Cyclic Peptides

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Expression of Fluorescent Cyclotides using Protein Trans-Splicing for Easy Monitoring of Cyclotide–Protein Interactions**

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Cyclotides are fascinating natural plant micro-proteins ranging from 28 to 37 amino acid residues long and exhibit various biological functions including anti-microbial, insecticidal, cytotoxic, antiviral (against HIV), protease inhibitory, and hormone-like activities.[1–4] They share a unique head-to-tail circular knotted topology of three disulfide bridges; one disulfide penetrates through a macrocycle formed by the other two disulfides, thereby interconnecting the peptide backbone to form what is called a cystine knot topology (Figure 1). This cyclic cystine knot (CCK) framework gives the cyclotides exceptional rigidity,[5] resistance to thermal and chemical denaturation, and enzymatic stability against degradation.[4,6] In fact, some cyclotides have been shown to be orally bioavailable. For example, the first cyclotide to be discovered, kalata B1, was found to be an orally effective uterotonic,[7] and other cyclotides have been shown to cross the cell membrane through macropinocytosis.[8–10] All of these features make cyclotides ideal substrates for drug development.[11–14]

Cyclotides have been isolated from plants in the *Rubiaceae*, *Violaceae*, *Cucurbitacea*,[4,15] and, most recently, *Fabaceae* families.[16–18] Around 200 different cyclotide sequences have been reported in the literature,[19,20] although it has been estimated that approximately 50 000 cyclotides may exist.[21,22] Despite sequence diversity, all cyclotides share the same CCK motif (Figure 1 A). Hence, these micro-proteins can be considered natural combinatorial peptide libraries that are structurally constrained by the cystine-knot scaffold[2] and head-to-tail cyclization, and in which, with the exception of the strictly conserved cysteine residues comprising the cysteine-knot, hypermutation of essentially all residues is permitted. Cyclotides can be chemically synthesized, thereby permitting the introduction of specific chemical modifications or biophysical probes.[14,23–25] More importantly, cyclotides can now be biosynthesized in bacterial cells using a biomimetic approach that involves the use of modified protein splicing units.[26–28] These characteristics make them ideal substrates for the production of genetically-encoded libraries based on the cyclotide framework. These cell-based libraries allow for in-cell molecular-evolution strategies to enable the generation and high-throughput selection of compounds with optimal binding and inhibitory characteristics. In contrast to chemically generated libraries, genetically encoded libraries enable the facile generation and screening of very large combinatorial libraries of molecules.

The genetic code of most organisms encodes only 20 canonical amino acid building blocks, with the rare exceptions

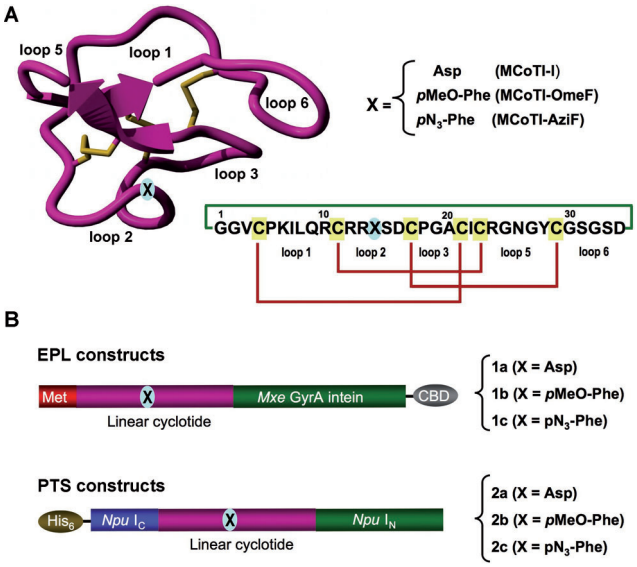

Figure 1. A) Tertiary structure of the cyclotide MCoTI-II (PDB code: 1IB9)[52] and the primary sequence of cyclotides used in this study. The backbone-cyclized peptide (connecting bond shown in green) is stabilized by the three disulfide bonds (shown in red). B) Intein precursors used for the expression of cyclotides. A non-natural amino acid (X) was introduced at position 14, which is in the middle of loop 2.

