

Expanded ribosomal synthesis of non-standard cyclic backbones *in vitro*

Corresponding Author: Professor Joongoo Lee

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications. Mentions of the other journal have been redacted.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I have reviewed the revised manuscript and read the authors' response letter to all reviewers. The authors did a substantial revision to their manuscript; however, I still have concerns about the revised version.

The main problem is that some of the key/important experiments suggested by the four reviewers were not performed. For example, the authors claimed that "...synthesizing such RiPPs extends beyond the scope of the current paper... the core focus of this paper is expanding the range of ribosome-mediated synthesis to include 5- and 6-membered rings." to Reviewer 1, "...this exploration goes beyond the scope of the current paper." to Reviewer 2, and "...demonstrating therapeutically relevant products is beyond the scope of this paper... To highlight, the core focus of this paper is to expand the ribosome's capacity to synthesize 5- and 6-membered rings *in vitro*" to Reviewer 4.

Without these experiments and controls required by the reviewers, the novelty and impact of the work are notably reduced. Because a similar work has been published previously using similar strategies, although in this work the authors claimed the synthesis of 5- and 6-membered rings peptides using ribosome. However, here is also the main concern (raised by all reviewers) that the second/cyclization reaction is not convincing without a clear experiment and mechanism. Based on this situation, the authors did not plan to demonstrate the application by synthesizing 'real' bioactive/ therapeutically relevant products (raised by the reviewers), which is not very understandable.

Reviewer #2

(Remarks to the Author)

The revised version of the manuscript addressed some of the issues.

However, two issues raised in my original comments (major comment 3) remain unresolved. In my view, this leaves the paper unsuitable for Nature Communications.

Specifically:

1) From the title of the paper, and throughout the text of the manuscript, the key message is that the ribosome can catalyze the formation of the cyclic structure. In my view, this message is principally wrong because the ribosome catalyzes formation of a single bond whereas the closure of the cycle occurs spontaneously. Conceptually, the reaction in their system is like the intramolecular formation of a disulfide bond within the protein molecule, but no one would claim that 'the ribosome catalyzes formation of the circle in the protein'! The message that the ribosome catalyzes formation of the cyclic structure is principally misleading. Here is an incomplete list of the statements in the manuscript that, in my view, are unacceptable: "This discovery represents the first example of the ribosome's ability to catalyze ring-closure reactions.", "show the ribosome's ability to directly synthesize new and diverse cyclic structures", "to further investigate the ribosome's ability to form cyclic structures", "this is the first demonstration of the ribosome's ability to modulate ring-closing reactions".

2) I remain absolutely unconvinced with the experiments on catalysis of peptide-like bond formation by the short RNA fragment, the P1c2 domain. The work this experiment relies upon (ref 17) remains highly controversial in the field. While claiming a dramatic result – catalysis of peptide bond formation by the fragment of rRNA - that work presented extremely

limited evidence of that and, similar to the current manuscript, lacked critical controls. Therefore, a simple reference to the results of that paper as an established fact, moreover, using those results as an indirect control for the new experiments, without even an attempt to reproduce the original findings are unacceptable. Furthermore, the authors claim that leaving out synthetic P1c2 RNA from the reaction is a sufficient negative control. I disagree. The relevant control would be the presence in the reaction of a different RNA (or better, the mutated P1c2 RNA) and demonstrating that no catalysis takes place. This control, however, was not done. Therefore, I remain convinced that this experiment must be left out in its entirety.

Reviewer #3

(Remarks to the Author)

This paper demonstrates that the ribosome is able to do a new type of chemical reaction leading to pyrazolidinedions or tetrahydropyridazinediones. This exact chemistry has not been reported before, and the authors do a nice job of showing that the initial attack of the alpha nitrogen on the ribose ester of the malonate or succinate followed by cyclization is the preferred mechanistic pathway. The chemistry, while novel, is not a huge leap from earlier work by Jewett (including some of the same co-authors) which uses the same hydrazine amino acid and reacts with a ketone rather than an ester.

This version of the manuscript is significantly improved based on addressing reviewer's comments. There are a couple of changes needed and additional experiments to do.

1. Using MALDI, the authors have quantified their yields relative to the standard peptides, and have found them to be ~15-fold worse. This is consistent with the poor signal to noise on many of the MALDI spectra. This is a significant drop and likely means that this technology is not amenable to library generation by mRNA display at least yet. The text in the article glosses over the reduced yields. They need to say in the text specifically that the yield drops 15-fold relative to all natural AAs.

2. The ribosome mimic experiments (Fig. 6), while creative, are not particularly convincing evidence for the ribosome-catalyzed nature of the cyclization for the following reasons:

- It's curious that they use the more cyclization competent malonate substrate for these studies, but the less efficient succinate substrates in Fig. 5. They should show that the succinate substrate is also able to cyclize with the ribosome mimic.
- As a control it is important to include a P1c mutant where some of the catalytic residues are mutated to reduce its similarity to the ribosome PTC and a mutant where the UGGU positioning residues are deleted to see if these are cyclization compatible.

Reviewer #4

(Remarks to the Author)

The authors have submitted an important paper that shows the ribosome can make peptides containing non-standard cyclic motifs such as 5- and 6-membered rings. In the revised version, they have addressed my concerns. I agree that efficiencies are low, which they acknowledge fairly, but that showing ribosome plasticity is important and so should be published.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed most of my concerns. I have no further comments.

Reviewer #2

(Remarks to the Author)

The revised version of the paper has adequately addressed one of my initial concerns. The presented data reasonably suggest that the ribosome facilitates cyclization following the first reaction step (the initial aminolysis catalyzed by the PTC). However, my second major concern remains unresolved. I remain unconvinced by the experiments involving the RNA-catalyzed reaction, as many critical claims in this section lack sufficient evidence. Below, I highlight a few of the specific issues:

1. Misleading Section Title (p.15):

The title, "Ribosomal catalysis is required for cyclization," is misleading. None of the experiments described in this section involve the ribosome.

2. Unsupported Structural Claim (p.15, l.5):

The statement, "P1c2 ... forming a dimer that creates a symmetrical three-dimensional structure similar to the ribosome's active site," is speculative. The authors provide no direct evidence supporting this claim. The referenced study (ref. 51) only indirectly suggests dimerization of this RNA fragment and does not provide data on its monomeric or dimeric structure. Furthermore, the active structure of the ribosomal catalytic center depends on numerous interactions with other rRNA

segments and ribosomal proteins. These stabilizing factors are absent in the isolated RNA construct, likely resulting in a drastically different structure.

3. Unsupported Structural Claim (p.15, l.8):

The assertion that “P2 represents an inactive version with an extended sequence that disrupts the formation of the stable ... dimeric structure” lacks experimental validation. The authors must either provide data proving P2 does not dimerize or reference prior work supporting this claim. Additionally, the reasoning that adding a small rRNA element (which interacts with the tRNA CCA end in the ribosome) renders the RNA construct inactive is unclear and unsubstantiated.

4. Unsupported and Contradictory Claims (p.15, l.15):

The claim that “analogues lacking the complementary UGGU sequence would fail to interact with tRNA, preventing the formation of a cyclic structure” contradicts the authors’ assertion that their RNA construct mimics the ribosome’s active site. Neither the A-site nor the P-site of the ribosome contains UGGU sequences that interact with tRNA. Notably, their inactive construct (P2) contains guanosines that position the P-site tRNA for catalysis, yet it is inactive, while the construct lacking these elements is claimed to be active. This inconsistency undermines their argument.

5. Unsupported Structural and Functional Claims (p.15, last lines):

The authors claim that the “P2c(UGGU) analogue ... retains activity for tRNA hybridization” without providing evidence. The presence of a UGGU sequence alone does not confirm tRNA hybridization. Data supporting this claim are necessary. These issues represent just a subset of the critical flaws in this section.

In summary, in my opinion, the inclusion of experiments involving the “catalytic RNA” is unnecessary and these experiments are tangential to the main focus of the paper. Rather than strengthening the manuscript, this section detracts from its otherwise compelling and well-supported findings.

In my opinion, the presented RNA-catalysis experiments render the paper incompatible with the standards of Nature Communications. Excluding this section would significantly enhance the manuscript’s overall quality and coherence.

Reviewer #3

(Remarks to the Author)

The new experiments in Fig. 6 support the ribosome-catalyzed mechanism and have addressed my major concern. This paper is now, in my opinion, suitable for Nature Communications.

Response to request to comment on reviewer #2’s ongoing concerns. Reviewer #2’s concerns are copied below, with comment from reviewer #3 indicated with “****”:

1. Misleading Section Title (p.15):

The title, “Ribosomal catalysis is required for cyclization,” is misleading. None of the experiments described in this section involve the ribosome.

