

Supporting Information:

Do plant cyclotides have a potential as immunosuppressant peptides?

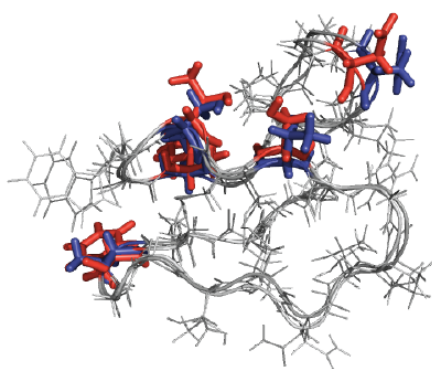
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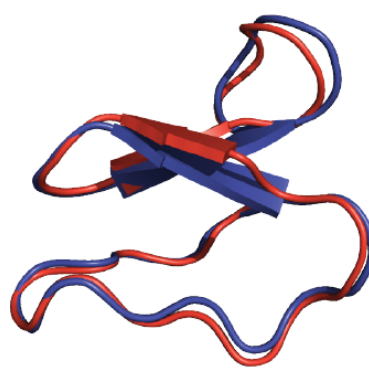
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17, A-1090 Vienna, Austria

Supplemental Figure S1. Structural alignment of kalata B1 and kalata B2. The NMR solution structures of kalata B1 shown in blue (PDB code: 1NB1) and kalata B2 shown in red (1PT4) were structurally aligned using PyMol. Alignment of all atoms (**A**) results in an RMSD of 0.725 Å (only the five differing residues are highlighted in colour, the remaining residues are shown in grey) and the backbone atoms (**B**) fit to a RMSD of 0.599 Å. The sequences of both cyclotides are shown below the aligned structures with differing residues indicated in grey boxes and different colours.

