# Dataset S1.

# (a) Summary of proteomic evidence supporting novel cyclotide sequences from C. ternatea.

The following MS spectra show the ProteinPilot-assigned peptide spectral matches (PSM) for full-length linearized cyclotides generated by endoproteinase Glu-C digestion (spectra named with suffix E), trypsin digestion (suffix T) and chymotrypsin digestion (suffix C). In many instances semi-endoGlu-C, semi-tryptic or semi-chymotryptic peptides were observed and the corresponding PSM have been included as additional lines of evidence supporting the full-length peptide assignments. In one case, Cter N, the assignment was performed using RapiDeNovo software (Bruker) after MS/MS performed on a Bruker UltrafleXtreme mass spectrometer. In all other cases, spectra were acquired on an Applied Biosystems QStar Elite mass spectrometer. As an example, the PSM of Cter M in spectrum E1a (Endo Glu-C digestion) shows the assignment of 25/29 amino acids. The unassigned amino acids were assigned in subsequent spectra (EndoGlu-C: E1b, E1c; tryptic: T1a-T1c). This is the case for each peptide sequenced. In the following dataset outputs from ProteinPilot, amino acid sequences coloured green represent PSMs identified with 95% confidence, yellow represent 50% confidence, red represent <50% confidence, and black are not matched. Sequence ion assignment is shown in each spectrum with y-ions shown in red and b-ions shown in green. The matched ions are also shown in the left panel, where green shading represents a matched ion.

# Endoproteinase Glu-C digest:

#### TCTLGTCYVPDCSCSWPICMKNGLPTCGE

NB: This peptide shows 75% sequence homology to kalata B7 (P58457) from Oldenlandia affinis.

- 1 GLPTCGETCTLGTCYVPDCSCSWPICMKN 29 Cter\_M
- 1 GLPVCGETCTLGTCYTQGCTCSWPICKRN 29 kB7

#### Fragmentation Evidence for Peptide

#### TC[CAM]TLGTC[CAM]YVPDC[CAM]SC[CAM]SWPIC[CAM]MKNGLPTC[CAM]GE Residue b b+2 y+2 У 1712.6988 102.0550 51.5311 3424.3903 260 v1 ¥2. y3 y4 V5 <u>γ6</u> C[CAM] 262.0856 131.5464 3323.3427 1662.1750 b3 b4 b2 b5|| b8 b9 b14 250 -563.22 y16+2 y17 b16+2 363.1333 182.0703 1582.1596 3163.3120 γ3+2 I γ5+2 y11+2 v13+2 y14+2 b18+2 y20+2 y22+2 v23+2 y25+2 y29+2 240 y21+2 y15+2 b19+2 476.2173 238.6123 3062.2643 1531.6358 b8+2 b9+2 b22+2 b24+2 b26+2 230 533.2388 267.1230 2949.1803 1475.0938 220 634.2865 317.6469 2892.1588 1446.5830 210 C[CAM] 794.3171 397.6622 2791.1111 1396.0592 200 1185.50 957.3805 479.1939 2631.0805 1316.0439 190 1056.4489 528.7281 1234.5122 2468.0172 180 1153,5016 577.2545 2368.9487 1184.9780 170 1268.5286 634,7679 2271.8960 1136.4516 160 1078.9382 C[CAM] 1428.5592 2156.8690 714.7833 150 998.9228 1515.5913 758.2993 1996.8384 2140 C[CAM] 1675.6219 838.3146 1909.8064 955.4068 ໍລິ້130 1762.6539 881.8306 1749.7757 875.3915 Ĕ 120 831.8755 1948.7333 974.8703 1662.7437 738.8358 2045.7860 1023.3966 1476.6644 110 1150 2158.8701 1079.9387 1379.6116 690.3094 100 1056.46 CICAMI 2318,9007 1159.9540 1266.5275 633.7674 90 2449.9412 1225.4742 1106.4969 553.7521 м 80 488.2318 2578.0362 1289.5211 975.4564 70 2692.0791 1346.5432 847.3614 424.1844 60 870 79 2749.1006 1375.0539 733.3185 367.1629 50 1432.14 2862.1846 1431.5960 676.2971 338.6522 738.86 40 282.1101 2959.2374 1480.1223 563.2130 30 1212.47 528173 038.44 365.13 634.29 1718.81 3060.2851 1530.6462 466.1602 233.5838 1235.03 20 1440 64 1476.72 205.08 973102 365.1125 183.0599 371.19 808.34 CICAM1 3220.3157 1610.6615 676.33 159.10 280.12 48.20 1611 21 1704.61 10 1315.96 1540.32 205.0819 103.0446 3277.3372 1639.1722 0 3406.3798 1703.6935 148.0604 74.5339 300 400 600 700 800 900 1000 1300 1400 100 200 500 1100 1200 1500 1600 1700 1800 m/z.Da

