR MS/MS spectrum of linear peptide **2**, with clear b and y fragments (see Table S1 for peak assignments). (ii) FT-ICR MS/MS of cyclic peptide **8**, showing that all expected b and y fragments for a linear peptide is absent. This assignment was further supported by proteolytic digestion and MS/MS of **8** (see Figure S4 and Table S2 for details).

E. NMR characterization of 8. (i) Proton NMR, (ii) COSY spectra, (iii) HSQC spectra, (iv) tabular interpretation of spectrum and (v) pure lyophilized **8**.

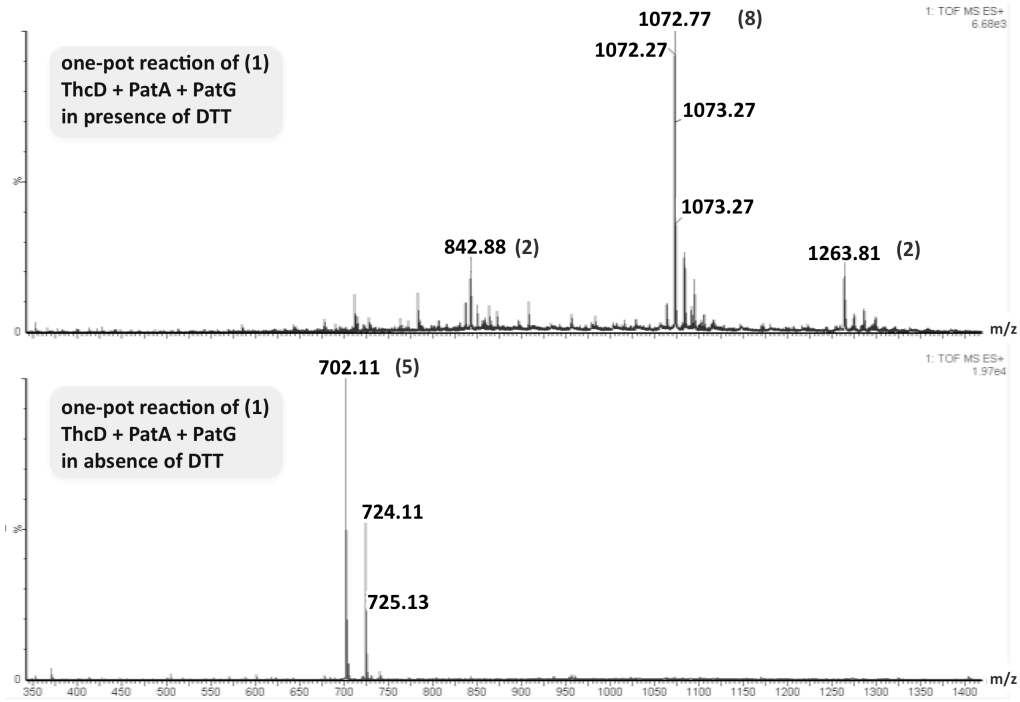
F. Quantification of yield. (i) HPLC trace of increasing amounts of purified **8**, (ii) a standard curve of the same that was used to compute yield of reaction.

G. A time-course of the one-pot reaction leading to 8 in comparison to the same (in presence of PatA) leading to 5. Each data point is an average of a set of duplicate reactions. Reactions were carried out with **1** (100 μM), ThcD (2 μM), PatA (2 μM, if present) and PatG (10 μM) in reaction mixtures. The X-axis represents time and the Y-axis represents area that is an absolute value obtained from the integration of the displayed mass peak from the total ion chromatogram of the mass spectrum, representing relative abundance of the product species.

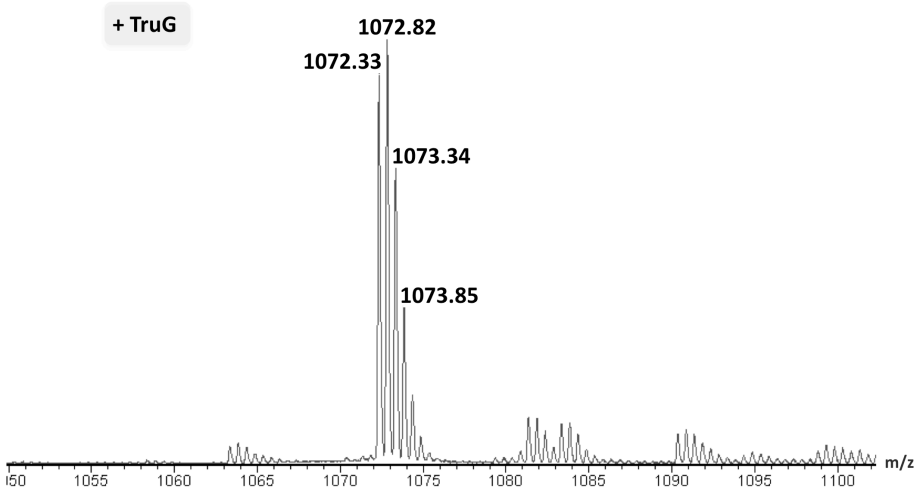
H. Formation of cyclic peptide 11. Formation of product **11** containing 5 amino acids from substrate **9** using ThcD and PatG. The reaction scheme is given on the right, whereas on the left the mass spectrum of the ThcD reaction with **9** (top) and the reaction after subsequent addition of PatG (bottom) is shown. Reactions were carried out with **9** (100 μM), ThcD (2 μM) and PatG (10 μM) for 18 h.

I. Possible macrocycle size of 43 residues. Predicted core peptide size of 43 residues from a precursor peptide sequence found in a cyanobactin biosynthetic gene cluster in *Calothrix* sp. PCC 7103.

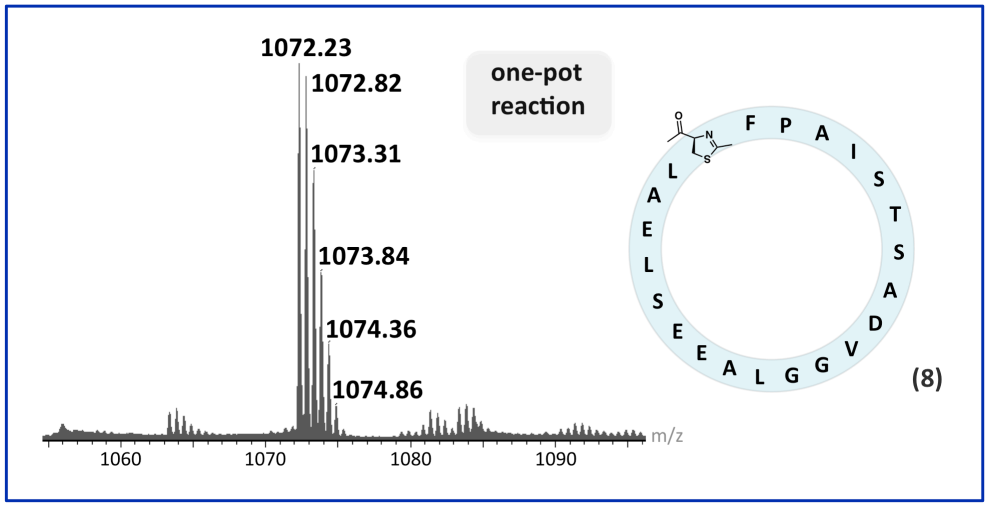
A


1: TOF MS ES+
6.68e3
1072.77 (8)
1072.27
1073.27
1073.27
842.88 (2)
1263.81 (2)
m/z
one-pot reaction of (1)
ThcD + PatA + PatG
in presence of DTT
1: TOF MS ES+
1.97e4
702.11 (5)
724.11
725.13
one-pot reaction of (1)
ThcD + PatA + PatG
in absence of DTT
m/z
350 400 450 500 550 600 650 700 750 800 850 900 950 1000 1050 1100 1150 1200 1250 1300 1350 1400

B

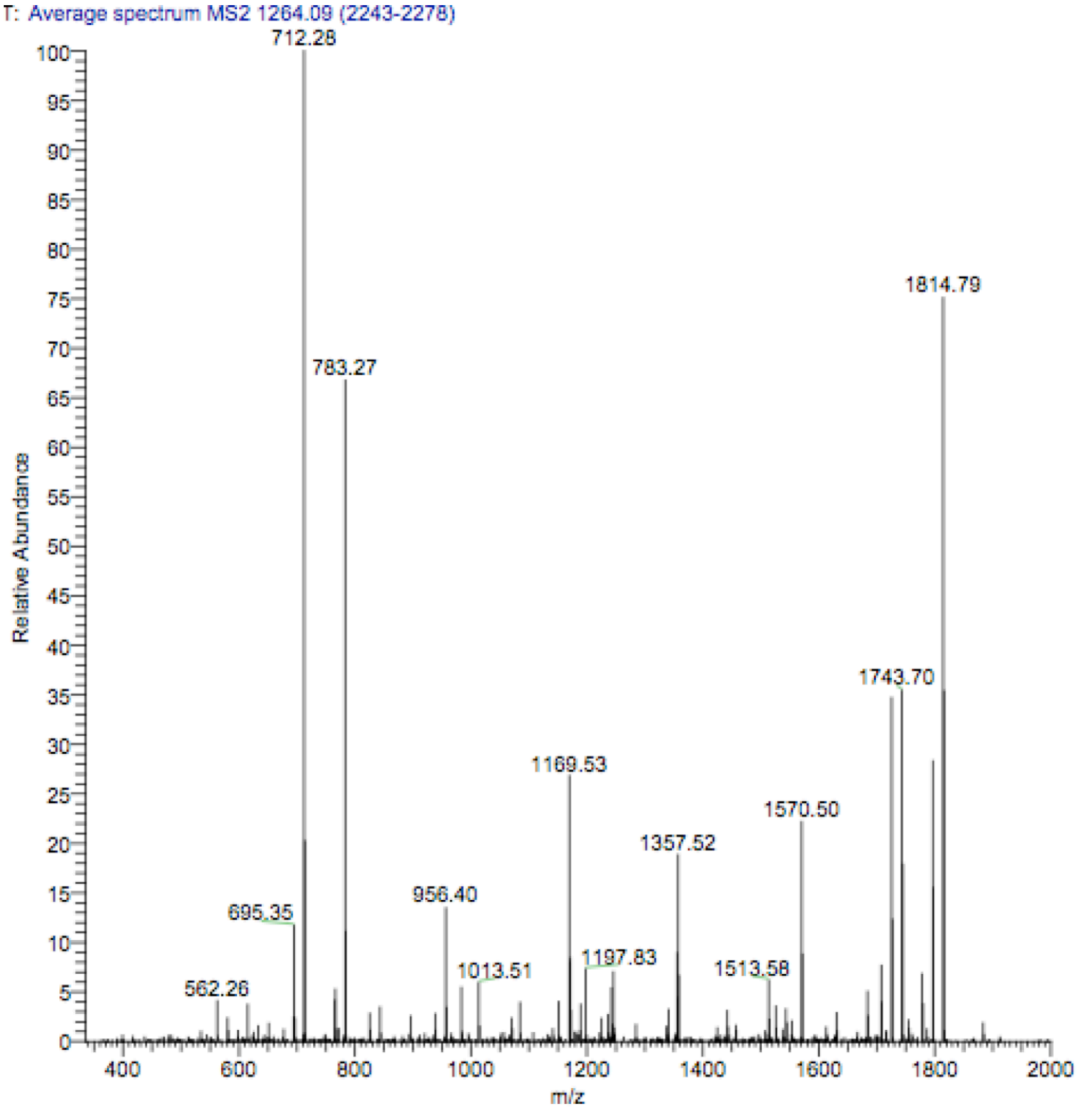

+ TruG
1072.82
1072.33
1073.34
1073.85
m/z
1050
1055
1060
1065
1070
1075
1080
1085
1090
1095
1100

C


1072.23
1072.82
1073.31
1073.84
1074.36
1074.86
m/z
1060
1070
1080
1090
one-pot reaction
F P A I S T S A D V G G A L E E S L E L A L
(8)

D

(i)


T: Average spectrum MS2 1264.09 (2243-2278)
712.28
783.27
1814.79
1743.70
1169.53
1570.50
1357.52
956.40
695.35
1197.83
1513.58
1013.51
562.26
Relative Abundance
m/z
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
400
600
800
1000
1200
1400
1600
1800
2000

(ii)

