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Supporting Information

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for

Immobilized Protease-Assisted Synthesis of Engineered Cysteine-Knot Microproteins

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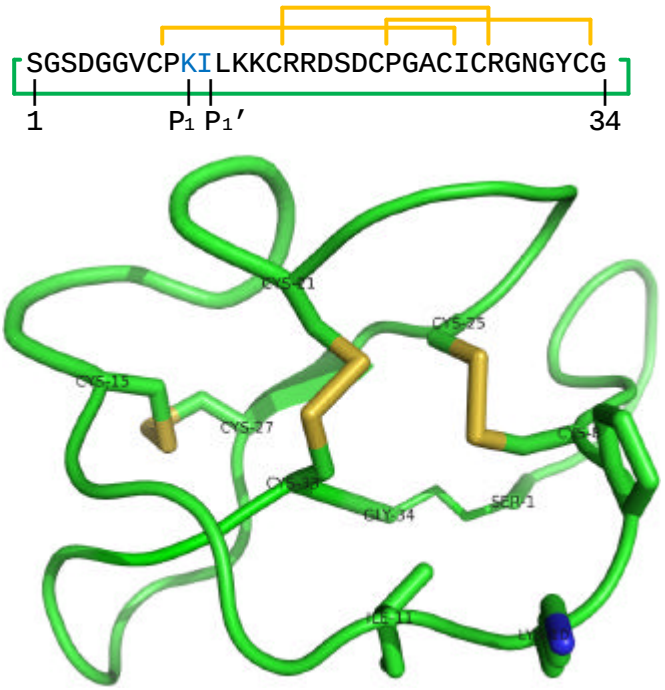
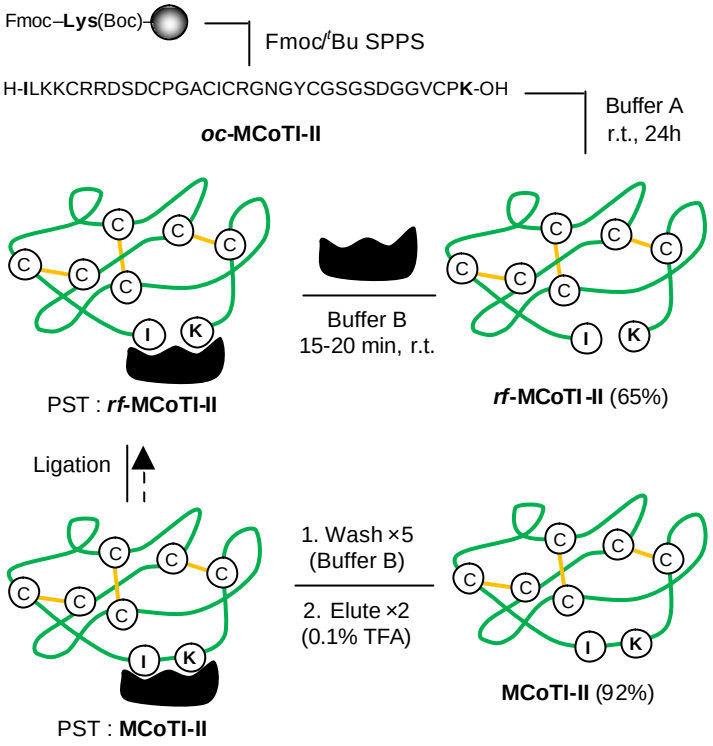
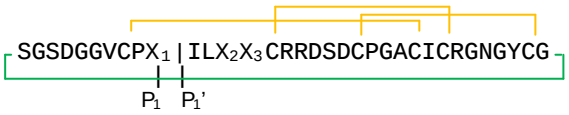


Figure 1. Sequence and solution structure f MCoTI-II.[8,9] side chains of Pro9, Lys10 (P_1) and Ile11 (P_1') are shown as sticks, disulfide bonds are shown in yellow. Presented with PyMol.



Scheme 1. Chemoenzymatic synthesis of MCoTI-II using SPPS and polymer-supported trypsin (PST). Buffer A: 0.1 M ammonium carbonate, pH 7.8 / acetonitrile 1:1, 1 mM glutathione [reduced form]; Buffer B: 100 mM sodium phosphate, pH 7.0.

Table 1. Sequence and structure of cyclotides synthesized in this study, and K_i values determined against trypsin and chymotrypsin.