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of selenocysteine[29] and pyrrolysine,[30] which limits the chemical complexity of genetically-encoded libraries that can be produced in living cells. The recent development of nonsense suppressing orthogonal tRNA/synthetase technology has allowed the genetic encoding of a large variety of non-natural amino acids.[31–34] Herein, we report the production of natively folded cyclotides containing non-natural amino acids by employing in vivo non-natural amino acid incorporation in combination with protein splicing to mediate intracellular backbone cyclization. To our knowledge, this is the first time that a natively folded cyclotide containing non-natural amino acids has been produced inside living cells. This approach makes possible in vivo generation of cyclotides containing non-natural amino acids with new or enhanced biological functions. For example, the ability to site-specifically introduce fluorescent probes should facilitate the in vivo production of fluorescently-labeled cyclotides for screening or probing molecular interactions using optical approaches.

To test the feasibility of introducing non-natural amino acids into folded cyclotides in vivo, we used the cyclotide MCoTI-I (Figure 1 A). This cyclotide is a powerful trypsin inhibitor ($K_i \approx 20$ pM)[27] that has been recently isolated from dormant seeds of *Momordica cochinchinensis*, a plant member of the *Cucurbitaceae* family.[35] Trypsin-inhibitor cyclotides are interesting candidates for drug design because their specificity can be altered and they can be used as natural scaffolds to generate novel binding activities.[36]

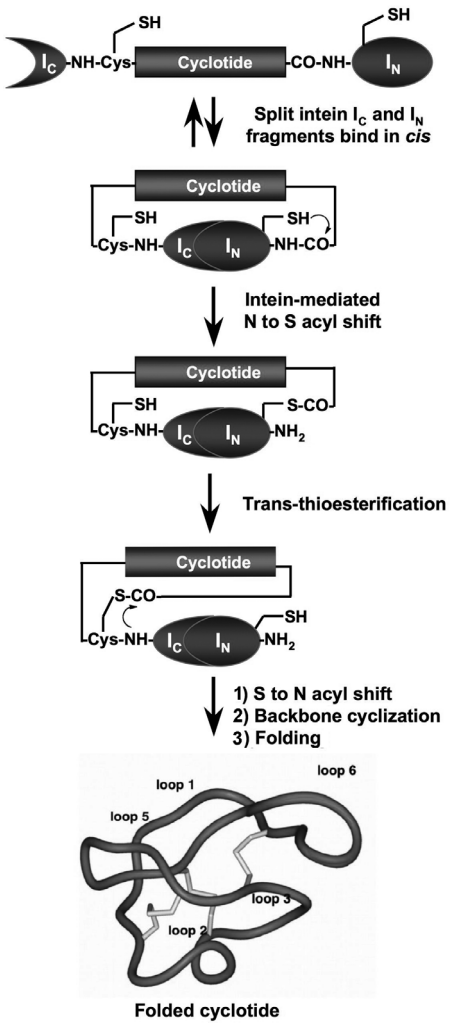
Because MCoTI cyclotides have been expressed inside *Escherichia coli* cells using an intramolecular version of expressed protein ligation (EPL),[27,28] we decided to try this approach first for in vivo generation of MCoTI-based cyclotides containing different non-natural amino acids. This method relies on the use of a protein splicing unit in combination with an in vivo intramolecular native chemical-ligation reaction to perform the backbone cyclization of the linear cyclotide precursor.[26,27] The amber stop codon TAG was used to encode a non-natural amino acid at the position corresponding to the residue Asp^{14} in MCoTI-I. This residue is located in the middle of loop 2 (Figure 1 A), which has been shown to be tolerant to mutations without affecting the structure and biological activity of the resulting cyclotide.[28] The incorporation of non-natural amino acids into the cyclotide framework was tested using *p*-methoxyphenylalanine (OmeF) and *p*-azidophenylalanine (AziF), which have been incorporated into various recombinant proteins.[33] More importantly, incorporation of AziF into the cyclotide framework should allow the site-specific incorporation of alkyne-containing fluorescent probes in vivo, using click chemistry.[37]

