Supplementary information for the manuscript: Circular permutation of the native enzyme-mediated cyclization position in cyclotides

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Peptide	Cyclic		Linear		Precursor	
	oxidised	reduced	oxidised	reduced	oxidised	reduced
$L5_{ALEG}$	3451.56	3457.56	3469.56	3475.56	3839.74	3845.74
$L5_{GLP}$	3451.56	3457.56	3469.56	3475.56	3736.72	3742.72
$[G1,G33]L5_{ALEG}$	3565.60	3571.60	3583.60	3589.60	3953.79	3959.79
$[G1]L5_{ALEG}$	3508.58	3514.58	3526.58	3532.58	3896.76	3902.76
$[G33]L5_{ALEG}$	3508.58	3514.58	3526.58	3532.58	3896.76	3902.76
$[G1,G33]L5\_Abl06_{ALEG}$	4095.94	4101.94	4113.94	4119.94	4484.12	4490.12
$kB1_{EII}$	2891.21	2897.21	2909.21	2915.21	3264.42	3270.42

Table S1. Calculated masses of peptides used in this study. Monoisotopic masses are presented in daltons (Da; $[M+H]^+$). Precursor masses represent the cyclotide sequence plus the C-terminal propeptide (CTPP). Linear masses represent removal of the CTPP, but without backbone cyclisation. Cyclic masses represent removal of the CTPP and backbone cyclisation.

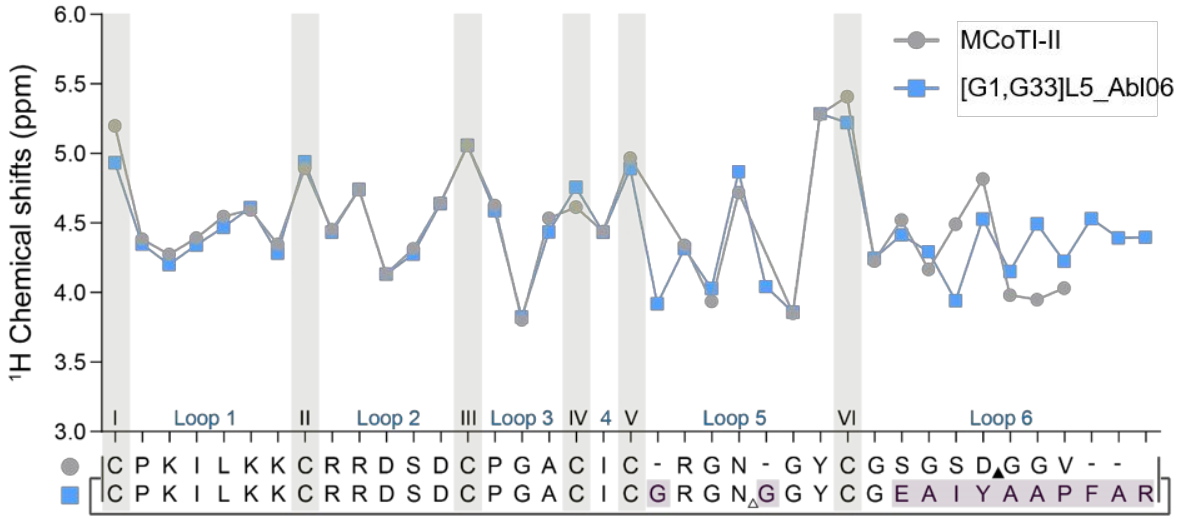

Figure S1. The comparison of Hα chemical shifts of [G1,G33]L5_Abl06 with the native scaffold MCoTI-II. A sequence alignment of the two peptides is shown below the panel, with the symbol used for each peptide present in front of them individually. Yellow stripes mark the positions of six cysteines (I to VI) and the segments between the cysteines are numbered loop 1 to 6. The mutated residues of the grafted peptide to MCoTI-II are highlighted in pink. The original cyclisation point in loop 6 of MCoTI-II is indicated by a solid triangle and the new cyclisation point in loop 5 of [G1,G33]L5_Abl06 by an empty triangle.

