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Spontaneous, co-translational peptide macrocyclization using *p*-cyanoacetylene-phenylalanine

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Peptide macrocycles (PMCs) are increasingly popular for the development of inhibitors of protein-protein interactions (PPIs). Large libraries of PMCs are accessible using display technologies like mRNA display and phage display. These technologies require macrocyclization chemistries to be compatible with biological milieu, severely limiting the types of technologies available for cyclization. Here, we introduce the novel non-canonical amino acid (ncAA) *p*-cyanoacetylene-L-Phe (pCAF), which facilitates spontaneous, co-translational cyclization through Michael addition with cysteine under physiological conditions. This new, robust chemistry creates stable macrocycles of a wide variety of ring sizes including bicyclic structures.

Protein-protein interactions are implicated in numerous diseases, but many of these are considered undruggable targets by small molecules due to their large and featureless surfaces. Peptides have emerged as a promising solution for the development of PPI inhibitors¹ due to their chemical composition, which can resemble the protein surface itself^{2,3}, and their larger size. Additionally, the capability to create diverse peptide libraries using mRNA Display^{4–6} (10¹³ molecules) or phage display (10⁹ molecules)^{7,8} affords a reliable way to find a potential hits for a given target. Peptides suffer some disadvantages as therapeutic candidates. They can be unstable due to protease digestion⁹ and often don't permeate the cellular membrane to reach their target^{10,11}. Peptide macrocycles (PMCs) offer a potential solution to these issues^{12–15}, as cyclization can confer inaccessibility to a protease's active site^{16,17}, or improve the cellular permeability^{11,13,18}.

Many strategies for peptide cyclization have been described^{13–15,19,20}; however, the majority of these require conditions (e.g. high temperatures²¹, usage of organic solvents²², heavy metal catalysis²³, etc.) that are incompatible with display techniques. Moreover, the majority of strategies for cyclization of displayed peptide libraries are applied post-translationally, adding to the complexity of the process. In contrast, reactions that lead to spontaneous co-translational cyclization of displayed peptide libraries represent a powerful, efficient and simple alternative, but few such chemistries have been described^{8,24–26}. Here we describe a new technology for the *in-vitro* translation of stable PMC using the novel non-canonical amino acid (ncAA) *p*-cyanoacetylene-phenylalanine (pCAF) capitalizing on the known reactivity of aryl cyanoacetylenes (also known as aryl propiolonitriles) with thiols²⁷. The spontaneous macrocycles formed will be ideal for the cyclization of peptide libraries for use in mRNA display and other display technologies.

We reasoned that the pCAF amino acid containing the cyanoacetylene functional group would spontaneously react intramolecularly with a cysteine in the peptide giving the cycle shown (Figure 1A). The *E. coli* phenylalanine aminoacyl-tRNA synthetase (PheRS) mutant A294G²⁸ is known to tolerate a number of 4-substituted Phe analogs²⁹, and we surmised that it would be able to charge pCAF on to tRNA^{Phe}, enabling the incorporation of this analog into peptides via translation.

4-cyanoacetylene-Phenylalanine (pCAF) was synthesized in 3 steps from Boc-4-iodophenylalanine, **1**, (Figure 1B). The first step involved a Sonogashira coupling with propargyl alcohol, giving compound **2**. The alcohol was converted to the nitrile through an oxidation-iminization-aldimine oxidation sequence³⁰, giving **3**. Protecting group removal led to the expected pCAF with high yields. Next, we evaluated the ability of PheRS A294G to charge pCAF. We performed a MALDI charging assay³¹

