**Supporting Information**

**Materials and instrumentation**

Analytical HPLC was performed on a HP1100 series instrument with 220 and 280 nm detection using a Vydac C18 column (5 µm, 2.1 x 100 mm) at a flow rate of 0.2 mL/min. All runs used linear gradients of 0.1% aqueous formic acid (solvent A) vs. 0.1% formic acid, 90% acetonitrile in $H_2O$ (solvent B). UV/Vis spectroscopy was carried out on an Agilent 8453 diode array spectrophotometer. ES-MS and LC-MS/MS was performed on an Applied Biosystems API 3000 triple quadrupole electrospray mass spectrometer using Analyst 1.4.2. Calculated masses were obtained using Analyst 1.4.2. Protein samples were run on 4-20% Tris-Glycine Gels (Lonza). The gels were then stained with Pierce Gelcode Blue (Pierce), photographed/digitized using a Kodak EDAS 290, and quantified using NIH ImageJ software (http://rsb.info.nih.gov/ij/). DNA sequencing was performed by Retrogen DNA facility (San Diego,CA), and the sequence data were analyzed with DNAStar Lasergene v5.5.2. All chemicals were obtained from Aldrich (Milwaukee, WI) or Novabiochem (San Diego, CA) unless otherwise indicated. Restriction enzymes were purchased from New England Biolabs. Primers were ordered from Integrated DNA Technologies. *Sacchraomyces cerevisiae* strains INVSc1 and W303-1a, and plasmids pYES2/NT, pYC2/NT, were purchased from Invitrogen. Plasmid p426GPD was purchased from ATCC (ATCC 87361). Plasmid propagations were done in *Escherichia coli* DH5$\alpha$ cells (Invitrogen) using LB Miller medium and the appropriate antibiotic.

**Cloning of *S. cerevisiae* expression plasmids**

**pYES2/NT-TS-MCoTI-I**

The gene fragment encoding the *Npu* DnaE $I_C$ and $I_N$ (residues 775-876, UniProtKB: B2J066) intein fragments fused the N- and C-terminus of cyclotide MCoTI-I, respectively, was cloned as previously described (1). Codon optimization of $I_C$-MCoTI-I-$I_N$ for *S. cerevisiae* was done by Genscript USA. The resulting gene was subcloned into a pUC57-Kan to provide the plasmid pUC57-TS-MCoTI-I. The DNA region encoding $I_C$-MCoTI-I-$I_N$ was amplified by PCR using plasmid pUC57-TS-MCoTI-I as a template. The 5'-primer (5'- AAA AAA AGC TTA TGG TTC TTC TCA CCA CCA CCA C -3') and 3'-primer (5'- AAA AAG GAT CCT TAT TAG TTT GGC AAG TTA TCA ACG CGC -3') introduced a *Hind* III and *BamH* I restricition sites respectively. The PCR product was purified, double digested with *Hind* III and *BamH* I, and ligated onto a *Hind* III and *BamH* I-treated pYES2/NT plasmid to give pYES2/NT-TS-MCoTI-I.

**pYC2/NT-TS-MCoTI-I**

The DNA region encoding $I_C$-MCoTI-I-$I_N$ was amplified by PCR and digested with *Hind* III and

*BamH* I as described above for pYES2/NT-TS-MCoTI-I. The digested amplicon was ligated onto a *Hind* III and *BamH* I-treated pYC2/NT plasmid to give pYC2/NT-TS-MCoTI-I.

**p426GPD-TS-MCoTI-I**

The gene encoding $I_C$-MCoTI-I-$I_N$ was amplified by PCR using plasmid pUC57-TS-MCoTI-I as a template. The 5'-primer (5'- AAA AAG GAT CCA TGG TTC TTC TCA CCA CCA CCA C -3') and 3'-primer (5'- AAA AAA AGC TTT TAT TAG TTT GGC AAG TTA TCA ACG CGC -3') introduced a *BamH* I and *Hind* III restricition sites respectively.

**p426GPD-TS-CP4**

p426GPD-TS-MCoTI-I was mutated to introduce *Afl* II and *Bgl* II restriction sites by site directed mutagenesis using the Quick Change Lightning Multi Site Directed Mutagenesis kit (Agilent technologies) as per the manufacturer's protocol using the mutagenic primers (5'- AC AAC TTC GCT ctt AAG AAC GGT TTC ATT GCT TCG AAC TG -3' and 5'- CGA AAC TGA gAT cTT GAC TGTTGAATACGG -3', respectively). The resulting plasmid was checked for expression and sequenced to confirm the mutations introduced. 5'-Phosphorylated oligonucleotides encoding the sequence of peptide CP4 (**Table S1**) were annealed in 20 mM sodium phosphate, 0.3 M NaCl buffer at pH 7.4 as described before (2). The resulting double stranded DNA (dsDNA) was purified using Qiagen's PCR Purification Kit. The resulting dsDNA was ligated to *Afl* II*, Bgl* II-treated p426GPD-TS-MCoTI-I to give plasmid p426GPD-TS-CP4. The sequence of the precursor $I_C$-CP4-$I_N$ precursor was confirmed by DNA sequencing.

**p426GPD-TS-MCoCP4**

5'-Phosphorylated oligonucleotides coding for the cyclotide MCoCp4 (**Table S1**) were synthesized and PAGE purified by IDT DNA. Complementary strands were annealed, purified, and ligated to *Afl* II*, Bgl* II-treated p426GPD-TS-MCoTI-I as described above to produce plasmid p426GPD-TS-MCo-Cp4. The sequence of the precursor $I_C$-MCoCp4-$I_N$ precursor was confirmed by DNA sequencing

**pRS306-α-syn-GFP**

Plasmid pRS306-α-syn-GFP (a kind gift from Dr. Susan Lindquist, Whitehead Institute for Biomedical Research, MIT) encodes human α-synuclein (α-syn, residues 1-140, UniProtKB: p37840) fused in frame to the C-terminus of GFP and it has been described previously (3).

