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Non-Canonical Amino Acids

Genetic Encoding of Cyanopyridylalanine for In-Cell Protein Macrocyclization by the Nitrile–Aminothiol Click Reaction

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Abstract: Cyanopyridylalanines are non-canonical amino acids that react with aminothiol compounds under physiological conditions in a biocompatible manner without requiring added catalyst. Here we present newly developed aminoacyl-tRNA synthetases for genetic encoding of *meta*- and *para*-cyanopyridylalanine to enable the site-specific attachment of a wide range of different functionalities. The outstanding utility of the cyanopyridine moiety is demonstrated by examples of i) post-translational functionalization of proteins, ii) in-cell macrocyclization of peptides and proteins, and iii) protein stapling. The biocompatible nature of the protein ligation chemistry enabled by the cyanopyridylalanine amino acid opens a new path to specific in vivo protein modifications in complex biological environments.

Bioorthogonal reactions for site-specific protein conjugation with chemical and biochemical tags have a wide range of applications in the material, biological, and health sciences.[1] The site-selective modification of a target protein can be used to confer specific biophysical properties[2] and install labels for spectroscopic imaging and tracking of proteins by fluorescence,[3] nuclear magnetic resonance,[4] or electron paramagnetic resonance spectroscopy techniques.[5]

Site-specific incorporation of noncanonical amino acids (ncAAs) by genetic encoding gives precise control of the sites, where new functional groups are installed in a target protein,[6] but there is only a very small number of established bioorthogonal reactions. To date, the most prominent examples are copper-catalysed azide-alkyne cycloadditions (CuAAC) and inverse-electron demand Diels–Alder reactions (IEDDA).[7] Copper catalysts are barely compatible with physiological conditions, and strain-promoted cycloaddition and IEDDA reactions add relatively large non-biological chemical moieties, the synthesis of which can be challenging. In the light of recent progress in genetic code reprogramming to incorporate multiple, distinct ncAAs with different bioorthogonal functionalities into a single protein,[8] there is an unmet demand for additional genetically encoded ncAAs that enable stable conjugations in a reaction that is not only biocompatible but fundamentally different from established bioorthogonal reactions.

The nitrile–aminothiol (NAT) click reaction is a condensation reaction between electrophilic nitriles and 1,2-aminothiols, which proceeds rapidly under biological conditions without the need of any added catalyst and has been shown to be compatible with all canonical amino acids within peptides, except cysteine at the N-terminus of a polypeptide chain.[9] The present work demonstrates its suitability for in vivo protein modification.

To enable site-specific NAT click reactions on proteins, we first identified pyrrolysyl-tRNA synthetase (PylRS) mutants specific for *meta*- and *para*-cyanopyridylalanines (mCNP and pCNP, Figure 1). The synthetases were selected from a library of PylRS mutants derived from the methanogenic archaeon ISO4-G1 (G1PylRS), using previously reported screening approach based on fluorescence-activated cell sorting (FACS),[10] and enabled site-specific incorporation of these amino acids in response to an amber stop codon.

In the absence of a crystal structure for G1PylRS, mutation sites were chosen based on the amino acid sequence alignment between G1PylRS and *Methanosarcina mazei* pyrrolysyl-tRNA synthetase (*Mm*PylRS) (Figure S1). By examining the crystal structure of *Mm*PylRS (PBD ID: 2Q7E),[11] seven residues in *Mm*PylRS (L305, Y306, N346, C348, Y384, V401, W417) were hypothesized to influence substrate recognition (Figure S2) and the corresponding residues were randomized in the G1PylRS library (Supporting Information).

The plasmid library encoding G1PylRS mutants under *GlnS′* promoter[12] and the orthogonal $^{G1Pyl}tRNA_{CUA}$ (pBK-G1RS) was co-transformed into *E. coli* DH10B cells with the selection plasmid pBAD-H6RFP, which contains the mCherry red fluorescent protein (RFP) gene preceded by a His_6 tag with an amber stop codon at position 8 (His6-TAG-RFP). The transformed cells were subjected to multiple

