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Ribosomal incorporation of cyclic β-amino acids into peptides using *in vitro* translation

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We demonstrate *in vitro* incorporation of cyclic β-amino acids into peptides by the ribosome through genetic code reprogramming. Further, we show that incorporation efficiency can be increased through the addition of elongation factor P.

Expanding nature's repertoire of ribosomal monomers could yield new classes of enzymes, medicines, and materials with diverse genetically encoded chemistry[1-5]. Already, efforts to expand the genetic code have shown that natural and engineered translation systems are capable of selectively incorporating a wide range of non-canonical monomers into polypeptides, especially at the N-terminus[6]. For example, genetic code reprogramming with the flexizyme system[7-9] (Fx, a transfer RNA(tRNA)-synthetase-like ribozyme that charges activated chemical substrates onto tRNAs) has shown incorporation of α-amino acids with non-canonical sidechains[10], β-amino acids[11-13], N-modified amino acids[14], hydroxyacids[15, 16], non-amino carboxylic acids[9, 17-19], thioacids[20], aliphatics[9], malonyl substrates[19], long-carbon chain amino acids (*e.g.*, γ-, etc.)[21], and even foldamers[22]. These achievements make possible novel peptide drugs[23-25] and new classes of sequence-defined polymeric materials, such as aramids[9, 19, 26].

While these works have deepened our understanding of molecular translation, they have also inspired continued studies. From a fundamental perspective, probing the limits of the natural translation apparatus will help determine the constraints on monomer size, shape, and chemistry that can be polymerized by the ribosome. From an application perspective, having access to an even broader repertoire of monomers for ribosome-mediated polymerization holds promise to further increase the number of bio-based products available through biomanufacturing.

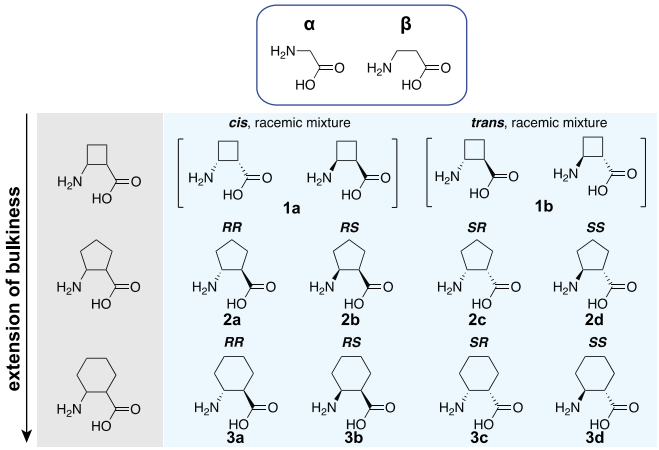

Fig. 1 Expanding the chemical substrate scope of ribosome-mediated polymerization to cyclic β-amino acid substrates. We explore the substrate specificity of the natural translation machinery for cyclic β-amino acid (cβAA) substrates using flexizyme-catalyzed acylation and ribosome-mediated incorporation. Ten non-canonical cβAAs comprising a variety of bulky cyclic structures are investigated.

Here, we set out to investigate the Fx-catalyzed tRNA charging of cyclic β-amino acids (cβAAs) and demonstrate subsequent *in vitro* incorporation of such amino acid derivatives into peptides by the ribosome. cβAAs were selected because, to our knowledge, they have not yet been incorporated into a growing polypeptide chain by the ribosome. Moreover, their rigid structure should produce different helix geometries and peptide turn characteristics that will help shed light on the limitations and monomer compatibility of the natural translation machinery. We specifically test three cyclic β-2,3-amino acid derivatives (2-aminocyclobutanecarboxylic acid, 2-aminocyclopentanecarboxylic acid, and 2-aminocylcohexane carboxylic acid) and their stereoisomers (**Fig. 1**). We first confirm that tRNA charging of cβAAs is possible. Then, we assess incorporation into either the N-terminus or C-terminus of a peptide using an *in vitro* ribosome-mediated protein synthesis platform (PURExpress™). Additionally, we investigate the effect of Elongation Factor P (EF-P), a bacterial protein translation factor, on C-terminal incorporation of different cβAA stereoisomers into a peptide in our reactions.