Cyclotide	Sequence			K_i values	
	X_1	X_2	X_3	Trypsin	Chymotrypsin
MCoTI-I	K	Q	R	**29** ± 2 **pM**	> 10 µM
MCoTI-II	K	K	K	**75** ± 5 **pM**	> 10 µM
MCoTI-II[K10R]	R	K	K	**85** ± 7 **pM**	> 10 µM
MCoTI-II[K10Q]	Q	K	K	5 µM	2 µM
MCoTI-II[K10F]	F	K	K	400 ± 42 nM	**9.8** ± 0.7 **nM**

Experimental Section

All general laboratory chemicals obtained from chemical suppliers (Novabiochem UK or Aldrich Chemical Co.) were used without further purification. Peptides were synthesised using an Advanced ChemTech Apex 396 multiple peptide synthesiser (Advanced Chemtech Europe, Cambridge, UK). Purification of crude peptides was performed on a Gilson semi-preparative RP-HPLC system (Anachem Ltd., Luton, UK) equipped with 306 pumps and a Gilson 155 UV/Vis detector. Analytical RP-HPLC was performed on a Gilson analytical HPLC system (Anachem Ltd., Luton, UK) equipped with a Gilson 151 UV/Vis detector and Gilson 234 auto injector. For both HPLC systems, the peptide bond absorption was detected at 223 nm. The following elution methods were used: Method A: 20 to 50% MeCN in H_2O over 40 min; Method B: 0 to 85% MeCN in H_2O over 40 min. All peptides and reaction mixtures were analysed using Method B unless otherwise specified.

Solid-phase resins: Preloaded Wang resin and 4-Sulfamylbutyryl AM resin were purchased from Novabiochem UK. The resin substitution ratios were as follows: Fmoc-Lys(Boc)-Wang : 0.69mmol/g; Fmoc-Phe-Wang : 0.74mmol/g; Fmoc-Gln(Trt)-Wang : 0.62mmol/g; Fmoc-Arg(Pbf)-Wang : 0.51mmol/g; 4-Sulfamylbutyryl AM resin : 1.1mmol/g.

Amino acids: N-α-9-Fluorenylmethoxycarbonyl (N-α-Fmoc) protected amino acid were obtained from Novabiochem UK with the following side chain protecting groups. Ala, Arg(Pbf), Asn(Trt), Asp(tBu), Cys(Trt), Gly, Ile, Leu, Lys(Boc), Pro, Ser(^{t}Bu), Tyr(^{t}Bu), Val and Fmoc-Asp(O^{t}Bu)-(Hmb)Gly-OH were purchased from Novabiochem UK.

Reagents: Peptide synthesis grade dimethylformamide (DMF) from Rathburn Chemicals UK. Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBop), N-hydroxybenzotriazole (HOBt), diisopropylethylamine (DIPEA) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU) were purchased from Novabiochem UK and Sigma Aldrich. Dry tetrahydrofuran (THF) and DMF, chloroform, dimethylsulfoxide (DMSO), dichloromethane (DCM), methanol, piperidine, thioanisole, *t*-butylmethylether (TBME), trifluoroacetic acid (TFA), ethanedithiol (EDT), iodoacetonitrile, 3-mercaptoethyl propionate, sodium thiophenolate, monobasic and dibasic sodium

phosphate, trimethylsilylisopropane (TIPS) were regent grade from Sigma Aldrich. A 'deprotection mixture' of TFA:H_2O:EDT:TIPS (94:2.5:2.5:1) was used for cleavage and deprotection reactions.

General procedures

Loading of Sulfamylbutyryl AM resin with Fmoc-Ala-OH

Sulfamylbutyryl AM resin (250 mg, 0.172 mmol) was swelled in DMF (3 mL) for 1 h at ambient temperature. To this solution were added Fmoc-Ala-OH (268 mg, 0.86mmol) and DIPEA (264 μL, 1.6 mmol). The reaction mixture was left to stir for 15 min followed by cooling to -20°C for 20 min. Then PyBOP (447mg, 0.86 mmol) was added and the reaction mixture stirred for 8 h at -20°C. The reaction mixture was then left to warm to ambient temperature overnight. The resin was recovered by filtration and washed with DMF (3 × 2 mL). The coupling was repeated using the same procedure and washed with DMF (3 × 2 mL), DCM (3 × 2 mL) and MeOH (3 x 2 mL), respectively. The loaded resin was dried in vacuo. The coupling efficiency was assessed as follows: typically 2 mg of thoroughly dried resin (or peptidyl resin) was incubated with a solution of 1 mL of 20% piperidine in DMF, and left for 1 h. The solution was then filtered off and the filtrate measured for its absorbance at 290 nm or 301 nm (ε_{290nm} = 5800 $M^{-1}cm^{-1}$, ε_{301nm} = 7800 $M^{-1}cm^{-1}$) against a blank of 20% piperidine in DMF. Loading efficiency was ~ 81%.