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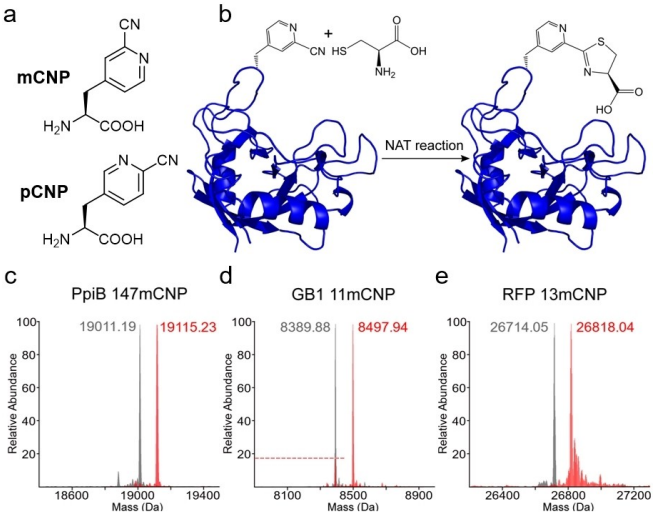

Figure 1. Structures and applications of the cyanopyridylalanines mCNP and pCNP. a) Chemical structures of mCNP and pCNP. b) Example of a NAT reaction between genetically incorporated mCNP and cysteine, which proceeds in aqueous solution at neutral pH and ambient temperature. c)–e) Intact protein mass spectrometry analysis of the reaction between genetically incorporated mCNP and cysteine; c) peptidyl–prolyl *cis*/*trans*-isomerase B (PpiB); d) streptococcal b1 immunoglobulin binding domain of protein G (GB1); e) red fluorescent protein (RFP). The grey and red spectra are of samples before and after the NAT reaction, respectively, and the red dashed line indicates the level of unreacted protein. Expected masses are reported in Table S4.

rounds of selection under positive (in the presence of the target ncAA) or negative (without the target ncAA) growth conditions. After each selection round, the FACS results showed clear enrichment of the target population. Cells containing tRNA synthetase candidates collected from the final round of selection were characterized individually (Figures S3 and S4, and Tables S1 and S2).

The best G1PylRS mutant for mCNP incorporation (G1mCNP34, in the following referred to as G1mCNPRS) featured the mutations L124A, Y125L, V167A, Y204W, and A221S. Despite randomization in the library, N165 and W237 were the same as in the wild-type sequence. The G1PylRS mutant for site-specific incorporation of pCNP (G1pCNP37, in the following referred to as G1pCNPRS) carries the mutations L124A, Y125F, Y204W, A221S, and W237Y, with N165 and V167 remaining unchanged. To gain an atomic level understanding of the mutations, we crystallized G1mCNPRS and solved its structure at a resolution of 2.2 Å (PDB ID: 7R6O; Figure S5; Table S8). As expected, the structure of the amino acid binding domain of the G1mCNP tRNA synthetase is highly conserved, but shows differences in the loop connecting β-strands 5 and 6, which contains Y204W as one of the key residues of the substrate binding site.

To produce proteins with cyanopyridylalanine residues in high yield, we used our previously developed two-plasmid system for in vivo incorporation of ncAAs via amber stop codon suppression.[10c] The gene of G1mCNPRS or G1pCNPRS, together with the orthogonal $^{G1Pyl}tRNA_{CUA}$, was cloned into a high-copy number pRSF plasmid to obtain the plasmids pRSF-G1mCNPRS and pRSF-G1pCNPRS, respectively, while the gene for the protein of interest was cloned into a low-copy number pCDF plasmid. Using the pCDF/pRSF system, mCNP and pCNP were incorporated with high fidelity and yield, for both single- and double-amber mutants, without any evidence of adduct formation with intracellular metabolites (Figure S6, Table S3). In addition, neither mCNP nor pCNP had any negative effect on *E. coli* cell growth (Figure S7 and S8). Next, we tested the reactivity of the incorporated cyanopyridylalanines in the NAT click reaction using L-cysteine as a model 1,2-aminothiol (Figure 1b). Different proteins containing mCNP or pCNP were incubated with 5 mM cysteine at 25 °C. Monitoring the reaction by intact protein mass spectrometry showed formation of the thiazoline product in greater than 90 % yield after 4 h (Figure 1c–e), in agreement with the reaction kinetics reported previously for peptides.[9b] The reaction was not impeded by the presence of 10 mM (tris(2-carboxyethyl)phosphine (TCEP) added to prevent oxidation of thiol groups during the reaction.