***I agree that this title is a bit misleading. It needs to include something about this being a ribosome PTC mimic. This just requires a small adjustment.

2. Unsupported Structural Claim (p.15, l.5):

The statement, “P1c2 ... forming a dimer that creates a symmetrical three-dimensional structure similar to the ribosome’s active site,” is speculative. The authors provide no direct evidence supporting this claim. The referenced study (ref. 51) only indirectly suggests dimerization of this RNA fragment and does not provide data on its monomeric or dimeric structure. Furthermore, the active structure of the ribosomal catalytic center depends on numerous interactions with other rRNA segments and ribosomal proteins. These stabilizing factors are absent in the isolated RNA construct, likely resulting in a drastically different structure.

***The P1c2 dimeric structure is derived from the structure of the ribosomal peptidyltransfer RNA. I agree that it is not exactly the same as a fully formed ribosome. However there are a couple of papers that have studied these PTC dimers, and I believe the peptidyltransfer catalysis shown by these papers is real. The reviewer argument seem to be based on lack of trust in the published work, rather than the work presented herein.

3. Unsupported Structural Claim (p.15, l.8):

The assertion that “P2 represents an inactive version with an extended sequence that disrupts the formation of the stable ... dimeric structure” lacks experimental validation. The authors must either provide data proving P2 does not dimerize or reference prior work supporting this claim. Additionally, the reasoning that adding a small rRNA element (which interacts with the tRNA CCA end in the ribosome) renders the RNA construct inactive is unclear and unsubstantiated.

***I agree with this critique. It would be simple for the authors to test this and my recommendation is to request them to do so. A better mutant would be one that isn’t dimerization deficient, but rather one that has one of the core peptidyltransferase residues necessary for catalysis mutated. But, there are very few papers that study these minimalist PTCs, and to my knowledge none have tested a mutant like this for reduced activity. So this would be a high burden to ask of the authors as they would have to make the mutant and prove its lack of activity first on other substrates. The P2 mutant seems like a reasonable compromise to me, if they can prove it still binds to the tRNAs but does not dimerize.

4. Unsupported and Contradictory Claims (p.15, l.15):

The claim that “analogues lacking the complementary UGGU sequence would fail to interact with tRNA, preventing the formation of a cyclic structure” contradicts the authors’ assertion that their RNA construct mimics the ribosome’s active site. Neither the A-site nor the P-site of the ribosome contains UGGU sequences that interact with tRNA. Notably, their inactive construct (P2) contains guanosines that position the P-site tRNA for catalysis, yet it is inactive, while the construct lacking these elements is claimed to be active. This inconsistency undermines their argument.

***I think this critique is unfair. The original papers studying these PTC systems use the same strategy to bring the tRNAs in proximity and the authors are just following suit. The refuting argument made about P2 by this reviewer only makes sense if the P2 properly dimerizes. The authors assume it does not and their results are consistent (if they indeed show that P2 does not dimerize, see #3 above).

5. Unsupported Structural and Functional Claims (p.15, last lines):

The authors claim that the “P2c(UGGU) analogue ... retains activity for tRNA hybridization” without providing evidence. The presence of a UGGU sequence alone does not confirm tRNA hybridization. Data supporting this claim are necessary.

***I agree that some sort of gel-shift showing binding would be helpful to bolster their conclusions. Showing that the UGGU construct forms a complex with the tRNA and the control sequence does not would be helpful.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

I am afraid the additional experiments have not clarified the issue regarding the purported RNA-catalyzed cyclic hydrazinedione formation.

First, the presented data on tRNA binding to their proposed dimer is unconvincing. The intensity of the rRNA fragment bands varies dramatically, as evidenced by the stark differences in signal strength (compare the bands marked by blue arrowheads in lanes 5 and 6 vs. 10 and 11). Given this inconsistency, how can we be certain that if the amount of the material in samples in lanes 5 and 6 was as high as those in samples in lanes 10 and 11, we would not observe the ‘purple and red arrowhead’ bands in lanes 5 and 6 as well?

Second, the experimental setup for the duplex formation and detection experiment is concerning. According to the newly added procedures section, the rRNA fragments were hybridized in the presence of 60 mM MgCl₂. However, the samples were then analyzed using what they call an “EMSA native gel,” which lacks Mg²⁺ and instead contains 1 mM EDTA. This is particularly notable because the native gels referenced in the NAR paper contained 15 mM MgCl₂. Such a discrepancy raises questions about the comparability and reliability of the results.

This lack of consistency undermines the validity of their conclusions.

As a result, I remain as unconvinced about the reliability of the experiments involving the rRNA constructs as I was before the last revision.

Reviewer #3

(Remarks to the Author)

In this latest revision, the authors worked to address critiques that:

- (1) the P2c minimalist ribosome mutant does not dimerize and therefore can serve as a negative control for catalysis
- (2) the minimalist ribosomes that have the complementary UGGU motif are able to bind to tRNAs with an acceptor stem encoding ACCA, but those without this motif cannot.

The gel shown in new Figure S18 does clearly demonstrate point #1 (compare lanes 5 and 6 vs. 7 and 8). However; I have a very hard time agreeing with the author's conclusions about point #2. The additional bands labeled with the red purple and orange arrows in lanes 10-12 that purport to demonstrate complex formation with the tRNAs are very faint, if not non-existent. Additional assays are needed to definitively show selective complex formation between the UCCU containing ribosome mimics and tRNAs.

Version 3:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

Although the bands are faint, I am now convinced from the data in supplementary Figure 18 that:

1. P1c2 UGGU dimerizes and forms a complexes with tRNA-fMet (lane 5 vs 7).
2. P2c UGGU dimerizes and forms a complex with tRNA-fMet (lane 9 vs. 12).

I'm a little less convinced that the P1c2 lacking UCCU does not form complexes with the tRNA (lane 6 vs. 8), but I understand the high level of difficulty of these experiments. Still, points 1 and 2 above support the overall conclusion that the ribosome is involved in catalysis of both the peptide bond formation and the cyclization. I support publication of this paper in its current form.

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Reviewer #1

I have reviewed the revised manuscript and read the authors' response letter to all reviewers. The authors did a substantial revision to their manuscript; however, I still have concerns about the revised version.

The main problem is that some of the key/important experiments suggested by the four reviewers were not performed. For example, the authors claimed that "...synthesizing such RiPPs extends beyond the scope of the current paper... the core focus of this paper is expanding the range of ribosome-mediated synthesis to include 5- and 6-membered rings." to Reviewer 1, "...this exploration goes beyond the scope of the current paper." to Reviewer 2, and "...demonstrating therapeutically relevant products is beyond the scope of this paper... To highlight, the core focus of this paper is to expand the ribosome's capacity to synthesize 5- and 6-membered rings in vitro" to Reviewer 4.

Without these experiments and controls required by the reviewers, the novelty and impact of the work are notably reduced. Because a similar work has been published previously using similar strategies, although in this work the authors claimed the synthesis of 5- and 6-membered rings peptides using ribosome. However, here is also the main concern (raised by all reviewers) that the second/cyclization reaction is not convincing without a clear experiment and mechanism. Based on this situation, the authors did not plan to demonstrate the application by synthesizing 'real' bioactive/ therapeutically relevant products (raised by the reviewers), which is not very understandable.

We may not have fully conveyed the innovation and specific focus of our work, potentially leading to a mismatch in expectations.

However, we are unable to address the reviewer's suggestion for several reasons:

- **Research scope:** Our system is not intended for the large-scale synthesis of therapeutically relevant materials. Instead, we aimed to demonstrate that the direct synthesis of cyclic structure using ribosome could diversify and enhance of peptide libraries, increasing the chance of discovering novel drugs with non-canonical cyclic backbones. For example, the RaPID system, developed for peptide drug selection based on mRNA display, is used to screen potential peptide candidates targeting specific proteins. However, it does not support scalable synthesis; once potential peptide drugs are identified through the system, they are chemically synthesized on a large scale using solid-phase methods for downstream applications, including binding affinity measurements, preclinical animal studies, and clinical trials.

For this, we have clearly stated in our previous manuscript: "*Direct production of non-ribosomal peptide-like materials may significantly enhance the diversity of peptide drug candidates in a library, particularly when prepared for screening via drug discovery platforms. For example, the RaPID (Random non-standard Peptides Integrated Discovery) system leverages the advantages of diverse cyclic structures, which are crucial for drug efficacy, with template-guided synthesis, inherent to ribosome-based synthesis.*"

- **Cost constraints:** As noted, our ribosome-mediated reactions occur on a 10 μ L scale, producing 1–10 ng/ μ L of peptide. A single ~\$600 kit yields approximately 250 ng of peptide, resulting in a cost of ~\$2.4/ng. Producing ~1 mg of peptide for bioactivity testing would require over 1,000,000 PURExpress reactions, exceeding \$2,400,000 in costs, excluding additional expenses for flexizyme reactions, tRNA synthesis, and other reagents. The time and financial resources necessary for such an experiment make it infeasible for us to perform.