**Supplementary spectrum E1a:** MS/MS of *m*/*z* 1142.13 (mass 3423.4 Da) showing sequence ion coverage. The derived sequence is TCTLGTCYVPDCSCSWPICMKNGLPTCGE.



Supplementary spectrum E1b: MS/MS of *m*/z 767.32 (mass 1532.62 Da) showing sequence ion coverage. The derived sequence is TCTLGTCYVPDCS.

3

c[c	CAM]SVVPIC[CA	MJMKNGLPTC	C[CAM]GE																				
Residue	b	b+2	У	y+2																			
C[CAM]	161.0379	81.0226	1909.8064	955.4068		260 - y	l.	y3		y5	l b	/6	y8			y10		y11		y13			
6	248.0700	124.5386	1749.7757	875.3915		250	b	2 y7+3	b3		b5			y16+2	059.00		b9	·   1	011				
V	434.1493	217.5783	1662.7437	831.8755		240									/ 000.90								
)	531.2020	266.1047	1476.6644	738.8358		230																	
	644.2861	322.6467	1379.6116	690.3094		220																	
[CAM]	804.3167	402.6620	1266.5275	633.7674		210																	
1	935.3572	468.1823	1106.4969	553,7521		200																	
	1063.4522	532.2297	975.4564	488.2318		100																	
	1177.4951	589.2512	847.3614	424.1844		190 ]																	
	1234.5166	617.7619	733.3185	367.1629		100 1																	
	1347.6006	674.3040	676.2971	338.6522		100																	
	1444.6534	722.8303	563.2130	282.1101		160 -																	
	1545.7011	773.3542	466.1602	233,5838		150 -																	
CAM]	1705.7317	853,3695	365.1125	183.0599	sitv	140 -																	
	1762.7532	881.8802	205.0819	103.0446	ten l	130 -																	
	1891.7958	946.4015	148.0604	74.5339	_	120																	
						110 -																	
						100																	
						90 -																	
						80 -																	
						70 -																	
						60 -																	
						50 -																	
						40																	
						30 -																	
						20				56.	3.22				964.51								
						10 - 14	1.05	09.12 <sub>36</sub>	512 434	1.13 518,20	040.07	737.	52 833.4	914.43	982.90	1105.92	,	1268.54	1349.63	1479.67	1637	T 5.4	
						م اسم	يستبيد والمراج	به و الم	يانيوسيان	وانعطيه ووجا	618.29	v06.37	أدأس موجعا فاور	المستقر ويتعالم	ب سور بالغال		سيو المارية الم	1200.34	يى يېغە يىيى <mark>ا</mark> يىلە	يعليم بطأك فريك	1007 أسغب جي	+ 	
						100	200	300	400	500	600	700	800	900	1000	1100	1200	1300	1400	1500	1600	1700	18
														m/z,	Da								

**Supplementary spectrum E1c:** MS/MS of *m*/z 955.41 (mass 1908.81 Da) showing sequence ion coverage. The derived sequence is SWPICMKNGLPTCGE.