We previously reported that mCNP installed in peptides by solid-phase peptide synthesis readily undergoes spontaneous cyclization with an N-terminal cysteine residue in aqueous buffers at pH 7.5.[9a] To test the viability of the cyclization reaction in a protein, we installed mCNP in the fusion protein NT-Ubi 7X (X indicating the position of the ncAA in the amino acid sequence), which comprised an N-terminal NT solubility tag[13] followed by a short linker containing the modified TEV protease recognition sequence ENLYFQC and human ubiquitin[14] at the C-terminal end (Figure 2a).[15] The fusion protein was readily expressed in *E. coli* and purified using Ni/nitrilotriacetic acid (Ni-NTA) resin. 256 and 162 mg of purified protein were obtained per 1 L cell culture with mCNP or pCNP, respectively, with high incorporation yield as indicated by intact protein mass spectrometry. The cyclization reaction was triggered by digestion with TEV protease to expose the cysteine residue of the TEV recognition sequence at the N-terminus. The cleaved protein product Cys-Ubi 7X cyclized within 15 minutes, as indicated by mass spectrometry, with mCNP and pCNP reacting with similar rates (Figure 2c and d). The TEV protease cleavage was complete after 4 hours, resulting in more than 75 % cyclized Cys-Ubi 7X (Figure 2c and d).

Encouraged by these results, we examined the utility of the intramolecular NAT click reaction for in vivo peptide cyclization. To generate the required N-terminal cysteine residue, the protein needs to be cleaved inside the bacterial cell. This was achieved by a 3-plasmid system comprising a pCDF plasmid for the expression of NT-Ubi 7X, the pRSF plasmid containing the orthogonal PylRS system, and a pBAD-TEV plasmid for co-expression of TEV protease. The results showed that Cys-Ubi 7X was produced in high yield (134 mg and 60 mg of purified protein per 1 L cell culture with mCNP or pCNP, respectively) and either sample was found to be cyclized quantitatively, regardless of the difference in structural constraints imposed on the 7-residue macrocycle by the two different cyanopyridylalanine residues (Figure 2e and f).