Solid-phase peptide synthesis

Peptide synthesis was carried out in peptide synthesis grade DMF. Resin preloaded with appropriate amino acid (25 μmol per well) was swelled in DMF for 60 min before coupling each cycle, *N*-α-amino Fmoc group was treated with 20% (*v*/*v*) piperidine in DMF for 15 min, with two further repetitions. A fivefold excess of amino acid over resin reactive groups (125 μmol, 250 μL of 0.5 M solution) was used for each coupling. In situ activation and coupling was carried out for 45 min with a mixture of HBTU/HOBt (125 μmol, 250 μL of 0.5 M solution) and DIPEA (125 μmol, 250 μL of 0.5 M solution). The peptide was washed with DMF (3 × 1 mL) between each deprotection and coupling step. A typical cycle consisted of deprotection, DMF wash, coupling and a further DMF wash. The *N*-*a*-Fmoc protection at the final residue was removed at the end of the synthesis

under the usual conditions. After synthesis was complete the fully protected MCoTI-bound resin was removed from the synthesiser, washed several times (3 × 2 mL DMF, 3x2 mL DCM, 3 × 2 mL methanol) and dried in vacuo. Asp7Gly6 was introduced as a dipeptide (Fmoc-Asp(O^tBu)-(Hmb)Gly-OH) and coupled for 2 h. Cys34 was introduced as Boc-Cys(Trt)-OH for *oc*-MCoTI thioester syntheses.

Activation and displacement from sulfamyl resin

Activation: fully protected MCoTI bound to sulfamyl resin (25 μmol) was swelled in 1.5 mL of dry NMP for 1 hour. To this solution was added DIEPA (200 μL, 1.1 mmol) followed by excess Iodoacetonitrile (180 μL, filtered through an alumina basic prior to use). The reaction flask was shielded from light and agitated for 24 h at ambient temperature. The resin was washed with NMP (5 × 5 mL), DCM (5 × 5 mL), MeOH (5 × 5 mL) and dried *in vacuo.* *Displacement:* to the activated resin were added a small amount of sodium thiophenolate (1.6mg), ethyl 3-mercaptopropionate (160 μL, 1.25 mmol) and DMF (500 μl). The reaction mixture was agitated overnight at ambient temperature. The resin was filtered and the filtrate was evaporated to dryness *in vacuo* to yield fully protected MCoTI thioester which was subsequently treated with 2 mL of deprotection mixture over 3 h to give the corresponding *oc*-MCoTI thioester. Precipitation and purification were conducted as described below.

Deprotection and Cleavage from Wang resin

Cleavage and deprotection of peptides was achieved by adding 1.5mL of deprotection mixture to dry peptide bound resin (25 μmol). The mixture was agitated on an orbital shaker for up to 3 h and then filtered. The resin was washed twice with a small volume of TFA and the combined washings and filtrate were precipitated with 10 mL ice cold *tert*-butylmethylether. The mixture was centrifuged at 5000 rpm for 20 minutes at 0°C, the supernatant discarded and the remaining peptide washed with a fresh aliquot of TBME. The process was repeated three times to ensure complete removal of all organic impurities. The crude peptide was dried in a desiccator over silica gel to yield an off-white solid.

Peptide purification

A small sample of crude peptide was analysed by analytical preparative RP-HPLC before purifying by preparative HPLC. The mobile phases contained 0.1% HPLC-grade TFA as an ion pairing agent and were degassed with helium. Peptide purification was achieved using preparative reverse-phase HPLC using a HICHROM C18 Column (250 x 21.2mm). Individual fractions from preparative reversed-phase HPLC were analysed using analytical reverse-phase HPLC, and pure fractions combined and diluted to <10% MeCN with deionised water. The peptide was obtained as a white solid after lyophilisation on a Christ Alpha 2-4 freeze dryer (Osterode am Harz Germany). Mass identification by Matrix-Assisted Laser Desorption/Ionisation Time of Flight Mass spectrometry (MALDI-TOF MS) was performed using *a*-Cyano-4-hydroxycinnamic acid as matrix.

Synthesis of cyclotides *via* thia-zip native chemical ligation

Glutathione (0.3 mg, 1 μmol) was dissolved in 1mL of 0.1 M carbonate buffer. This solution was added to *oc*-MCoTI thioester (0.28 μmol) which was dissolved in 1 mL of acetonitrile and the reaction mixture left for 24 h at room temperature. The reaction mixture was then diluted twofold in water and purified by preparative RP-HPLC using Method A. Fractions were collected, analysed by analytical HPLC, pure fractions pooled and lyophilized to yield the product cyclotide.