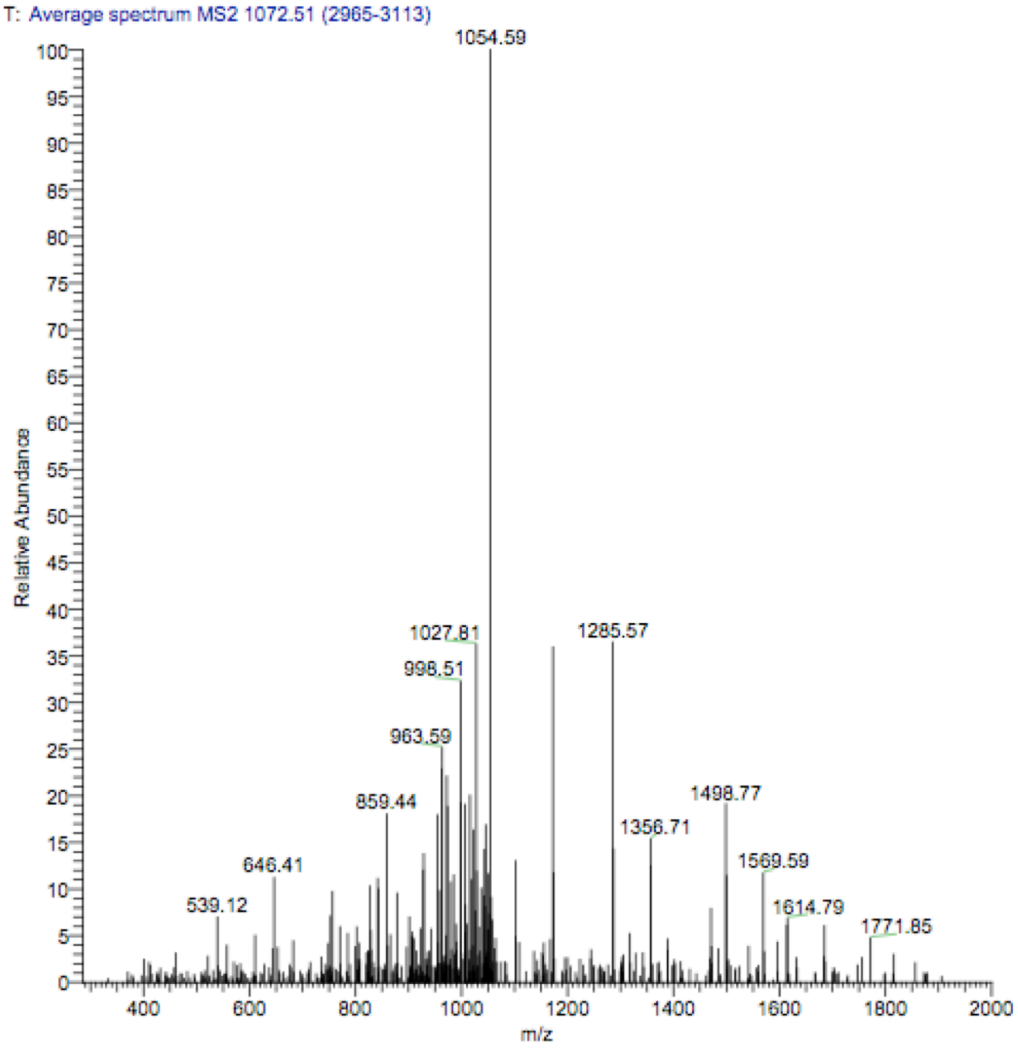

T: Average spectrum MS2 1072.51 (2965-3113)
Relative Abundance
m/z
1054.59
1027.81
1285.57
998.51
963.59
859.44
1498.77
1356.71
1569.59
646.41
539.12
1614.79
1771.85

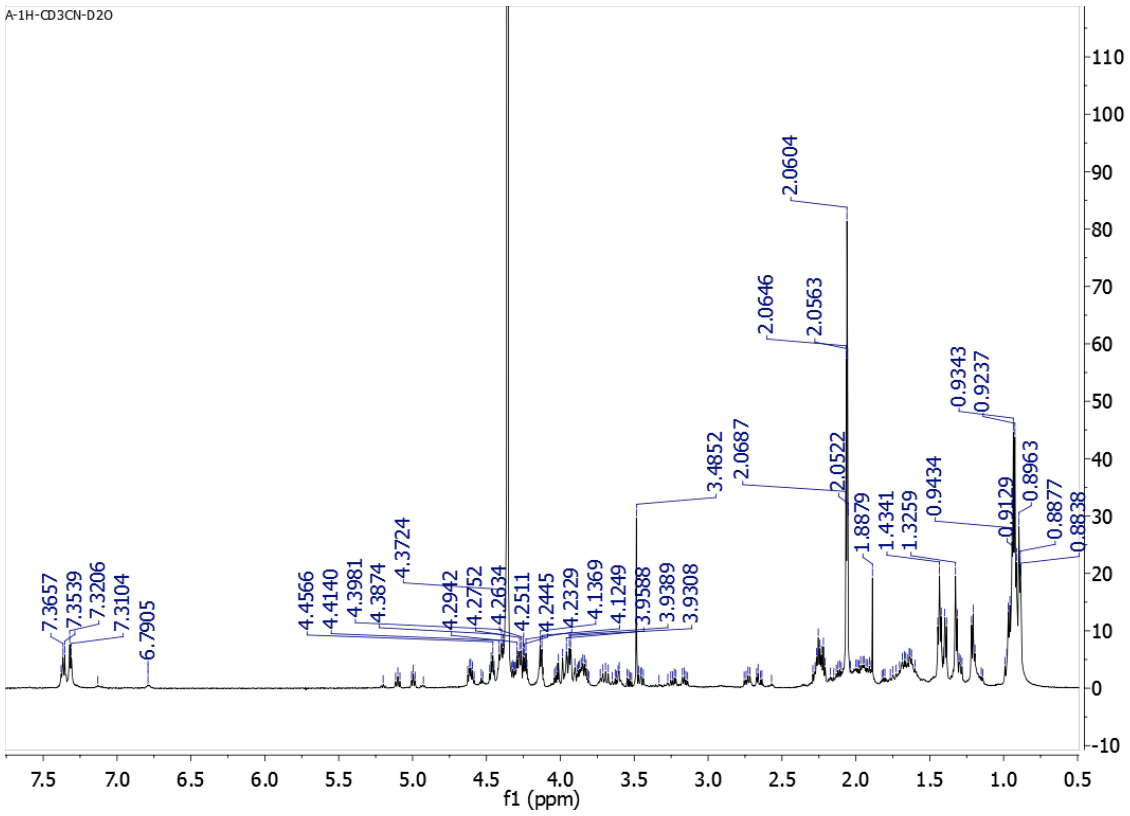
Table S1, related to Figure 3

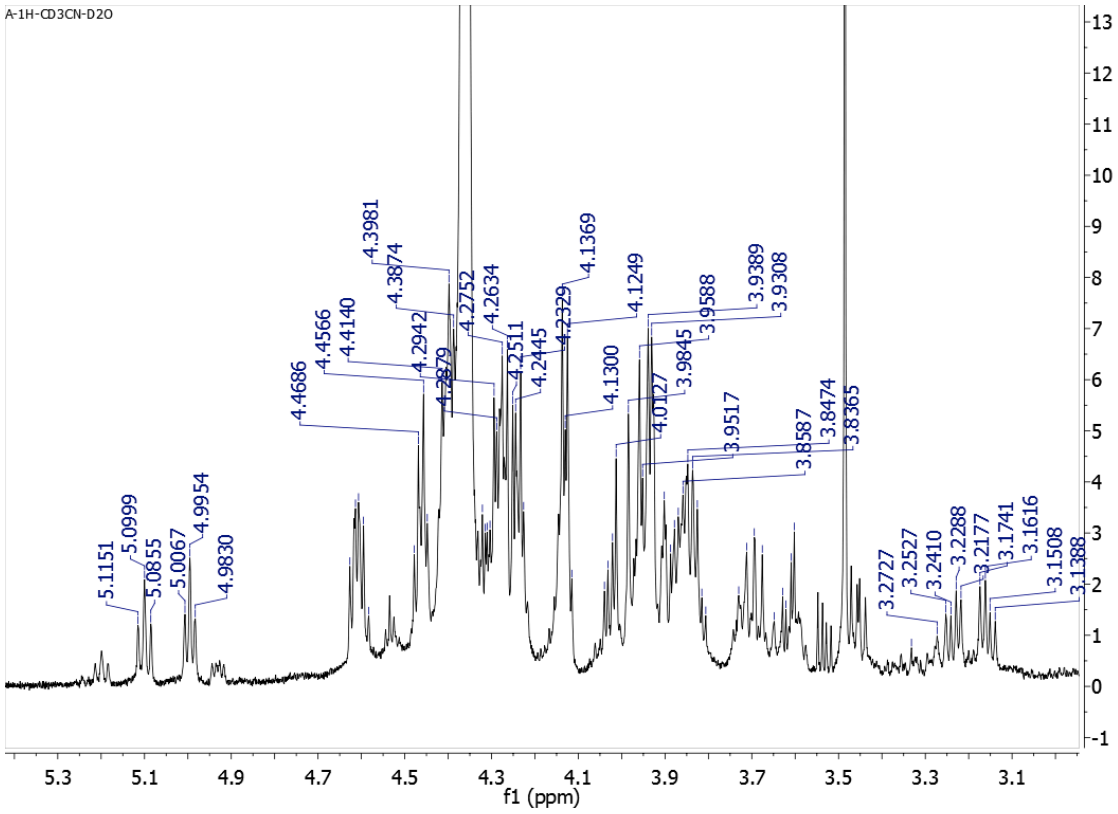
Interpretation of FT-ICR MS/MS spectrum shown in Fig. S3-D (i).