- **Ambiguity in 'bioactive' definition:** The terms 'bioactive' and 'therapeutically relevant' that the reviewer suggested are very broad, encompassing numerous biological functions. Even narrowing the scope to antibacterial activity, it is nearly impossible to identify an effective bioactive molecule without systematic selection or prediction tools.
- **Challenges in ad hoc synthesis:** Although cyclic structures are essential for the bioactivity of natural products like NRP, PKs, and RiPP, their bioactivities arise from the interplay of the cyclic scaffold with specific substituents that influence binding affinity or structural stability. For instance, the antibacterial activity of penicillin V is due to its unique bicyclic structure; a β -lactam ring fused to a thiazolidine ring. Isolated β -lactam (4-membered) or thiazolidine (5-membered) rings lack the stability and specificity to function as antibiotics. [Rolinson GN, Stevens S. 6-Aminopenicillanic acid IV. Antibacterial activity. Proceedings of the Royal Society of London Series B Biological Sciences 154, 509-513 (1961)]
- Nevertheless, to address the reviewer's suggestion, we synthesized and tested a cyclic motif for antibacterial activity. We benchmarked the molecular structure of 1-phenylpyrazolidine-3,5-dione (PZD, shown in panel A, Figure I), which is known to inhibit tyrosine kinase in *E. coli* and *P. aeruginosa*.

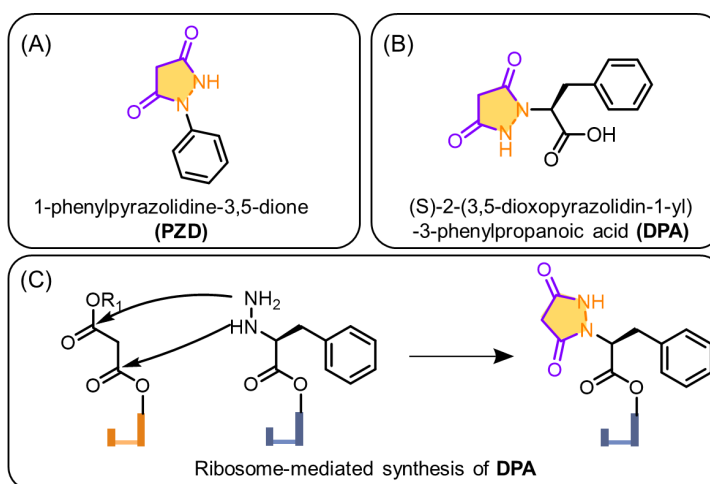


Figure I. (A) currently available antibacterial reagent (PZD). (B) The chemically synthesized derivative of PZD with a similar molecular structure. (C) A scheme showing that PZD can be synthesized using our ribosome-mediated approach.

- We designed and chemically synthesized (S)-2-(3,5-dioxypyrazolidin-1-yl)-3-phenylpropanoic acid (DPA, panel B, Figure I), a compound with structural similarity to PZD, because while unscalable, this could potentially be synthesized via our ribosome-mediated method using malonate and amino-L-phenylalanine (HzPhe) substrates.

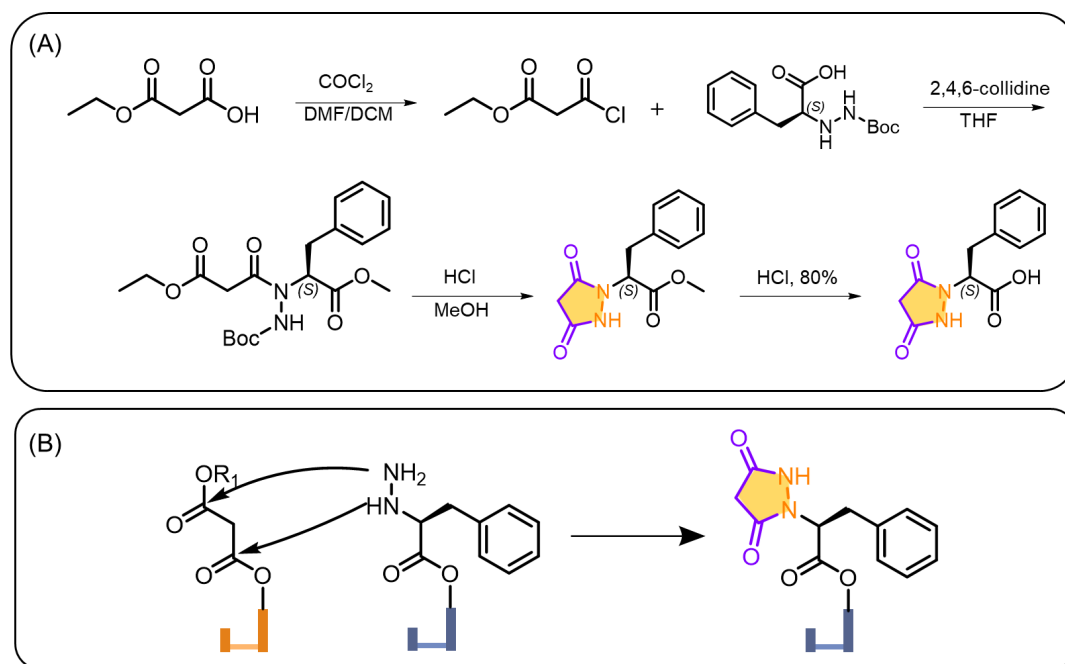


Figure II. (A) We performed the four chemical reactions to synthesize DPA. (B) In contrast, the ribosome-mediated synthesis would undergo a single-step to produce the same compound.

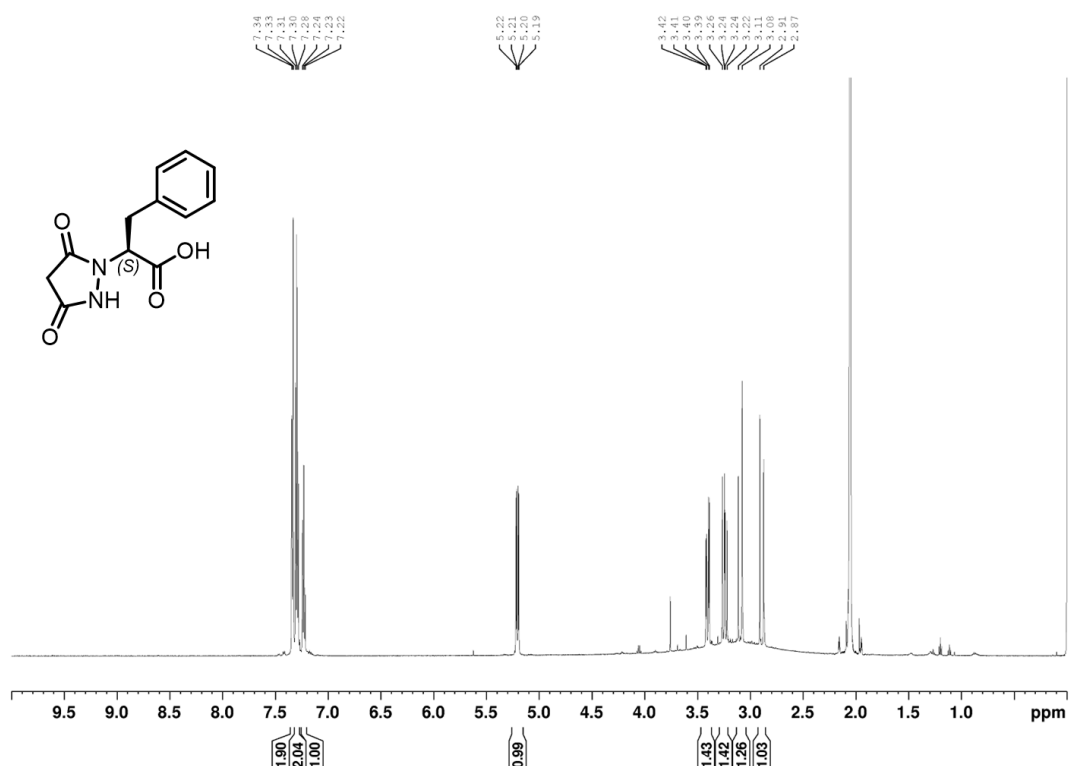


Figure III. NMR characterization of DPA

- Using the NMR-characterized DPA (Figure III), we performed bacterial growth inhibition assay on *E. coli* and *B. subtilis* (Figure IV). Treatment with kanamycin (red) fully inhibited bacterial growth, but DPA showed no inhibition for either gram-negative

or gram-positive bacteria (yellow). This result supports that while the cyclic motif is valuable, its standalone use is insufficient for providing 'bio (antimicrobial)' activity. Effective drug development requires understanding the interactions between the cyclic core and additional structural elements, alongside target mechanisms of action.