***S. cerevisiae* transformation and selection**

Expression plasmids derived from plasmids pYC2/NT and pYES2/NT, and p426GPD and pRS306 were transformed into *S. cerevisiae* strains INVSc1 and W303-1a, respectively, by electroporation. Briefly, cells were first treated with filter sterilized LiAc/DTT/TE buffer (1 mM

EDTA, 10 mM DTT, 10 mM Tris•HCl and 100 mM LiAcO) at pH 7.5. 10-100 ng of DNA in 1-5 µL of water was placed in a sterile screw cap microfuge tube. Electrocompetent cells (40 µL) were added and mixed. The mixture was transferred to a pre-chilled 0.2 mm Biorad electroporation cuvette. Electroporation was performed with a Gene Pulser Xcell electroporator (Bio-Rad) set at 2.0 kV, 200 Ω and 25 µF. The electroporated samples were immediately diluted with 1 mL of ice-cold sterile 1 M sorbitol and returned to ice. Aliquots of the transformation mix were either plated directly or diluted as needed with additional 1 M sorbitol and spread onto synthetic complete media lacking uracil (SC-U) agar plates.

**Expression of MCoTI-I in *S. cerevisiae***

Expression using an inducible promoter (GAL1) was carried out in INVSc1 cells (Invitrogen) at 30° C in SC-U media containing 2 % galactose. Single colonies were grown into 15 mL of SC-U medium containing 2% glucose and allowed to grow overnight at 30° C with shaking. Cells were diluted to an optical density at 600 nm ($OD_{600}$) of 0.4 into SC-U medium containing 2% galactose for 48 h at 30° C. Expression using a constitutive promoter (GPD) was performed using W303-1a cells at 30° C in SC-U media containing 2% glucose for 48 h. In both cases cells were harvested by centrifugation, resuspended in 30 mL of lysis buffer (1 mM PMSF, 50 mM Tris•HCl, 100 mM protease cocktail mixture (Thermo Scientific), 150 mM NaCl) at pH 7.4 containing 1% nonidet P-40. After sonication, the lysate was clarified by centrifugation at 15,000 rpm in a sorval SS-34 rotor for 30 min. The clarified supernatant was incubated with Ni-NTA beads (Thermo Scientific), previously equilibrated with lysis buffer at pH 7.4 containing 10 mM imidazole, for 1 h at 4°C with gentle rocking. The beads were extensively washed with 50 bed-volumes of washing buffer (20 mM imidazole, 50 mM Tris•HCl, 150 mM NaCl) at pH 7.4. Quantification of the split intein fragments was performed by SDS-PAGE gel. Folded cyclotide MCoTI-I was identified and quantified by LC-MS/MS (**Fig. S1**).

**Expression of CP4 in *S. cereivisiae***

Cyclic peptide Cp4 was expressed using a GPD constitutive promoter as described above for MCoTI-I. Cyclic peptide CP4 was identified and quantified by LC-MS/MS (**Fig. S2**)

**Expression of MCoCP4 in *S. cerevisiae***

Cyclotide MCo-Cp4 was expressed using a GPD constitutive promoter as described above for MCoTI-I. Folded cyclotide MCoCP4 was identified and quantified by LC-MS/MS (**Fig. S3**).

**Genomic integration and expression of human α-syn in *S. cerevisiae***

About 10 µg of *Pst* I treated pRS306-α-syn fusion with GFP was combined with 90 µL competent *S. cerevisiae* W303-1a cells and electroporation was achieved as described above. Cells were immediately recovered in 1 M sorbitol for 2 h at 30° C. Transformants were selected by plating onto SC-U agar plates. Expression of α-syn was performed by plating the cells in 2% galactose media and confirmed by western blot (**Fig. S4**).

**Co-expression of CP4 or MCoCP4 and α-syn in *S. cerevisiae***

The yeast α- syn uses the GAL1 promoter to tightly control expression of human α- syn, so that no α-syn is produced until the yeast are grown on galactose medium. We co-transformed 100 ng of p426GPD-TS-MCoCp4 or p426GPD-TS-CP4 into 40 µL of competent cells of α- syn w303 yeast culture and plated it on media lacking uracil to select for transformed cells.

**Immunoblotting**

*S. cerevisiae* cells expressing α-syn were grown to mid-log phase, harvested, washed with water and lysed in ethanol, with glass beads. The precipitated proteins were spun down at 13,000 rpm for 15 min, at 4° C, and the pellets resuspended in standard SDS-sample buffer. Proteins were resolved by SDS-PAGE, transferred to PVDF membranes and immunoblotting was performed following standard procedures. Syn-1 antibody (Transduction Laboratories) was used to check α-syn expression (**Fig. S4**).

**Spotting Experiments**

Cells carrying the α-syn-GFP fusion protein and plasmids p426GPD-TS-MCoCP4 or p426GPD-TS-CP4 were grown overnight at 30° C in liquid media containing 2% raffinose until they reached log or mid-log phase. Cultures were then normalized for $OD_{600}$, serially diluted and spotted onto SC-U agar media containing either 2% glucose or 2% galactose, after which they were grown at 30° C for 2-3 days.