The goal of this work was to assess ribosomal synthesis of peptides with site-specifically introduced cβAAs. A key question

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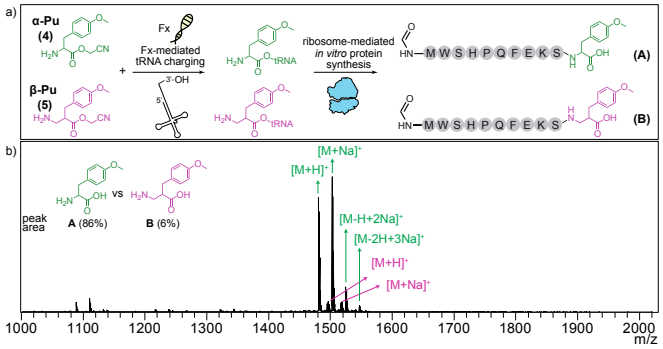

Fig. 2 Ribosomal incorporation of α- and β-amino acids. The peptides were prepared in the PURExpress™ system using Fx-mediated tRNA^{Pro1E2}(GGU), purified via the Strep tag (WSHPQFEK), and characterized by MALDI. The peptide containing α-Pu was found 14 times higher than the peptide with β-Pu at the C-terminus when the same amount of tRNA^{Pro1E2}(GGU) charged with α- and β-Pu was added to the PURE reaction, presumably because of the preference for L-α-amino acids of the natural translational machinery. The observed masses for the peptide with α-Pu incorporated at the C-terminus are 1481 $[M+H]^+$, 1503 $[M+Na]^+$, 1525 $[M-H+2Na]^+$, 1547 $[M-2H+3Na]^+$ Da and the peptides with β-Pu are 1496 $[M+H]^+$, 1518 $[M+Na]^+$ Da, respectively. Data are representative of three independent experiments.

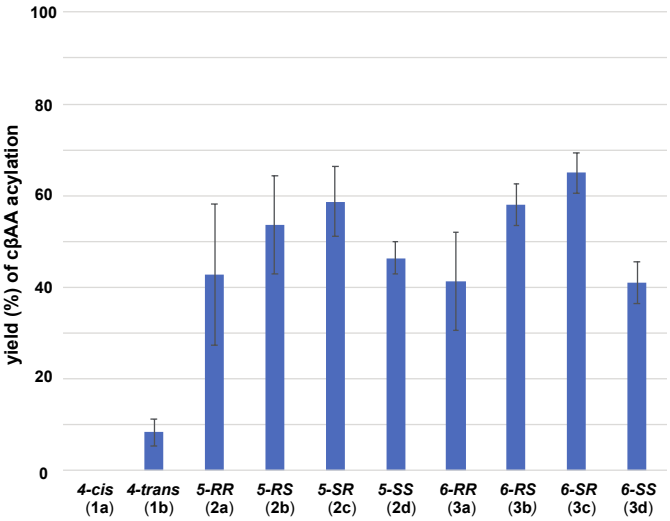

Fig. 3 The yield (%) of flexizyme-mediated acylation for the 10 cβAAs. The acylation reactions were performed using 6 different conditions (2 different pH (7.5 and 8.8) and 3 different Fx (e, d, aFx)) to find an optimized reaction condition. 4-cβAAs (**1a**-**1b**) were charged inefficiently presumably because of their propensity to form a cyclic product, lactam, while 5-cβAAs (**2a**-**2d**) and 6-cβAAs (**3a**-**3d**) were charged in high yield (40-60 %, n=3; mean values, where n is the number of independent experiments. See **Fig. S1**).