First, we explored the expression level of the corresponding intein precursors (**1b** and **1c**, Figure 1 B) in BL21(DE3) cells. Expression of the intein precursors of MCoTI-OmeF and MCoTI-AziF was performed in cells co-transformed with a plasmid encoding the corresponding MCoTI-intein precursor for EPL-mediated cyclization and the plasmid encoding the orthogonal amber-suppressing $tRNA_{CUA}$/aminoacyl-tRNA synthetase pair specific for OmeF (pVLOmeRS) or AziF (pERAzi), respectively. In both cases, the expression level of the intein precursors (**1b** and **1c**) was similar (Supporting Information, Figure S1). The suppression efficiency was estimated to be approximately 10% for **1b** and 20% for **1c**, compared to the expression of the wild-type MCoTI-I intein precursor **1a** (approximately 40 mg L^{-1}).

Under these expression conditions, all intein precursors showed around 60% in vivo cleavage, indicating that the intein was active and unaffected by the incorporation of the non-natural amino acid (Figure S1). Second, we tested the ability of the different intein-MCoTI precursors to produce the corresponding folded cyclotide by treatment with reduced glutathione (GSH) at pH 7.2 following the conditions optimized for MCoTI-cyclotides.[38] In both cases, the in vitro reaction was clean and efficient in providing the properly folded cyclotides MCoTI-OmeF and MCoTI-AziF (Figure S1). The final yield after purification was 4 μg L^{-1} (MCoTI-OmeF) and 14 μg L^{-1} (MCoTI-AziF). The expression yield for the wild-type MCoTI-I using these expression and cyclization conditions was approximately 48 μg L^{-1} after purification. Next, we explored the expression of the MCoTI-OmeF and MCoTI-AziF cyclotides inside bacterial cells using EPL-mediated cyclization.[27,28,38] When we tried this approach with MCoTI-OmeF and MCoTI-AziF, however, the amount of folded cyclotides was below the detection limit.

To boost the expression of cyclotides in vivo we explored the use of protein trans-splicing (PTS) to facilitate the in vivo cyclization process and to improve the yield of non-natural amino acid-containing cyclotides (Scheme 1). PTS is a post-translational modification similar to protein splicing with the difference that the intein self-processing domain is split into N- (I_N) and C-intein (I_C) fragments. The split-intein fragments are not active individually, however, they can bind to each other with high specificity under appropriate conditions to form an active protein-splicing or intein domain in *trans*.[39] PTS-mediated backbone cyclization can be accomplished by rearranging the order of the intein fragments. By fusing the I_N and I_C fragments to the C- and N-termini of the polypeptide for cyclization, the trans-splicing reaction yields a backbone-cyclized polypeptide (Scheme 1).[40] This approach has recently been used for the biosynthesis of cyclic hexapeptides containing non-natural amino acids.[41] Herein, in vivo cyclization was performed using the naturally occurring *Synechocystis sp.* (*Ssp*) PCC6803 DnaE split intein.[42] However, the *Ssp* DnaE intein requires specific amino acid residues at both intein–extein junctions for efficient protein splicing.[43] To overcome this problem we used the *Nostoc puntiforme* PCC73102 (*Npu*) DnaE split-intein. This DnaE intein has the highest reported rate of protein trans-splicing ($\tau_{1/2} \approx 60$ s)[44] and has a high splicing yield.[44,45] We explored the ability of the *Npu* DnaE split-intein to produce folded wild-type MCoTI-I cyclotide in *E. coli* cells. To accomplish this, we designed the split-intein construct **2a** (Figure 1 B). In this construct, the MCoTI-I linear precursor was fused in-frame at the C- and N-termini directly to the *Npu* DnaE I_N and I_C polypeptides. None of the additional native C- or N-extein residues were added in this construct. We used the native Cys residue located at the beginning of loop 6 of MCoTI-I (Figure 1 A) to facilitate backbone cyclization. A histidine tag was also added at the N-terminus to facilitate purification.