***S. cerevisiae* transformation of plasmid mixtures and phenotypic screening**

High efficient *S. cerevisiae* transformations were performed as previously described (4). Briefly, cells expressing α-syn-GFP were grown to log phase in 200 mL SC-U liquid medium with 2% glucose at 30° C, pelleted and washed twice times with ice cold water (100 ml) and with ice-cold electroporation buffer (1 mM $CaCl_2$, 1 M sorbitol in water) (100 mL). Pellets were resuspended

in 40 mL of 10 mM DTT, 100 mM LiAc in water for 30 min at 30° C with shaking. Cells were then spun down, washed with ice-cold electroporation buffer (100 mL) and resuspended in 400 µL of electroporation buffer to reach a final volume of 2 mL. The pure plasmids or plasmid mixture encoding cyclotide precursors MCoTI-I (10 µg) and MCoCP4 (0.2 ng) was mixed with 400 µL of competent cells (10 µL) and added to 400 µL of electrocompetent cells. Electroporation was performed with an electroporator 2510 (Eppendorf) set at 2.5 kV, 200 Ω and 25 µF. The electroporated cells were immediately diluted 8 mL of 1 M sorbitol:YPD media (1:1 vol.) and incubated for 1 h at 30° C with shaking. Transformed cells were collected by centrifugation, resuspended in 50 mL of SC-U media containing 2% glucose and grown at 30° C for 8 h with shaking. Aliquots containing ≈$10^6$-$10^7$ cells were plated onto SC-U agar media containing either 2% glucose or 2% galactose at 30° C for 2-3 days.

**Quantification by LC-MS/MS**

Cell lysates were extracted using C18 SepPak cartridges (Waters) with elution in MeCN:$H_2O$ (3:2 v/v) containing 0.1% TFA and lyophilized. The samples were dissolved in $H_2O$ containing 0.1% formic acid and analyzed by HPLC-tandem mass spectrometry using a C18-HPLC column (5 mm, 2.1 × 100 mm), and $H_2O$-MeCN buffers containing 0.1% formic acid as mobile phase. Typical analysis used a linear gradient of 0-90% MeCN in $H_2O$ over 10 min. Detection was performed on an API 3000 ES-MS using a multiple reaction-monitoring (MRM) mode. Data were collected and processed using Analyst software (Applied Biosystems). The calibration curve using pure MCoTI-I was found to be linear in the range of 50–250 ng. Loss of MCoTI-I during extraction was quantified by spiking a control sample with a known amount of purified MCoTI-I and analysis by HPLC-MS/MS. The recovery was found to be approximately 50%. A similar approach was used to quantify cyclotide MCoCP4 and cyclic peptide CP4.

**Capture of in-cell generated MCoTI-I cyclotide using trypsin-immobilized shepharose beads**

Trypsin-immobilized agarose beads were prepared as previously described (1). Briefly, NHS-activated Sepharose was washed with 15 volumes of ice-cold 1 mM HCl. Each volume of beads was incubated with an equal volume of coupling buffer (200 mM sodium phosphate, 250 mM NaCl buffer at pH 6,) containing 2-4 mg of porcine pancreatic trypsin type IX-S (14,000 units/mg)/mL for 3 h with gentle rocking at room temperature. The beads were then rinsed with 10 volumes of coupling buffer, and incubated with excess coupling buffer containing 100 mM ethanolamine (Eastman Kodak) for 3 hours with gentle rocking at room temperature. Finally,

the beads were washed with 50 volumes of washing buffer (200 mM sodium acetate, 250 mM NaCl buffer at pH 4.5) and stored at 4° C until use. The sepharose-trypsin beads are stable for a month under these conditions. Affinity purification of MCoTI-cyclotides was carried out as follows: 30 mL of clarified lysate was incubated with 500 µL of trypsin-sepharose for one hour at room temperature with gentle rocking, and centrifuged at 3,000 rpm for 1 min. The beads were washed with 50 volumes of column buffer containing 0.1% Tween-20 and then rinsed with 50 volumes of column buffer without detergent. The sepharose beads were treated with 3 x 0.5 mL of 8 M Gdm•Cl at room temperature for 15 min and then eluted by gravity. The eluted fractions were analyzed by LC-MS/MS as described above.

**Chemical synthesis of cyclic peptide CP4**

Solid-phase synthesis was carried out on an automatic peptide synthesizer ABI433A (Applied Biosystems) using the Fast-Fmoc chemistry with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activation protocol at 0.1 mmole scale on a Fmoc-Gly-sulfamylbutyryl AM resin loaded as previously described (2). Side-chain protection was employed as previously described for the synthesis of peptide α-thioesters by the Fmoc-protocol (5), except for the N-terminal Cys residue, which was introduced as Boc-Cys(Trt)-OH. After chain assembly, the alkylation, thiolytic cleavage and deprotection were performed as previously described (5,6). Briefly, 50 mg of protected peptide resin were first alkylated two times with $ICH_2CN$ (174 µL, 2.4 mmol; previously filtered through basic silica) and DIEA (82 µL, 0.46 mmol) in N-methylpyrrolidone (NMP) (2.2 mL) for 12 h. The resin was then washed with NMP (3 x 5 mL) and DCM (3 x 5 mL). The alkylated peptide resin was cleaved with $HSCH_2CH_2CO_2Et$ (200 µL, 1.8 mmol) in the presence of a catalytic amount of sodium thiophenolate (NaSPh, 3.5 mg, 22 µmol) in dimethylformamide (DMF):DCM (3:4 v/v, 1.4 mL) for 24 h. The resin was then dried at reduced pressure. The side-chain protecting groups were removed by treating the dried resin with trifluoroacetic acid (TFA):$H_2O$:tri-isopropylsilane (TIS) (95:2:3 v/v, 5 mL) for 3 h at room temperature. The resin was filtered and the linear peptide thioester was precipitated in cold $Et_2O$. The crude material was dissolved in the minimal amount of $H_2O$:MeCN (4:1) containing 0.1% TFA and characterized by HPLC and ES-MS. Cyclization was accomplished by flash dilution of linear precursor α-thioester TFA crude to a final concentration of ≈ 50 µM into freshly degassed 2% sodium 2-mercaptoethanesulfonate (MESNA), 100 mM sodium phosphate buffer at pH 7.5 for 2 h. Cyclized CP4 was purified by semi-preparative HPLC using a linear gradient of 18-40% solvent B over 30 min. Pure cyclic CP4 was characterized by HPLC and ES-MS (**Fig. S5**).