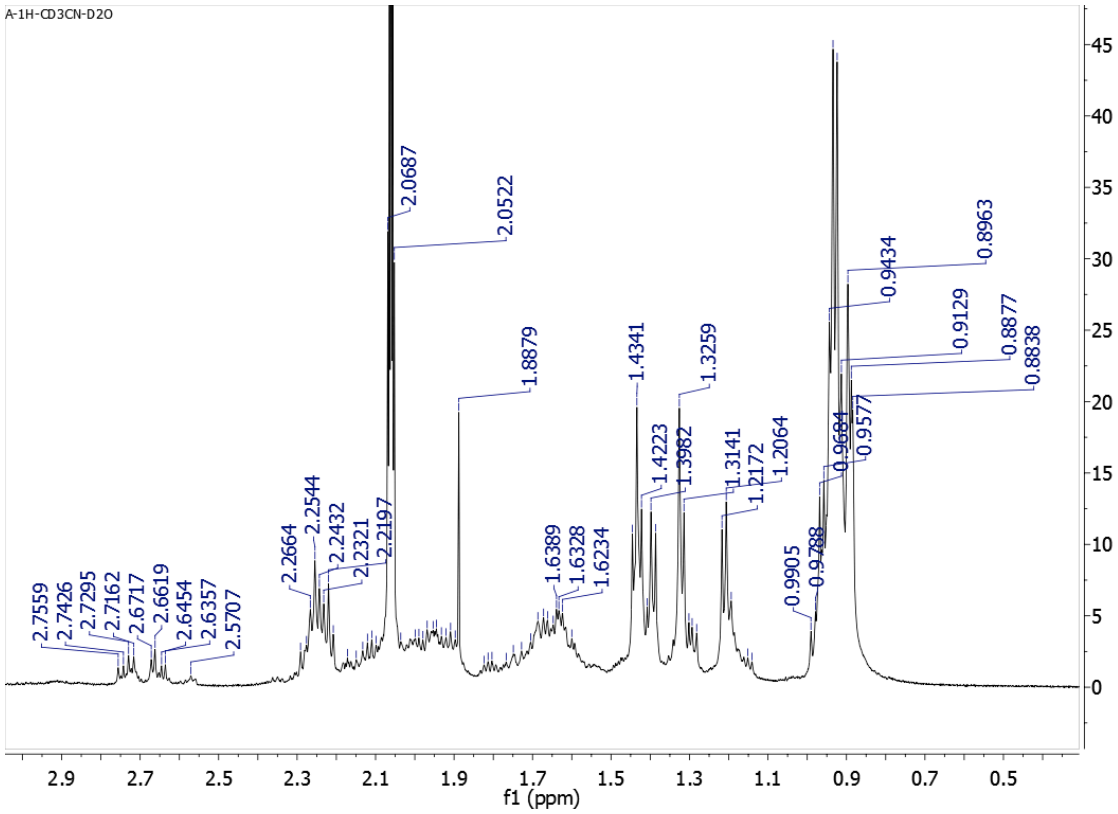
Sequence	b	b^{++}	b^{++} -H_2O	b -H_2O	Sequence	y	y^{++}	y^{++} -H_2O	y -H_2O
L	-	-	-	-	LAELSEEALGGVDASTSIAPFC*SYD	-	-	-	-
LA	-	-	-	-	AELSEEALGGVDASTSIAPFC*SYD	-	-	-	-
LAE	-	-	-	-	ELSEEALGGVDASTSIAPFC*SYD	-	-	-	-
LAEL	-	-	-	-	LSEEALGGVDASTSIAPFC*SYD	-	1107.13	-	-
LAELS	-	-	-	-	SEEALGGVDASTSIAPFC*SYD	-	-	-	-
LAELSE	-	-	-	-	EEALGGVDASTSIAPFC*SYD	-	-	-	-
LAELSEE	-	-	-	-	EALGGVDASTSIAPFC*SYD	1883.78	-	-	-
LAELSEEA	843.37	-	-	825.34	ALGGVDASTSIAPFC*SYD	1754.68	-	-	-
LAELSEEAL	956.41	-	-	938.38	LGGVDASTSIAPFC*SYD	1683.68	-	-	-
LAELSEEALG	1013.51	-	-	995.52	GGVDASTSIAPFC*SYD	1570.51	-	-	-
LAELSEEALGG	1070.52	-	-	-	GVDASTSIAPFC*SYD	1513.58	-	-	-
LAELSEEALGGV	1169.53	-	-	1151.57	VDASTSIAPFC*SYD	1456.56	-	-	-
LAELSEEALGGVD	1284.55	-	-	-	DASTSIAPFC*SYD	1357.52	-	-	-
LAELSEEALGGVDA	1355.69	-	-	1337.61	ASTSIAPFC*SYD	1242.55	-	-	-
LAELSEEALGGVDAS	1442.46	-	-	1424.55	STSIAPFC*SYD	1171.53	-	-	-
LAELSEEALGGVDAST	1543.57	-	-	1525.63	TSIAPFC*SYD	1084.46	-	534.27	-
LAELSEEALGGVDASTS	1630.57	-	-	1612.82	SIAPFC*SYD	983.35	-	-	-
LAELSEEALGGVDASTSI	1743.71	-	-	1725.84	IAPFC*SYD	896.31	-	-	-
LAELSEEALGGVDASTSIA	1814.79	-	-	1796.75	APFC*SYD	783.27	-	-	765.43
LAELSEEALGGVDASTSIAP	-	957.64	-	-	PFC*SYD	712.28	-	-	695.35
LAELSEEALGGVDASTSIAPF	-	-	-	-	FC*SYD	-	-	-	-
LAELSEEALGGVDASTSIAPFC*	-	-	-	-	C*SYD	-	-	-	-
LAELSEEALGGVDASTSIAPFC*S	-	-	-	-	SYD	-	-	-	-
LAELSEEALGGVDASTSIAPFC*SY	-	1197.83	-	-	YD	-	-	-	-
LAELSEEALGGVDASTSIAPFC*SYD	-	-	1245.78	-	D	-	-	-	-

E

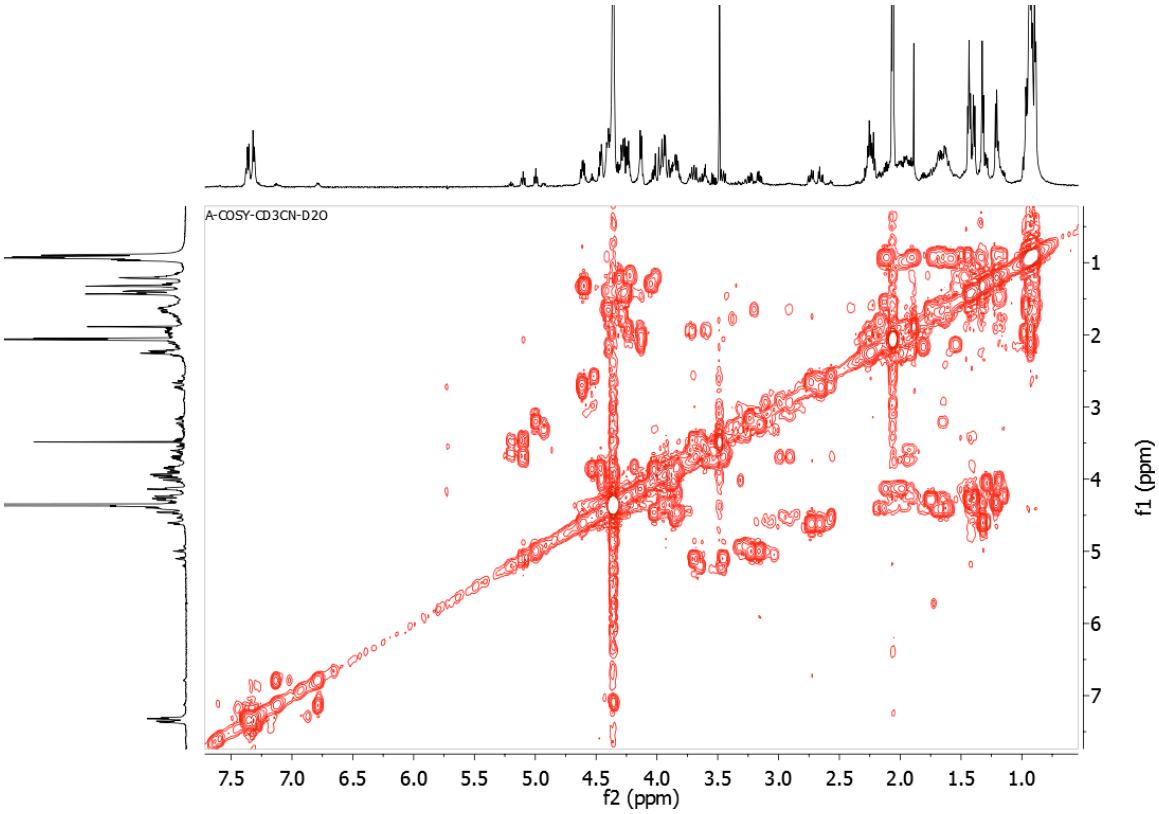
(i)


A-1H-CD3CN-D2O
7.3657
7.3539
7.3206
7.3104
6.7905
4.4566
4.4140
4.3981
4.3874
4.3724
4.2942
4.2752
4.2634
4.2511
4.2445
4.2329
4.1369
4.1249
3.9588
3.9389
3.9308
3.4852
2.0687
2.0604
2.0646
2.0563
2.0522
1.8879
1.4341
1.3259
0.9434
0.9343
0.9237
0.9129
0.8963
0.8877
0.8838
f1 (ppm)

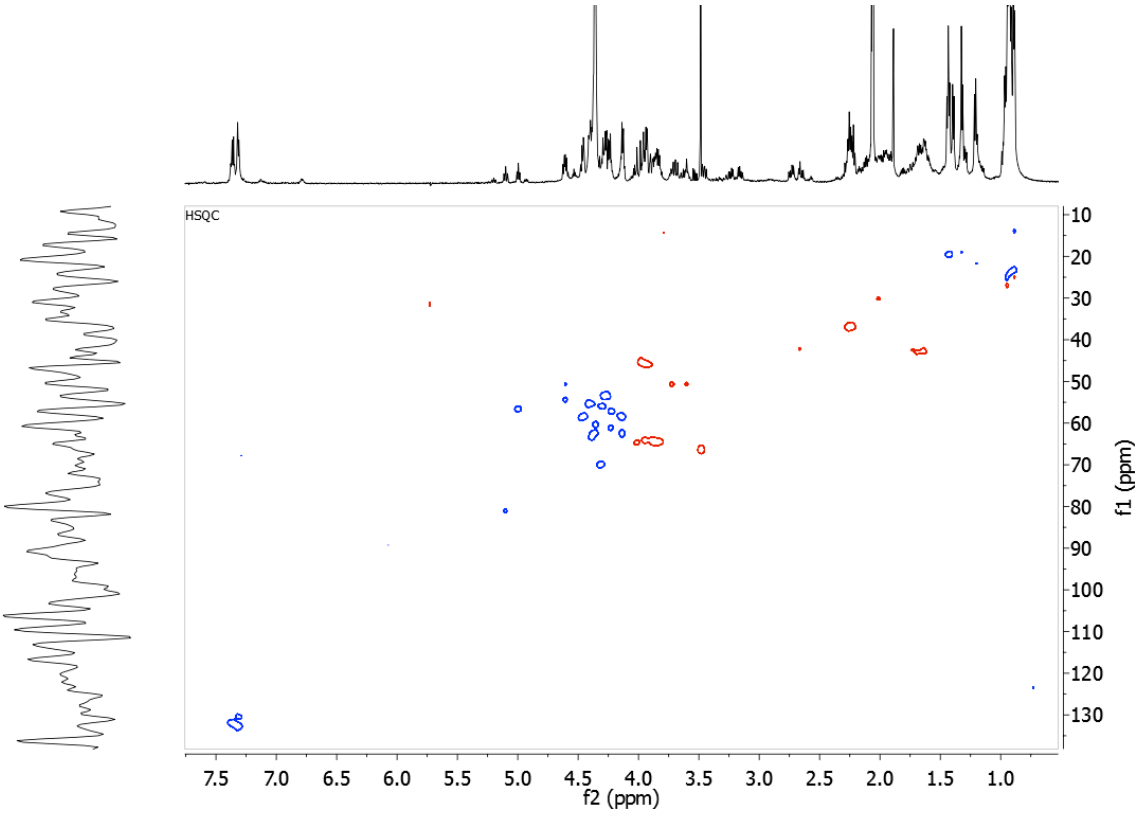

A-1H-CD3CN-D2O
5.1151
5.0999
5.0855
5.0067
4.9954
4.9830
4.4686
4.4566
4.4140
4.3981
4.3874
4.2942
4.2879
4.2752
4.2634
4.2511
4.2445
4.2329
4.1369
4.1300
4.1249
4.0127
3.9845
3.9588
3.9517
3.9389
3.9308
3.8587
3.8474
3.8365
3.2727
3.2527
3.2410
3.2288
3.2177
3.1741
3.1616
3.1508
3.1388
f1 (ppm)


A-1H-CD3CN-D2O
2.7559
2.7426
2.7295
2.7162
2.6717
2.6619
2.6454
2.6357
2.5707
2.2664
2.2544
2.2432
2.2321
2.2197
2.0687
2.0522
1.8879
1.6389
1.6328
1.6234
1.4341
1.4223
1.3982
1.3259
1.3141
1.2172
1.2064
0.9905
0.9788
0.9684
0.9577
0.9434
0.9129
0.8963
0.8877
0.8838
f1 (ppm)

(ii)


A-COSY-CD3CN-D2O
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
f2 (ppm)
1
2
3
4
5
6
7
f1 (ppm)

(iii)


HSQC
10
20
30
40
50
60
70
80
90
100
110
120
130
f1 (ppm)
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
f2 (ppm)

(iv)