was to explore the possibility of incorporating such monomers at the C-terminus of a peptide. Before starting our investigation of cβAAs, we compared the translation machinery's compatibility of non-cyclic β-amino acids to that of α-amino acids in C-terminus incorporation. Two cyanomethylester (CME) substrates derived from α- and β-puromycin (Pu) containing a methoxybenzyl group on the α-carbon (**Fig. 2a**) were prepared. We intentionally avoided using a naturally occurring functional group (hence the methoxybenzyl group) in the comparison to eliminate any bias the translation machinery may have towards a naturally occurring amino acid (both carbon chain and functional group), allowing more direct comparison of the monomer backbones.

We used a short tRNA mimic (22nt), called the microhelix tRNA (mihx), to determine and optimize the yields of the Fx-mediated charging of the α- and β-Pu analogues[7, 8]. Yields were determined using an acidic polyacrylamide gel (**Fig. S1**). We found that both monomers were charged, with efficiencies of 31% and 87%, for the α- and β- substrates, respectively.

Next, we investigated whether the Fx substrates charged to tRNAs were accepted by the natural protein translation machinery. Given previous work with β-amino acids [11, 13], we expected this to occur. We performed Fx-mediated acylation of tRNA^{Pro1E2}(GGU) under the same reaction conditions obtained from the mihx experiment. Unreacted monomers were separated from tRNAs using ethanol precipitation[27] and the resulting tRNA fraction, which includes the substrate-charged tRNA (α-Pu:tRNA vs. β-Pu:tRNA), was added to the *in vitro* ribosome-mediated incorporation reaction (**Fig. 2a**). To normalize for differences in acylation yields, 2.8 times higher amounts of the α-Pu:tRNA ethanol precipitation sample was added to the final reaction (**Fig. S1d**). For ribosome-catalyzed incorporation, we used the PURExpress™ system (ΔtRNA, Δaa, NEB), which contains a minimal set of components required for protein translation. We supplemented into the reaction only the 9 amino acids required to express a Streptavidin tag (amino acid sequence M+ WSHPQFEK) with the puromycin-derivative substrates incorporated downstream of the tag at the ACC codon on the template messenger RNA (mRNA). After incubation, we isolated the resulting peptide using affinity-based purification and analyzed the peptide by mass spectrometry using MALDI. As expected, the peak corresponding to the theoretical mass of the peptide containing α-puromycin was higher than the peptide containing β-puromycin (**Fig. 2b**). It was ~14 times higher, indicating the natural translation system can incorporate monomers with α-amino acid backbones at higher efficiencies compared to β-amino acid backbones, which requires an engineered ribosome[12, 28, 29] for efficient incorporation.

Next, we sought to examine the natural ribosome's tolerance for different levels of steric bulkiness around the amine group. To test this, we designed three cβAAs containing a cyclobutyl, cyclopentyl, and cyclohexyl backbone with different stereoisomeric characteristics (**Fig. 1**). In a previous study[26], we synthesized two cyclopropyl ester substrates for Fx-mediated acylation using 2-aminocylcopropanecarboxylic acid (3-cβAA), however, the substrates were not able to be charged to tRNA by Fx presumably due to γ-characteristics in cyclic chain driving lactam formation. In this study, we synthesized 10 additional dinitrobenzyl (DNB) ester substrates using cyclobutyl β-amino acids (4-cβAA) with two isomers (*cis* and *trans*), and cyclopentyl β-amino acids (5-cβAA) and cyclohexyl β-amino acids (6-cβAA) with 4 different stereoisomeric configurations (*1R,2R*, *1R,2S*, *1S,2R*, and *1S,2S*) on the α and β carbon, respectively. Fx-mediated acylation using mihx (**Fig. S1a-c**) was carried out and the best reaction conditions giving high acylation yields were determined. The acylation yields for 4-cβAA were observed to be low (0-9%, **Fig. 3**), presumably due to the δ-characteristics of the amine on the substrates, which can efficiently form a lactam with a 6-membered ring (see **Fig. S1e** for a proposed mechanism). This result is consistent with