A

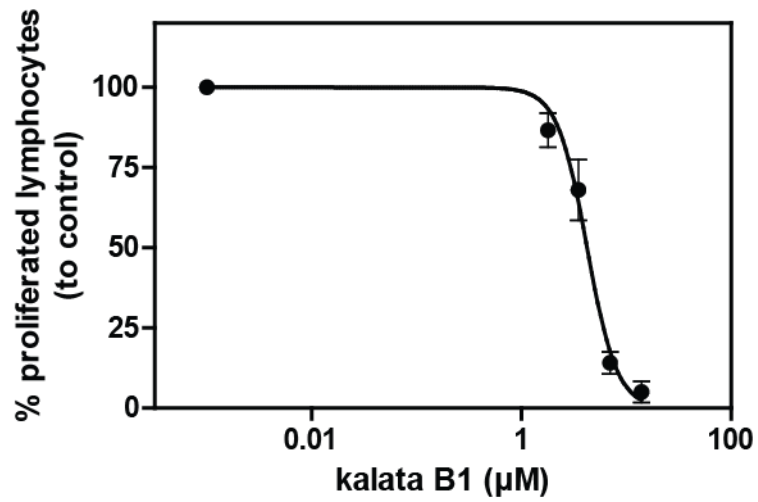


B



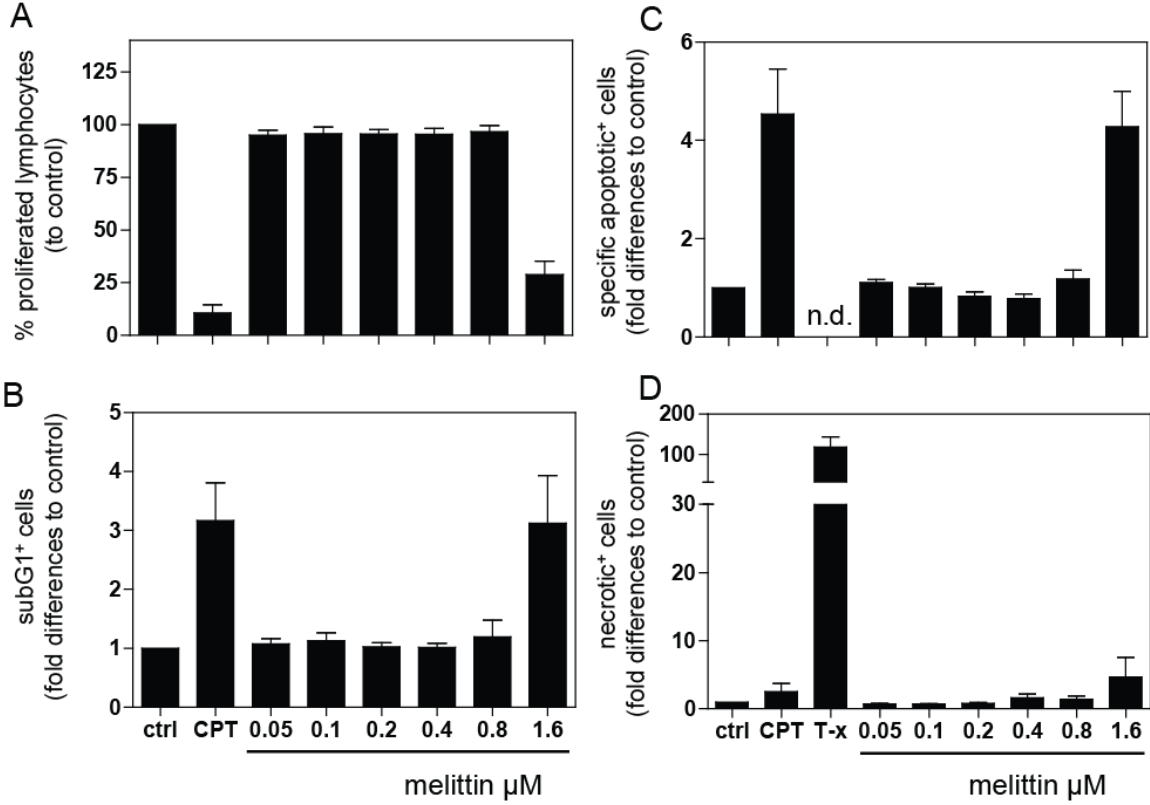
kalata B1	GLPVCGETC	V	GGTCNTPGC	T	S	W	V	CTR	N
kalata B2	GLPVCGETC	F	GGTCNTPGC	S	T	W	I	CTR	D

Supplemental Figure S2. Determination of IC₅₀ for anti-proliferative effect of kalata B1 on activated human PBMC. The IC₅₀ of the anti-proliferative effects of kalata B1 (see Figure 4) has been determined by non-linear regression analysis (log inhibitor vs. normalized response) using GraphPad Prism.



Supplemental Figure S3. Effects of melittin on cell proliferation and cytotoxicity of activated human PBMC.

Antibody (anti-CD3/CD28 mAbs)-activated human primary lymphocytes were cultured in the presence of medium (ctrl), camptothecin (CPT, 30 $\mu\text{g/mL}$), Triton-X 100 (T-x) or different concentrations of melittin (0.05-1.6 μM) for flow cytometric analysis of cell division (A), “subG1” DNA content (B) or apoptotic (C) and necrotic (D) cell content. For apoptotic and necrotic detection cells were stained with annexin V and propidium iodide to assess the percentages of viable (annexin V⁻/PI⁻), apoptotic (annexin V⁺/PI⁻ or annexin V⁺/PI⁺) and necrotic (annexin V⁻/PI⁺) cells. Data are presented as mean \pm SEM of three to four independent experiments. n.d. = not detectable.



Supplemental Table S1. Comparison of kalata B1 (and other cyclotides) inhibitory effects in various test systems.

Assay system	Cells	IC ₅₀ (μM)	Reference [#]
kalata B1			
Anti-proliferative activity	human peripheral blood mononuclear cells	3.9 ± 0.5	this study
Nematocidal activity	<i>H. contortus</i> nematodes	2.7	Huang et al., 2010
	<i>T. colibriformis</i> nematodes	4.5	Huang et al., 2010
Cytotoxicity	human T-lymphoblast cells	3.5	Daly et al., 2004
other cyclotides*			
Cytotoxicity	human lymphoma cell line (U-937)	0.6 - 6	Svangard et al., 2004
		0.3 - 7	Lindholm et al., 2002
Cytotoxicity	human myeloma cell line (RPMI-8226/s)	1 - 4	Svangard et al., 2004
		0.1 - 6	Lindholm et al., 2002

*Activity was reported of various cyclotides (varv A, varv E, varv F, vitri A, cycloviolacin O2) from *Viola arvensis*, *V. odorata* and *V. tricolor*; References can be found in the main manuscript

Supplemental File 1. LC-MS reconstruct of *O. affinis* cyclotides. Excel spreadsheet (XLS) containing the raw (labelled) data of LC-MS reconstruct of *O. affinis* extracts as analysed by nano LC-MS. Presented as Adobe Acrobat file (PDF).