**Chemical synthesis of cyclotide MCoCP4**

Cyclotide MCoCP4 was chemically produced as previously described (2,7). Folded MCoCP4 was purified by semi-preparative HPLC using a linear gradient of 22.5-40% solvent B over 30 min. Cyclotide MCoCP4 was characterized by HPLC and ES-MS (**Fig. S6**), and by NMR (see below).

**NMR spectroscopy**

NMR samples were prepared by dissolving MCoCP4 cyclotide into 80 mM potassium phosphate pH 6.0 in 90% $H_2O$/10% $^2H_2O$ (v/v) or 100% $D_2O$ to a concentration of approximately 0.2 mM. NMR data was recorded on a Bruker Avance II 700 MHz spectrometer equipped with a TXI cryoprobe. Data were acquired at 27 °C, and 2,2-dimethyl-2-silapentane-5-sulfonate, DSS, was used as an internal reference. The carrier frequency was centered on the water signal, and the solvent was suppressed by using WATERGATE pulse sequence. TOCSY (spin lock time 80 ms) and NOESY (mixing time 150 ms) spectra were collected using 1024 $t_2$ and 256 $t_1$ points with 64 transients. Spectra were processed using Topspin 2.1 (Bruker). Each 2D-data set was apodized by 90°-shifted sinebell-squared in all dimensions, and zero filled to 1024 x 512 points prior to Fourier transformation. Chemical shifts were assigned by using CARA software (8).

**Table S1.** Forward (p5) and reverse (p3) 5'-phosphorylated oligonucleotides used to produce plasmids expressing polypeptides CP4 and MCoCP4.

| DNA fragment | | Oligonucleotide sequence |
|---|---|---|
| ***CP4*** | p5 | 5'- TTA AGA ACG GTT TCA TTG CTT CGA ACT GTC TTG CTA CCT GGG CTG TTG GTT GTT TGT CTT ACG AAA CTG A -3' |
| | p3 | 5'- GAT CTC AGT TTC GTA AGA CAA ACA ACC AAC AGC CCA GGT AGC AAG ACA GTT CGA AGC AAT GAA ACC GTT C -3' |
| ***MCoCP4*** | p5 | 5'- ttA AGA ACG GTT TCA TTG CTT CGA ACT GTG GTT CTG GTT CTT GTC TTG CTA CCT GGG CTG TTG GTG GTG GTG TTT GTC CAA AGA TTT TGC AAC GCT GTC GCC GCG ATT CTG ATT GTC CAG GTG CTT GTA TTT GTC GCG GTA ACG GTT ACT GTT TGT CTT ACG AAA CTG A -3' |
| | p3 | 5'- GAT CTC AGT TTC GTA AGA CAA ACA GTA ACC GTT ACC GCG ACA AAT ACA AGC ACC TGG ACA ATC AGA ATC GCG GCG ACA GCG TTG CAA AAT CTT TGG ACA AAC ACC ACC ACC AAC AGC CAG GTA GCA AGA CAA GAA CCA GAA CCA CAG TTC GAA GCA ATG AAA CCG TTC -3' |

**Table S2.** Chemical shifts of the backbone amide ($H^N$) and alpha ($H^\alpha$) protons of MCoCP4 and their differences from that of the wild type MCoTI-I.

| Residue | $H^N$ (ppm) | $H^\alpha$ (ppm) | $\Delta H^N$ (ppm) | $\Delta H^\alpha$ (ppm) |
|---|---|---|---|---|
| **V3** | 8.31 | 4.30 | 0.01 | 0.48 |
| C4 | 8.63 | 4.45 | -0.01 | 0 |
| K6 | 8.12 | 4.15 | 0 | 0 |
| I7 | 7.55 | 4.26 | -0.01 | 0 |
| L8 | 8.59 | 4.41 | 0.02 | 0 |
| Q9 | 8.86 | 4.45 | 0.04 | 0.02 |
| R10 | 8.15 | 4.26 | -0.39 | 0.18 |
| C11 | 8.15 | 4.69 | -0.06 | 0 |
| R12 | 9.28 | 4.32 | 0 | 0 |
| R13 | 7.99 | 4.64 | 0 | 0 |
| D14 | 9.06 | 4.67 | -0.02 | 0 |
| S15 | 8.03 | 4.09 | -0.02 | -0.06 |
| D16 | 7.79 | 4.83 | 0.04 | -0.02 |
| C17 | 8.01 | 4.83 | -0.01 | 0.08 |
| G19 | 8.41 | 3.67 | -0.02 | 0 |
| A20 | 8.25 | 4.34 | 0 | 0.1 |
| C21 | 8.25 | 4.45 | 0 | -0.01 |
| I22 | 8.86 | 4.70 | 0.19 | -0.14 |
| C23 | 9.28 | 4.85 | 0 | 0 |
| **R24** | 7.99 | 4.44 | -0.01 | 0.37 |
| G25 | 8.86 | 3.80 | 0 | 0 |
| N26 | 7.69 | 4.57 | 0 | 0 |
| G27 | 8.30 | 3.56 | 0 | 0 |
| Y28 | 7.18 | 5.13 | -0.03 | 0 |
| C29 | 8.67 | 5.29 | -0.01 | 0 |
| G30 | 9.68 | 4.34 | 0 | 0 |
| S31 | 8.67 | 4.46 | 0 | 0 |
| G32 | 8.86 | 4.30 | 0 | -0.01 |

Residues of MCoCP4 with the chemical shift differences above 0.2 ppm in $H^\alpha$ are in bold.
Residues G1 and G2 are broadened in MCoCP4.