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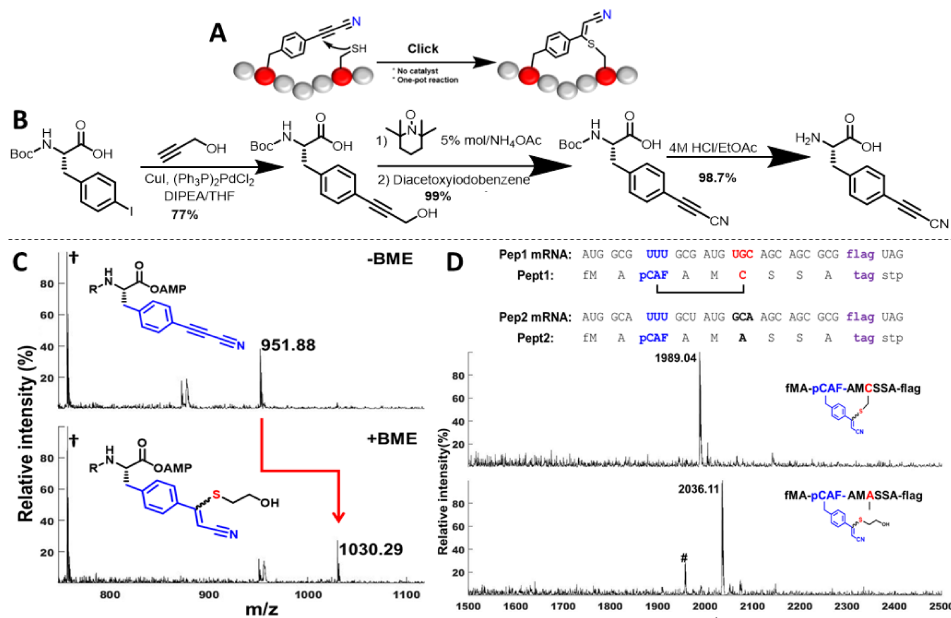


Fig. 1 Synthesis and cyclization chemistry of pCAF in the presence of thiols. (A) Concept of cyclization; pCAF should spontaneously react with cysteine via a Michael addition. (B) Synthesis of pCAF. (C) MALDI charging assay of pCAF with *E. coli* total tRNA and FRS(A294G). In the top panel, BME was excluded from the reaction (calc: 952.30); the bottom panel contained 200mM BME (calc: 1030.31). †Derivatized AMP which is used as an internal standard for the assay (calc: 756.23, obsd: 756.26) (D) *In-vitro* translation of mRNAs encoding cysteine (top) or alanine (bottom) lead to different outcomes. The cysteine-containing mRNA led to a peptide mass consistent with intramolecular cyclization (calc: 1989.73) and the alanine-containing mRNA led to a mass consistent with BME attack on pCAF (calc: 2035.77). #peptide with unreacted pCAF (calc: 1957.76, obsd: 1958.11).

with pCAF and PheRS A294G in the presence and absence of beta-mercaptoethanol (BME) (Figure 1C). In the absence of BME, we identified a peak that matched the expected mass of the derivatized pCAF-AMP ester, validating that it is a substrate for PheRS A294G. In the presence of BME, the observed peak shifted by 78 Da, which matches the expected BME adduct, validating the expected reactivity of pCAF towards thiols.

To analyze the behavior of pCAF in the context of *in vitro* translation, we designed mRNA templates that encode pCAF in the presence or absence of an encoded cysteine (Figure 1D). Considering the sensitivity of pCAF to thiols as observed in our AARS charging assay (Figure 1C), we prepared a version of the PURE system that replaces the standard thiol reducing agent (DTT) with the phosphine triscarboxyethyl phosphine (TCEP). This swap reduced the efficiency of the system but did not affect the fidelity of the translation (Fig. S1 in ESI). We hoped that residual BME present in the translation system (from the enzyme storage buffers) would not be sufficient to react with the translated pCAF prior to reaction with a cysteine. Gratifyingly, the cysteine-encoded template gave a single mass matching the cyclized product; while the alanine containing template gave the mass of the BME adduct. We attempted to react the putative cyclized peptide with thiols, amines, and se-

lenides, but in each case no reaction was observed (Fig. S2 in ESI), which speaks to the stability of the cyclized peptide and the irreversibility of the reaction.

The lack of a BME adduct in the cysteine-containing template suggests that the thiol attack on the pCAF-containing peptide occurs only after the pCAF amino acid is incorporated into the peptide. To investigate this, we pre-charged pCAF onto tRNA^{Phe} in the presence of BME and added the resulting Michael addition product BME-pCAF-tRNA^{Phe} to the translation with a cysteine encoding mRNA template. The product showed no evidence of cyclization, and instead showed the incorporation of BME-pCAF (Figure S3 in ESI). This highlights the inertness of the thiol adducts of pCAF and supports post-translational cyclization.