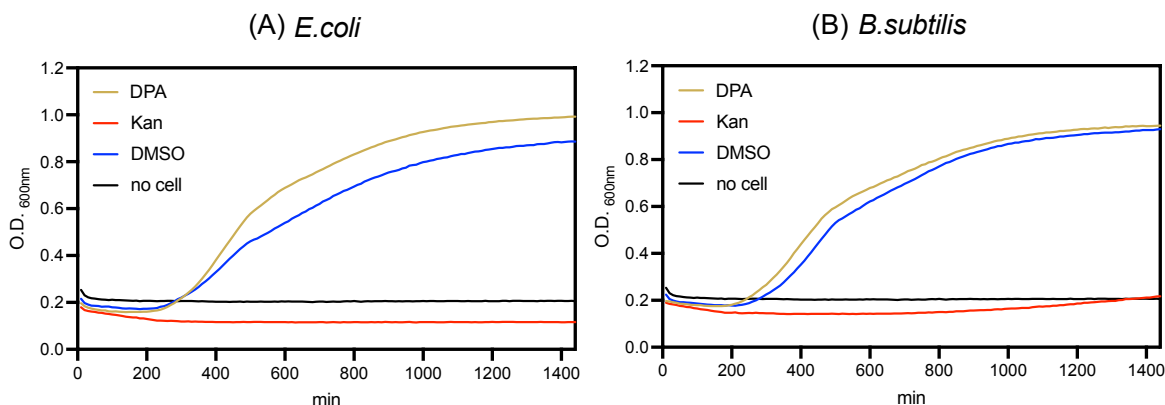


Figure IV. The growth rate assay for *E. coli* (A) and *B. subtilis* (B) were conducted using a final concentration of 1 mM DPA. Kanamycin (red line) served as the positive control, and all samples were dissolved in 4% DMSO. Treatment with 4% DMSO alone (blue line) showed no inhibition of cell growth.

- We do not conclude DPA is inherently 'bio-inactive' from these results. Instead, our findings indicate that DPA lacks antimicrobial activity against *E. coli* and *B. subtilis* under the specific culture condition we tested. While our studies provide a solid foundation for cyclic structure formation, further efforts in rational design, optimization, and functional validation of entire peptide structures are essential to discover therapeutically viable drugs.
- To reiterate, our results highlight the plasticity of the ribosome's ancient bond-formation mechanism and its ability to expand the range of cyclic backbones that can be synthesized. This catalytic plasticity has been observed in both remnants of the evolution of the genetic code (e.g., acceptance of both 2' and 3' tRNA esters with equal facility), and modern efforts to reprogram the genetic code (e.g., ribosomal incorporation of backbone-extended amino acids and ribosomal ester formation). Despite these advances, the boundaries of ribosome-mediated biosynthesis remain underexplored.
- Building upon the earlier work, we present, for the first time, evidence of ribosome-mediated synthesis of 5- and 6-membered hydrazinedione backbones through multiple aminolysis reactions. In addition, as Reviewer #3 noted, 'this exact chemistry has not been reported before' and we found that 'the initial attack of the alpha nitrogen on the ribose ester of the malonate or succinate, followed by cyclization, is the preferred mechanistic pathway'. We believe these findings open new avenues for understanding and leveraging ribosomal mechanisms to synthesize novel cyclic structures.
- To address the question about cyclization raised by other reviewers, we conducted additional experiments using multiple ribosome's peptidyl transferase center (PTC) analogues, including catalytically active and inactive variants. As expected, the desired product was synthesized only in the presence of an active PTC analogue. Based on these results, we conclude that the distinct RNA sequences in the active and inactive PTC analogues likely play a critical role in the second cyclization

mechanism. For further details, please refer to our response to Reviewer #2. While additional insights into the second cyclization reaction could be obtained through structural studies, we believe such studies lie beyond the scope of the present work.

Reviewer #2

The revised version of the manuscript addressed some of the issues. However, two issues raised in my original comments (major comment 3) remain unresolved. In my view, this leaves the paper unsuitable for Nature Communications.

We appreciate your thorough review of our revised manuscript, which was transferred from [Redacted], as well as the concerns you have raised. However, we believe one of the concerns may stem from a misunderstanding of the mechanism underlying our findings related to a post-translational modification reaction. We have clarified and addressed the issues below

Specifically:

1) From the title of the paper, and throughout the text of the manuscript, the key message is that the ribosome can catalyze the formation of the cyclic structure. In my view, this message is principally wrong because the ribosome catalyzes formation of a single bond whereas the closure of the cycle occurs spontaneously. Conceptually, the reaction in their system is like the intramolecular intramolecular formation of a disulfide bond within the protein molecule, but no one would claim that 'the ribosome catalyzes formation of the circle in the protein!' The message that the ribosome catalyzes formation of the cyclic structure is principally misleading. Here is an incomplete list of the statements in the manuscript that, in my view, are unacceptable: "This discovery represents the first example of the ribosome's ability to catalyze ring-closure reactions.", "show the ribosome's ability to directly synthesize new and diverse cyclic structures", "to further investigate the ribosome's ability to form cyclic structures", "this is the first demonstration of the ribosome's ability to modulate ring-closing reactions".

The reviewer appears to have overlooked key principles of our experiment, which we thoroughly described in the manuscript. Specifically, there seems to be a misunderstanding regarding the mechanism of one of the post-translational modifications (disulfide bond formation) and our intramolecular cyclization reaction. While both involve intramolecular reactions, they are fundamentally different:

- **Disulfide bond formation does not occur within the ribosome:** This post-translational modification involves the formation of a bond between two sulfhydryl groups of cysteine residues within a peptide, occurring after the peptide is synthesized. Importantly, this reaction takes place outside the ribosome.
- **The cyclization reaction does not occur spontaneously in solution, unlike disulfide bond formation:** As demonstrated by extensive experimental evidence provided in **Figures 5–7** (pages 14–18) and **Supplementary Figures 16–18**, the intramolecular ring-closing reaction occurs exclusively within the ribosome. Our data clearly show that this reaction does not occur spontaneously in solution or through a post-translational mechanism.
- The reviewer's misunderstanding of this fundamental distinction appears to have led to an underestimation of the significance of our findings. To address any remaining confusion and further emphasize the importance of our work, we illustrate the differences between disulfide bond formation and our findings (Figure V). We hope this clarification resolves any misunderstandings and highlights the novelty and significance of our study.

Reviewer's misunderstanding of disulfide bond formation vs. our ring-closing reaction

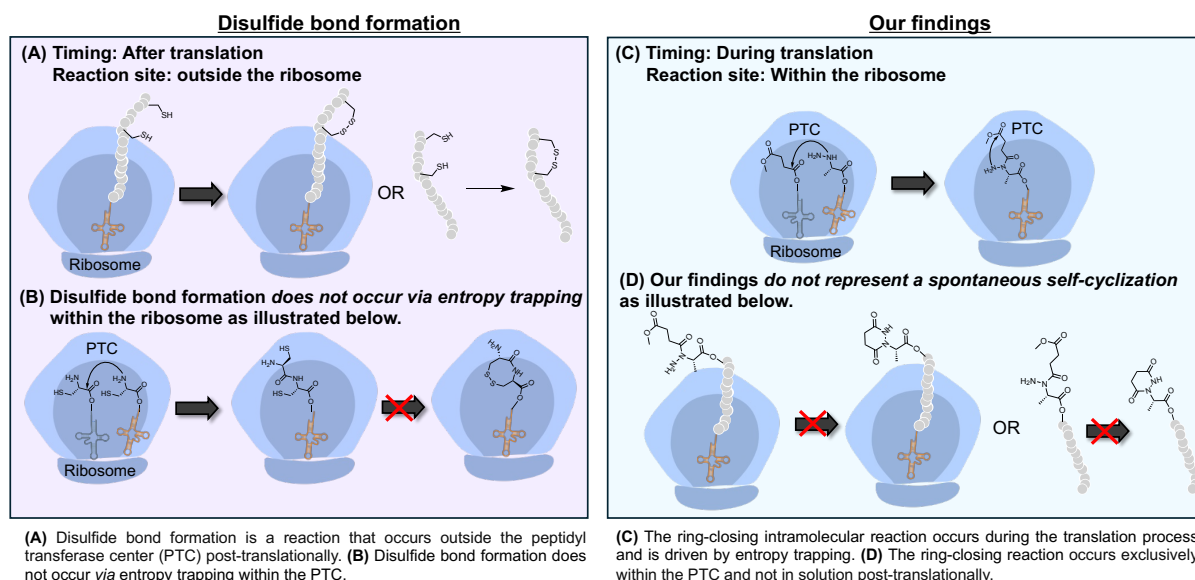


Figure V. Comparison disulfide bond to cyclization reaction within the ribosome

2) I remain absolutely unconvinced with the experiments on catalysis of peptide-like bond formation by the short RNA fragment, the P1c2 domain. The work this experiment relies upon (ref 17) remains highly controversial in the field. While claiming a dramatic result – catalysis of peptide bond formation by the fragment of rRNA - that work presented extremely limited evidence of that and, similar to the current manuscript, lacked critical controls. Therefore, a simple reference to the results of that paper as an established fact, moreover, using those results as an indirect control for the new experiments, without even an attempt to reproduce the original findings are unacceptable. Furthermore, the authors claim that leaving out synthetic P1c2 RNA from the reaction is a sufficient negative control. I disagree. The relevant control would be the presence in the reaction of a different RNA (or better, the mutated P1c2 RNA) and demonstrating that no catalysis takes place. This control, however, was not done. Therefore, I remain convinced that this experiment must be left out in its entirety.