In vivo expression of wild-type MCoTI-I using PTS-mediated backbone cyclization was achieved by transforming



Scheme 1. In vivo expression of native folded cyclotides using intein-mediated protein trans-splicing.

the plasmid encoding the split-precursor **2a** into Origami-(DE3) cells to facilitate folding.[46] The MCoTI-precursor split-intein was over expressed for 18 hours at room temperature, showing very high levels of expression (approximately 70 mg L^{-1}) and almost complete cleavage (more than 95% in vivo, Figure 2A). The high reactivity of this precursor prevented us from performing a full characterization. Next, we tried to isolate the natively folded MCoTI-I generated in vivo by incubating the soluble fraction of a fresh cell lysate with trypsin immobilized on sepharose beads. Correctly folded MCoTI-cyclotides are able to bind trypsin with high affinity ($K_i \approx 20$–30 pM). Therefore, this step can be used for affinity purification and to test the biological activity of the recombinant cyclotides.[28] After extensive washing, the absorbed products were eluted with a solution containing 8 M guanidinium chloride (GdmCl) and analyzed by HPLC. The HPLC analysis revealed the presence of a major peak that had the expected mass of the native MCoTI-I fold (Figure 2B; Figure S2). Recombinant MCoTI-I produced by PTS-mediated cyclization was also natively folded, as characterized by NMR spectroscopy (Figure S2 and Table S2).[5] The in vivo expression level of folded MCoTI-I produced by PTS-mediated cyclization was estimated to be 70 μg L^{-1} of bacterial culture, which corresponds to an intracellular concentration of approximately 7 μM.

In vivo expression of folded MCoTI cyclotides by PTS was about seven times more efficient than intramolecular EPL-mediated backbone cyclization. This improvement may be explained by our choice of the split-intein *Npu* DnaE. This split-intein is extremely efficient; it exhibits fast kinetics with a good yield of protein trans-splicing. Differences in the cyclization process between the PTS and EPL methods may also contribute to the improvement in the cyclization yield. In PTS, the cyclization is driven by the affinity between the two-intein fragments, I_N and I_C, which in the case of the *Npu* DnaE intein is very high ($K_D \approx 3$ nM).[47] Once the intein complex is formed, the trans-splicing reaction is also extremely fast ($\tau_{1/2} \approx 60$ s for the *Npu* DnaE intein).[48] In contrast, EPL-mediated cyclization follows a slightly more complex mechanism that relies on the formation of the C-terminal thioester at the N-extein junction and the removal of the N-terminal leading sequence (a Met residue in this case) to provide an N-terminal Cys. These two groups then react to form a peptide bond between the N- and C-termini of the polypeptide. It is also worth noting that in contrast with the *Ssp* DnaE intein, which requires at least 4 native residues at the N- and C-terminal extein–intein junctions to work efficiently,[49] the *Npu* orthologue used herein tolerates different sequences at both junctions as demonstrated by the efficient trans-splicing of precursor **2a** (Figure 2A). The tetrapeptide sequences at both intein–extein junctions in construct **2a** have only 20% sequence homology with the native sequences of both *Npu* DnaE exteins.

Encouraged by these results, we decided to try in vivo expression of cyclotides MCoTI-OmeF and MCoTI-AziF using PTS. For this purpose, precursors **2b** and **2c** (Figure 1B) were overexpressed in Origami (DE3) cells by transforming the plasmids pVLOmeRS and pERAzi and growing the bacterial cells in the presence of OmeF or AziF, respectively. Constructs **2b** and **2c** are similar to **2a** but were designed to incorporate non-natural amino acids instead of residue Asp^{14} in MCoTI-I (Figure 1B). The expression levels of the intein precursors **2b** and **2c** were approximately 7 mg L^{-1} (10% suppression in comparison to wild-type precursor **2a**) and approximately 20 mg mL^{-1} (25% suppression), respectively. In vivo trans-splicing for **2b** and **2c** was also similar ($\geq 95\%$, Figure 2A) to that of the wild-type PTS construct **2a**. Cyclotides MCoTI-OmeF and MCoTI-AziF were purified by affinity chromatography using trypsin sepharose beads from fresh soluble cell lysates, and the trypsin-bound fractions were analyzed by LC-MS/MS and ES-MS (Figure 2C; Figure S3). Cyclotide MCoTI-OmeF generated in vivo by PTS was also characterized by NMR, confirming the adoption of a native cyclotide fold (Figure 2D; Figure S4). The expression levels for cyclotides MCoTI-OmeF and MCoTI-AziF were estimated to be 1 μg L^{-1} and 2 μg L^{-1}, corresponding to an intracellular concentration of approximately 0.1 μM and 0.17 μM, respectively.