The Michael addition cyclization reaction does not lead to a mass change, so to further confirm cyclization and check the stability of our macrocycles to digestion, we designed a protease digestion assay. mRNAs encoding a Factor Xa cleavage site in between the pCAF and either a cysteine or serine were created (Figure 2 A). Our expectation was that cyclization would lead to a resistance to Factor Xa cleavage, while linear peptides would be digested efficiently. Both *in vitro* translated peptides were subjected to intensive digestion with Factor Xa followed by re-capture onto anti-FLAG resin. The cysteine-containing

peptide gave a mass consistent with cyclization, and was protease-resistant, remaining largely intact after the digestion (Figure 2B). On the other hand, the serine containing peptide showed the mass of the BME adduct prior to digestion and was completely degraded. Using an N-terminal ^{35}S -Met label, we quantified the fraction of the input peptide that could be recovered after digestion. 31% of the cysteine containing peptide was recovered, but none of the serine containing peptide could be recovered.

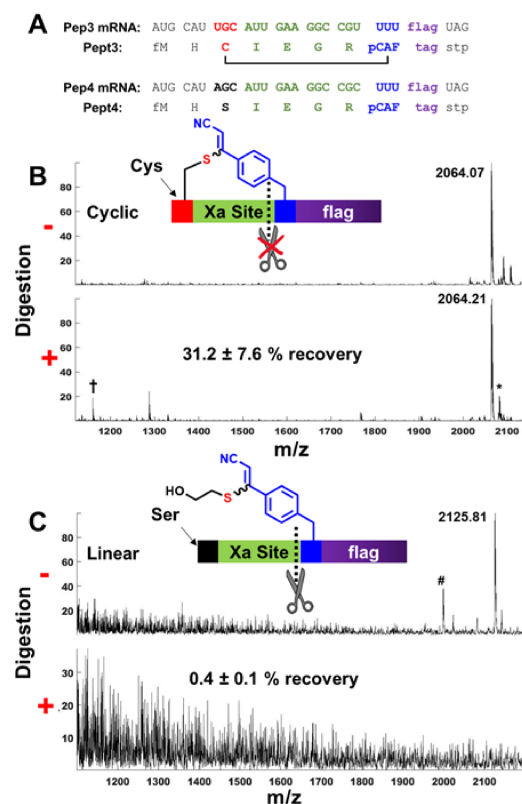


Fig. 2 Cyclization leads to protease resistance. After *in vitro* translation of templates shown in (A) the anti-FLAG purified peptides were digested for 18 h with Factor Xa, followed by re-capture with anti-FLAG resin. Panel B shows the masses of Pept3 before (top) and after (bottom) digestion. 31% of the peptide was recovered in the re-capture assay. (C) The same recapture assay was applied to Pept4, giving predominantly the BME adduct (calc: 2125.86) as well as a small amount of the Phe substitute # (calc: 1998.85, obsd: 1998.77). †cleaved Phe substituted Pept3 (calc: 1160.47, obsd: 1160.68.) *hydrolyzed but otherwise intact Pept3 (calc: 2081.83, obsd: 2082.22 Da).

We then investigated the effect of peptide size on macrocyclization. We translated several mRNAs with increased ring size ranging from 2 to 10 AA residues between the cyclization centers. The MALDI-MS data for each peptide lacked any evidence of BME adducts and were consistent with efficient cyclization (Figure 3A). The peptides were also synthesized in reasonable yield (Figure 3B), compa-

table to translations using phenylalanine in place of pCAF (Figure S4 in ESI).