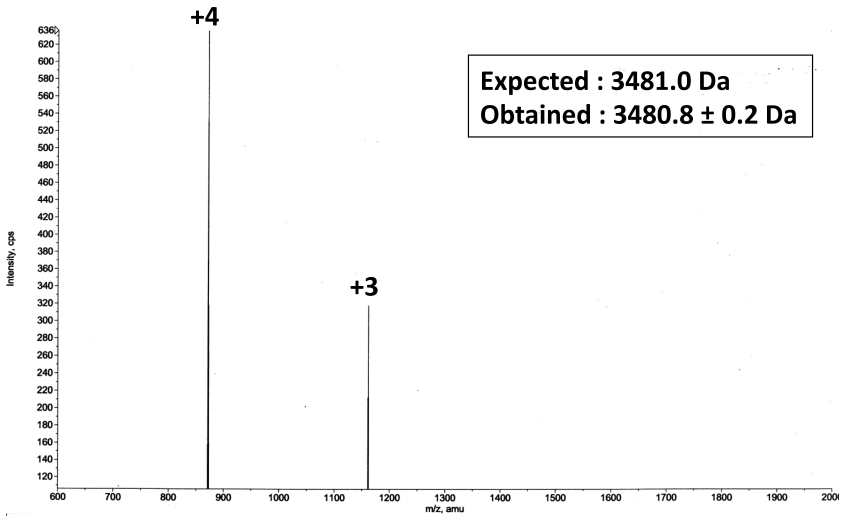


**Figure S1.** Mass spectrum of in-cell generated cyclotide MCoTI-I. Precursor ion scan was performed using the ion (m/z = 871.6) in Q3.

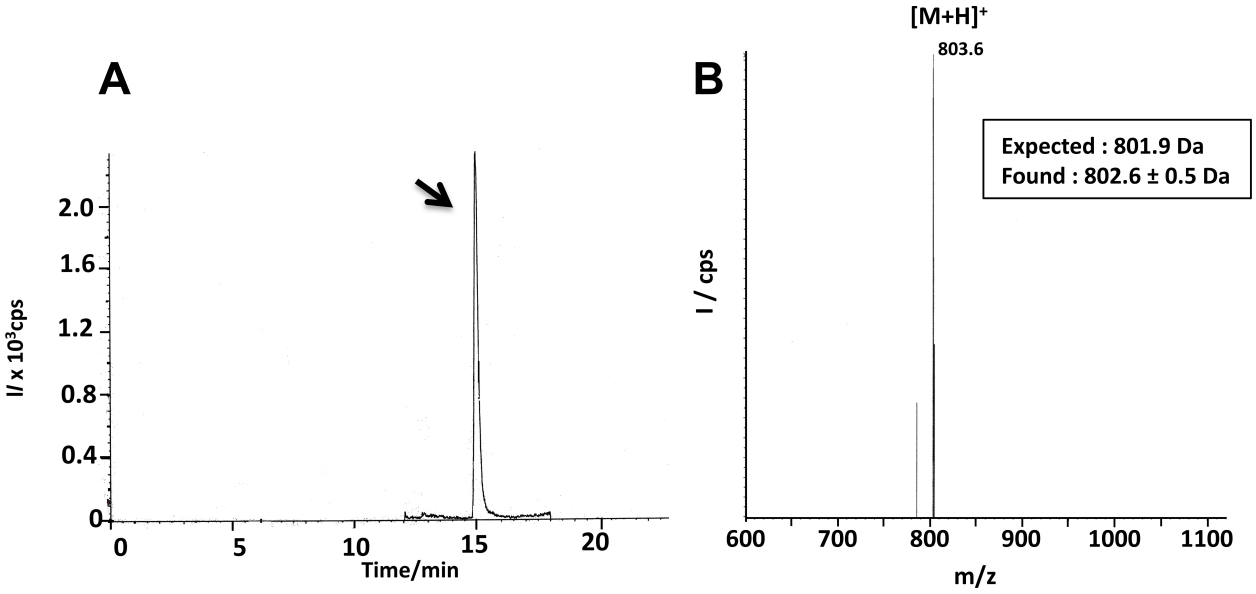


**Figure S2.** Mass spectrometry analysis of in-cell generated cyclic peptide CP4. **A.** LC-MS/MS analysis of in-cell produced cyclic peptide CP4. Quantification was performed using the molecular ion (m/z =803.2) in Q1 and the fragment (m/z =785.5) in Q3. **B.** Mass spectrum of in-cell generated cyclic peptide CP4. Precursor ion scan was performed using the fragment (m/z = 785.5) in Q3.

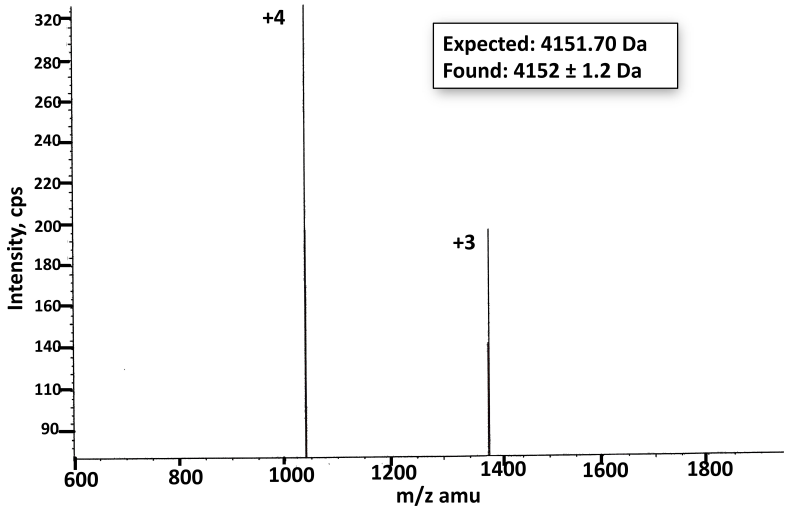


**Figure S3.** Mass spectrum of in-cell generated cyclotide MCoCP4. Precursor ion scan was performed using the ion (m/z = 1038.9) in Q3.