This is a reasonable concern, and we appreciate the reviewer for raising this important question. We also considered the possibility that the peptide bond formation and cyclization could occur solely due to the proximity of the two substrates on tRNA, rather than the catalytic activity of P1c2. To investigate this, we conducted additional experiments using a different set of PTC analogues, including P2c, an inactive version with an extended sequence that disrupts the formation of stable and functional dimeric structure required for its catalytic activity.

- The experiment was performed using a set of four different PTC analogues: P1c2^{UGGU}, P1c2, P2c^{UGGU}, and P2c. The UGGU sequence of P1c2^{UGGU} and P2c^{UGGU} corresponds to the complementary sequence (ACCA) of 3'-end of tRNA. This pairing positions the substrates in close proximity and aligns them precisely for peptide bond formation. Conversely, analogues lacking the complementary UGGU sequence would fail to interact with tRNA, preventing the critical alignment required for cyclic structure formation.
- We first synthesized the RNA constructs using in vitro transcription with T7 RNA

polymerase. We then separately charged substrates **m**, **s**, and **H_z** to tRNA^{ACCA} via Fx, purified them, and mixed **m** with **H_z** and **s** with **H_z** in separate solutions containing P1c2^{UGGU}.

- The extracted ion chromatogram for each reaction yielded a single peak at 5.0 and 3.3 min corresponding to the mass of the 5- and 6-membered hydrazinedione, respectively (calculated [M+H]⁺: 422.141, observed [M+H]⁺: 422.145 in **Figure VI-A** (blue) and calculated [M+H]⁺: 436.158, observed [M+H]⁺: 436.158 in **Figure VI-B** (blue)). Notably, no peaks corresponding to the 5- or 6-membered rings were observed in the experiments containing P1c2 (**Figures VI-A and B** (green)). This indicates that the bond formation reaction does not occur without the interaction of tRNAs through the UGGU and ACCA sequence, which are essential for bringing the substrate into proximity at the catalytic site of P1c2.

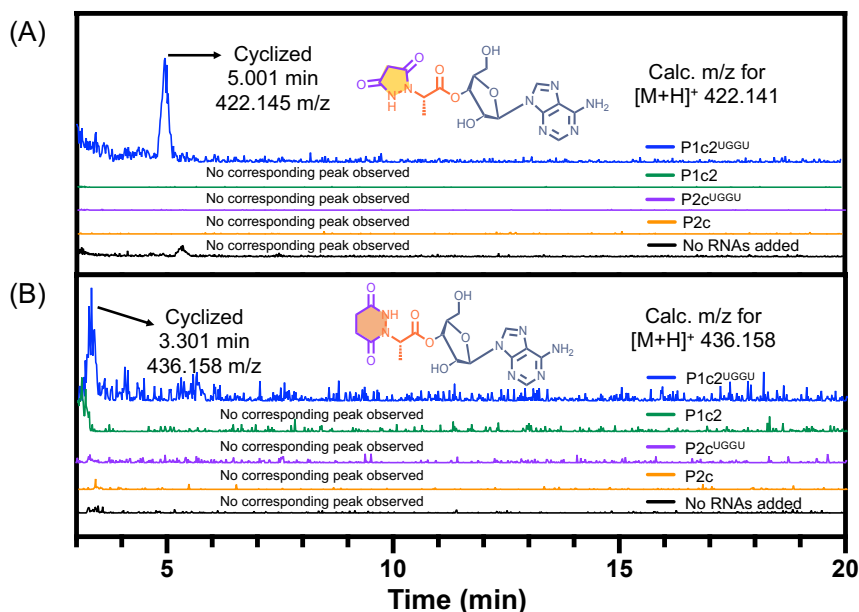


Figure VI. The extracted ion chromatograms show a single peak corresponding to the theoretical mass of a 5- and 6-membered ring linked to the 3'-OH of adenosine, only in the presence of catalytically functional P1c2UGGU in the solution (blue, [M+H]⁺ = 422.141 (cal), [M+H]⁺ = 422.145 (obs) for 5-membered ring and [M+H]⁺ = 436.158 (cal), [M+H]⁺ = 436.158 (obs) for 6-membered ring). P1c2, lacking the interaction of tRNA due to the elimination of the UGGU sequence, does not exhibit the ability to catalyze the peptide bond formation (green). Similarly, reactions with P2cUGGU, of which structure is mutated to be inactive, produce no cyclic products (purple), suggesting that simple proximity is insufficient for cyclic structure formation. Additionally, reactions with P2c (orange), which lacks both catalytic activity and tRNA interaction, yield no corresponding products. No corresponding products were observed in the absence of PTC analogues (black).

- Importantly, the cyclic structure formation could potentially occur simply due to the proximity of the two substrates on tRNA, rather than the catalytic activity of P1c2. To address this possibility, we conducted additional experiments using the P2c^{UGGU} analogue, which is inactive for peptide bond formation but retains activity for tRNA hybridization. No peaks corresponding to the masses of 5- or 6-membered rings were detected in samples catalyzed by the P2c^{UGGU} domain (**Figures VI-A and B**, purple), strongly suggesting that proximity alone is insufficient to drive peptide and ring formation. Similarly, the P1c2 analogue lacking the UGGU sequence also failed to

produce the expected product (Figures VI-A and B, orange). These findings suggest that the distinctive sequences of P1c2 and P2c may include key residues required for the catalytic activity in peptide bond formation. Not surprisingly, no peak for the 5- and 6-membered cyclic hydrazinedione was detected in the absence of PTC analogues (Figures VI-A and B, black).

- Shown below in Figure VII is the sequence alignment results and a 2D schematic of the secondary structure below that highlights the differences between P1c2 (70-mer) and P2c (97-mer).