NGLPTC[CAM]GETC[CAM]TLGTC[CAM]YVPDC[CAM]SC[CAM]SVVPIC[CAM]MK



Supplementary spectrum T1a: MS/MS of m/z 1142.13 (mass 3423.38 Da) showing sequence ion coverage. The derived sequence is NGLPTCGETCTLGTCYVPDCSCSWPICMK.



Supplementary spectrum T1b: MS/MS of *m*/z 981.90 (mass 1961.78 Da) showing sequence ion coverage. The derived sequence is CYVPDCSCSWPICMK.



Supplementary spectrum T1c: MS/MS of *m*/z 741.33 (mass 1480.65 Da) showing sequence ion coverage. The derived sequence is NGLPTCGETCTLGT.

7

# Endoproteinase Glu-C digest:

### TCVLGTCYTPDCSCTALVCLKNGSAFCGE

 NB: This peptide shows homology to kalata B9 (P85127) from Oldenlandia affinis.

 1
 GSAF-CGETCVLGTCYTPDCSC-TALVCLKN 1
 29
 Cter\_N 31

 kB9



Supplementary spectrum E2a: MS/MS of *m*/z 3301.42 (mass 3300.42 Da) showing sequence ion coverage. The derived sequence is TCVLGTCYTPDCSCTALVCLKNGSAFCGE.



Supplementary spectrum T2a: MS/MS of *m*/z 3301.42 (mass 3300.42 Da) showing sequence ion coverage. The derived sequence is NGSAFCGETCVLGTCYTPDCSCTALVCLK.

# Endoproteinase Glu-C digest:

#### SCVFIPCITGIAGCSCKSKVCYRNGIPCGE

NB: This peptide shows 93% sequence homology to circulin-C (P84641) from Chassalia parvifolia.

1 GIPCGESCVFIPCITGIAGCSCKSKVCYRN 30 Cter\_0

1 GIPCGESCVFIPCITSVAGCSCKSKVCYRN 30 Circulin-C

#### Fragmentation Evidence for Peptide



Supplementary spectrum E3a: MS/MS of *m*/z 863.41 (mass 3449.62 Da) showing sequence ion coverage. The derived sequence is SCVFIPCITGIAGCSCKSKVCYRNGIPCGE.



**Supplementary spectrum E3b:** MS/MS of *m*/z 821.39 (mass 1640.77 Da) showing sequence ion coverage. The derived sequence is CVFIPCITGIAGCS.



Supplementary spectrum E3c: MS/MS of m/z 609.94 (mass 1826.81 Da) showing sequence ion coverage. The derived sequence is CKSKVCYRNGIPCGE.

# Chymotrypsin digest:

# RNGIPCGESCVFIPCITGIAGCSCKSKVCY

### Fragmentation Evidence for Peptide



**Supplementary spectrum C3a:** MS/MS of *m*/*z* 698.31 (mass 1394.60 Da) showing sequence ion coverage. The derived sequence is RNGIPCGESCVF.



Supplementary spectrum C3b: MS/MS of *m*/z 531.25 (mass 1060.49 Da) showing sequence ion coverage. The derived sequence is IPCITGIAGC.

# Endoproteinase Glu-C digest:

SCVFIPCITAAIGCSCKSKVCYRNGIPCGE

NB: This peptide shows 96% sequence homology to cyclotide A (D2WPK4) from *Gloeospermum blakeanum*.

1 GIPCGESCVFIPCITAAIGCSCKSKVCYRN 30 Cter\_P

1 GIPCGESCVFIPCITAAIGCSCKTKVCYRN 30 Cyclotide-A

#### Fragmentation Evidence for Peptide



Supplementary spectrum E4a: MS/MS of m/z 866.90 (mass 3463.58 Da) showing sequence ion coverage. The derived sequence is SCVFIPCITAAIGCSCKSKVCYRNGIPCGE.