Synthesis of *rf*-MCoTI-I, II and analogues by oxidative refolding

oc-MCoTI acid (0.28μmol) was dissolved in 1 mL of 0.1 M of carbonate buffer. This solution was added glutathione (0.3 mg, 1 μmol), which was dissolved in acetonitrile (1 mL). The reaction mixture was allowed for 48 h at room temperature, diluted 2-fold in water, loaded onto the preparative HPLC, and peptide eluted using Method A. Fractions were collected, analysed by using Method B on the analytical HPLC, pooled and lyophilised.

Synthesis of cyclotides *via* ligation by immobilised protease

Cyclisation using immobilised trypsin: immobilised trypsin (500μL of resuspended beads) was washed with 0.1 M phosphate buffer (5 x 1mL, pH 7.4) before use. *rf*-MCoTI peptide (0.03 μmol) was dissolved in 500 μL 0.1 M phosphate buffer pH 7.4 and added to immobilised trypsin. The reaction mixture was incubated at 37°C for 15 min, mixed on a vortex mixer for 10 s and centrifuged at 14000rpm

for 5 minutes. The supernatant was removed from immobilised trypsin which was subsequently washed with water (5 x 1 mL); the reaction mixture was mixed and centrifuged as above at each washing. 0.1% TFA in water was added and the mixture mixed and left to stand for 5 minutes, the supernatant was recovered, and this procedure was repeated. The combined TFA washings were analysed by analytical HPLC and MALDI-MS.

Cyclisation using immobilised chymotrypsin: the reaction was performed exactly as for immobilised trypsin, except that 25 mg immobilised chymotrypsin was weighed out and washed as above.

Peptide concentration determination for MCoTI-I, II and MCoTI-II [K10R]

The concentration of active MCoTI cyclotide was determined by titration with trypsin (adapted from ref. [12]). Microprotein at a range of concentrations (1 µM to 0.1 nM, 50 µL) in TBS pH 7.6 (Tris buffered saline: 50 mM Tris.HCl, 150 mM NaCl, 0.01% Tritron-X-100, 0.02% sodium azide) was incubated with trypsin (25 nM, 50µL) for 30 min at 37^{o}C in a 96-well plate (Falcon Microtest^{TM} 96). Carbobenzoxy-L-arginine-7-amino-4-methyl coumarin (75 µM, 100 µL) was added to the solution and the absorbance measured at room temperature with excitation and emission wavelengths at 360 and 465 nm, respectively. The concentration of active cyclotide was determined from inhibition curves obtained in two separate experiments assuming a 1:1 interaction between inhibitor and trypsin.

Peptide concentration determination for MCoTI-II [K10F] and MCoTI-II [K10Q]

These MCoTI-II analogues have relatively low inhibition activity against trypsin, and so a standard addition method was used to determine peptide concentration using analytical HPLC. A standard addition graph from MCoTI-I, II and MCoTI-II[K10R] was plotted by varying the active peptide concentration (200 to10 µM) versus peak area measured at 223 nm and, by comparison of peak areas, used to determine the concentration of microproteins MCoTI-II[K10F] and MCoTI-II[K10Q].

Inhibition kinetics

All inhibition kinetics were determined by competitive binding assays at 37°C on the Cytofluor series 400 microplate reader (Perseptive Biosystem, Warrington,

UK), using Falcon microtest™ 96-well plates. TBS pH 7.6 was prepared for trypsin and chymotrypsin assays. The equilibrium dissociation constant (K_i) for the complex between trypsin or chymotrypsin and cyclotide was determined by using enzyme concentration lower than K_i. Thus protease (0.005 nM, 50 µL for trypsin or 0.5 nM, 50 µL for chymotrypsin) was incubated with a range of concentrations of inhibitor (1 nM to 0.01 pM, 50 µL for trypsin and 70 nM to 0.05 pM, 50 µL for chymotrypsin) in TBS for 1 h. The measurement was started by the addition of substrate: *N*-*p*-tosyl-Glysine-Proline-arginine-7-Amido 4-methyl coumarin (5 µM, 100 µL) for trypsin assays and Suc-Ala-Ala-Pro-Phe-AMC (10 µM, 100 µL) for chymotrypsin assays. The initial rate of substrate hydrolysis was monitored by the cleavage of AMC from substrate at an excitation of 360 nm and emission of 460 nm. Initial rate data were then fitted using the GraFit Software package (http://www.erithacus.com/grafit). All assays were reproduced 3 times with fresh solutions of all reagents.