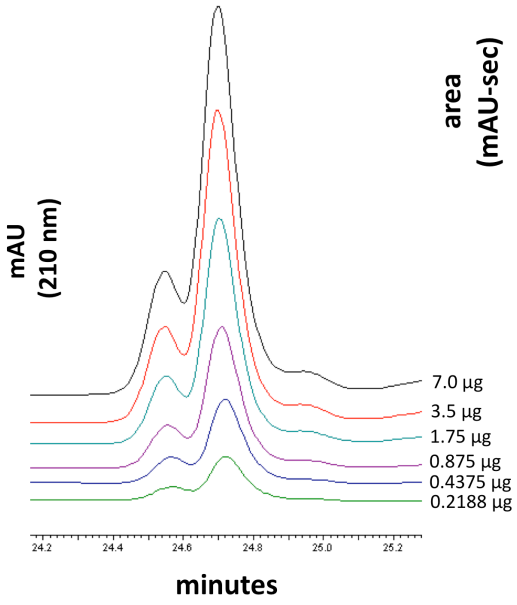
		^{1}H NMR	HSQC
Ile	α	4.23 m	61.4
	β	1.93 m	ND
	γ	0.94 m; 1.89 m	ND
	δ	0.90 m	ND
Ser-1	α	4.45 m	58.3
	β	3.87 m; 3.82 m	64.5
Ser-2	α	4.47 m	58.7
	β	4.00 m; 3.85 m	64.3
Ser-3	α	4.36 m	60.3
	β	3.95 m	64.2
Thr	α	4.22 m	57.4
	β	4.31 m	70.0
	γ	1.21 m	22.0
Ala-1	α	4.60 m	54.5
	β	1.31 m	19.6
Ala-2	α	4.27 m	55.9
	β	1.43 m	19.8
Ala-3	α	4.28 m	55.9
	β	1.42 m	19.8
Ala-4	α	4.26 m	55.9
	β	1.39 m	19.8
Asp	α	4.61 m	54.3
	β	2.74 m; 2.66 m	41.9
Val	α	4.12 m	62.3
	β	2.10 m	ND
	γ	0.94m; 0.95m	ND
Gly-1	α	3.93 m	46.1
Gly-1	α	3.94 m	45.8
Leu-1	α	4.40 m	55.2
	β	1.64 m	43.0
	γ	1.60 m	ND
	δ	0.98 m	ND
Leu-2	α	4.41 m	55.2
	β	1.66 m	43.0
	γ	1.63 m	ND
	δ	0.95 m	ND
Leu-3	α	4.27 m	53.5
	β	1.73 m	42.5
	γ	1.68 m	ND
	δ	0.93 m	ND

Glu-1	α	4.11 m	58.5
	β	1.96 m	30.8
	γ	2.23 m	37.2
Glu-1	α	4.11 m	58.2
	β	2.00 m	30.8
	γ	2.23 m	37.2
Glu-1	α	4.11 m	58.3
	β	1.98 m	30.8
	α	2.23 m	37.2
Thiazoline	β	5.10 d (9.2, 9.0)	81.0
	β	3.69 m; 3.45 m	38.8
Phe	α	4.99 dd (7.0, 6.9)	56.4
	β	3.23 m, 3.15 m	ND
	Benzene ring	7.30~7.38 m	130.1 ~134.1
Pro	α	4.23 m	61.3
	β	1.92 m, 1.29 m	ND
	γ	2.04 m, 1.91 m	ND
		3.72 m, 3.59 m	50.7

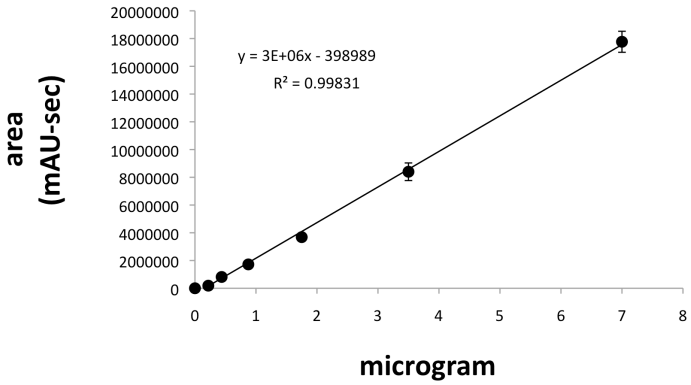
(v)

F

(i)



(ii)



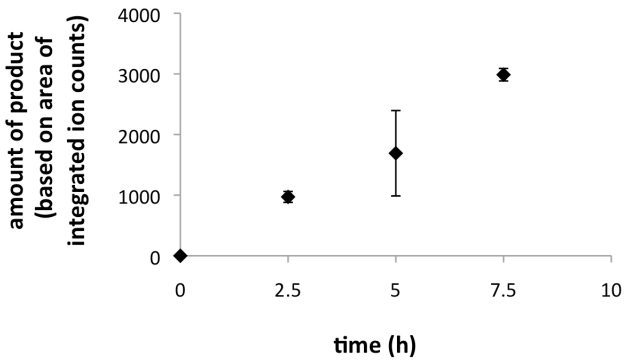
Yield calculation:

y= 7976775
x= 2.8 ug

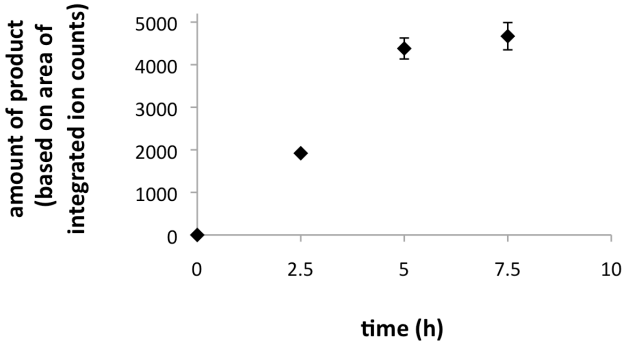
$x_{observed}$= 2.8 ug => 1.3 nmol
$x_{expected}$= 3.5 ug => 1.6 nmol
(if yield is 100%)

yield= 81.3%

G

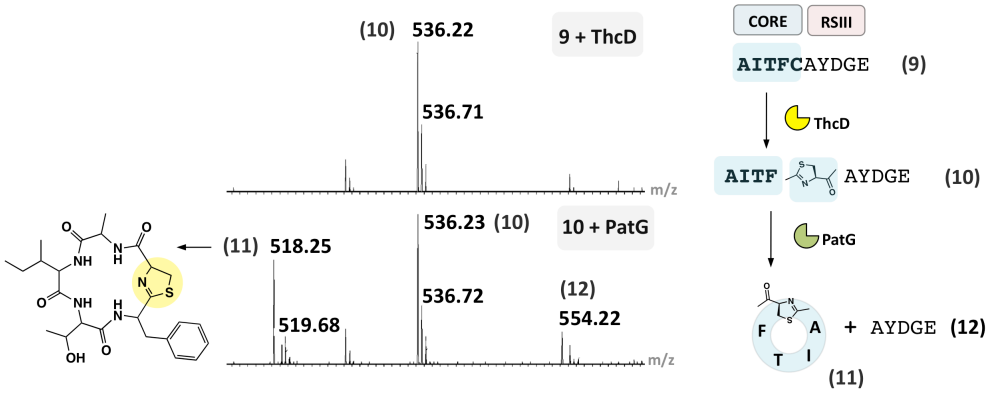


Product formation of (8) in one-pot reaction starting from (1)



Product formation of (5) in one-pot reaction starting from (1)

H


(10) 536.22
9 + ThcD
536.71
m/z
536.23 (10)
10 + PatG
(11) 518.25
536.72
(12)
554.22
519.68
m/z
CORE
RSIII
AITFCAYDGE (9)
ThcD
AITF AYDGE (10)
PatG
F A T I
+ AYDGE (12)
(11)

I

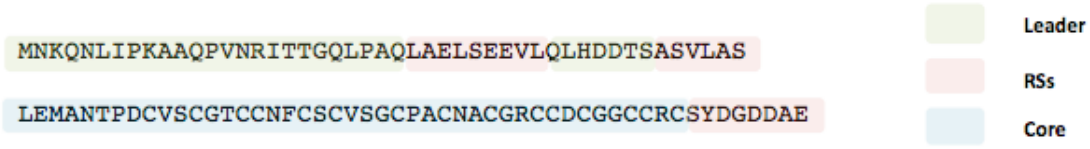
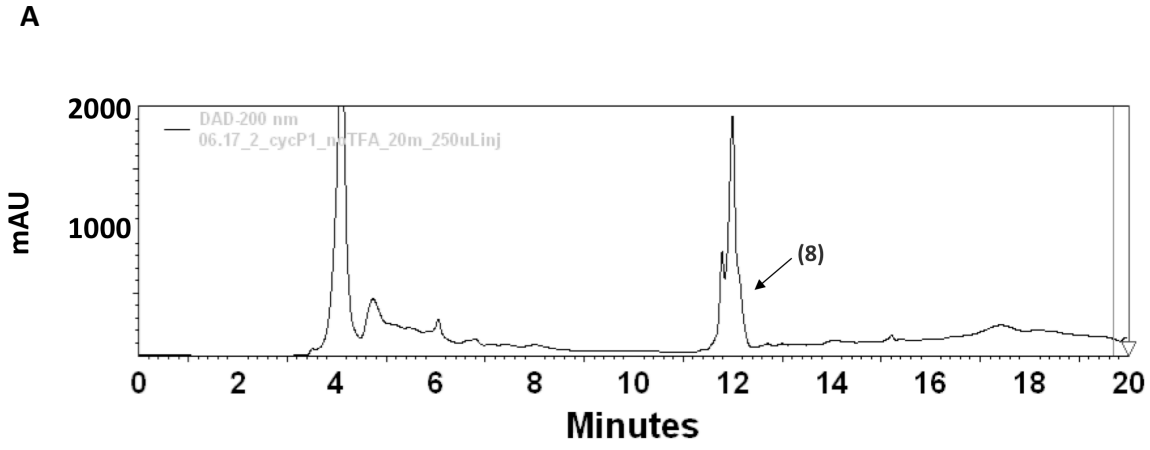

MNKQNLIPKAAQPVNRITTGQLPAQLAELSEEVLQLHDDTSASVLAS
LEMANTPDCVSCGTCCNFCSCVSGCPACNACGRCCDCGGCCRCSYDGDDAE
Leader
RSs
Core

Figure S4 (A-C), related to Figure 4 and Results (Enzymatic reactions in designed unnatural synthesis)

A. Purification of macrocycle 8. HPLC trace (UV was monitored at 200 nm) of the purification of **8** from a one-pot reaction of **1** (100 µM) with ThcD (2 µM) and PatG (10 µM) in 1.0 mL reaction volume for 18 h.

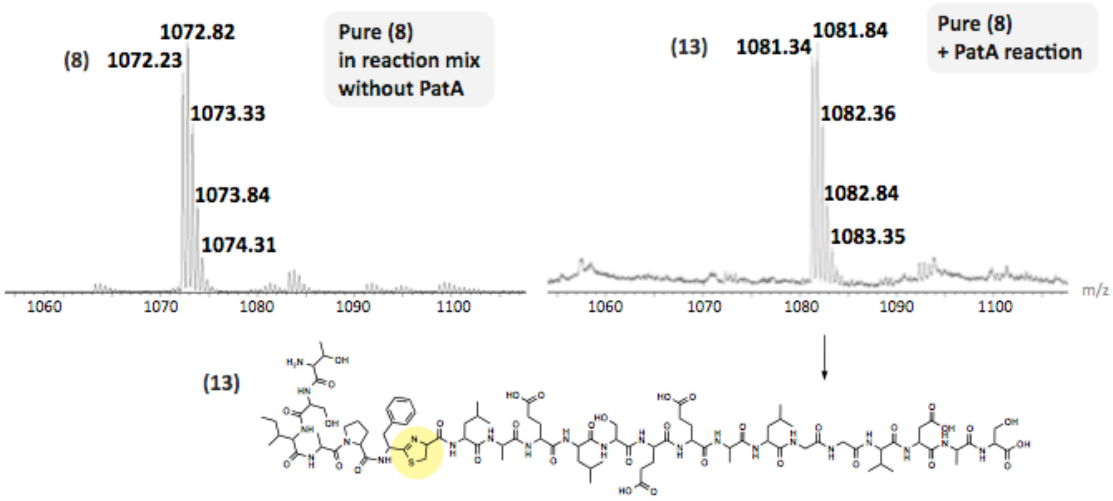
B. Reaction of 8 (200 µM) with PatA (7 µM) in optimised assay conditions. (i) Mass spectrum of the product **13** formed in this reaction in comparison to a negative control reaction lacking PatA (on the left). (ii) FT-ICR MS/MS of product **13** the interpretation of which is given in Table S2. (iii) The high-resolution MS spectrum of **13** was recorded with an error of less than 2.5 ppm. It is to be noted that ring opening of **8** further confirmed the structure of **8** to be a N-C macrocycle.