LC-MS reconstruct, EMS 1000 Da/sec

Reconstruct 2700-3500 Da, signal-to-noise: 4, 25, 50; combined datasets from at least 3 independent LC-MS runs
 CyBase comparison: MW +/- 1 Da

*=other cyclotide detected (not Oaffinis)

**=MW +/-2 Da

No.	cyclotide	Mass		Score	Evidence	Theoretical Mass	Δ Mass
		Da (avg.)	Da (mono.)				
1	new	2706,63	2704,37	0,9997	I		
2	new	2723,22	2721,27	1	I		
3	kalata b1-1	2724,12	2722,28	1	IC	2724,18	0,0559
4	new	2821,78	2819,36	1	IC		
5	new	2822,30	2820,55	0,9995	I		
6	new	2833,30	2831,41	1	I		
7	[L2A] kalata B1	2851,88	2849,54	1	IC	2850,25	1,6322
8	Ac-[desGly]-KB1-Am	2854,31	2851,68	0,9996	I	2853,3	1,0072
9	new	2873,73	2871,13	0,9996	I		
10	kalata S	2878,81	2875,93	0,9993	I	2878,30	0,5141
11	new	2879,82	2877,46	1	IC		
12	kalata B12**	2882,30	2880,83	0,9969	I	2880,27	2,0256
13	kalata B11	2884,48	2881,44	0,9999	I	2884,26	0,2236
14	new	2891,45	2888,50	1	IC		
15	kalata B1	2892,85	2890,39	1	IC	2892,33	0,5228
16	kalata B4	2893,24	2890,56	1	I	2893,31	0,0718
17	new*	2896,70	2894,45	0,9999	I		
18	new	2897,11	2894,57	1	IC		
19	[G-A] kalata B1	2906,47	2904,75	0,9995	I	2906,35	0,1191
20	new	2909,53	2906,90	1	IC		
21	acyclic kalata B1	2911,32	2908,36	1	IC	2910,35	0,9675
22	new*	2912,90	2911,43	0,9999	I		
23	new*	2922,95	2920,48	1	IC		
24	new*	2925,32	2922,40	1	IC		
25	new*	2926,80	2923,70	1	IC		
26	new	2927,30	2924,66	1	IC		
27	new	2937,91	2935,50	0,9998	I		
28	new	2942,99	2940,48	1	IC		
29	kalata B2	2956,14	2953,74	1	IC	2955,38	0,7637
30	new*	2959,95	2957,56	1	IC		
31	new	2960,36	2958,24	0,9996	I		
32	new	2969,25	2968,13	0,9997	I		
33	new*	2971,44	2969,50	1	IC		
34	new	2973,80	2970,55	1	IC		
35	new	2974,14	2971,51	1	IC		
36	new*	2975,38	2973,49	1	IC		
37	kalata B15	2977,00	2974,56	1	IC	2976,40	0,602
38	new*	2986,57	2983,80	1	I		
39	new	2988,28	2985,60	1	IC		
40	new	2990,37	2987,51	1	IC		
41	new	2994,11	2991,86	1	IC		
42	new*	3006,25	3003,50	1	IC		
43	new*	3010,97	3008,88	1	IC		
44	kalata B14	3023,74	3021,17	0,9987	I	3022,43	1,3147
45	new*	3028,61	3025,92	0,9998	I		
46	kalata B6	3029,96	3027,66	0,9999	I	3029,42	0,5381
47	kalata B10	3030,21	3027,53	1	IC	3030,41	0,2028
48	Oak6 cyclotide 1	3035,87	3033,49	1	IC	3035,47	0,398
49	kalata B13	3036,06	3033,58	1	IC	3036,46	0,4018
50	new	3039,91	3037,45	1	IC		
51	new	3040,05	3036,62	1	IC		
52	new*	3045,78	3043,50	1	IC		
53	new*	3046,32	3044,95	1	IC		
54	new*	3047,97	3046,60	0,9999	I		
55	kalata B10 lin	3048,54	3046,50	1	IC	3048,43	0,1091
56	new*	3051,82	3048,48	1	IC		
57	new*	3052,72	3049,57	1	IC		
58	new*	3065,79	3063,36	0,9997	I		
59	kalata B7	3072,26	3069,74	0,9998	I	3071,59	0,67
60	new*	3073,89	3072,70	0,9999	I		
61	kalata B3	3083,31	3080,64	1	IC	3082,48	0,8309
62	new*	3087,22	3084,61	1	IC		
63	new	3089,27	3086,96	0,9997	I		

