## **Supplementary Information**

## An environmentally sustainable biomimetic production of cyclic disulfide-rich peptides

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served nass 1+H] <sup>x</sup>	Theoretical mass [M+H] <sup>X</sup>
6.85*	836.85*
4.89*	894.86*
08.90*	1108.92*
9.61#	739.62#
24.97*	1224.92*
6.98#	816.95#
51.19#	1151.19#
53.64 <sup>\$</sup>	863.64\$
1.51@	691.11@
67.25#	1267.19#
0.69 <sup>\$</sup>	950.64 <sup>\$</sup>
0.25@	760.71 <sup>@</sup>
	served nass I+H]× 6.85* 4.89* 08.90* 9.61# 24.97* 6.98# 51.19# 53.64\$ 1.51@ 57.25# 50.69\$ 0.25@

Table S1. Masses of cyclic peptide and reduced and alkylated (RA) peptides in this study

Reduced and alkylated peptides are indicated by <sup>RA</sup>. Bold and underlined <u>C</u> indicates S-carbamidomethylated cysteines; \* indicates  $[M+H]^{2+}$ ; # indicates  $[M+H]^{3+}$ ; \$ indicates  $[M+H]^{4+}$  and @ indicates  $[M+H]^{5+}$ .

**Table S2.** Summary of amount and cost of materials, reagents, solvents and water used in the synthesis of 100 mg of MCoTI-II using benchmark SPPS

Materials, reagents, solvents and water	Weight (kg)	Cost (AUD)
$\Sigma$ weight of amino acids	0.192	118.20
$\Sigma$ weight of resin	0.002	8.77
$\Sigma$ weight of DMF	44.000	420.96
$\Sigma$ weight of DCM	1.203	7.63
$\Sigma$ weight of piperidine	0.958	89.18
$\Sigma$ weight of HCTU	0.154	92.62
$\Sigma$ weight of DIPEA	0.142	55.28
$\Sigma$ weight of MeOH	0.031	0.19
$\Sigma$ weight of MeCN	1.144	13.93
$\Sigma$ weight of TIPS	0.006	13.34
$\Sigma$ weight of TFA	0.533	93.74
$\Sigma$ weight of H <sub>2</sub> 0	11.039	0.00
Total	59.403	913.84

**Table S3.** Summary of amount and cost of materials, reagents, solvents and water used in the recombinant production of 112 mg MCoTI-II. The calculations include *P. pastoris* production and purification of MCoTI-II CPs as well as production and purification of *MCo*AEP2.

Materials, reagents, solvents and water	Weight (kg)	Cost (AUD)
$\Sigma$ weight of yeast extract	0.030	8.94
$\Sigma$ weight of peptone	0.060	7.10
$\Sigma$ weight of glycerol	0.082	1.29
$\Sigma$ weight of methanol	0.438	1.83
$\Sigma$ weight of biotin%	0.000	0.10
$\Sigma$ weight of yeast nitrogen base	0.027	38.01
$\Sigma$ weight of methanol	0.003	0.06
$\Sigma$ weight of MeCN	0.401	4.88
$\Sigma$ weight of TFA%	0.000	0.00
$\Sigma$ weight of imidazole%	0.001	0.50
$\Sigma$ weight of H <sub>2</sub> 0	4.595	0.00
Total	5.637	62.71

%Trace amounts used in downstream purification buffers.



**Fig. S1** High resolution MS analysis of recombinant cyclic peptides and reduced- alkylated variants of (a) SFTI-KLK5, (b) [G22N]cVc1.1 and (c) MCoTI-II.



**Fig. S2** Peptide mass fingerprinting of reduced and alkylated cyclic peptide of SFTI-KLK5. Alkylated cysteines are shown in the S-carbamidomethylated (cam) form. Red underlined segments represent the cyclization site after AEP treatment. The precursor mass of fragments selected for MS/MS are shown.



**Fig. S3** 1D proton NMR spectra of cyclic peptides produced recombinantly. (a) [G22N]cVc1.1 and (b) MCoTI-II.



**Fig. S4** Bioreactor production of MCoTI-II CPs. (a) Fermentation parameters displaying dissolved oxygen (DO), temperature and pH for growth phases of glycerol batch, glycerol-fed batch and methanol fed-batch. (b) SDS-PAGE analysis of MCoTI-II CP accumulation at different points post methanol induction. (c) QTRAP-MS quantification of IMAC purified MCoTI-II precursors. Three technical replicates were performed (n = 3). Data presented as means  $\pm$  SEM.



**Fig. S5** Flowchart comparison of benchmark SPPS and *P. pastoris* platform production of MCoTI-II. (a) SPPS of MCoTI-II with cEF and associated costs on a 100 mg scale (b) *P. pastoris* platform production of MCoTI-II with cEF and associated costs on a 112 mg scale. Based on the current exchange rate of 1 AUD = 0.70 USD the benchmark SPPS cost is 639 USD and the *P. pastoris* platform cost is 43 USD. \*cEF (complete E factor) = ( $\Sigma m$  (solvents) +  $\Sigma m$  (reagents) - m (product))/ m (product)



**Fig. S6** UPLC-UV-HRMS chromatograms of (a) combined IMAC purified fractions containing MCoTI-II precursor prior to AEP cyclization, (b) final purified *P. pastoris* platform produced MCoTI-II and (c) synthetic MCoTI-II produced by SPPS. <sup>#</sup> unidentified *P. pastoris* protein and \*  $\beta$  - aspartyl isomers.<sup>1</sup>

1. Hernandez, J.-F.; Gagnon, J.; Chiche, L.; Nguyen, T. M.; Andrieu, J.-P.; Heitz, A.; Trinh Hong, T.; Pham, T. T. C.; Le Nguyen, D., Squash trypsin inhibitors from Momordica cochinchinensis exhibit an atypical macrocyclic structure. *Biochemistry* **2000**, *39*, 5722-5730.