C. Time-course of formation of PatA products 13 and 4. Substrate **1** (50 µM) was converted to heterocyclized product **2** with ThcD (2 µM). Subsequent addition of PatG (10 µM) afforded the macrocycle **8**. To this reaction PatA (2 µM) was added and formation of the linearized product **13** was followed with time (bottom). In a control reaction, boiled PatG was added to the ThcD reaction before addition of PatA, such that the linear species **2** was the substrate for PatA, and the formation of the subsequent product **4** was followed with time (top). The X-axis represents time and the Y-axis represents area that is an absolute value from the integration of the displayed mass peak from the total ion chromatogram of the mass spectrum, representing relative abundance of the product species.

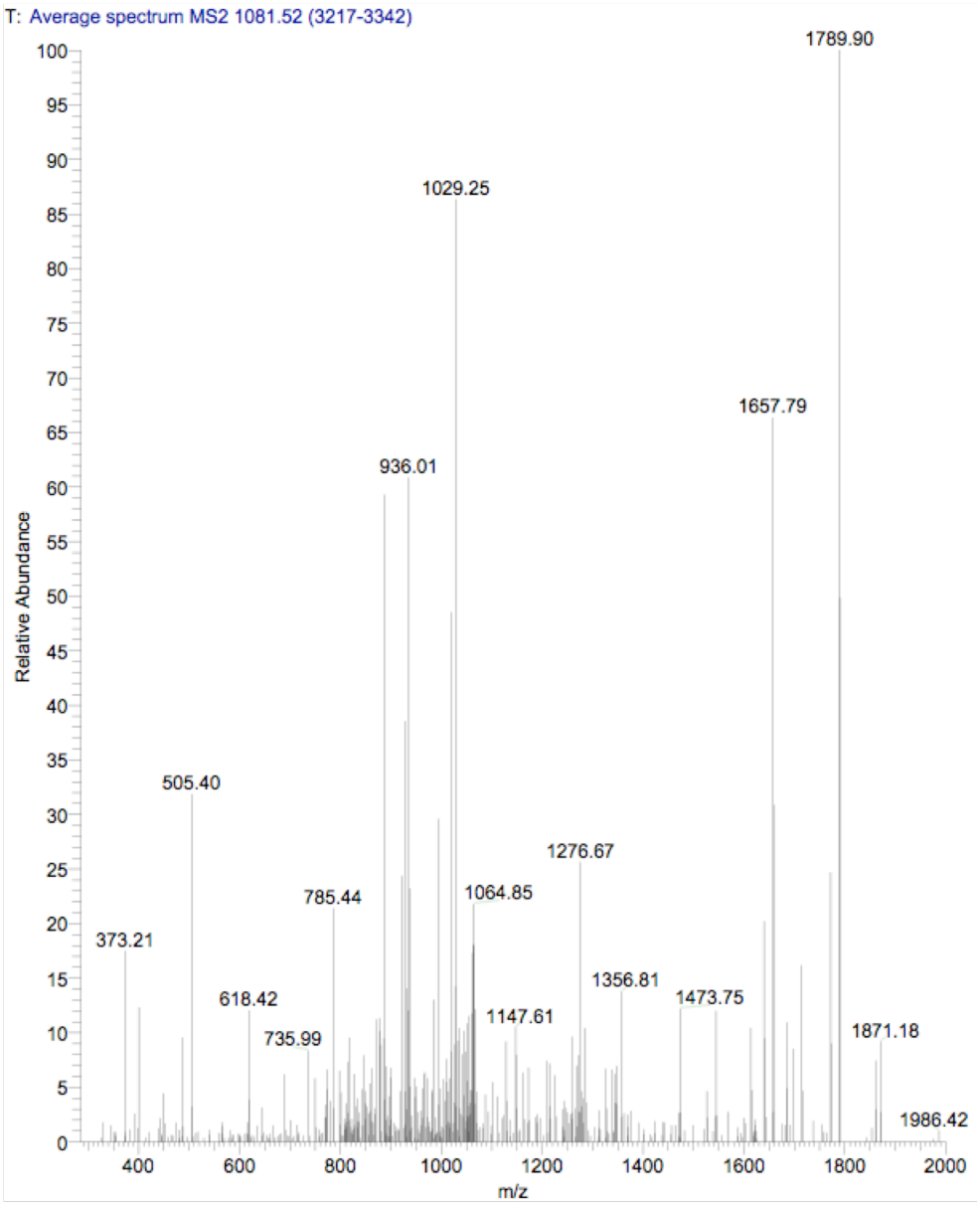


B

(i)


(8) 1072.23
1072.82
1073.33
1073.84
1074.31
Pure (8)
in reaction mix
without PatA
(13) 1081.34
1081.84
1082.36
1082.84
1083.35
Pure (8)
+ PatA reaction
1060
1070
1080
1090
1100
m/z
(13)

(ii)


T: Average spectrum MS2 1081.52 (3217-3342)
Relative Abundance
m/z
1789.90
1029.25
1657.79
936.01
505.40
1276.67
785.44
1064.85
373.21
1356.81
1473.75
618.42
1147.61
735.99
1871.18
1986.42

(iii)

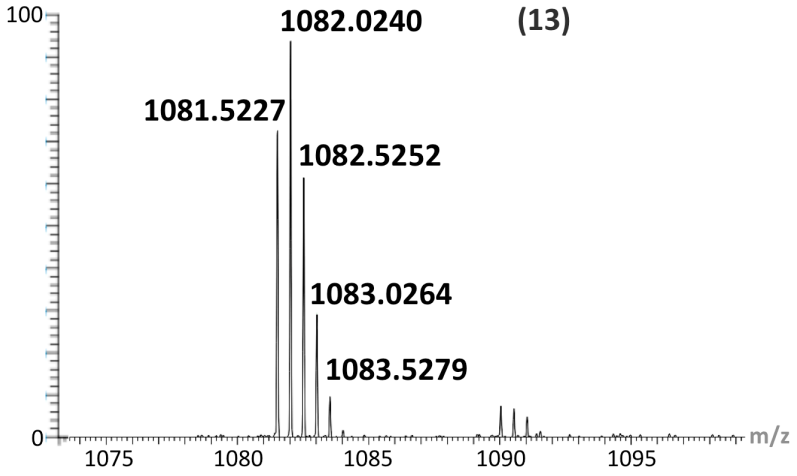
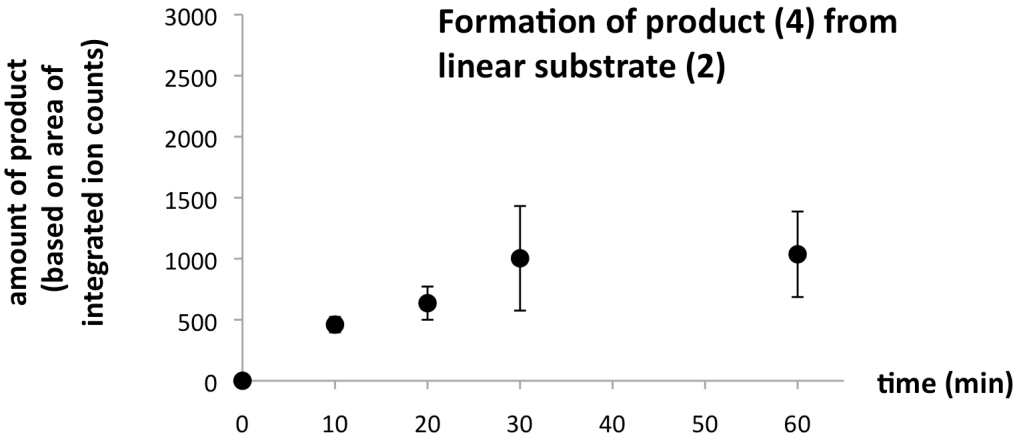


Table S2, related to Figure 4

Interpretation of FT-ICR MS/MS spectrum shown in Figure S4-B (ii).

Sequence	b	b^{++}	b^{++} -H_2O	b -H_2O	Sequence	y	y^{++}	y^{++} -H_2O	y -H_2O
T	-	-	-	-	TSIAPFC*LAELSEEALGGVDAS	-	-	-	-
TS	-	-	-	-	SIAPFC*LAELSEEALGGVDAS	-	-	-	-
TSI	-	-	-	-	IAPFC*LAELSEEALGGVDAS	-	-	-	-
TSIA	373.21	-	-	-	APFC*LAELSEEALGGVDAS	1861.80	-	-	-
TSIAP	-	-	-	-	PFC*LAELSEEALGGVDAS	1789.90	-	-	-
TSIAPF	-	-	-	-	FC*LAELSEEALGGVDAS	-	-	-	-
TSIAPFC*	-	-	-	-	C*LAELSEEALGGVDAS	-	-	-	-
TSIAPFC*L	-	-	-	-	LAELSEEALGGVDAS	-	-	-	-
TSIAPFC*LA	886.68	-	-	-	AELSEEALGGVDAS	-	-	-	-
TSIAPFC*LAE	-	-	-	-	ELSEEALGGVDAS	1276.67	-	-	-
TSIAPFC*LAEL	1128.59	-	-	-	LSEEALGGVDAS	1147.61	-	-	-
TSIAPFC*LAELS	-	-	-	-	SEEALGGVDAS	-	-	-	-
TSIAPFC*LAELSE	-	-	-	-	EEALGGVDAS	-	-	-	-
TSIAPFC*LAELSEE	1473.75	-	-	-	EALGGVDAS	818.52	-	-	-
TSIAPFC*LAELSEEA	1544.82	-	-	1526.29	ALGGVDAS	689.38	-	-	-
TSIAPFC*LAELSEEAL	1657.79	-	-	1693.95	LGGVDAS	618.42	-	-	-
TSIAPFC*LAELSEEALG	1714.97	-	-	-	GGVDAS	505.40	-	-	487.29
TSIAPFC*LAELSEEALGG	1771.91	886.68	877.39	-	GVDAS	-	-	-	-
TSIAPFC*LAELSEEALGGV	1871.18	936.01	927.34	-	VDAS	-	-	-	-
TSIAPFC*LAELSEEALGGVD	1986.42	993.55	984.89	-	DAS	-	-	-	-
TSIAPFC*LAELSEEALGGVDA	-	1029.25	1020.19	-	AS	-	-	-	-
TSIAPFC*LAELSEEALGGVDAS	-	-	1064.85	-	S	-	-	-	-

C


Formation of product (4) from linear substrate (2)
amount of product (based on area of integrated ion counts)
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
time (min)

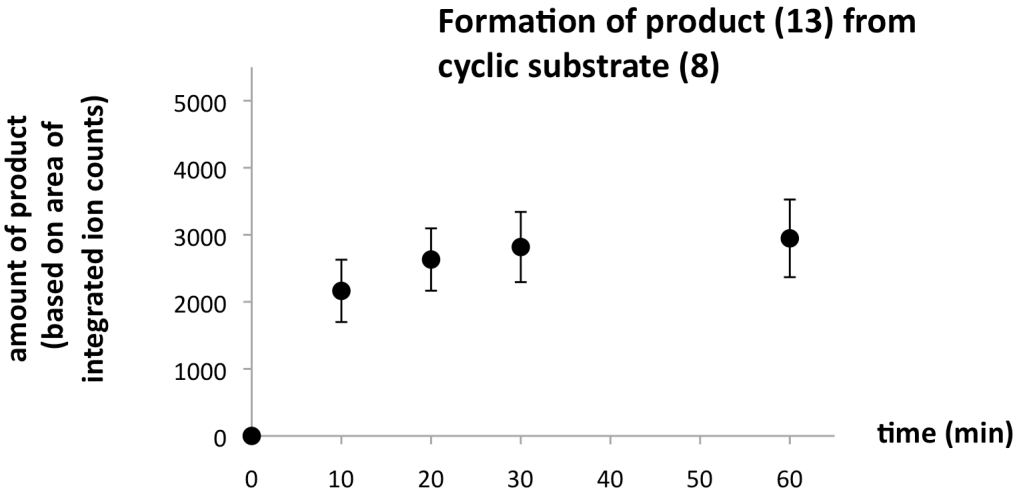
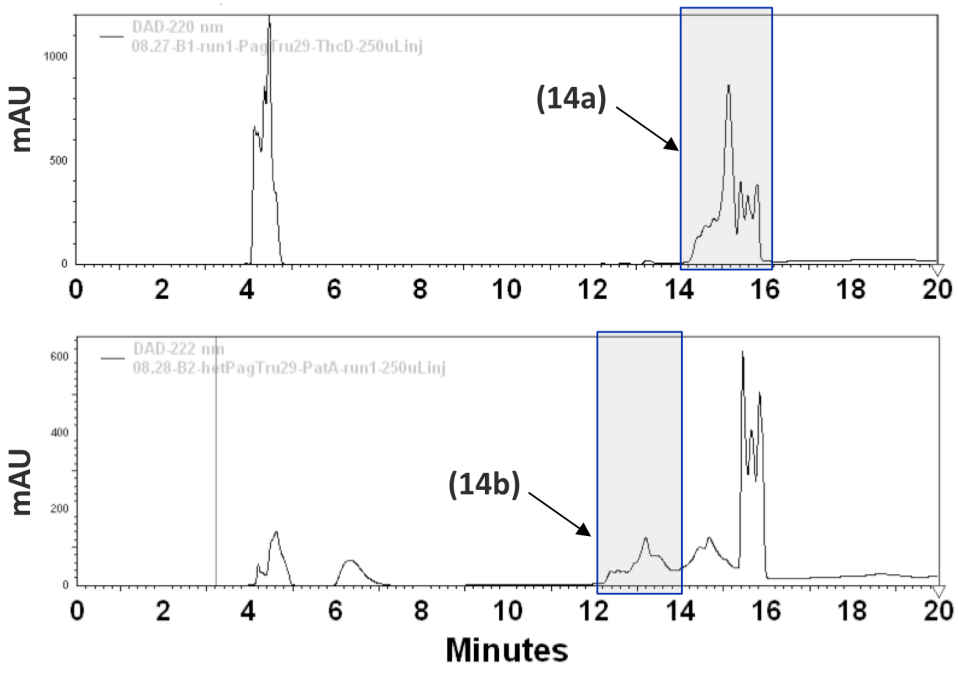