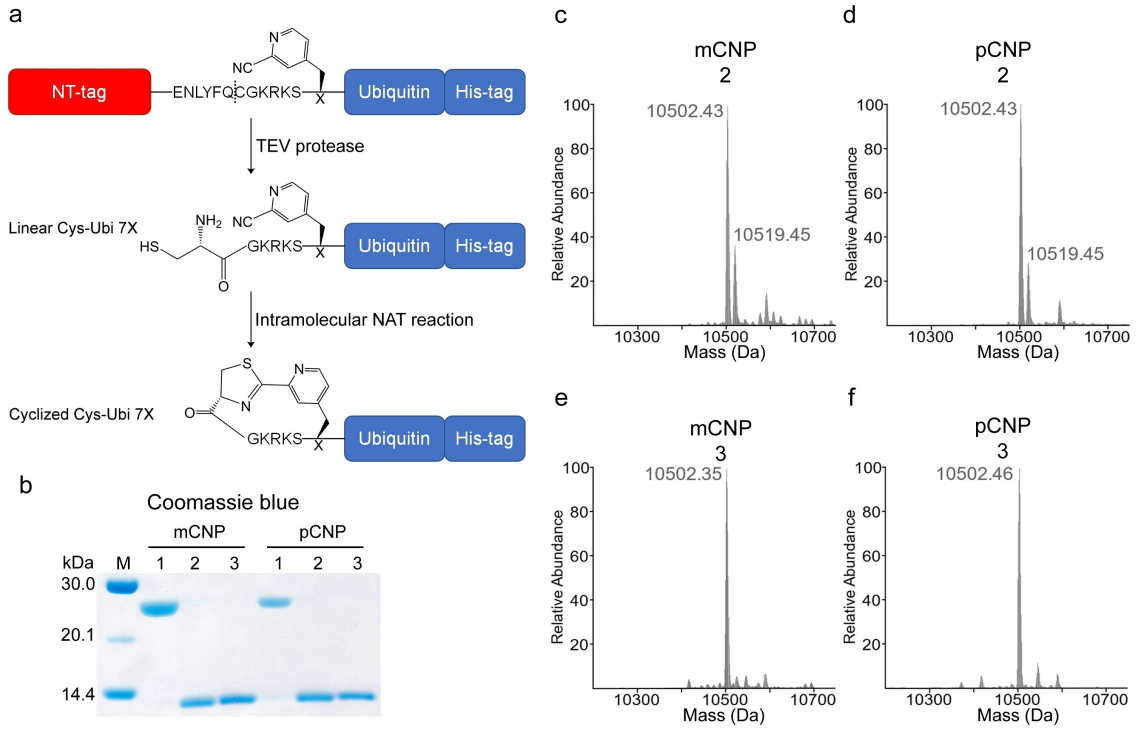

Figure 2. In vivo protein macrocyclization via intramolecular NAT click reaction. a) Design of the NT-Ubi 7X fusion protein used in the current study. X indicates the position of the ncAA. b) SDS-PAGE analysis: M, protein molecular weight marker (the size of each band is indicated on the left); lane 1, NT-Ubi 7X before TEV protease cleavage; lane 2, NT-Ubi 7X after TEV protease cleavage for 4 h at 25 °C; lane 3, purified Cys-Ubi X7 expressed using the 3-plasmid system for in vivo protein macrocyclization. c–f) Intact protein mass spectra of the Cys-Ubi X7 samples shown in lanes 2 and 3 of (b). Expected masses of linear and cyclized Cys-Ubi X7 are 10519.94 and 10502.91 Da, respectively.

An alternative way of polypeptide cyclization is achieved by a tandem NAT click reaction between a polypeptide containing two cyanopyridylalanine residues and a bi-functional aminothiol reagent. We used ethylenediamine dicysteine (EDDC), where the carboxyl groups of two cysteine residues are linked by an ethylenediamine moiety (Figure 3a, Supporting Information). The approach takes advantage of the difference in reaction rate between the slower intermolecular and faster intramolecular NAT reaction, where EDDC first reacts with a single cyanopyridylalanine moiety to form a singly tagged protein and this monofunctionalized intermediate undergoes a fast, spontaneous intramolecular NAT reaction with the second cyanopyridylalanine moiety. As a result, formation of the cyclized product is strongly favoured over doubly tagged uncyclized product even in the presence of a large excess of the diaminothiol reagent. The scheme was successful with all three proteins tested, which were produced with two mCNP residues (GB1 A24X/K28X, RFP 237X/243X and RFP A204X/237X, where X = mCNP). Following incubation with 5 mM EDDC (10 mM for RFP 237X/243X) at 25 °C, the reactions were complete after 4 h in near-quantitative yields as indicated by intact protein mass spectrometry (Figure 3b–d and S9).

The success of our system for genetic encoding of cyanopyridylalanines in high yield is based on i) our PylRS library derived from the methanogenic archaeon ISO4-G1, which proved exceptionally adaptable for encoding aromatic ncAAs, and ii) a two-plasmid selection system established previously for a chimeric PylRS variant tailored to the genetic encoding of lysine-based ncAAs.[10c] The versatility of G1PylRS systems has been demonstrated previously in bacteria,[16] mammalian cells,[17] and cell-free protein synthesis.[10c]

The ncAAs mCNP and pCNP present a balanced compromise between biocompatibility and reactivity. They are non-toxic in vivo and the activated nitrile functionality does not react with functional groups found in cellular biopolymers. In contrast, the activated nitrile functionality ligates readily and in high yield with 1,2-aminothiol compounds, provided they are present in high local concentration, as achieved in, e.g., intramolecular reactions. In *E. coli*, the cyanopyridylalanine ncAAs appear to be resistant against reaction with metabolic compounds, including cysteine present at natural intracellular concentrations. This is an advantage over ncAAs with a azide group, which are susceptible to chemical reduction in bacterial cells,[4b,18] or *trans*-cyclooctene (TCO) groups, which have been shown to be prone to isomerization to the non-reactive *cis*-cyclooctene isomer in vivo.[19] Over the last decade, PylRS mutants have been developed to genetically encode more than 100 ncAAs,[20] but previous attempts to genetically encode the 1,2-aminothiol group as a reactive conjugation group proved unsuccessful due to reaction of the aminothiol moiety with pyruvate, which is abundant in cells.[21] A genetic encoding system subsequently developed for a