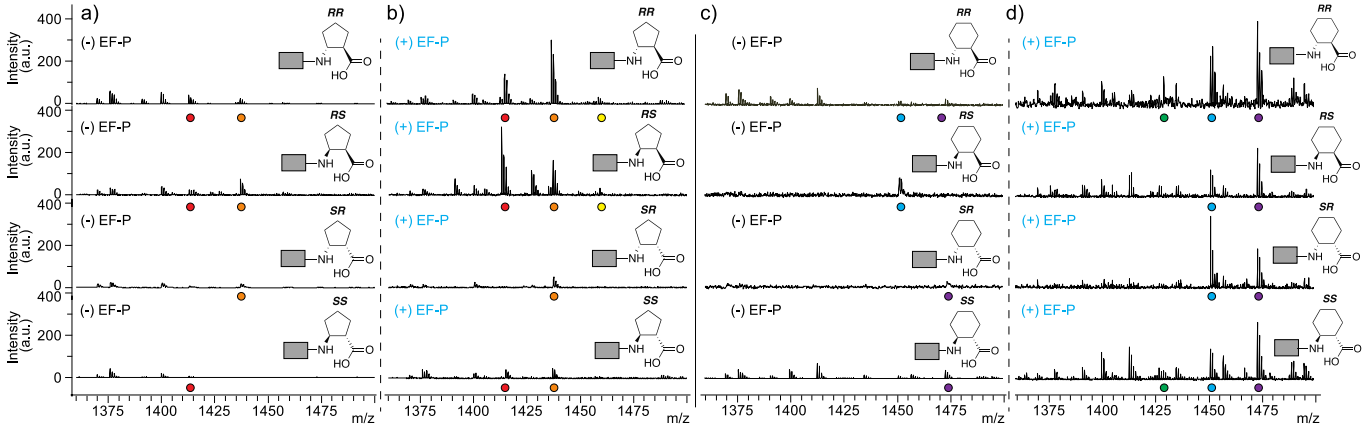

Fig. 4. Incorporation of bulky cβAAs in the presence of EF-P. 10 μM (in final) of EF-P in the *in vitro* protein translation system yields higher intensity of peptide containing a 5- and 6-cβAA at the C-terminus. The red, orange, and yellow circles represent the mass of peptide containing a 5-cβAA at the C-terminus, corresponding to $[M+H]^+$ = 1415, $[M+Na]^+$ = 1437, and $[M-H+2Na]^+$ = 1459, respectively. The green, blue, and purple represent the peptide containing a 6-cβAA with a mass of $[M+H]^+$ = 1429, $[M+Na]^+$ = 1451, $[M-H+2Na]^+$ =1473, respectively. See SI for full spectrum. The grey bar represents the peptide with a sequence of fMWSHPQFEKST, where fM is formylated Met.

our previous observation, where only 8% acylation onto mihx was observed for 5-aminopentanoic acid[26]. In contrast, the other eight 5- and 6-cβAA substrates showed high acylation yield (~30 to 67 %) as the formation of lactam via the intramolecular nucleophilic attack by the primary amine is significantly slowed. Interestingly, the yields of acylation varied by the configuration of the substrates even under the same reaction condition, indicating stereoisomers have different interactions with tRNA and the active site of Fx.