Formation of product (13) from cyclic substrate (8)
amount of product (based on area of integrated ion counts)
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
60
time (min)

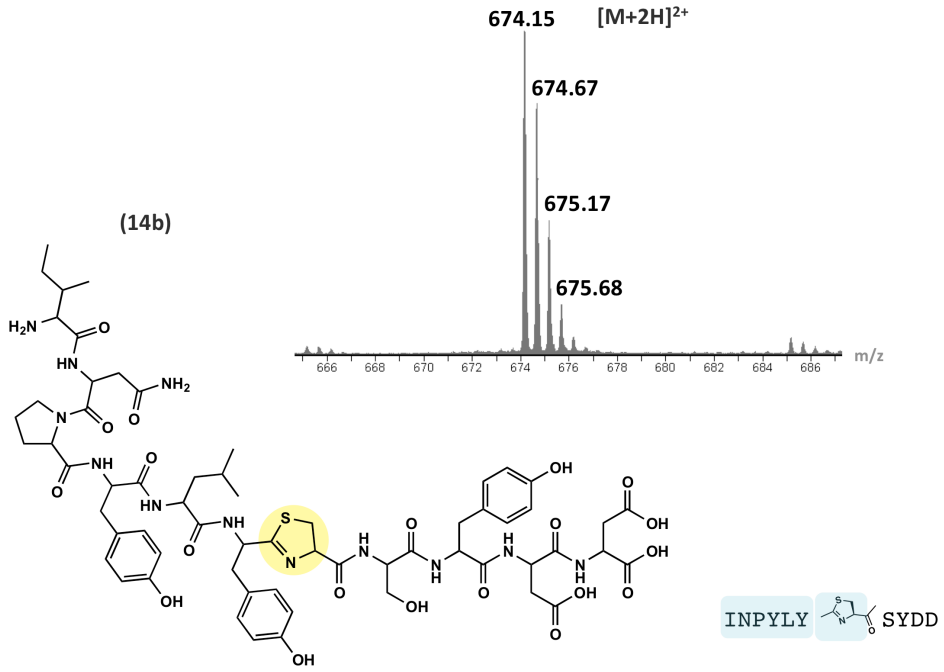
Figure S5, related to Figure 5 and Results (Cyclic peptides from full-length artificial precursor peptides)

Formation of cyclic peptide 14c (cyc-INPYLYC* where C* is a thiazoline). (i) On the left is HPLC trace of ThcD-treated **14** (top) and PatA-treated **14a** (bottom). In the top panel, the peak between 14 min to 16 min is the **14a** product, whereas in the bottom panel the peak between 12 min to 14 min is **14b**. (ii) Structure and mass spectrum of **14b**, which is a result of ThcD and PatA modification on substrate **14**. (iii) FT-ICR MS of cyclic peptide **14c** with an error of 4.2 ppm. (iv) Mass spectrum of the formation of **14c** over a 72 h time-period.

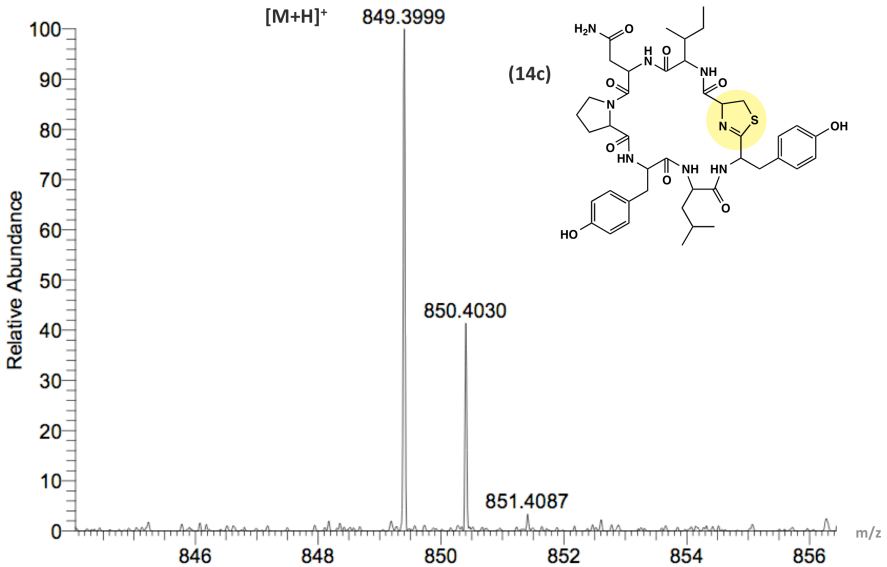
(i)



(ii)


674.15
[M+2H]2+
674.67
675.17
675.68
m/z
(14b)
INPYLY
SYDD

(iii)


[M+H]+
849.3999
(14c)
Relative Abundance
850.4030
851.4087
m/z

(iv)

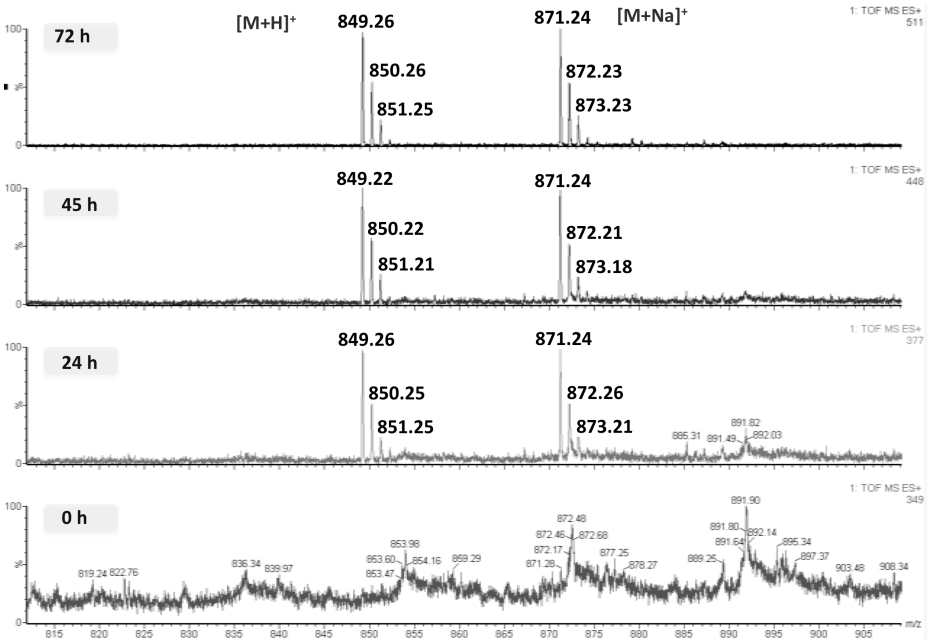
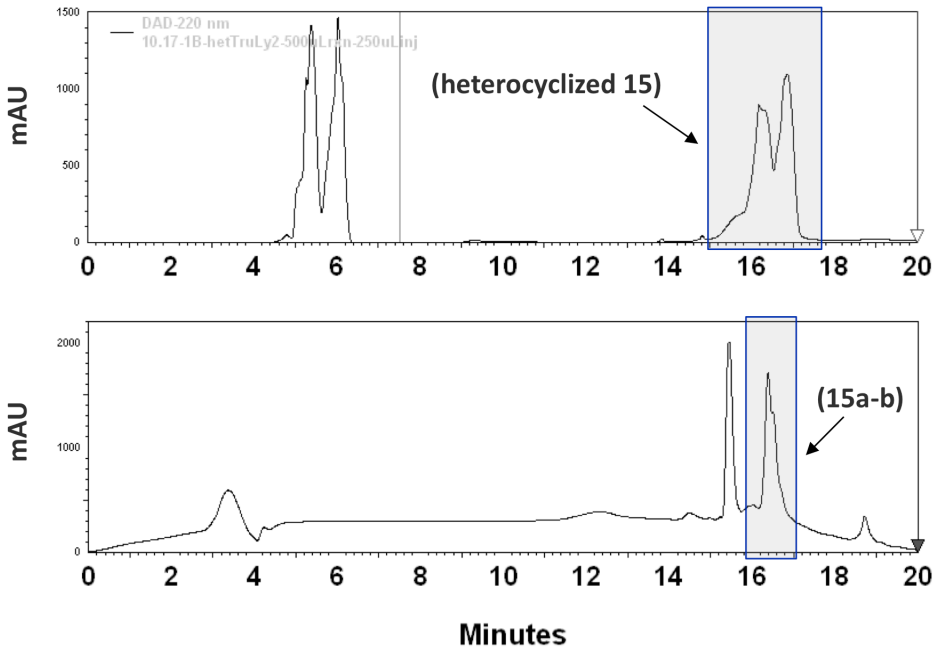

72 h
[M+H]+
849.26
850.26
851.25
871.24
[M+Na]+
872.23
873.23
45 h
849.22
850.22
851.21
871.24
872.21
873.18
24 h
849.26
850.25
851.25
871.24
872.26
873.21
0 h
m/z

Figure S6, related to Figure 6 and Results (Multiple modified cyclic peptides in a single reaction batch from a precursor peptide carrying multiple cores)
Formation of the cyclic peptides 15c-d (cyc-AC*MPC*YP and cyc-TFPVPTVC* where C* is a thiazoline) (i) HPLC trace of ThcD-treated **15** (top) and PatA reaction of the same (bottom). In the top panel, the peak between 15 min to 17.5 min is the heterocyclized **15** product, whereas in the bottom panel the peak between 16 min to 17 min contains the intermediates **15a-b**. (ii) Structure and mass spectra of **15a-b**. The PatG cyclic products of these intermediates (**15c-d**) is shown in Figure 6.