Characterisation data

***oc*-MCoTI-I, II acids and analogues** Peptide sequence: H-IL**X_2X_3**CRRDSDCPGACICRGNGYCGSGSDGGVCP**X_1**-OH							
Name	**X_1**	**X_2**	**X_3**	**Doubly coupled residues**	**Yield**	**MALDI-MS**	**Retention time (t_R), Method B**
oc-MCoTI-I acid	K	Q	R	Ala21, Gly22	7%	3507 $[M+H]^+$ 1754 $[M+2H]^{2+}$	12.36
oc-MCoTI-II acid	K	K	K	Ala21, Gly22 and Ile34	8%	3478 $[M+H]^+$	12.3
oc-MCoTI-II[K10R] acid	R	K	K	Ala21, Gly22 and Ile34	13%	3505 $[M+H]^+$ 1753 $[M+2H]^{2+}$	12.03
oc-MCoTI-II[K10F] acid	F	K	K	Ala21, Gly22 and Ile34	10%	3497 $[M+H]^+$	14.4
oc-MCoTI-II[K10Q] acid	Q	K	K	Ala21, Gly22 and Ile34	5%	3478 $[M+H]^+$ 1739 $[M+2H]^{2+}$	12.25

***oc*-MCoTI-I, II thioesters and analogues** Peptide sequence: H-CICRGNGYCGSGSDGGVCP**X_1**IL**X_2X_3**CRRDSDCPGA-S$(CH_2)_2$COOEt							
Name	**X_1**	**X_2**	**X_3**	**Doubly coupled residues**	**Yield**	**MALDI-MS**	**Retention time (t_R): Method B**
oc-MCoTI-I thioester	K	Q	R	Val18	6%	3622 $[M+H]^+$	14.8
oc-MCoTI-II thioester	K	K	K	Ile14, Lys15 and Val18	5%	3594 $[M+H]^+$ 1798 $[M+2H]^{2+}$	14.35
oc-MCoTI-II[K10R] thioester	R	K	K	Ile14, Arg15	7%	3622 $[M+H]^+$	14.1
oc-MCoTI-II[K10F] thioester	F	K	K	Ile14, Phe15	4%	3613 $[M+H]^+$ 1807 $[M+2H]^{2+}$	15.4
oc-MCoTI-II[K10Q] thioester	Q	K	K	Ile14, Gln15	4%	3593 $[M+H]^+$ 1797 $[M+2H]^{2+}$	14.55

Cyclotides synthesised *via* thia-zip NCL			
Cyclotide	**Yield**	**MALDI-MS**	**Retention time (t_R): Method B**
MCoTI-I	68%	3482 $[M+H]^+$ 1741 $[M+2H]^{2+}$	12.3
MCoTI-II	72%	3454 $[M+H]^+$ 1727 $[M+2H]^{2+}$	11.5
MCoTI-II[K10R]	72%	3483 $[M+H]^+$ 1742 $[M+2H]^{2+}$	11.8
MCoTI-II[K10F]	64%	3474 $[M+H]^+$ 1737 $[M+2H]^{2+}$	15.2
MCoTI-II [K10Q]	64%	3455 $[M+H]^+$ 1727 $[M+2H]^{2+}$	12.5

Refolded cyclotides			
Name	**Yield**	**MALDI-MS**	**Retention time (t_R): Method B**
rf-MCoTI-I	72%	3501 $[M+H]^+$	10.5
rf-MCoTI-II	65%	3472 $[M+H]^+$ 1737 $[M+2H]^{2+}$	10.4
rf-MCoTI-II[K10R]	59%	3500 $[M+H]^+$ 1751 $[M+2H]^{2+}$	10.5
rf-MCoTI-II[K10F]	60%	3491 $[M+H]^+$ 1746 $[M+2H]^{2+}$	12.2
rf-MCoTI-II [K10Q]	64%	3473 $[M+H]^+$ 1736 $[M+2H]^{2+}$	10.63

Cyclotides synthesised by immobilised protease ligation			
Name	**Yield by HPLC, based on conversion of *rf*-cyclotide**	**MALDI-MS**	**Retention time (t_R): Method B**
MCoTI-I	93 %	3482 $[M+H]^+$ 1742 $[M+2H]^{2+}$	12.35
MCoTI-II	92 %	3454 $[M+H]^+$ 1728 $[M+2H]^{2+}$	11.4
MCoTI-II[K10R]	94 %	3481 $[M]^+$ 1741 $[M+2H]^{2+}$	11.8
MCoTI-II[K10F]	90 %	3474 $[M+H]^+$	15.4