64	new	3091,00	3089,03	0,9997	I		
65	Oak6 cyclotide 2	3093,29	3090,61	1	IC	3092,56	0,7328
66	new*	3097,63	3094,57	1	IC		
67	new*	3099,85	3096,60	1	IC		
68	kalata B18**	3147,33	3145,02	0,9977	I	3145,67	1,6615
69	new*	3266,81	3264,99	0,9997	I		
70	kalata B8	3284,34	3281,75	1	IC	3283,79	0,5453
71	new*	3300,96		1	C		
72	new	3446,88	3444,98	0,9998	I		

Total:	72
New:	25
New*:	24

Supplemental File 2. *O. affinis* database search results following digests and LC-MS/MS analysis. Excel spreadsheet (XLS) containing the Protein Pilot™ database search results of the cyclotide LC-MS/MS analysis. Presented as Adobe Acrobat file (PDF).

N	Unused	Total	%Cov	%Cov(50)	%Cov(95)	Accession	Name	Peptides(95%)
1	6,43	6,43	100,00	85,48	82,26	cb P85175	kalata B8/1-31 cybaseid=168 organism=Oldenlandia affinis	4
2	6,00	6,00	100,00	51,72	51,72	cb P58457	kalata B7/1-29 cybaseid=26 organism=Oldenlandia affinis	6
3	5,91	5,91	100,00	98,28	96,55	cb P58454	kalata B2/1-29 cybaseid=4 organism=Oldenlandia affinis	4
4	2,75	2,75	100,00	98,28	72,41	cb P83938	kalata B4/1-29 cybaseid=30 organism=Oldenlandia affinis	2
5	2,63	2,63	93,33	88,33	88,33	cb P58456	kalata B5/1-30 cybaseid=59 organism=Oldenlandia affinis	5
6	2,00	3,75	100,00	98,28	72,41	cb P85133	kalata B15/1-29 cybaseid=253 organism=Oldenlandia affinis	3
7	2,00	2,00	100,00	50,00	50,00	cb P85128	kalata B10/1-30 cybaseid=246 organism=Oldenlandia affinis	1
7	0,00	2,00	100,00	50,00	50,00	cb 247	kalata B10 linear/1-30 cybaseid=247 organism=Oldenlandia affinis	1
8	2,00	2,00	85,48	85,48	43,55	cb P85127	kalata B9/1-31 cybaseid=244 organism=Oldenlandia affinis	1
8	0,00	2,00	85,48	85,48	43,55	cb 245	kalata B9 linear/1-31 cybaseid=245 organism=Oldenlandia affinis	1
9	2,00	2,00	98,21	50,00	50,00	cb P85130	kalata B12/1-28 cybaseid=250 organism=Oldenlandia affinis	2
10	1,06	2,00	100,00	50,00	50,00	cb P58455-b3	kalata B6/1-30 cybaseid=24 organism=Oldenlandia affinis	1
10	0,00	2,00	100,00	50,00	50,00	cb 247	kalata B10 linear/1-30 cybaseid=247 organism=Oldenlandia affinis	1
11	0,63	2,01	98,28	98,28	72,41	cb P56254	kalata B1/1-29 cybaseid=1 organism=Viola odorata; Oldenlandia affinis; Viola baoshanensis; Viola ye	4
12	0,20	0,20	100,00	61,67	0,00	cb P58455-b6	kalata B3/1-30 cybaseid=25 organism=Oldenlandia affinis	0