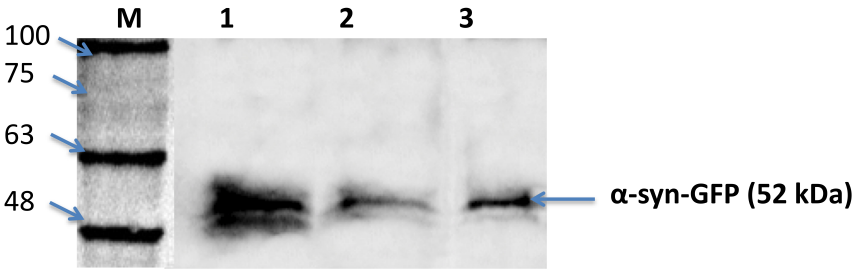


**Figure S4.** Western-blot analysis of α-syn-GFP protein expressed in *S. cerevisiae* transformed with plasmid pRS306-α-syn. Clone #1 was used for the experiments described in the manuscript (M: protein markers; lanes labeled 1, 2 and 3 stand for cell lysates from clones #1, #2 and #3).

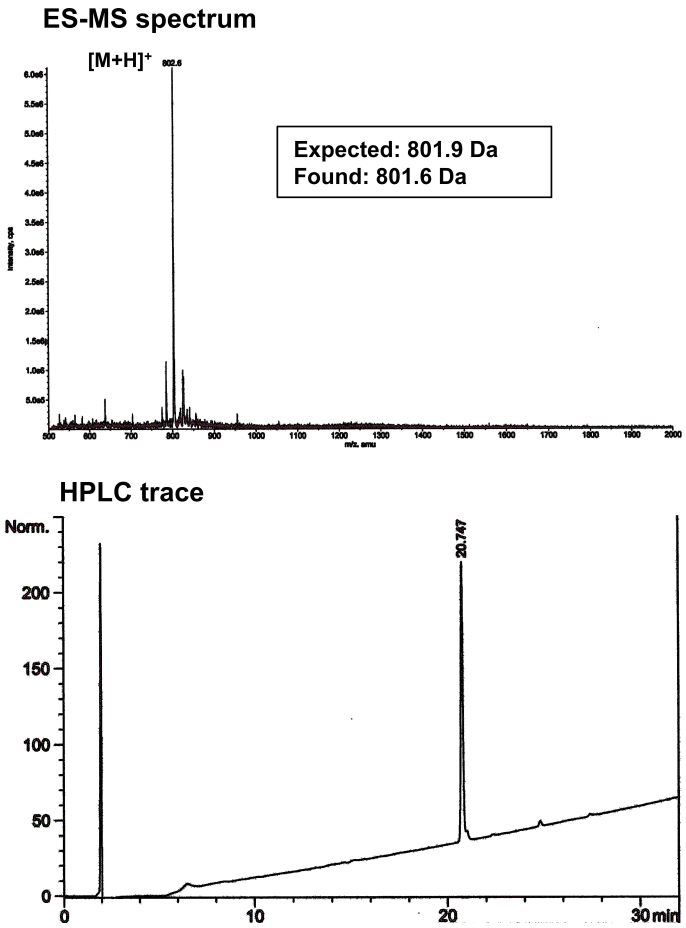


**Figure S5.** HPLC and ES-MS characterization of synthetic cyclic peptide CP4. Expected mass of cyclized peptide was 801.9 Da while the observed mass was 801.6 Da.