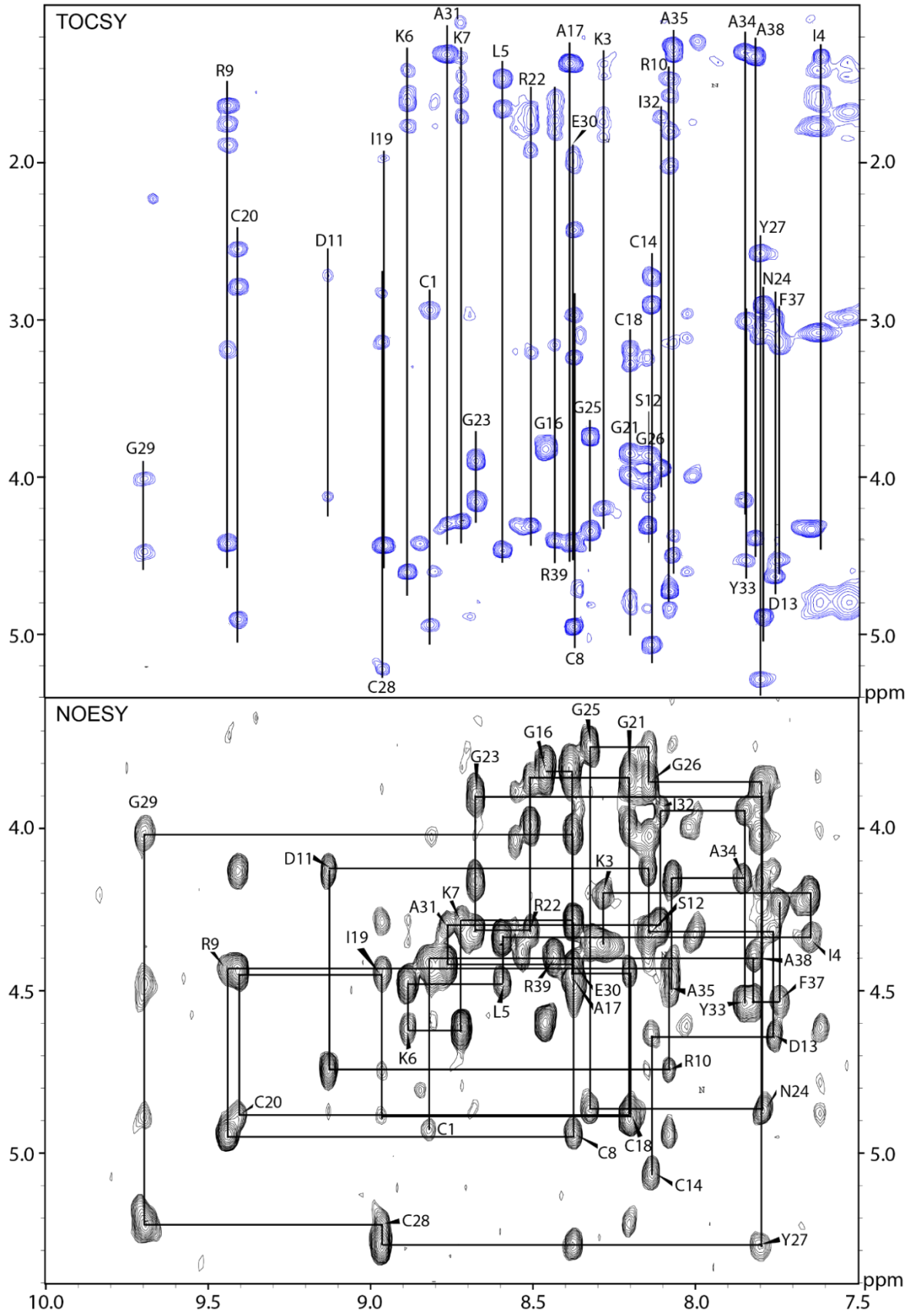

Figure S2. The TOCSY and NOESY spectra of L5[G1,G33]Abl06. The spin systems of all residues (except for Pro-2, Pro-15 and Pro-36) are shown in the TOCSY spectrum (top panel) and the sequential connectivities in the NOESY spectrum (bottom panel).