(i)



(ii)

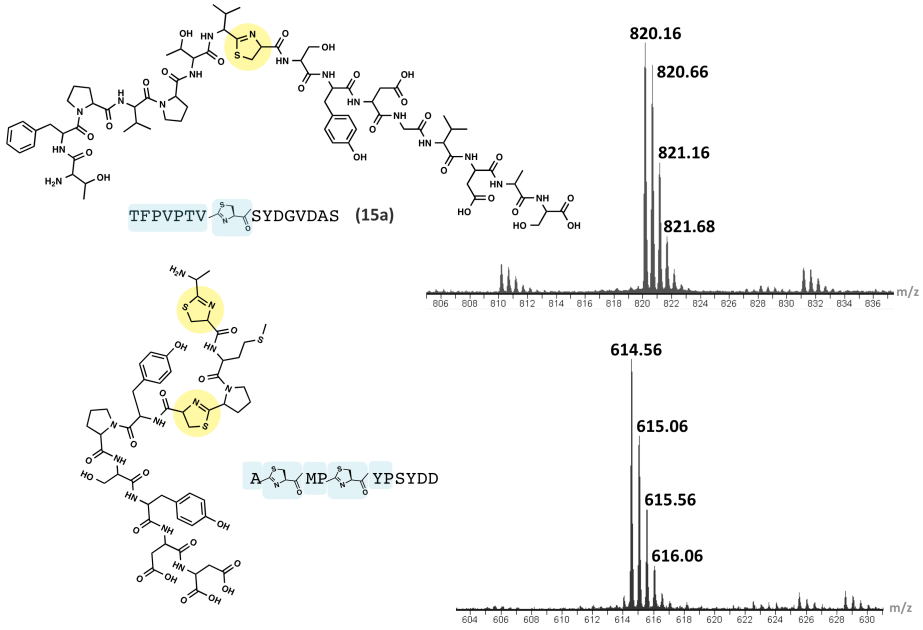

TFPVPTV SYDGVDAS (15a)
820.16
820.66
821.16
821.68
m/z
806 808 810 812 814 816 818 820 822 824 826 828 830 832 834 836
A MP YPSYDD
614.56
615.06
615.56
616.06
m/z
604 606 608 610 612 614 616 618 620 622 624 626 628 630

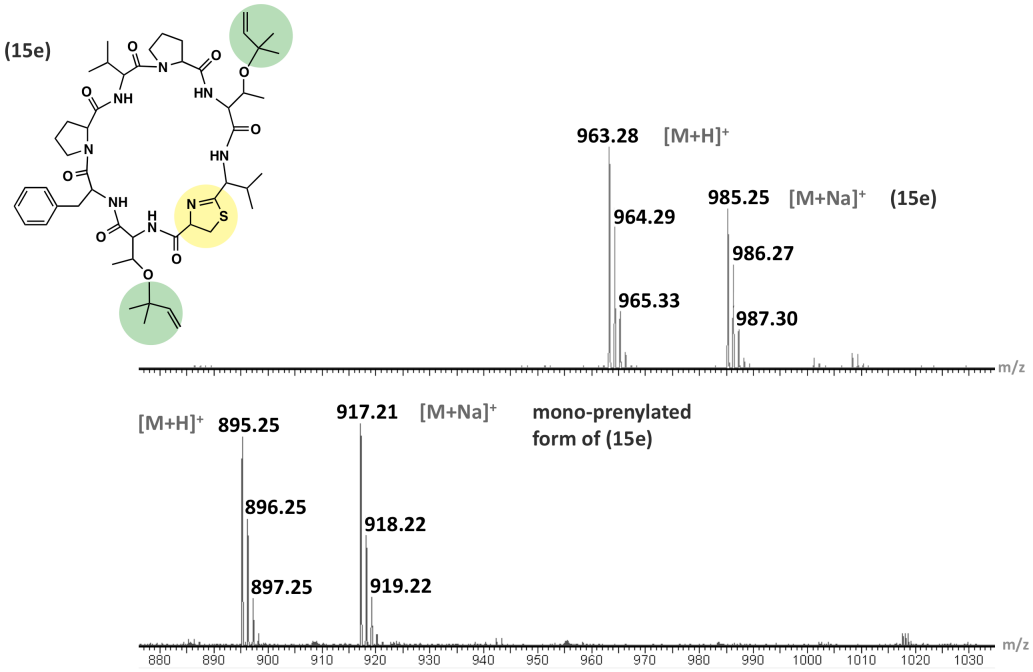
Figure S7, related to Figure 7 and Results (Combining posttranslational modifications from multiple pathways)

A. Mass spectra of the prenylated products. (i) cyc-TFPVPTVC* and (ii) cyc-AC*MPC*YP where C* represents a thiazoline and an underlined residue represents prenylation at that position. See Figure 7 for mass spectrum of cyc-INPYLYC*. (iii) Accurate mass measurement of monoprenylated form of **15e**, **15e** and **15f** with an error of 4.9 ppm, 3.6 ppm and 1.9 ppm respectively.

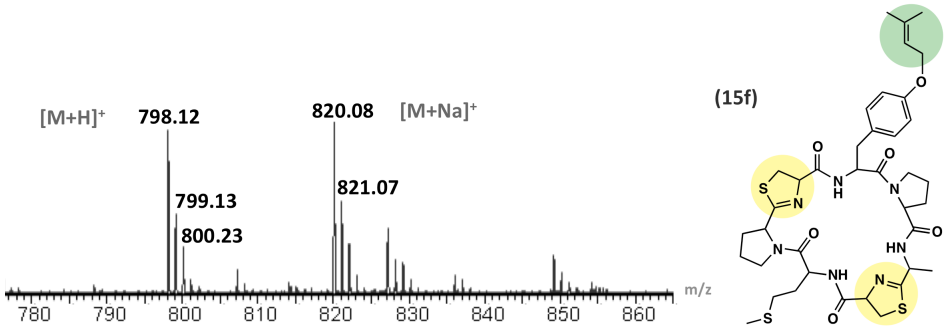
B. Formation of prenylated cyclic products. (i) LC trace of TruF1-PagF prenylation on a reaction mixture containing cyclic products **15c-d** (top) showing formation of prenylated products in comparison to the LC trace of the same reaction mixture before addition of the prenyltransferase (bottom). (ii-iii) In the top panel mass spectrum of TruF1-PagF reaction containing the prenylated products (masses shown in blue) is given in comparison to the mass spectrum of the same reaction before addition of the prenyltransferase, showing absence of the prenylated products of **15**. (iv) LC trace of PagF reaction on a reaction mixture containing the cyclic product **14c** (left) showing formation of the prenylated product **14d**, in comparison to the same reaction before addition of PagF. The mass spectrum of the same set of reaction is on the right, with the mass of the prenylated **14d** shown in blue, which is absent in the reaction before the addition of PagF. (v) FTMS-MS fragmentation spectra of monoprenylated **15e** show loss of prenyl group.

A

(i)

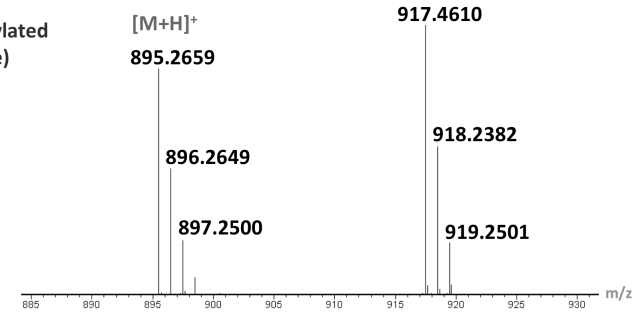

(15e)
963.28 [M+H]+
964.29
965.33
985.25 [M+Na]+ (15e)
986.27
987.30
m/z
[M+H]+ 895.25
896.25
897.25
917.21 [M+Na]+
mono-prenylated form of (15e)
918.22
919.22
880 890 900 910 920 930 940 950 960 970 980 990 1000 1010 1020 1030
m/z

(ii)

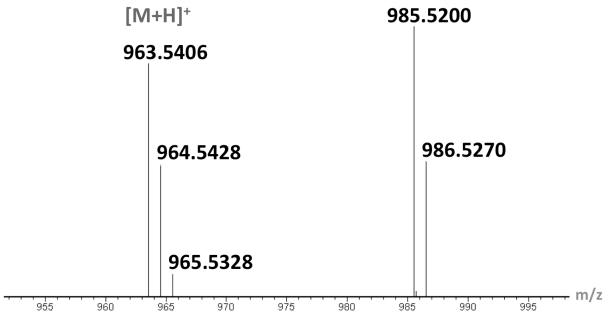

[M+H]+ 798.12
799.13
800.23
820.08 [M+Na]+
821.07
780 790 800 810 820 830 840 850 860
m/z
(15f)

(iii)

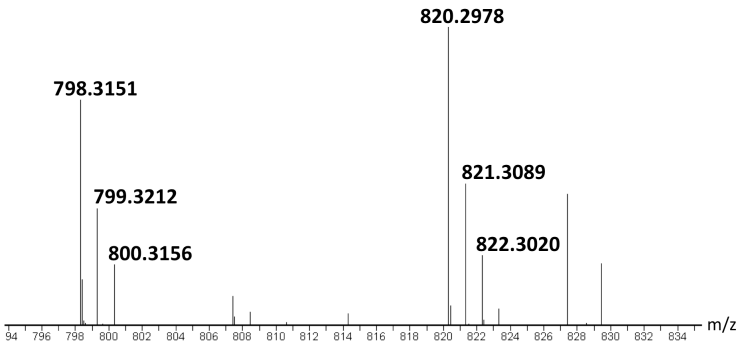
mono-prenylated form of (15e)



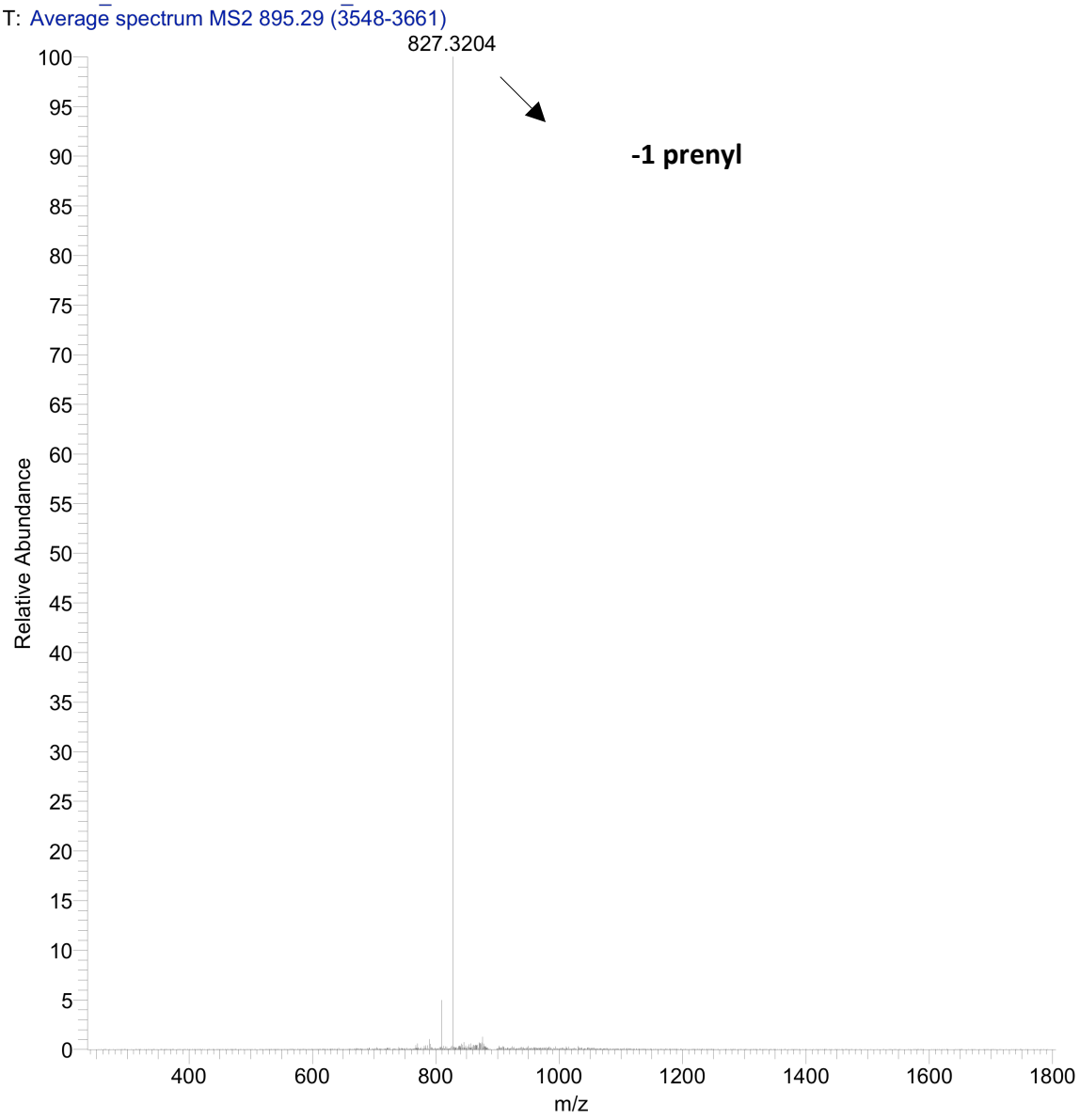
(15e)