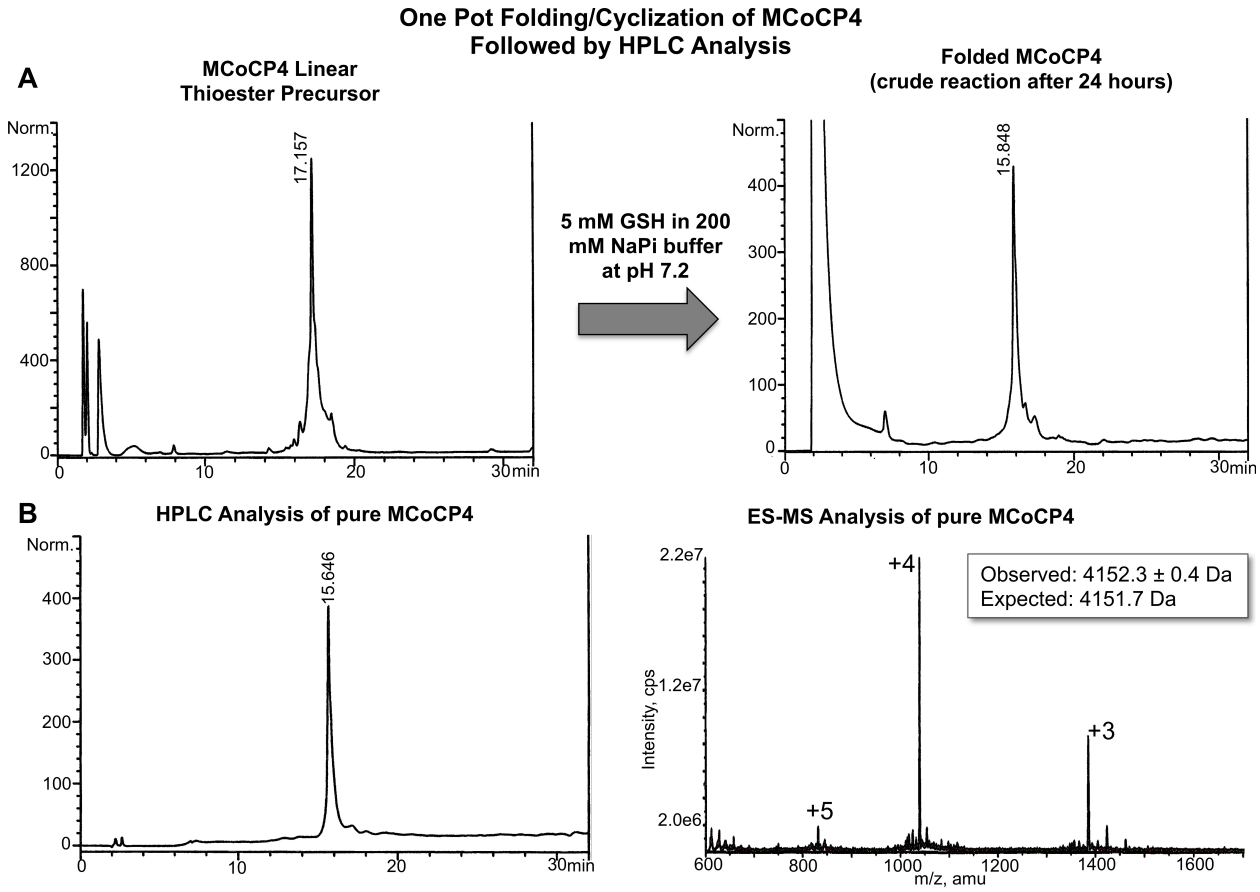


**Figure S6**. Chemical production and characterization of cyclotide MCoCP4. **A.** Cyclization and folding of cyclotide MCoCP4. **B.** Characterization by HPLC and ES-MS of purified cyclotide MCoCP4.

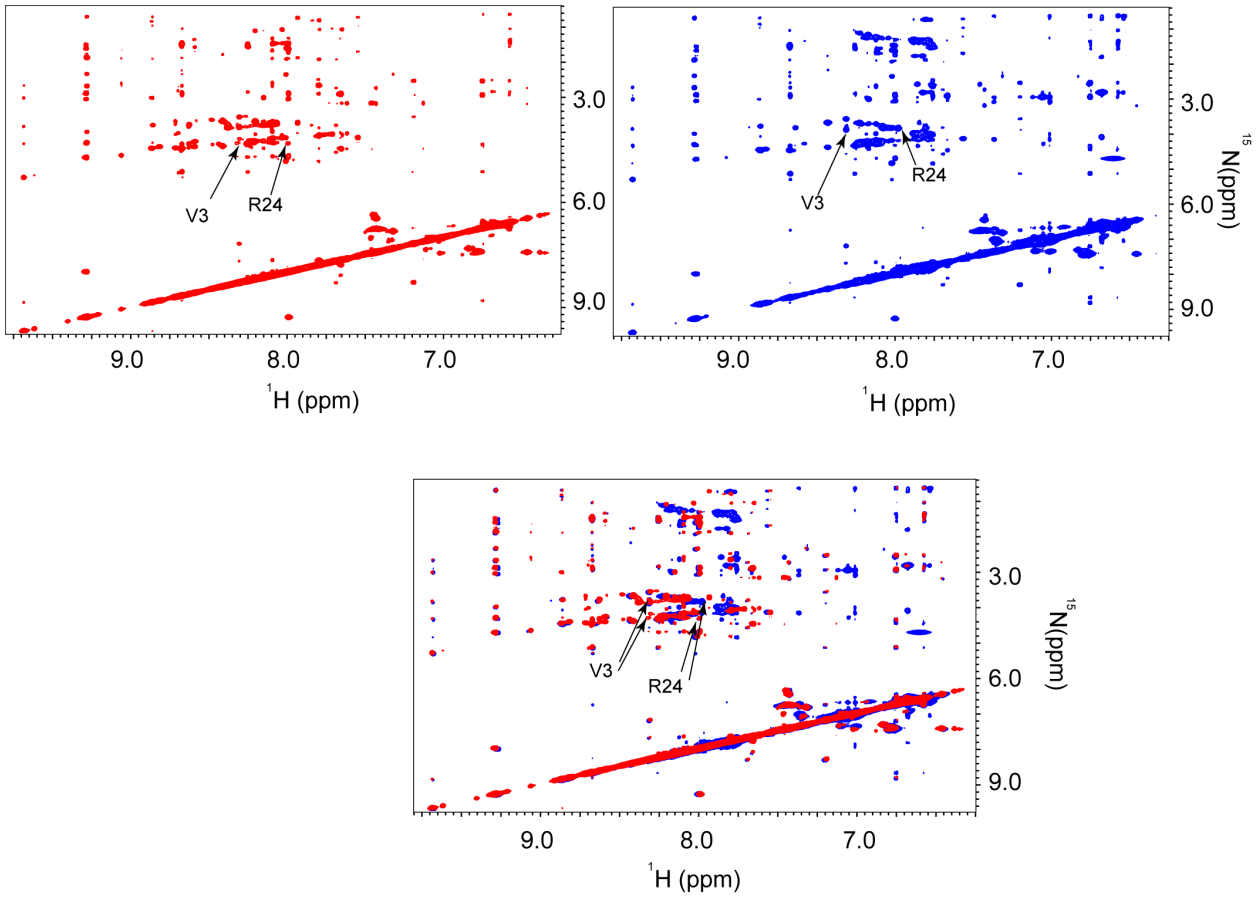


**Figure S7.** NMR characterization of cyclotide MCoCP4. Overlay of a selected region of 2D [$^1$H-$^1$H] NOESY spectra of MCoCP4 (top right panel, red) with MCoTI-I (top left panel, blue). Residues R24 and G32, which show prominent changes in $H^{\alpha}$ chemical shifts, are indicated.