SC	CAM]VFIPC[CA	M]LTAAIGC[0	CAM]S																		
Residue	b	b+2	У	y+2																	
S	88.0393	44.5233	1655.7590	828.3831	440 -	N	2  y3	y4	y5	y6	y7	yB		γ9	y10	l I y	ahl L	y12			
C[CAM]	248.0700	124.5386	1568.7270	784.8671	400	b2	6	3	b4	b5		b14+2y1	5+2 b7	· ·	b8	b9 b1	0 b11	b12			
V	347.1384	174.0728	1408.6963	704.8518	420					607	.30										
F	494.2068	247.6070	1309.6279	655.3176	400																
1	607.2908	304.1491	1162.5595	581.7834	380 -																
P	704.3436	352.6754	1049.4754	525.2414	360																
C[CAM]	864.3743	432.6908	952.4227	476.7150	240																
L	977.4583	489.2328	792.3920	396.6996	340 -																
Т	1078.5060	539.7566	679.3080	340.1576	320																
A	1149.5431	575.2752	578.2603	289.6338	300 -																
A	1220.5802	610.7938	507.2232	254.1152	280																
I	1333.6643	667.3358	436.1860	218.5967	260																
G	1390.6858	695.8465	323.1020	162.0546	2000																
C[CAM]	1550.7164	775.8618	266.0805	133.5439	<u>≳</u> 240 1																
S	1637.7484	819.3779	106.0499	53.5286	ja 220 −								829.40		105	1.48					
	I				200								023.40								
					180																
					160 -																
					140 -																
					120 -																
					100																
					°° -		323.1	1													
					60 -			0.17.15													
					40 -			347.15	494.23	;	679.3	:4									
					20	218.06 248	.07	371.19 436	.20 507.:	23 579.30		776 9	11	855.85	978.46		1164 59	1335	.72		
						المعالي معالمة المعالم			- NAL				l	1			1107.00				
					10	0 200	300	400	500	60	0 7	00 8	300	900	1000	1100	1200	1300	1400	1500	1600
													r	n/z, Da							

**Supplementary spectrum E4b:** MS/MS of *m/z* 828.39 (mass 1654.78 Da) showing sequence ion coverage. The derived sequence is SCVFIPCITAAIGCS.

Fragmentation Evidence for Peptide



**Supplementary spectrum E4c:** MS/MS of *m*/*z* 609.94 (mass 1826.81 Da) showing sequence ion coverage. The derived sequence is CKSKVCYRNGIPCGE.

# Chymotrypsin digest:

# **RNGIPCGESCVFIPCLTAAIGC**SCKSKVCY

### Fragmentation Evidence for Peptide



Supplementary spectrum C4a: MS/MS of *m*/*z* 698.31 (mass 1394.60 Da) showing sequence ion coverage. The derived sequence is RNGIPCGESCVF.



Supplementary spectrum C4b: MS/MS of *m*/*z* 531.25 (mass 1060.49 Da) showing sequence ion coverage. The derived sequence is IPCITGIAGC.

20

# Endoproteinase Glu-C digest:

SCVFIPCISTVIGCSCKNKVCYRNGIPCGE

NB: This peptide shows 93% sequence homology to cyclotide I (Q30CB5) from Hybanthus floribundus.

1 GIPCGESCVFIPCISTVIGCSCKNKVCYRN 30 Cter\_Q

1 GIPCGESCVFIPCISGVIGCSCKSKVCYRN 30 Cyclotide-I

#### Fragmentation Evidence for Peptide



Supplementary spectrum E5a: MS/MS of *m*/z 884.60 (mass 3534.61 Da) showing sequence ion coverage. The derived sequence is SCVFIPCISTVIGCSCKNKVCYRNGIPCGE.



Supplementary spectrum E5b: MS/MS of *m*/z 850.41 (mass 1698.81 Da) showing sequence ion coverage. The derived sequence is SCVFIPCISTVIGCS.



**Supplementary spectrum E5c:** MS/MS of *m*/*z* 618.95 (mass 1853.84 Da) showing sequence ion coverage. The derived sequence is CKNKVCYRNGIPCGE.