N	Unused	Total	%Cov	%Cov(50)	%Cov(95)	Accession	Name	Peptides(95%)
1	0,88	0,88	100	87,92999983	0	cb P58454	kalata B2/1-29 cybaseid=4 organism=Oldenlandia affinis	0
2	0,52	0,52	100	50	50	cb P58457	kalata B7/1-29 cybaseid=26 organism=Oldenlandia affinis	1
3	0,21	0,21	100	25	0	cb P58455-b6	kalata B3/1-30 cybaseid=25 organism=Oldenlandia affinis	0

3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,003507	TCFGGTCNTPGCTCD	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Carbamidomethyl(C)@12; Carbamidomethyl(C)@cleaved D-P@C-term	0,0259826	1706,605347	427,6586	1706,579346	427,6520996	4	3	1.1.1.527.3	62,4578
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,003391	TCFGGTCNTPGCTCD	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Carbamidomethyl(C)@12; Carbamidomethyl(C)@cleaved D-P@C-term	0,426373005	1707,005737	427,7587	1706,579346	427,6520996	4	3	2.1.1.376.4	68,8942
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,002554	TCFGGTCNTPGCTCD	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Carbamidomethyl(C)@12; Carbamidomethyl(C)@cleaved D-P@C-term	0,508404016	1707,087769	427,7792	1706,579346	427,6520996	4	3	1.1.1.528.4	62,6258
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,002429	TCFGGTCNTPGCTCD	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Carbamidomethyl(C)@12; Carbamidomethyl(C)@cleaved D-P@C-term	0,315533032	1706,894897	427,731	1706,579346	427,6520996	4	3	1.1.1.560.4	66,261
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,00238	TCFGGTCNTPGCTCD	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Carbamidomethyl(C)@12; Carbamidomethyl(C)@cleaved D-P@C-term	4,146339893	1710,725708	428,6887	1706,579346	427,6520996	4	3	2.1.1.436.4	79,0321
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,00356	TCFGGTCNTPGCTCD	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Carbamidomethyl(C)@12; Carbamidomethyl(C)@cleaved D-P@C-term	3,811630011	1710,390991	428,605	1706,579346	427,6520996	4	3	1.1.1.562.5	66,4919
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,0008264	TCFGGTCNTPGCTCD	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Carbamidomethyl(C)@12; Carbamidomethyl(C)@cleaved D-P@C-term	-0,022452701	1706,556885	854,2857	1706,579346	854,296936	2	3	1.1.1.121.3	21,0624
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,0005564	TCFGGTCNTPGCTCD	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Carbamidomethyl(C)@12; Carbamidomethyl(C)@cleaved D-P@C-term	1,644760013	1708,224121	428,0633	1706,579346	427,6520996	4	3	2.1.1.324.3	60,3719
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,0005812	TCFGGTCNTPGCTCD	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Carbamidomethyl(C)@12; Carbamidomethyl(C)@cleaved D-P@C-term	-0,0111002	1706,568237	854,2914	1706,579346	854,296936	2	3	1.1.1.155.3	20,999
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,0001157	TCFGGTCNTPGCTCD	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Carbamidomethyl(C)@12; Carbamidomethyl(C)@cleaved D-P@C-term	1,671609998	1708,250977	428,07	1706,579346	427,6520996	4	3	3.1.1.328.3	50,558
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	8,25E-05	TCFGGTCNTPGCTCD	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Deamidated(N)@8; Carbamidomethyl(C)@12; C-cleaved D-P@C-term	-1,480370045	1706,083008	427,528	1707,563354	427,8881018	4	3	1.1.1.139.4	19,0583
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,013688	TCFGGTCNTPGCTCDPWPICTRD	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Carbamidomethyl(C)@12; Carbamidomethyl(C)@14; Carbamidomethyl(C)@20	-1,055250049	2730,999268	911,3404	2732,054688	911,6921387	3	7	4.1.1.183.5	27,9217
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,01821	TCFGGTCNTPGCTCDPWPICTRD	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Carbamidomethyl(C)@12; Carbamidomethyl(C)@14; Carbamidomethyl(C)@20	-2,06855011	2729,986084	911,0026	2732,054688	911,6921387	3	7	4.1.1.184.5	28,0711
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,01433	TCFGGTCNTPGCTCDPWPICTRD	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Carbamidomethyl(C)@12; Carbamidomethyl(C)@14; Carbamidomethyl(C)@20	-0,147958994	2731,906738	911,6428	2732,054688	911,6921387	3	4	1.1.1.211.5	27,0104
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,054360001	TCFGGTCNTPGCTCDPWPICTRDGLPTCGE	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Deamidated(N)@8; Carbamidomethyl(C)@12; C-missed D-G@23	-0,88845998	3446,450195	1149,824	3447,339355	1150,120361	3	8	4.1.1.192.5	29,305
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,01474	TCFGGTCNTPGCTCDPWPICTRDGLPTCGE	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Carbamidomethyl(C)@12; Carbamidomethyl(C)@missed D-G@23	0,197362006	3446,552246	1149,858	3446,355225	1149,792358	3	6	3.1.1.176.3	28,8826
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,008241	TCFGGTCNTPGCTCDPWPICTRDGLPTCGE	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Carbamidomethyl(C)@12; Carbamidomethyl(C)@missed D-G@23	0,137669995	3446,492188	1149,838	3446,355225	1149,792358	3	5	1.1.1.236.3	29,675
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,005676	TCFGGTCNTPGCTCDPWPICTRDGLPTCGE	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Carbamidomethyl(C)@12; Carbamidomethyl(C)@missed D-G@23	0,187107995	3446,543213	1149,855	3446,355225	1149,792358	3	6	1.1.1.234.3	29,4281
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,0006526	TCFGGTCNTPGCTCDPWPICTRDGLPTCGE	Carbamidomethyl(C)@2; Carbamidomethyl(C)@7; Carbamidomethyl(C)@12; Carbamidomethyl(C)@missed D-G@23	0,127416	3446,483154	1149,835	3446,355225	1149,792358	3	4	2.1.1.150.4	29,2597
3	0,21	0,21	100	25	0 cb PS8455-b6 kalata B3/1-30 cybaseid=25 organism=Olderlandia affinis	0	0,01839	WPICTRDGLPTCGE	Carbamidomethyl(C)@4; Carbamidomethyl(C)@12 cleaved P-W@N-term; missed D-G@7	0,157080993	1660,890869	831,4527	1660,733765	831,3741455	2	6	3.1.1.177.3	29,0288