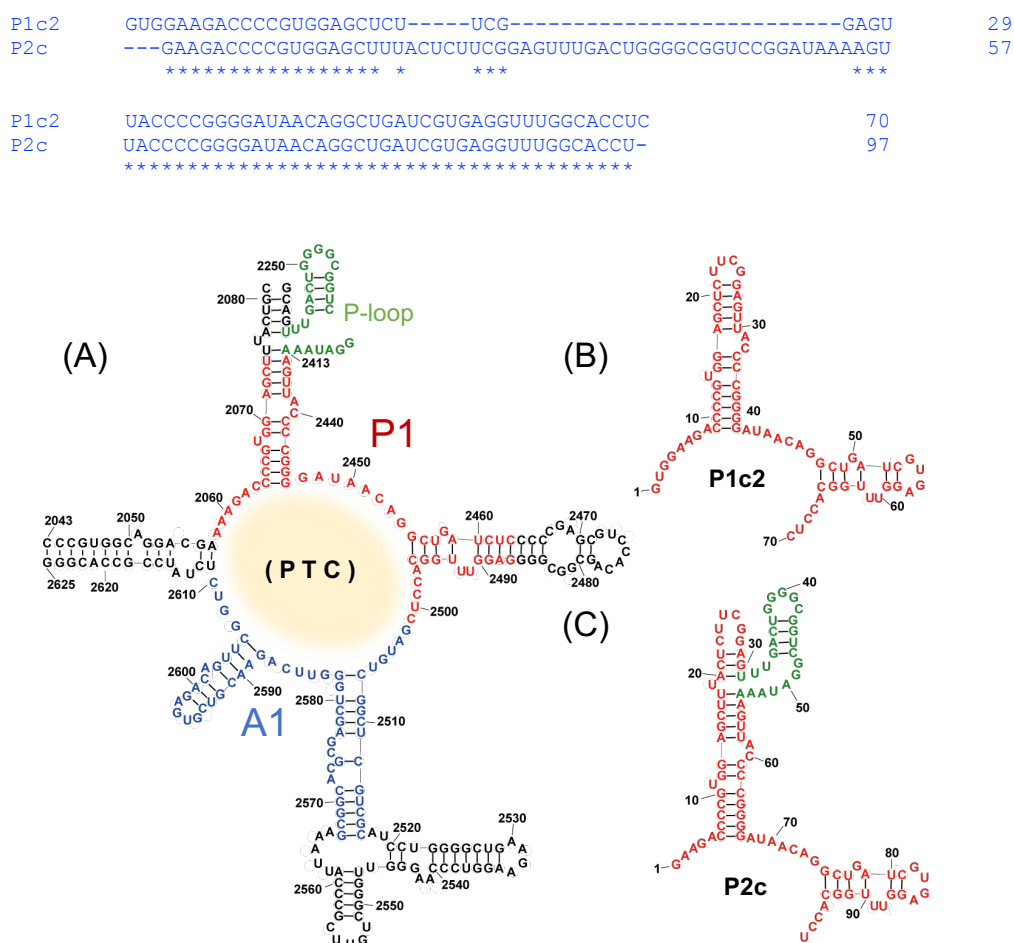


Figure VII. Sequence comparison of the two PTC analogues, P1c2 and P2c. (A) The peptidyltransferase center (PTC) in the ribosome's large subunit is the catalytic center that facilitates peptide bond formation. The PTC is structurally composed of two major regions: P1 and A1. (B) The P1c2 domain (70-mer) is a functional RNA molecule that mimics the PTC of *T. thermophilus* 23S ribosomal RNA. It is derived from the P1 region within domain V of the ribosome. This domain forms a dimer, creating a symmetrical 3D structure resembling the ribosome's active site and capable of catalyzing peptide bond formation. (C) The P2c domain (97-mer) is also derived from the P1 within domain V of the ribosome, however, it includes an additional P-loop (green), which disrupts the formation of a stable dimeric structure and prevents it from catalyzing peptide bond formation.

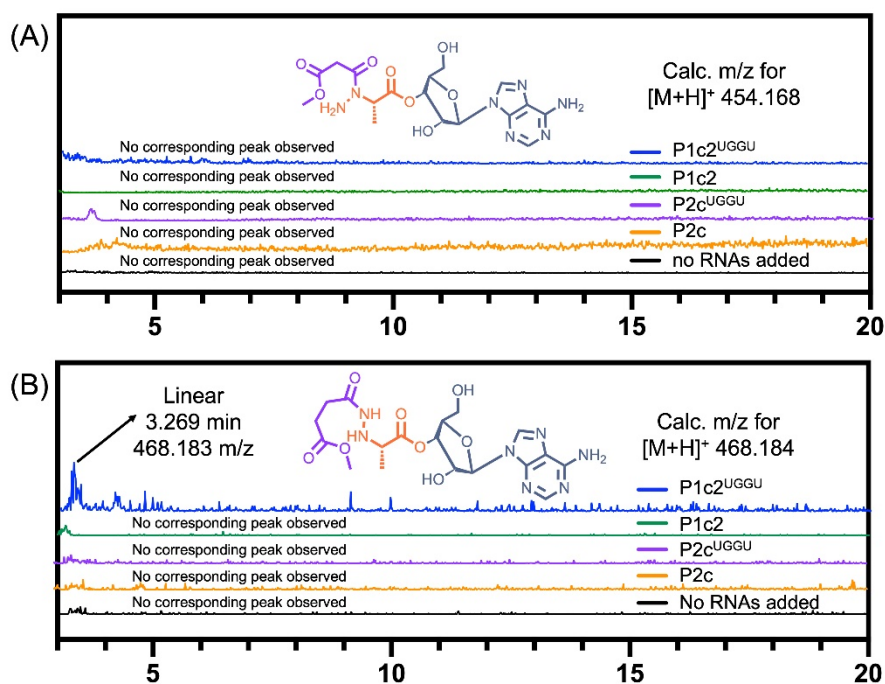


Figure VIII. Monitoring linear product formation through ribosome's catalytic domain analogues (P1c2^{UGGU}, P1c2, P2c, and P2c^{UGGU}). We used extracted ion chromatograms to analyze the formation of linear products within the ribosome's catalytic domain analogues (P1c2^{UGGU}, P1c2, P2c^{UGGU}, and P2c) for the reaction of malonate ester (**m**) and succinate ester (**s**) with hydrazine ester (**Hz**). (A) Consistent with the reactions of **m** and **Hz** with the full ribosome shown in **Figure 3C**, no linear product linked to the 3'-OH of adenosine was detected (blue). (B) Similarly, consistent with the reactions of **s** and **Hz** with the full ribosome shown in **Figure 3D** yielding a linear product, a single peak at 3.269 min corresponding to the theoretical mass of a linear product linked to the 3'-OH of adenosine was detected ($[M+H]^+ = 468.184$ (cal), $[M+H]^+ = 468.189$ (obs)). No such peaks were detected in any of the control experiments (green, purple, orange, and black). These findings suggest that P1c2^{UGGU} exhibits biochemical properties comparable to those of the full ribosome, reinforcing its catalytic similarity in facilitating specific reactions.

- The reactions products produced in the presence of P1c2^{UGGU} are consistent with the products obtained in the experiments conducted with the full ribosome (Figure VIII). While no linear product (calculated $[M+H]^+ : 454.168$) was detected in any of the 5-membered hydrazinedione formation reactions of **m** and **Hz**, a linear product (calculated $[M+H]^+ : 468.183$) was observed in the 6-membered formation reaction of substrate **s** and **Hz**.
- We have incorporated these changes into pages 15-18 of the revised manuscript accordingly.

Reviewer #3

This paper demonstrates that the ribosome is able to do a new type of chemical reaction leading to pyrazolidinediones or tetrahydropyridazinediones. This exact chemistry has not been reported before, and the authors do a nice job of showing that the initial attack of the alpha nitrogen on the ribose ester of the malonate or succinate followed by cyclization is the preferred mechanistic pathway. The chemistry, while novel, is not a huge leap from earlier work by Jewett (including some of the same co- authors) which uses the same hydrazine amino acid and reacts with a ketone rather than an ester.

This version of the manuscript is significantly improved based on addressing reviewer's comments. There are a couple of changes needed and additional experiments to do.

- We appreciate the positive assessment for our work.

1. Using MALDI, the authors have quantified their yields relative to the standard peptides, and have found them to be ~15-fold worse. This is consistent with the poor signal to noise on many of the MALDI spectra. This is a significant drop and likely means that this technology is not amenable to library generation by mRNA display at least yet. The text in the article glosses over the reduced yields. They need to say in the text specifically that the yield drops 15-fold relative to all natural AAs.

- We have added that the yield of product produced through this system is 15-fold lower compared to the yield of peptide composed of all natural amino acids.

We now write on page 21:

"Admittedly, the product yield in the PURExpress system is low (~10 ng per 10 μ L reaction scale, 15-fold lower to the typical amount of peptide composed of natural amino acids)."

2. The ribosome mimic experiments (Fig. 6), while creative, are not particularly convincing evidence for the ribosome-catalyzed nature of the cyclization for the following reasons:

a. It's curious that they use the more cyclization competent malonate substrate for these studies, but the less efficient succinate substrates in Fig. 5. They should show that the succinate substrate is also able to cyclize with the ribosome mimic.

b. As a control it is important to include a P1c mutant where some of the catalytic residues are mutated to reduce its similarity to the ribosome PTC and a mutant where the UGGU positioning residues are deleted to see if these are cyclization compatible.

- This is an excellent point highlighting the catalytic activity of the ribosome's RNA domain in forming peptide and cyclic products. We sincerely appreciate the reviewer's effort to enhance the significance of our study.
- As suggested by the reviewer, we conducted additional experiments using a different set of substrates and PTC analogues. For Question (a), we designed the experiment for the formation of 6-membered ring using substrates **s** and **H_z**. For Question (b), we synthesized a set of 4 different PTC analogues including P2c, an inactive version with an extended sequence that disrupts the formation of stable and functional dimeric structure required for its catalytic activity.

- The experiments were performed using 4 different RNAs: P1c2^{UGGU}, P1c2, P2c^{UGGU}, and P2c. The UGGU sequence of P1c2^{UGGU} and P2c^{UGGU} corresponds to the complementary sequence (ACCA) of 3'-end of tRNA. This pairing positions the substrates in close proximity, aligning them precisely for peptide bond formation. Conversely, analogues lacking the complementary UGGU sequence would fail to interact with tRNA, preventing the critical alignment required for cyclic structure formation. By comparing the results from these analogues, we aim to determine whether peptide bond formation and cyclization occurs due to the catalytic activity of PTC analogues or the proximity of the two substrates on tRNA.
- From the extracted ion chromatogram for the 6-membered ring formation, we identified a single peak at 3.3 min, corresponding to the mass of the 6-membered hydrazinedione, (calculated [M+H]⁺: 436.158, observed [M+H]⁺: 436.158 in Figure IX-B (blue)). Notably, no peaks corresponding to the 6-membered rings were observed in the experiments containing P1c2 (Figure IX-B (green)). This indicates that the bond formation reaction does not occur without the interaction of tRNAs through the UGGU and ACCA sequence, which are essential for bringing the substrate into proximity at the catalytic site of P1c2.