# Chymotrypsin digest:

# **RNGIPCGESCVFIPCISTVIGC**SCKNKVCY

### Fragmentation Evidence for Peptide



Supplementary spectrum C5a: MS/MS of *m*/*z* 698.31 (mass 1394.60 Da) showing sequence ion coverage. The derived sequence is RNGIPCGESCVF.



**Supplementary spectrum C5b:** MS/MS of *m*/*z* 560.28 (mass 1118.54 Da) showing sequence ion coverage. The derived sequence is IPCISTVIGC.



Supplementary spectrum C5c: MS/MS of *m*/*z* 666.03 (mass 1995.09 Da) showing sequence ion coverage. The derived sequence is IPCISTVIGCSCKNKVC.

### **Evidence for Cter R:**

# Endoproteinase Glu-C digest:

SCVFIPCTVTALLGCSCKDKVCYKNGIPCGE

NB: This peptide shows 93% sequence homology to Cyclopsychotride-A (P56872) from Psychotria longipes.

1 GIPCGESCVFIPCTVTALLGCSCKDKVCYKN 31 Cter\_R

#### **Fragmentation Evidence for Peptide**



Supplementary spectrum E6a: MS/MS of *m*/z 899.17 (mass 3592.66 Da) showing sequence ion coverage. The derived sequence is SCVFIPCTVTALLGCSCKDKVCYKNGIPCGE.



Supplementary spectrum E6b: MS/MS of *m*/z 892.95 (mass 1783.88 Da) showing sequence ion coverage. The derived sequence is SCVFIPCTVTALLGCS.



**Supplementary spectrum E6c:** MS/MS of *m*/*z* 609.95 (mass 1826.83 Da) showing sequence ion coverage. The derived sequence is CKDKVCYKNGIPCGE.

# Chymotrypsin digest:

## KNGIPCGESCVFIPCTVTALLGCSCKDKVCY

## Fragmentation Evidence for Peptide



**Supplementary spectrum C6a:** MS/MS of *m*/*z* 684.81 (mass 1367.61 Da) showing sequence ion coverage. The derived sequence is KNGIPCGESCVF.

# (b) SignalP analyses of Cter M and kalata B1 alongside selected albumin-1 precursors from Fabaceae.

Panel A- SignalP (1) analysis of Cter M precursor protein predicts signal peptidase cleavage at the proto-N-terminus of the mature cyclotide sequence, between precursor protein residues 24 and 25 (72.9% probability). Panel B- SignalP analysis of kalata B1 precursor protein predicts signal peptidase cleavage between precursor protein residues 22 and 23 (82.5% probability). As in all previously characterized cyclotide genes, a pro-region and an N-terminal repeat region are encoded prior to the start of the first cyclotide domain. Panels C through F- Respective SignalP analyses of albumin-1 precursor proteins from *Pisum sativum*, *Medicago truncatula*, *Phaseolus vulgaris*, and *Glycine max* predict signal peptidase cleavage at the proto-N-termini of mature PA1b peptide sequences. Cleavages are predicted between residues 26 and 27 (53.0%), 22 and 23 (51.1%), 27 and 28 (69.7%), and 19 and 20 (98.6%) respectively.

1. Bendtsen JD, Nielsen H, von Heijne G, & Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* 340:783-795.

SignalP-NN result:

Α

SignalP-HMM result:





#### # <u>data</u>

><	CterM			leng	yth = 70					
#	Measu	ıre	Position	n Value	Cutoff	signa	1 ]	pept:	ide?	
	max.	С	25	0.802	0.32	YES				
	max.	Y	25	0.801	0.33	YES				
	max.	s	14	0.994	0.87	YES				
	mean	s	1-24	0.916	0.48	YES				
		D	1-24	0.858	0.43	YES				
#	Most	lik	ely cleav	vage site	e between	pos. 3	24	and	25:	TEG-GI

Clitoria ternatea Cter M SignalP analysis.