(15f)

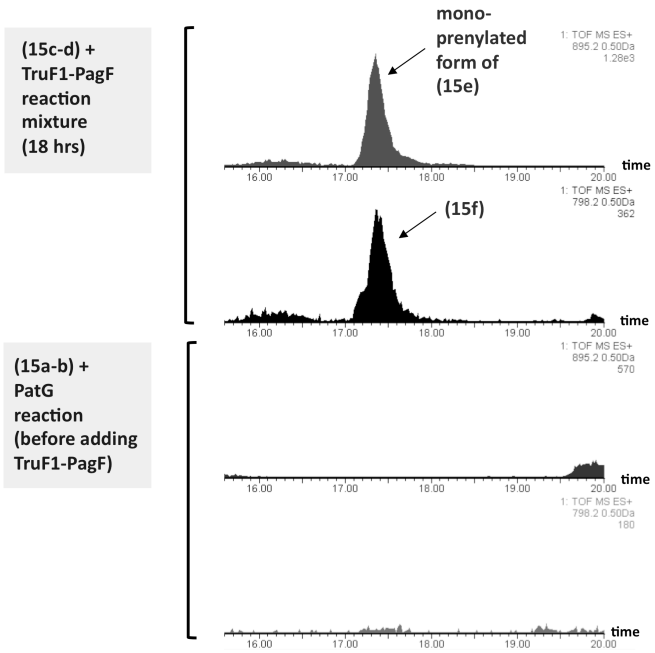


(v)

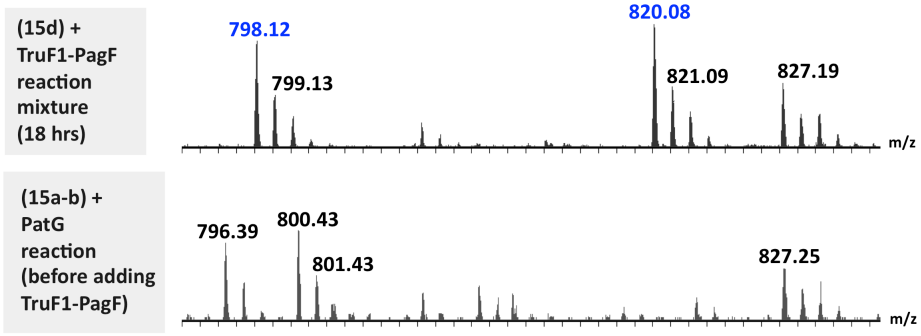

T: Average spectrum MS2 895.29 (3548-3661)
827.3204
-1 prenyl
Relative Abundance
m/z
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
400
600
800
1000
1200
1400
1600
1800

B

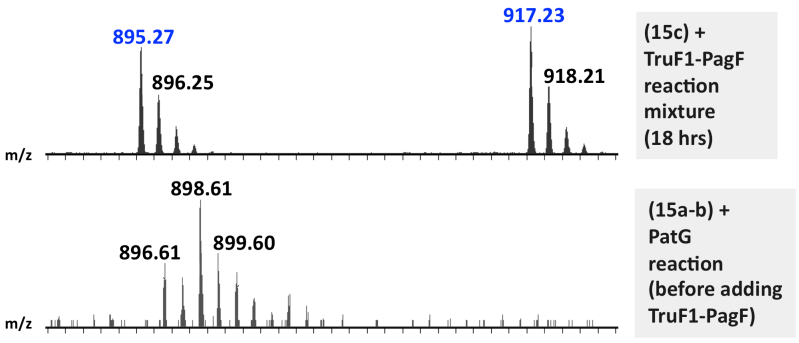
(i)


(15c-d) + TruF1-PagF reaction mixture (18 hrs)
mono-prenylated form of (15e)
1: TOF MS ES+ 895.2 0.50Da 1.28e3
time
16.00
17.00
18.00
19.00
20.00
1: TOF MS ES+ 798.2 0.50Da 362
(15f)
time
(15a-b) + PatG reaction (before adding TruF1-PagF)
1: TOF MS ES+ 895.2 0.50Da 570
time
1: TOF MS ES+ 798.2 0.50Da 180
time

(ii)


(15d) + TruF1-PagF reaction mixture (18 hrs)
798.12
799.13
820.08
821.09
827.19
m/z
(15a-b) + PatG reaction (before adding TruF1-PagF)
796.39
800.43
801.43
827.25
m/z

(iii)


895.27
896.25
917.23
918.21
m/z
(15c) + TruF1-PagF reaction mixture (18 hrs)
898.61
896.61
899.60
m/z
(15a-b) + PatG reaction (before adding TruF1-PagF)

(iv)

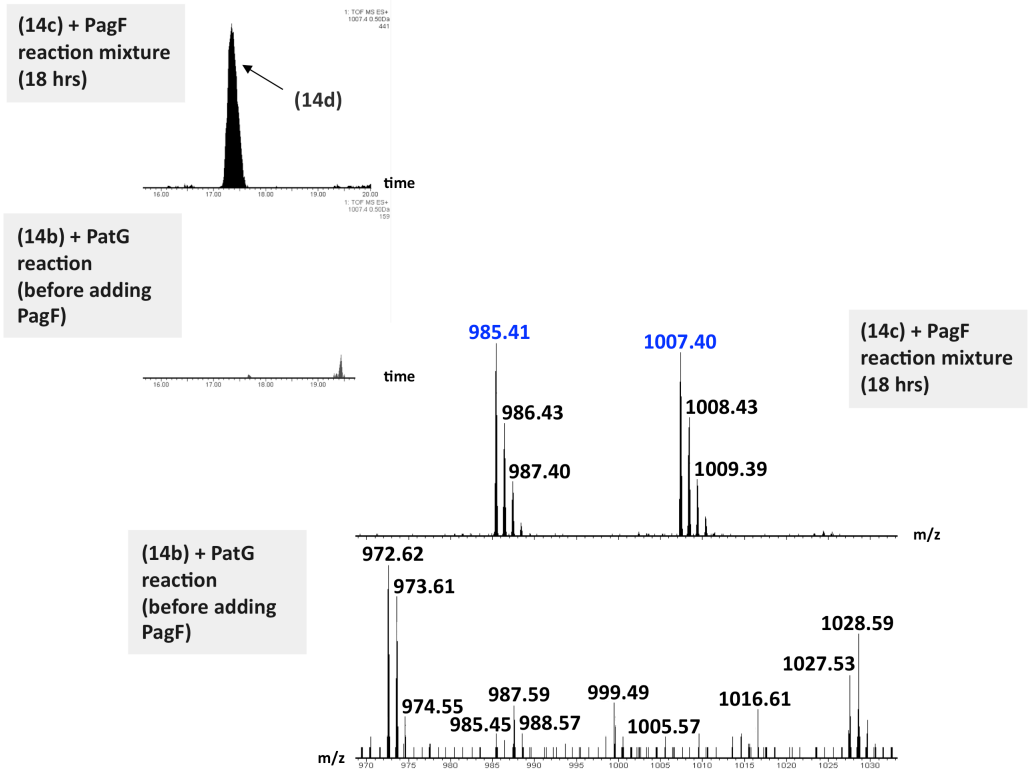

(14c) + PagF reaction mixture (18 hrs)
(14d)
time
(14b) + PatG reaction (before adding PagF)
time
985.41
1007.40
(14c) + PagF reaction mixture (18 hrs)
986.43
1008.43
987.40
1009.39
m/z
(14b) + PatG reaction (before adding PagF)
972.62
973.61
1028.59
1027.53
974.55
987.59
999.49
1016.61
985.45
988.57
1005.57
m/z

Table S3, related to Methods

A list of all substrates, intermediates and products made in this study. Substrates **14** and **15** are His-tagged. The expected masses of each species are given in Table S4. The symbols indicate the following posttranslational modifications: C* is a thiazoline, cyc is N-C macrocyclization, and Y and T are forward and reverse prenylations respectively.

1	LAELSEEALGGVDASTSIAPFCSYD
1a	TSIAPFPSYD
2	LAELSEEALGGVDASTSIAPF**C***SYD
3	LAELSEEALGGVDAS
4	TSIAPF**C***SYD
5	**cyc**-TSIAPF**C***
5a	**cyc**-TSIAPFP
6	**cyc**-**TS**IAPF**C***+isoprene
7	TSIAPFCSYD
8	**cyc**-LAELSEEALGGVDASTSIAPF**C***
9	AITFCAYDGE
10	AITF**C***AYDGE
11	**cyc**-AITF**C***
12	AYDGE
13	TSIAPF**C***LAELSEEALGGVDAS
14	MTKKNLKPQQAAPVQREINTTSSESLAELSEEALGGVDASINPYLYCSYDD
14a	MTKKNLKPQQAAPVQREINTTSSESLAELSEEALGGVDASINPYLY**C***SYDD
14b	INPYLY**C***SYDD
14c	**cyc**-INPYLY**C***
14d	**cyc**-INP**Y**L**Y**C*
15	MNKKNILPQLGPVIRLTAGQLAELSEEALGGVDASTFPVPTVCSYDGVDASACMPCYPSYDD
15a	TFPVPTV**C***SYDGVDAS
15b	A**C***MP**C***YPSYDD
15c	**cyc**-TFPVPTV**C***
15d	**cyc**-A**C***MP**C***YP
15e	**cyc**-**T**FPVP**T**V**C***
15f	**cyc**-A**C***MP**C***YP

Table S4, related to Methods

Expected masses of the different species listed in Table S3.

	$[M+H]^+$	$[M+2H]^{2+}$	$[M+3H]^{3+}$
1	-	1272.5946	848.7324
1a	1096.5309	-	-
2	-	1263.5893	842.7288
3	1460.7114	730.8598	-
4	1084.4768	542.7425	-
5	702.3279	-	-
5a	714.3821	-	-
6	770.39053	-	-
7	1102.4873	-	-
8	-	1072.5149	-
9	1089.4557	-	-
10	1071.4451	546.2267	-
11	518.2431	-	-
12	554.2093	-	-
13	-	1081.5202	-
14	-	-	-
14a	-	-	-
14b	1347.5561	674.2822	-
14c	849.3963	-	-
14d	985.5215	-	-
15	-	-	-
15a	-	820.8735	-
15b	-	614.7095	-
15c	827.4119	-	-
15d	730.2509	-	-
15e	963.5372	-	-
15f	798.3136	-	-

Supplemental Experimental Procedures

Detailed assay conditions for stepwise enzyme addition

1. Stepwise synthesis of product 5

All reactions were performed in Tris buffer pH 7.5 (50 mM) at 37°C. Peptide **1** (200 μM) was mixed with ThcD (2 μM), ATP (1 mM) and $MgCl_2$ (5 mM) to a total volume of 50 μL. After incubation for 2 h, PatA (2 or 5 μM) and $CaCl_2$ (10 mM) were added and the volume adjusted to 100 μL. After another 2 h incubation, 50 μL of this reaction was mixed with PatG protease/macrocyclase domain or full-length TruG (10 μM) and $MgCl_2$ (5 mM) to a total volume of 100 μL for 18 h. Since the reaction reaches completion by this time it can be assumed that 50 μM of the PatG product **5** was present in the reaction mixture. This was further diluted such that 25 μM of the cyclic peptide **5** was made available to TruF1 (10 μM) and DMAPP (10 mM) in a final reaction volume of 50 μL for another 18 h.

2. Stepwise synthesis of products 14c and 15c-d

All reactions were performed in Tris buffer pH 7.5 (50 mM) at 37°C. His-tagged peptides **14** and **15** (70-80 μM) was mixed with ThcD (2 μM), ATP (1 mM) and $MgCl_2$ (5 mM) to a total volume of 500 μL for 18 h. The reaction was purified by HPLC. The fractions containing the heterocyclized intermediate was collected and dried down, followed by subsequent addition of PatA (2 μM) and $CaCl_2$ (10 mM) in a 300 μL reaction volume for another 18 h. The reaction was further purified by HPLC, and the fractions containing the PatA product was dried and added to reaction mixtures containing PatG (20 μM) in a volume of 50-100 μL for 24-72 h. For subsequent prenylation step, the PatG reaction mixture was not purified. Instead the crude mixture was dried down and made upto a reaction volume of 50 μL with TruF1 and/or PagF along with DMAPP (10 mM) in optimised reaction conditions for 18 h.