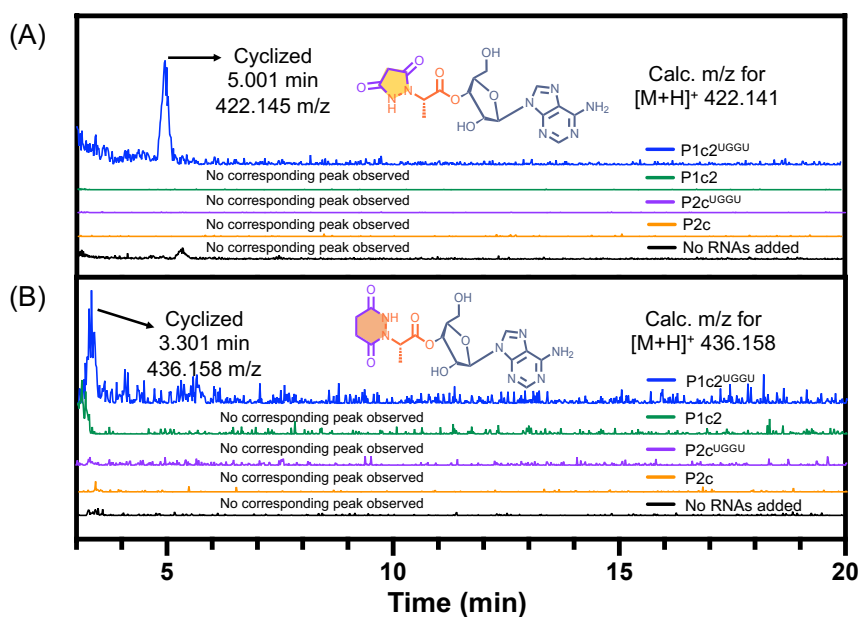


Figure IX. The extracted ion chromatograms show a single peak corresponding to the theoretical mass of a 5- and 6-membered ring linked to the 3'-OH of adenosine, only in the presence of catalytically functional P1c2^{UGGU} in the solution (blue). [M+H]⁺ = 422.141 (cal), [M+H]⁺ = 422.145 (obs) for 5-membered ring, [M+H]⁺ = 436.158 (cal), [M+H]⁺ = 436.158 (obs) for 6-membered ring. P1c2, lacking the interaction of tRNA due to the elimination of the UGGU sequence, does not exhibit the ability to catalyze the peptide bond formation (green). Similarly, reactions with P2c^{UGGU}, which is mutated to be inactive, produce no cyclic products (purple), suggesting that simple proximity is insufficient for cyclic structure formation. Additionally, reactions with P2c (orange), which lacks both catalytic activity and tRNA interaction, yield no corresponding products. Reactions without PTC analogues did not produce any corresponding products (black).

- Importantly, the cyclic structure formation could potentially occur simply due to the proximity of the two substrates on tRNA, rather than the catalytic activity of P1c2. To

address this possibility, we conducted additional experiments using the P2c^{UGGU} analogue, which is inactive for peptide bond formation but retains activity for tRNA hybridization. No peaks corresponding to the masses of 5- or 6-membered rings were detected in samples catalyzed by the P2c^{UGGU} domain (Figures IX-A and B, purple), strongly suggesting that proximity alone is insufficient to drive peptide and ring formation. Similarly, the P2c analogue lacking the UGGU sequence also failed to produce the expected product (Figures IX-A and B, orange). These findings suggest that the distinctive sequences of P1c2 and P2c may include key residues required for the catalytic activity in peptide bond formation. Not surprisingly, no peak for the 5- and 6-membered cyclic hydrazinedione was detected in the absence of PTC analogues (Figures IX-A and B, black).

- We show sequence alignment results and a 2D schematic of the secondary structure below to highlight the differences between P1c2 (70-mer) and P2c (97-mer) (Figure X).

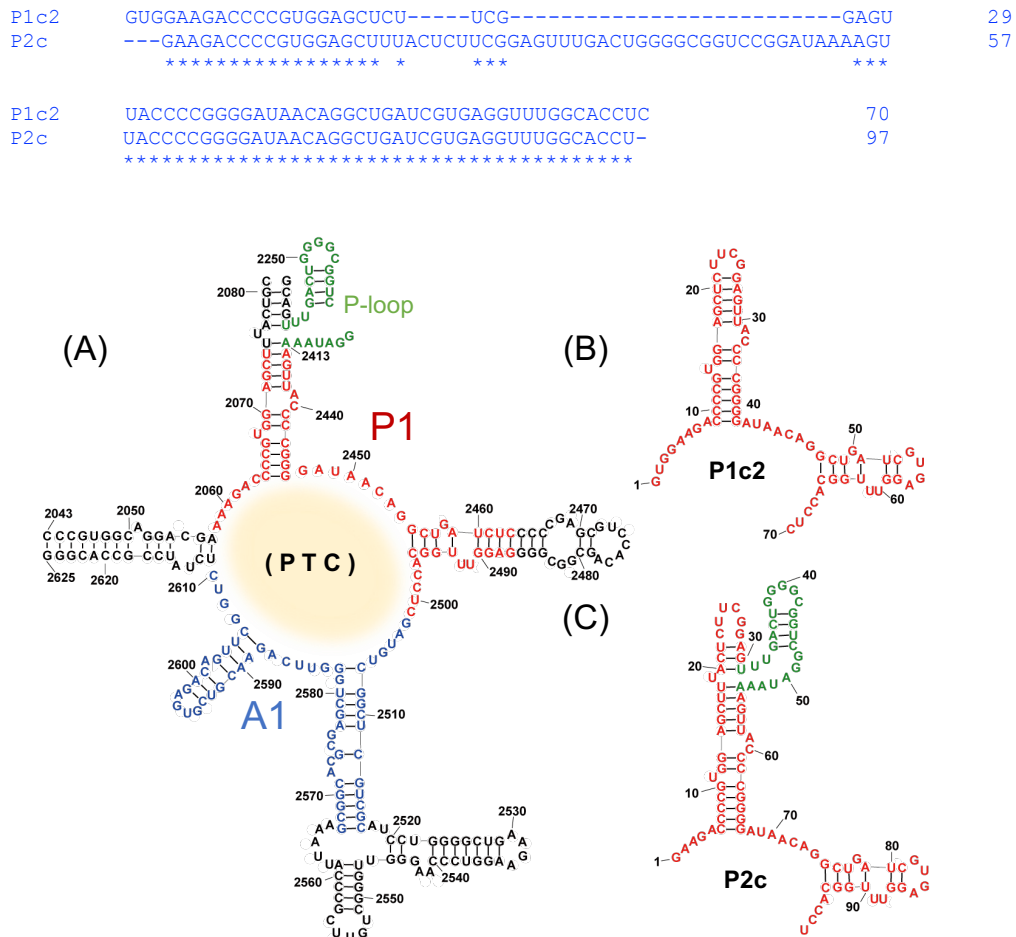


Figure X. Sequence comparison of the two PTC analogues, P1c2 and P2c. (A) The peptidyltransferase center (PTC) in the ribosome's large subunit is the catalytic center that facilitates peptide bond formation. The PTC is structurally composed of two major regions: P1 and A1. (B) The P1c2 domain (70-mer) is a functional RNA molecule that mimics the PTC of *T. thermophilus* 23S ribosomal RNA. It is derived from the P1 region within domain V of the ribosome. This domain forms a dimer, creating a symmetrical 3D structure resembling the ribosome's active site and capable of catalyzing peptide bond formation. (C) The P2c domain (97-mer) is also derived from the P1 within domain V of the ribosome, however, it includes an additional P-loop

(green), which disrupts the formation of a stable dimeric structure and prevents it from catalyzing peptide bond formation.