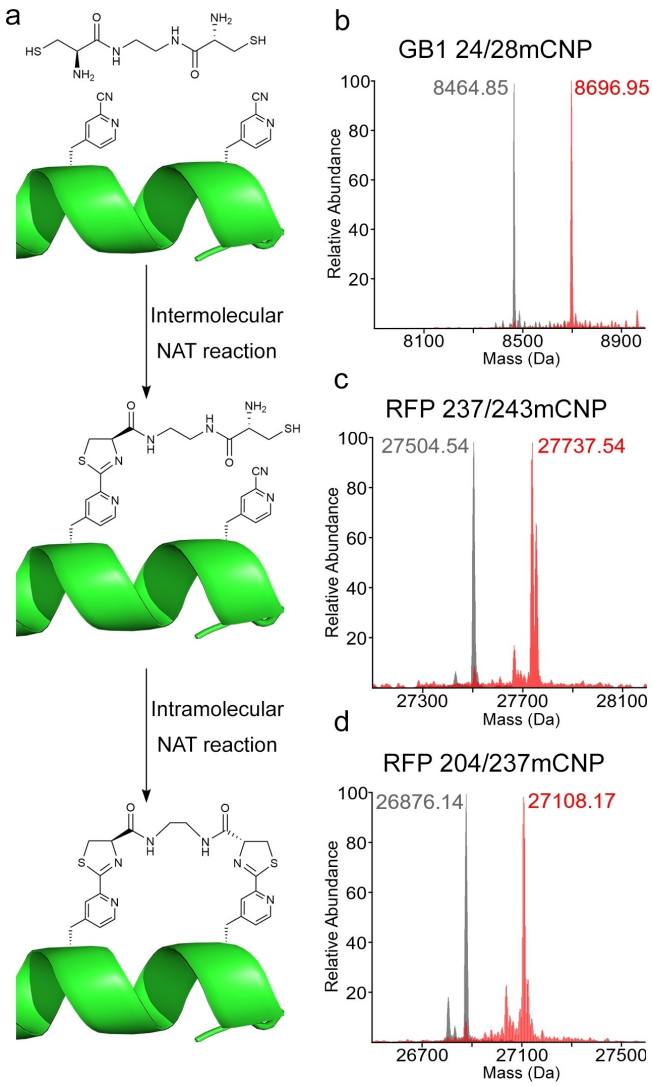

Figure 3. Stapling by tandem intermolecular-intramolecular NAT reaction. a) Intermolecular NAT reaction of EDDC results in the formation of singly tagged GB1 sample, which spontaneously undergoes an intramolecular NAT reaction to form the cyclized product (PDB: 1PGB[23]). b)–d) Intact protein mass spectrum analysis of the reaction between GB1 A24X/K28X, RFP 237X/243X, or RFP A204X/237X after incubation with EDDC for 4 h at 25 °C. Monoisotopically deconvoluted mass spectra of RFP samples (Figure 1e, c, d) with annotation of the minor peaks due to oxidation and chromophore hydrolysis are shown in Figure S9. Expected masses of the unreacted proteins and the cyclized products are reported in Table S4.

chemically caged 1,2-aminothiol functionality depends on the provision of chemicals for decaging, compromising in vivo applications.[22]

The present work expands the number of genetically encoded noncanonical amino acids by two different versions of cyanopyridylalanine, which are privileged for NAT click reactions owing to a reactive nitrile group, which spontaneously reacts with 1,2-aminothiol compounds. Incorporation of mCNP or pCNP residues in a protein offers a multitude of possibilities for its site-specific conjugation with different tags under physiological conditions, which may contain fluorescent or other reporter groups. The PylRS-based system is particular attractive, as it is fully orthogonal in prokaryotic and also eukaryotic cells.[24] As the cyanopyridylalanine residues react with N-terminal cysteine, they afford a new approach for peptide and protein cyclisation. Two cyanopyridylalanine residues in a polypeptide chain provide a means for polypeptide stapling.

The NAT click reaction between cyanopyridylalanine residues and 1,2-aminothiols proceeds spontaneously after simple mixing. Unlike the copper catalyst in CuAAC reactions, which tends to generate reactive oxygen species that degrade proteins and are toxic to cells,[25] the absence of added catalyst in the NAT click reaction is a considerable advantage in modifying proteins within living cells. Beyond protein tagging, cyanopyridylalanines enable efficient protein macrocyclization in vitro and in cells, which we envisage to afford a convenient protein engineering tool to enhance the thermal and proteolytic stability of proteins, as well as for the in vivo generation of libraries of macrocyclic peptides and proteins.[26]

In conclusion, the biocompatible NAT click reaction between genetically encoded cyanopyridylalanine ncAAs and aminothiols offers an attractive tool for intra- and extracellular bioconjugation. The advantages offered by the NAT click reaction over previously reported bioorthogonal reactions makes it a highly valuable alternative to available protein ligation tools. The plasmids pRSF-G1mCNPRS and pRSF-G1pCNPRS have been deposited at Addgene (Watertown, MA) to support wide distribution (Addgene #174718 and #174719, respectively).

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Bioorthogonal Reaction · Cyanopyridylalanine · Genetic Encoding · Noncanonical Amino Acids · Protein Conjugation

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