Next, we explored the possibility of using fluorescently-labeled cyclotides to perform screening of protein–cyclotide

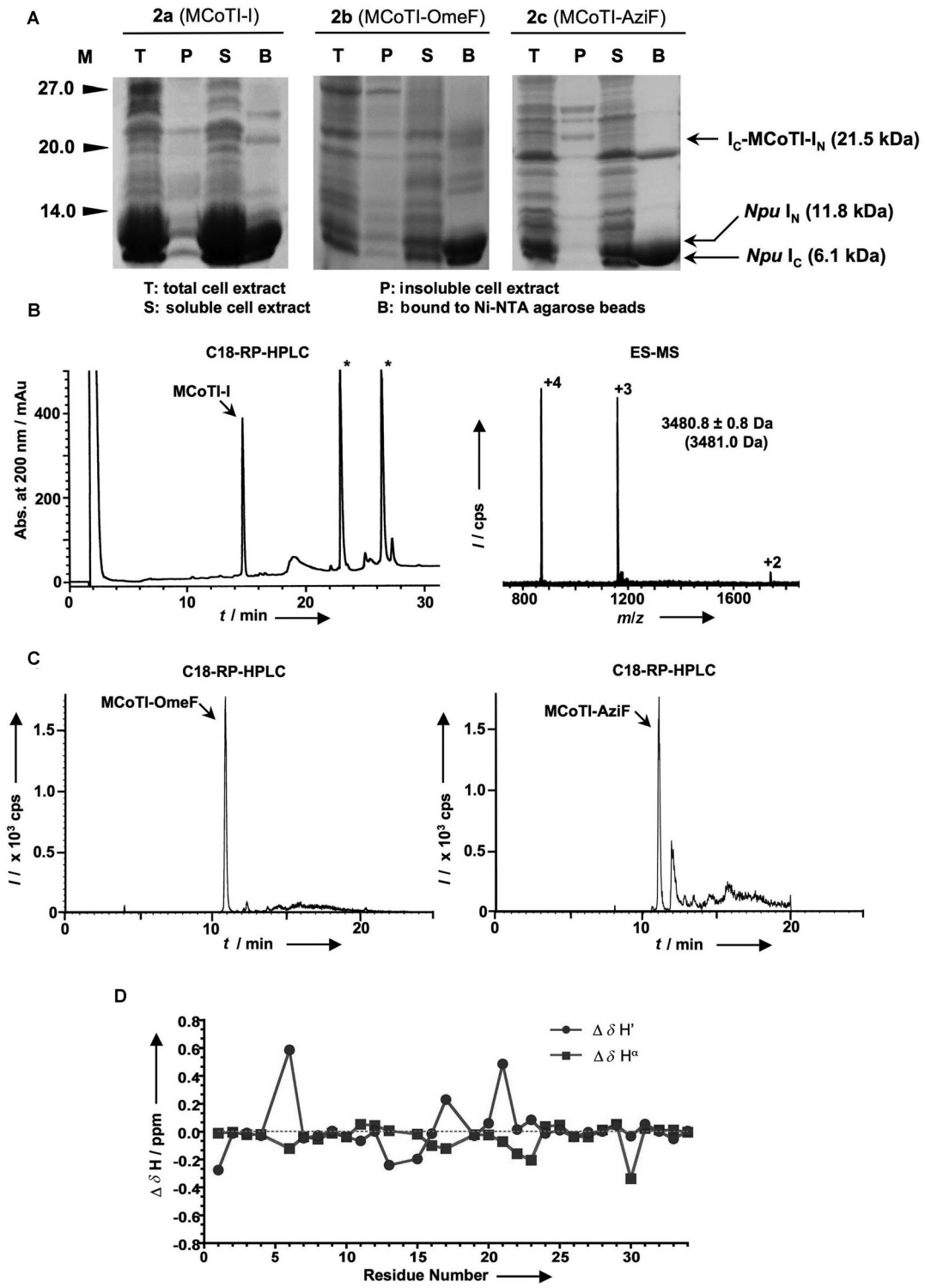


Figure 2. In vivo expression of MCoTI-I based cyclotides in *E. coli* cells using *Npu* DnaE intein-mediated PTS. A) SDS-PAGE analysis of the recombinant expression of cyclotide precursors **2a**, **2b**, and **2c** in Origami2(DE3) cells for production of the cyclotides MCoTI-I and MCoTI-OmeF, respectively. B) Analytical HPLC trace (left) of the soluble cell extract from bacterial cells expressing the precursor **2a** (MCoTI-I) after purification by affinity chromatography on a trypsin-sepharose column. Endogenous bacterial proteins that bind trypsin are marked with asterisks. Mass spectrum (right) of affinity purified MCoTI-I. The expected average molecular weight is shown in parentheses. C) LC-MS/MS analysis of the soluble cell extract of bacterial cells expressing precursors **2b** (MCoTI-OmeF) and **2c** (MCoTI-AziF). D) Summary of the backbone ^{1}H NMR assignments for the backbone protons of MCoTI-OmeF produced by PTS in vivo: $\Delta\delta(^1H)$ are the deviations in the chemical shifts of the main chain protons between the values obtained for MCoTI-OmeF and MCoTI-I (Table S2). Assignments for residue 14 were not included in the graph.

interactions. To accomplish this, we used MCoTI-AziF and trypsin as a model system. Preliminary results showed that MCoTI-AziF can be efficiently (almost quatitatively) labeled in vitro with a dibenzylcyclooctyne (DBCO)-derivative of the fluorescent dye aminomethyl coumarin acetate (AMCA) through copper-free click chemistry (Figure S5). In vivo labeling of MCoTI-AziF with DBCO-AMCA was also very efficient (Figure 3A; Figure S7). No unreacted