Supplemental File 3. Cyclotide quantification data. Excel spreadsheet (XLS) containing the data of five independent experiments of cyclotide quantification. Presented as Adobe Acrobat file (PDF).

Identified cyclotide	MW calculated (Da)	SEM	Δ MW (Da)	MW LC-MS reconstruct (Da)	SEM	Δ MW (Da)	MW theoretical (Da)	RT (min)	SEM	Area (mAU*min)	SEM	Height (mAU)	SEM	Rel.Area %	SEM
kalata B8	3283,91	0,15	0,12	3284,24	0,19	0,45	3283,79	29,95	0,01	20,55	0,57	28,01	0,72	5,6	0,2
kalata B7	3071,83	0,19	0,24	3072,27	0,17	0,68	3071,59	37,54	0,04	7,89	0,22	41,43	0,21	2,2	0,1
kalata B1	2892,27	0,27	0,06	2892,98	0,21	0,65	2892,33	45,48	0,02	50,81	0,84	96,96	0,61	13,9	0,2
kalata B6	3029,60	0,28	0,18	3029,84	0,12	0,42	3029,42	46,50	0,03	29,63	0,46	46,88	0,38	8,1	0,1
kalata B13	3035,56	0,20	0,90	3035,89	0,12	0,57	3036,46	50,32	0,03	14,88	1,16	28,75	0,38	4,1	0,3
kalata B2	2955,76	0,12	0,38	2955,90	0,09	0,52	2955,38	51,82	0,03	72,86	3,17	103,65	0,86	20,0	0,6
kalata B3	3082,72	0,21	0,24	3083,04	0,08	0,55	3082,48	52,59	0,07	11,00	1,40	23,02	1,27	3,0	0,4

*n=5, HPLC quantification (area under curve) of five independent experiments

**MW average mass

***MW calculated from +2 or +3 ion