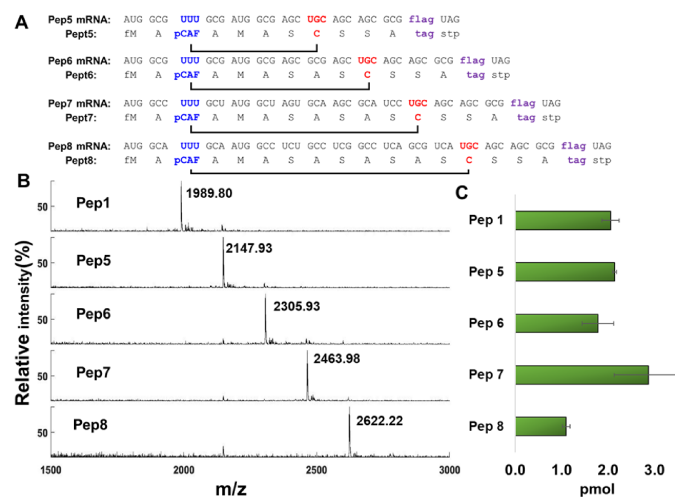


Fig. 3 Cyclization occurs at a wide variety of ring sizes. (A) mRNAs and peptides produced. (B) MALDI-evidence for cyclization at various ring sizes. The expected molecular weights from top to bottom are: 1989.73 Da, 2147.80 Da, 2305.87 Da, 2463.94 Da, 2622.01 Da. (C) Yields of the produced PMC per 30 μL of translation. Error bars signify the range of duplicate experiments.

Finally, we tested the pCAF cyclization chemistry on a peptide containing two cysteines and two pCAF residues. *In-vitro* translation of this peptide gave a MS corresponding to the double-cyclization product with only small peaks corresponding to BME adduct formation, demonstrating that this chemistry is compatible with the spontaneous creation of two independent macrocycles (Figure 4).

Here we have described a novel nCAA, pCAF that can be incorporated in place of phenylalanine into *in-vitro* translated peptides and leads to efficient, spontaneous intramolecular cyclization to give PMCs. Peptides that contain other potentially reactive residues (e.g. Lys, Arg, Tyr, Glu, Asp and His) and lack cysteine are inert to cyclization and instead react with residual BME in the translation system to form linear adducts. There are only a few spontaneous and irreversible cyclization chemistries that are compatible with translation, Suga's α -chloroacetamide chemistry^{24,32} or an alternative benzyl halide variant^{26,33}, Fasan's MOrPh-PhD^{8,34}, and the cyclization of an N-terminal cysteine with cyanothioazole recently reported by Hohsaka²⁵. Suga's chemistry requires Flexizyme charging of the α -chloroacetamide-AA onto tRNAs followed by reaction with cysteine²⁴. MOrPh-PhD requires addition of a tyrosine bearing an electrophile using stop codon suppression which also cyclizes with cysteine^{8,34}. Our system offers direct AARS-mediated charging and higher effi-

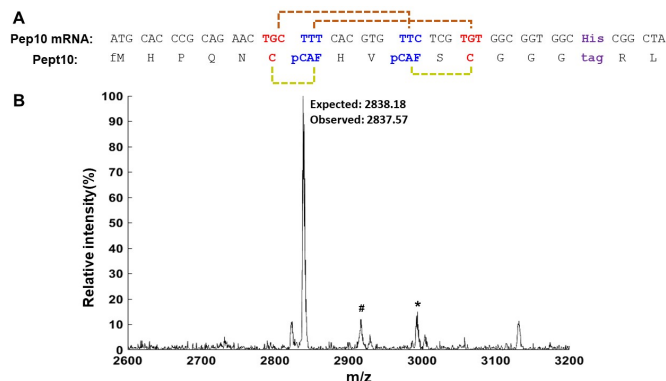


Fig. 4 A peptide with two cysteines and two pCAF residues leads to double-cyclization. (A) mRNA sequence encoding two cysteines and two pCAF molecules. The two possible doubly-cyclized peptide topologies are shown. (B) MALDI-MS spectrum of the *in vitro* translated peptide product (calc: 2838.18, obsd: 2837.57). # Monocyclized peptide with BME added through Michael addition with one pCAF (calc: 2916.19, obsd: 2916.52). * Monocyclized peptide with two BME additions one through Michael addition with pCAF and one as a disulfide with cysteine (calc: 2992.18, obsd: 2992.41). The yield (duplicate trials) was 8.2 ± 1.0 pmols in a 100 μ L reaction.

ciency than stop-codon suppression, and this has allowed the spontaneous formation of a doubly-cyclized peptide product. The strategy could potentially be implemented with phage display through breaking the degeneracy of the Phe codon box³⁵ to allow simultaneous encoding of both pCAF and Phe. Moreover, the Michael adduct formed with our cycle could potentially be elaborated to do further transformations. Such studies are underway.

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Conflict of Interest

The authors declare no conflicts of interest.

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