MCoTI-AziF was found after treatment of the cells with DBCO-AMCA, as determined by LC-MS/MS (data not shown). As expected, the resulting AMCA-labeled MCoTI-AziF was able to bind commercial porcine pancreatic trypsin efficiently (Figure S8), indicating that introduction of the fluorophore in loop 2 of MCoTI-AziF did not have a detrimental effect on its biological activity. To monitor this interaction in vitro and in vivo, we used rat anionic trypsin, which can be expressed more efficiently in bacterial expression systems.[50] We decided to use fluorescence resonance energy transfer (FRET) to visualize the interaction between trypsin and AMCA-labeled MCoTI-AziF. For this purpose, the protease was fused to the N-terminus of the enhanced green fluorescent protein (EGFP). AMCA and EGFP show a good overlap between the emission band of the donor (AMCA) and the absorption band of the acceptor (EGFP), thus allowing the visualization of their interaction using FRET.[51] Moreover, structural analysis of a MCoTI-II–trypsin complex model[28] reveals that the distance between the C-terminus of trypsin and the Cα of residue 15 in MCoTI-I is approximately 35 Å. This distance is well within the range for the visualization of complex formation by FRET.[51] The catalytic residue Ser[195] in trypsin was also mutated to Ala to facilitate the recombinant expression of trypsin-S195A-EGFP by preventing its cellular toxicity. As shown in Figure 3B,C, AMCA-labeled cyclotide MCoTI-AziF was able to efficiently bind trypsin-S195A-EGFP (K_D of 1.8 ± 0.7 nM) in vitro, and more importantly, the cyclotide–protein interaction could be easily monitored by intermolecular FRET shown by the simultaneous decrease and increase of the fluorescence signals at 445 nm and 515 nm, respectively.