Next, we acylated the 4, 5, and 6-cβAAs onto $tRNA^{fMet}$, which decodes the AUG codon on mRNA, allowing incorporation of substrates at the N-terminus. Following acylation, purified tRNAs were for ribosome-mediated incorporation in the PURExpress™ translation reaction and the resulting peptides were analyzed by mass spectrometry as described above. A peak corresponding to the theoretical mass of peptides containing 4-cβAAs was not observed, most likely due to substrate limitations arising from low acylation yields. However, we found that 5- and 6-cβAAs that could be charged onto $tRNA^{fMet}$(CAU) were successfully incorporated into a peptide at the N-terminus (**Fig. S2 and S3**), which is in good agreement with the previous observation that the natural translational machinery is flexible towards extended backbone monomers for N-terminal incorporation[4, 6, 9, 22, 23, 30]. Full incorporation of the cβAAs was not observed and semi-quantitative analysis suggests a range of incorporation efficiencies between 7-64% **(Fig. S2 and S3)**. To test C-terminal incorporation, we repeated the experiment described above with 5- and 6-cβAAs acylated onto $tRNA^{Pro1E2}$(GGU) decoding a Thr (ACC) codon. Although the mass spectrometry data revealed limited yields of the desired product, all 5-cβAAs were found to be incorporated (**Fig. 4a**, peaks marked as red and orange circles), while corresponding peaks for (*1S*, *2R*)-6-cβAA were not found (**Fig. 4c**). These results (**Fig. S4** and **S5**) suggest that the natural ribosome is limited in elongation with substrates featuring modified backbones, where not only the position of the primary amine but the overall steric bulkiness around the amine may be relevant.

To address poor monomer compatibility with the translation apparatus, recent works have demonstrated the importance of optimizing translation factor concentrations and in particular EF-P[31]. EF-P is a bacterial translation factor that accelerates peptide bond formation between consecutive prolines, and has been shown to help alleviate ribosome stalling. In the case of β-amino acids, the use of engineered β-aminoacyl-tRNAs based on $tRNA^{Pro}$ in which the sequence of the T-stem and D-arm motifs, interacting with EF-Tu and EF-P, respectively, have been optimized increases incorporation efficiency[31].

Based on previous work, we hypothesized that EF-P would similarly enable higher incorporation of the cβAAs that are charged to $tRNA^{Pro1E2}$ bearing an engineered D-arm and T-stem[13]. To test this hypothesis, active EF-P was prepared by co-expressing three accessory genes, YjeA, YjeK, YfcM in *E. coli* as previously described[32] (see **SI** for detailed preparation). Purified EF-P (10 μM final concentration) was then supplemented into the PURE system containing the substrates charged to $tRNA^{Pro1E2}$(GGU). In the resulting MALDI spectra, we discovered peaks corresponding to the theoretical mass of a peptide containing all tested 5- and 6-cβAAs with significantly enhanced intensity (**Fig. 4b**,**d**, and **Fig. S6**) compared to the experiments performed without EF-P (**Fig. 4a**,**c**, and **Fig. S6**).

In summary, our work expands the range of backbone-extended amino acid substrates for molecular translation. Specifically, we showed that a diverse repertoire of 10 cβAAs amino acids could be acylated to tRNA by the Fx system and that these acylated tRNA-monomers could be used in ribosome-mediated polymerization using the wild type ribosome. We observed different levels of incorporation efficiency based on stereoisomeric properties and demonstrated that the combination of an engineered tRNA and additional EF-P improves cβAA incorporation. Our observations suggest opportunities for ribosome engineering[33].

Taken together, our results unlock the use of cβAAs for molecular translation. As such, we expect this work to motivate new directions in repurposing the translation machinery for monomers bearing such non-canonical structures. Ribosomally synthesized polymers containing site-specifically introduced cβAAs could lead to novel peptide drugs and peptide-based polymers that require programmed stereochemistry.

Conflicts of interest

M.C.J. and J.L. are co-inventors on the US provisional patent that incorporates discoveries described in this manuscript. All other authors declare no competing interests.

Acknowledgments

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Contributions

J.L., R.T., D.S.K., A.D.E., and M.C.J. planned and designed the research. J.L. and R.T. performed experiments. D.S.K. and M.B. prepared critical reagents and optimizations. J.L., D.S.K., and M.C.J wrote the manuscript. M.C.J. supervised this study.

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