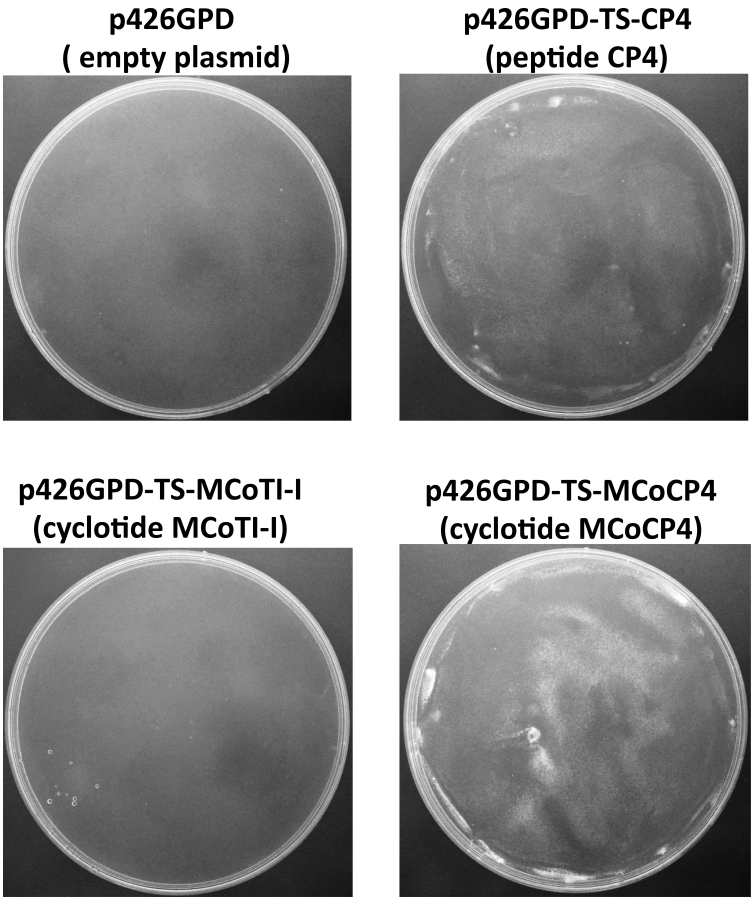


**Figure S8**. Cells encoding the α-syn-GFP fusion protein and plasmids p426GPD, p426GPD-TS-MCoTI-I, p426GPD-TS-MCoCP4 or p426GPD-TS-CP4 were grown overnight at 30°C in liquid media containing 2% raffinose until they reached log or mid-log phase. Cultures were then normalized for $OD_{600}$ and around $10^5$-$10^6$ cells were spotted onto SC-$U^-$ agar media containing 2% galactose, after which they were grown at 30°C for 2-3 days. Only cells encoding peptide CP4 and cyclotide MCoCP4 were able to grow in the presence of galactose.

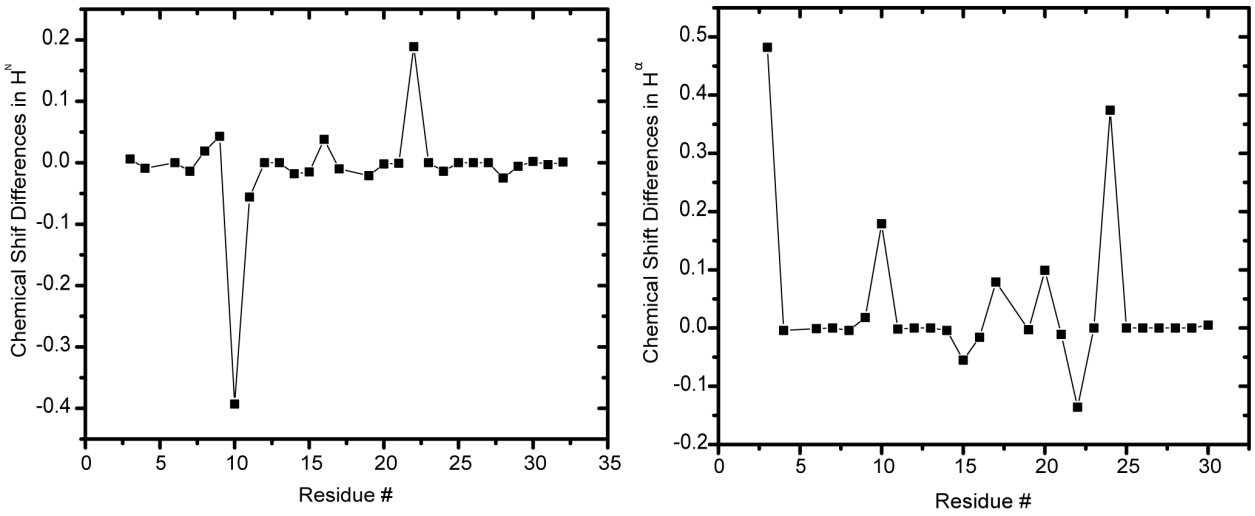


**Figure S9.** Chemical shift differences of backbone amide ($H^N$) and alpha ($H^\alpha$) protons of MCoCP4 with respect to that of the wild-type MCoTI-I, for the residues described in Table S2. All proton chemical shift differences are below 0.5 ppm suggesting that the McoTI-I scaffold is largely unperturbed in MCoCP4.

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