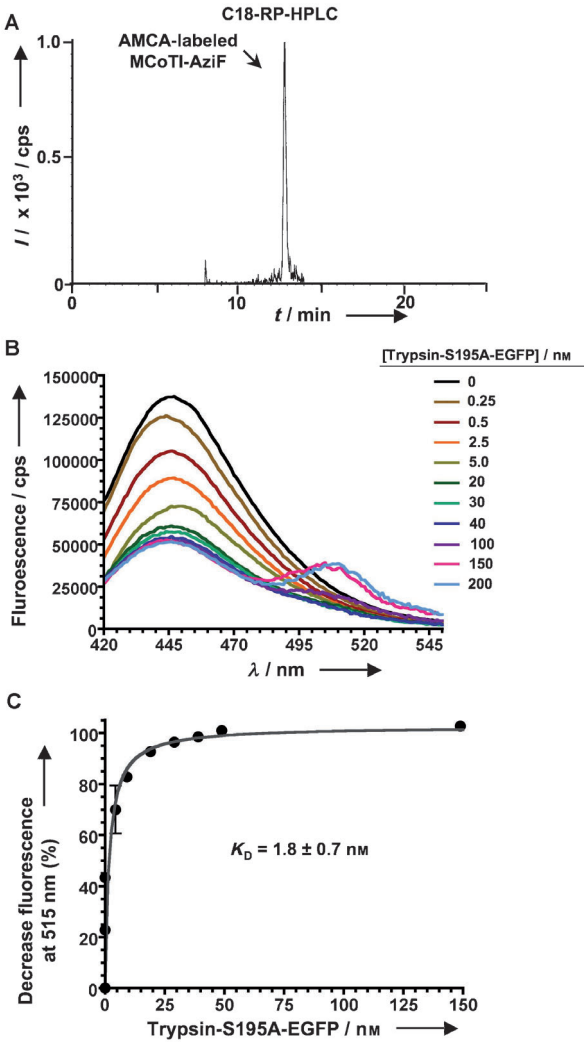


Figure 3. In vivo production and biological activity of AMCA-labeled MCoTI-AziF. A) LC-MS/MS trace of the soluble cell extract of bacterial cells indicating the presence of AMCA-labeled MCoTI-AziF. B) Titration of AMCA-labeled MCoTI-AziF (2 nM) with increasing amounts of trypsin-S195A-EGFP (0–200 nM) monitored by fluorescence spectroscopy. Excitation was performed at 360 nm. Background signal from EGFP in the absence of AMCA-labeled MCoTI-AziF was subtracted in all spectra. C) Binding isotherm of AMCA-labeled MCoTI-AziF and trypsin-S195A-EGFP by plotting the decrease in fluorescence signal at 445 nm. The dissociation constant (K_D) was calculated assuming a 1:1 molecular complex.

In summary, we have shown that the biosynthesis of cyclotides containing non-natural amino acids can be achieved by using different intein-based methods. EPL-backbone cyclization can provide non-natural amino acid containing cyclotides when the cyclization is carried out in vitro by GSH-induced cyclization and folding of the corresponding precursor. In vivo production, however, is less efficient using this method. We have shown that PTS-mediated backbone cyclization using the highly efficient *Npu* DnaE split-intein can be used for the efficient production of cyclotides inside live *E. coli* cells. We estimate that the in vivo production of MCoTI-I was around seven times more efficient using *Npu* DnaE PTS than EPL, thereby providing an attractive alternative for the production of these types of polypeptides in bacterial cells. The high efficiency of PTS-mediated cyclization combined with nonsense-suppressing orthogonal tRNA/synthetase technology made the in vivo production of cyclotides containing non-natural amino acids possible. Of particular interest is the introduction of azido-containing amino acids, which can react with DBCO-containing fluorescent probes to provide in vivo fluorescently labeled cyclotides. The classical approach for in vivo production of fluorescent-labeled proteins by fusing a fluorescent protein to the target protein is not applicable to cyclotides, owing to their small size and restricted backbone-cyclized topology. We have shown that cyclotides containing the non-natural amino acid AziF can be expressed in live bacterial cells and easily labeled with DBCO-AMCA to monitor cyclotide–protein interactions. This finding opens the possibility for in vitro and potentially also in vivo screening of genetically encoded libraries of cyclotides for the rapid selection of novel cyclotide sequences able to bind a specific

bait protein using high-throughput cell-based optical screening approaches.

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