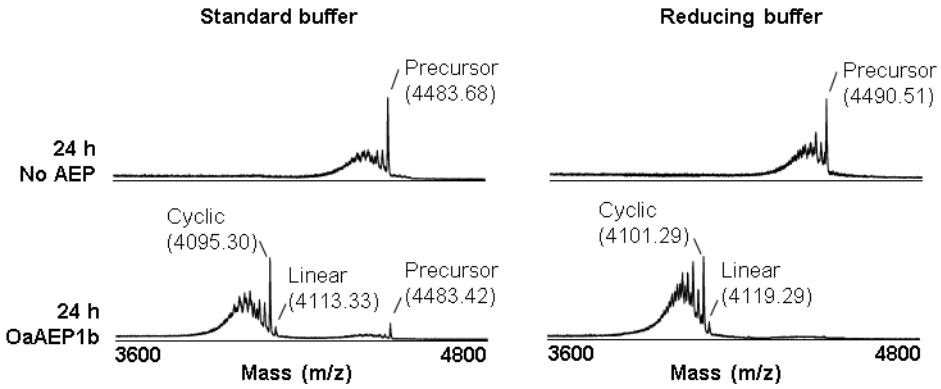

Figure S3. Cyclisation of [G1,G33]L5_Abl06 in presence of reducing agent. MALDI-MS traces showing peptide masses in a standard sodium acetate buffer (left panels) or in the same buffer with 1 mM TCEP (reducing buffer, right panels). Observed peptide m/z are labelled at 24 h without AEP (top panels) and with addition of 200 nM OaAEP1b (bottom panels). Expected monoisotopic masses (Da; $[M+H]^+$) are: 4484.12 (precursor, oxidised), 4490.12 (precursor, reduced), 4113.94 (linear, oxidised), 4119.94 (linear, reduced), 4095.94 (cyclic, oxidised), and 4101.94 (cyclic, reduced).

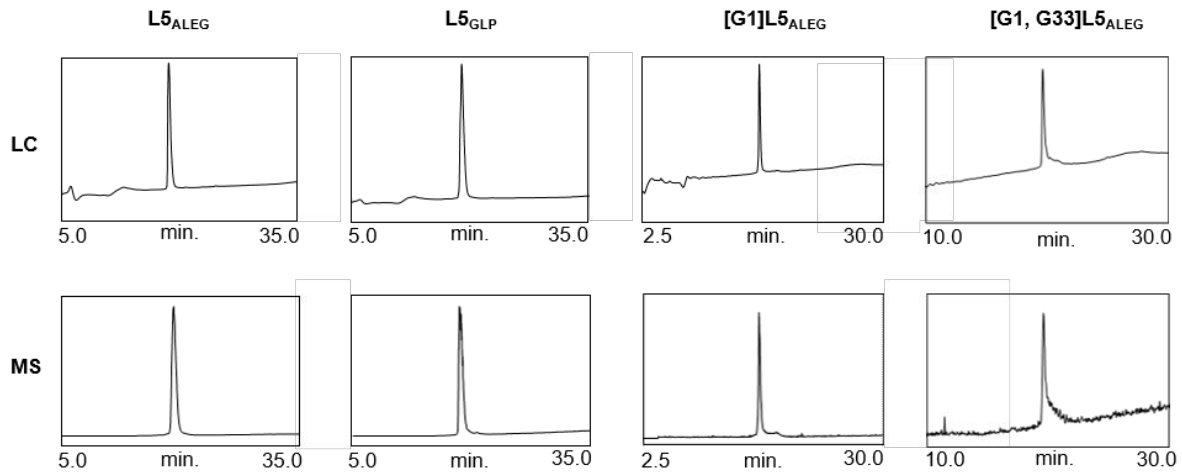

Figure S4. LC-MS traces for peptides used in the study. The LC (top row of panels) and MS (bottom row of panels) are shown for each peptide. LC-MS data were not available for [G33]$L5_{ALEG}$; readers are referred to Figure S5 for 1D NMR spectra of this peptide as an indicator of peptide quality.