#### # data

>CterM Prediction: Signal peptide Signal peptide probability: 0.993 Signal anchor probability: 0.006 Max cleavage site probability: 0.729 between pos. 24 and 25 В

### SignalP-NN prediction (euk networks): Sequence C score -1.0 S score Y score -0.8 0.6 ຍ ວິດ ທີ່ 0.4 0.2 0.0 MAKFTVCLLLCLLLAAFVGAFGSELSDSHKTTLVNEIAEKMLQRKILDGVEATLVTDVAEKMFLRKMKA**t** 10 20 30 50 60 0 40 70 Position



#### # <u>data</u>

>2	Sequer	ice		leng	th = 70				
#	Measu	ire	Position	Value	Cutoff	signal	pept:	ide?	
	max.	С	23	0.793	0.32	YES			
	max.	Y	23	0.826	0.33	YES			
	max.	s	13	0.998	0.87	YES			
	mean	s	1-22	0.959	0.48	YES			
		D	1-22	0.893	0.43	YES			
#	Most	lik	ely cleava	ge site	between	pos. 2	2 and	23:	AFG-SE

Oldenlandia affinis kalata B1 SignalP analysis.

### # <u>data</u>

>Sequence Prediction: Signal peptide Signal peptide probability: 1.000 Signal anchor probability: 0.000 Max cleavage site probability: 0.825 between pos. 22 and 23

# С





### # <u>data</u>

>P62929.1 Pisum sativ length = 70 # Measure Position Value Cutoff signal peptide? 0.388 0.32 YES max. C 27 max. Y 27 0.560 0.33 YES max. S 0.995 YES 14 0.87 mean S 1-26 0.929 0.48 YES 1-26 D 0.745 0.43 YES # Most likely cleavage site between pos. 26 and 27: VGA-AS

Pisum sativum Albumin-1 SignalP analysis.

#### # data

>P62929.1\_Pisum\_sativum\_Albumin-1\_D
Prediction: Signal peptide
Signal peptide probability: 0.991
Signal anchor probability: 0.008
Max cleavage site probability: 0.530 between pos. 26 and 27

# D





SignalP-HMM prediction (euk models): CAE00461.1 Medicago truncatula Albumin-1

><	CAE004	461.	1_Medicago	lengt	th = 70				
#	Measu	ıre	Position	Value	Cutoff	signal	pepti	ide?	
	max.	С	23	0.483	0.32	YES			
	max.	Y	23	0.596	0.33	YES			
	max.	s	6	0.994	0.87	YES			
	mean	s	1-22	0.957	0.48	YES			
		D	1-22	0.777	0.43	YES			
#	Most	lik	ely cleava	ge site	between	pos. 22	2 and	23:	VPT-KK

Medicago truncatula Albumin-1 SignalP analysis.

#### # <u>data</u>

>CAE00461.1\_Medicago\_truncatula\_Albumin-1
Prediction: Signal peptide
Signal peptide probability: 0.981
Signal anchor probability: 0.017
Max cleavage site probability: 0.511 between pos. 22 and 23

# Ε





Phaseolus vulgaris Albumin-1 SignalP analysis.



SignalP-HMM prediction (euk models): GW884643.1 Phaseolus vulgaris Albumin

#### # <u>data</u>

>GW884643.1\_Phaseolus\_vulgaris\_Albumin Prediction: Signal peptide Signal peptide probability: 0.977 Signal anchor probability: 0.011 Max cleavage site probability: 0.697 between pos. 27 and 28

# F





SignalP-HMM prediction (euk models): Q39837.3 Glycine max Albumin-1

>Q39837.3 Glycine max length = 70 # Measure Position Value Cutoff signal peptide? 0.932 0.32 YES max. C 20 max. Y 0.761 YES 20 0.33 max. S YES 2 0.950 0.87 mean S 1-19 0.735 0.48 YES D 1-19 0.748 0.43 YES # Most likely cleavage site between pos. 19 and 20: IEA-AD

Glycine max Albumin-1 SignalP analysis.

#### # data

>Q39837.3\_Glycine\_max\_Albumin-1 Prediction: Signal peptide Signal peptide probability: 0.991 Signal anchor probability: 0.001 Max cleavage site probability: 0.986 between pos. 19 and 20