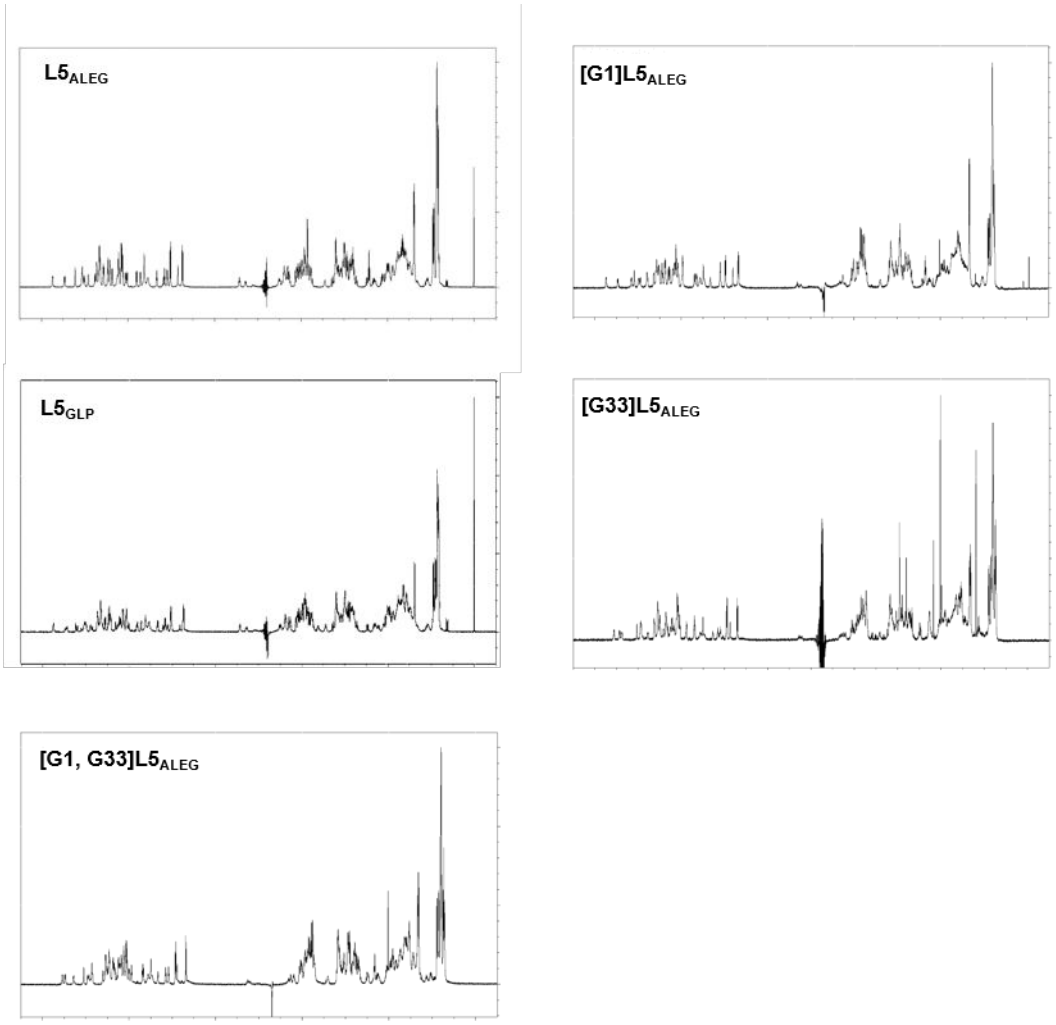

Figure S5. 1D NMR for peptides used in cyclisation assays. Each peptide shows well-dispersed peaks in the amide region of the spectra.

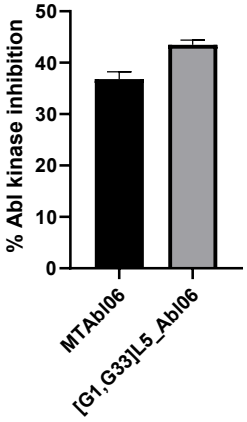

Figure S6. The percentage of Abl kinase inhibition of MTAbl06 (36.8%) and [G1,G33]L5_Abl06 (43.7%) upon 30 min incubation. The replicates (n= 2) were done independently and the error bars represent SD.

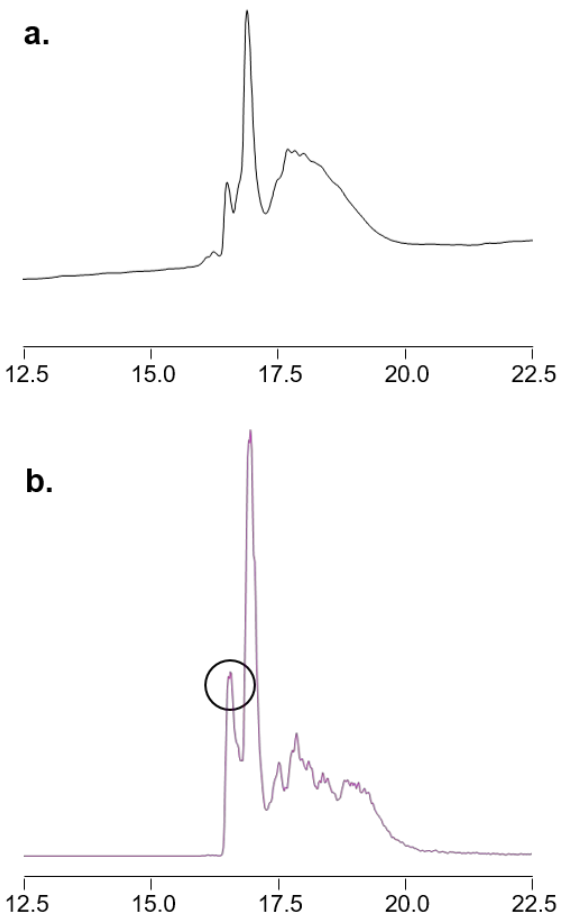

Figure S7. Oxidative folding of cyclic [G1,G33]L5_Abl06. **a.** HPLC showing the peptide after oxidative folding. **b.** Extracted mass of [G1,G33]L5_Abl06 after oxidative folding. The peak that corresponds to the correctly folded product (circled) accounts for 5.6% of the total product in the sample.