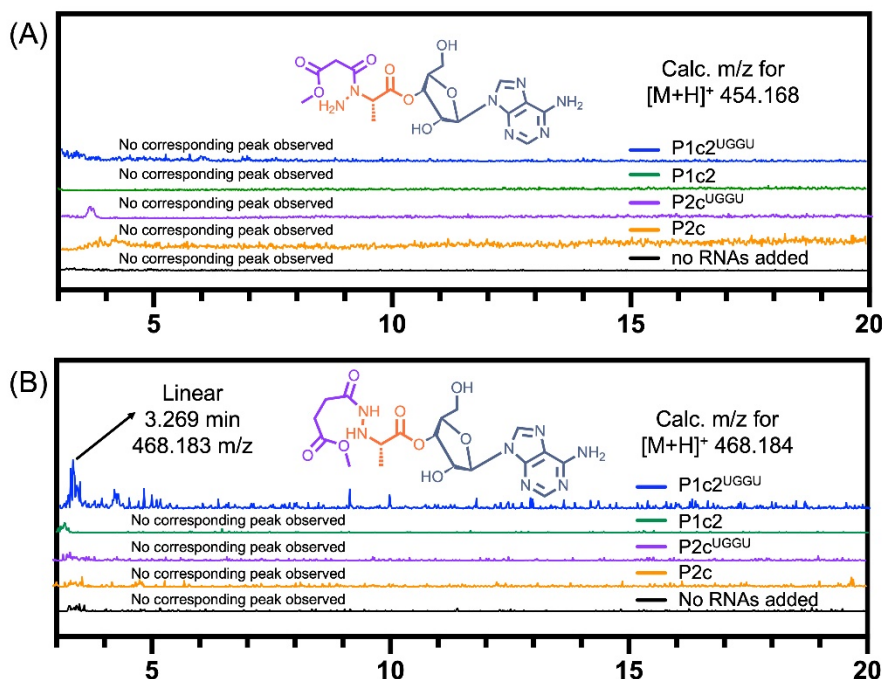


Figure XI. Monitoring linear product formation through ribosome's catalytic domain analogues (P1c2^{UGGU}, P1c2, P2c^{UGGU}, and Pc2). We used extracted ion chromatograms to analyze the formation of linear products within the ribosome's catalytic domain analogues (P1c2^{UGGU}, P1c2, P2c^{UGGU}, and Pc2) for the reaction of malonate ester (**m**) and succinate ester (**s**) with hydrazine ester (**Hz**). (A) Consistent with the reactions of **m** and **Hz** with the full ribosome shown in **Figure 3C**, no linear product linked to the 3'-OH of adenosine was detected (blue). (B) Similarly, consistent with the reactions of **s** and **Hz** with the full ribosome shown in **Figure 3D** yielding a linear product, a single peak at 3.269 min corresponding to the theoretical mass of a linear product linked to the 3'-OH of adenosine was detected ($[M+H]^+ = 468.184$ (cal), $[M+H]^+ = 468.189$ (obs)). No such peaks were detected in any of the control experiments (green, orange, purple, and black). These findings suggest that P1c2^{UGGU} exhibits biochemical properties comparable to those of the full ribosome, reinforcing its catalytic similarity in facilitating specific reactions.

- While no linear product (calculated $[M+H]^+ : 454.168$) was detected in any of the 5-membered hydrazinedione formation reactions, a single peak corresponding to the theoretical mass of a linear product was observed exclusively in the 6-membered formation reaction of substrate **s** and **Hz** in the presence of P1c2^{UGGU} (Figure XI). These findings are consistent with the result obtained in the experiments conducted with the full ribosome.
- We have incorporated these changes into pages 15-18 of the revised manuscript accordingly.

Reviewers #2 and #3

#3. Unsupported Structural Claim (p.15, l.8):

The assertion that “P2 represents an inactive version with an extended sequence that disrupts the formation of the stable ... dimeric structure” lacks experimental validation. The authors must either provide data proving P2 does not dimerize or reference prior work supporting this claim. Additionally, the reasoning that adding a small rRNA element (which interacts with the tRNA CCA end in the ribosome) renders the RNA construct inactive is unclear and unsubstantiated.

***I agree with this critique. It would be simple for the authors to test this and my recommendation is to request them to do so. A better mutant would be one that isn't dimerization deficient, but rather one that has one of the core peptidyltransferase residues necessary for catalysis mutated. But, there are very few papers that study these minimalist PTCs, and to my knowledge none have tested a mutant like this for reduced activity. So this would be a high burden to ask of the authors as they would have to make the mutant and prove its lack of activity first on other substrates. The P2 mutant seems like a reasonable compromise to me, if they can prove it still binds to the tRNAs but does not dimerize.

#5. Unsupported Structural and Functional Claims (p.15, last lines):

The authors claim that the “P2c(UGGU) analogue ... retains activity for tRNA hybridization” without providing evidence. The presence of a UGGU sequence alone does not confirm tRNA hybridization. Data supporting this claim are necessary.

***I agree that some sort of gel-shift showing binding would be helpful to bolster their conclusions. Showing that the UGGU construct forms a complex with the tRNA and the control sequence does not would be helpful.

- We thank the reviewers for their constructive critiques. We especially appreciate reviewer #3 for addressing reviewer #2's concerns.
- As suggested by the editor, we conducted additional experiments to address points #3 and #5. Consistent with previous results, we observed that the PTC analogues dimerize and hybridize with tRNAs, indirectly supporting our claim that peptide bond and cyclic structure formation is catalyzed by the ribosome. These results have been incorporated into the revised manuscript (main text and supplementary information (SI)).
- On page 15 of the main text, we now write:

“PTC analogues exhibit catalytic activity for cyclization.

To directly demonstrate that the cyclic hydrazinedione formation results from the ribosome's catalytic activity, we conducted an additional experiment using functional ribosome analogues that mimic the peptidyl transferase center (PTC) components of the bacterial 23S ribosomal RNA (**Figure 6A**). Specifically, we designed two PTC analogues, P1c2 (70-mer) and P2c (97-mer; Supplementary Information). P1c2 incorporates elements from the P1 of the ribosome's domain V (**Figure 6B**),^{55, 56} forming a dimer that creates a symmetrical three-dimensional structure similar to the ribosome's active site and is capable of catalyzing peptide bond formation (**Figure 6C**). In contrast, P2c represents an inactive version with an extended sequence that disrupts the formation of the stable and functional dimeric structure required for its

catalytic activity. We synthesized a set of four different PTC analogues (P1c2^{UGGU}, P1c2, P2c^{UGGU}, and P2c) and tRNA^{ACCA}. The UGGU sequence at the 3'-end of the PTC analogues was specifically designed to pair with tRNA molecules bearing a complementary 3'-ACCA sequence. To test whether the RNA constructs produced *in vitro* were functional, we first incubated the synthesized PTC analogues for 48 hours at 0 °C and observed that P1c2^{UGGU} and P1c2 formed a dimeric construct, while P2c^{UGGU} and P2c did not (**Supplementary Figure 18A**), consistent with previously reported results. Additionally, we incubated the PTC analogues with tRNA^{ACCA} and found that only P1c2^{UGGU} and P2c^{UGGU}, which contain the UGGU sequence, produced a new construct (**Supplementary Figure 18B**), likely indicating a hybridized RNA construct formed through UGGU-ACCA pairing.⁵⁷ We hypothesized that this pairing would position the substrates in close proximity for peptide bond formation. Conversely, analogues lacking the complementary UGGU sequence would fail to interact with tRNA, preventing the formation of a cyclic structure.

- On page 6 of SI, we added the procedures for the new experiments.

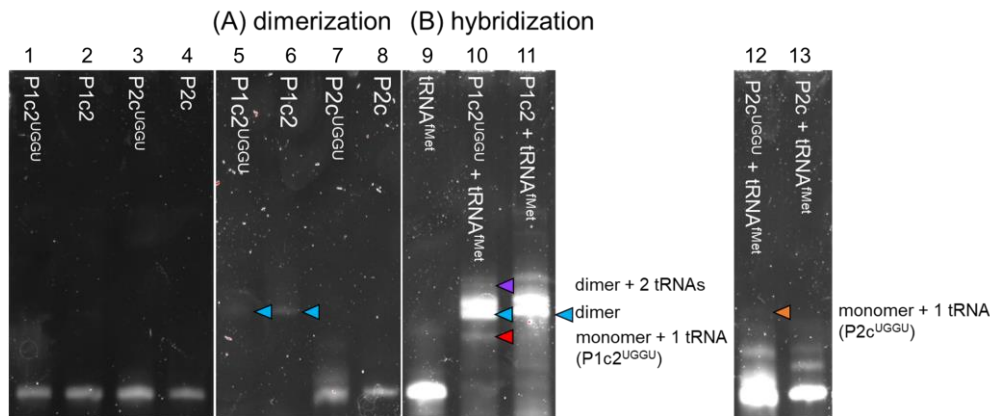
Dimerization and hybridization assays of PTC analogues

For dimerization of PTC analogues, 1 µL of 5 µM PTC analogues was mixed with 7 µL of 71.4 mM HEPES (pH 7.5). To the mixture, 2 µL of 300 mM MgCl₂ was added, and the solution was incubated on ice for 48 h in cold room to allow self-dimerization. For hybridization of PTC analogues with tRNAs, 10 µL of 0.5 µM tRNA^{Met}(CAU) was added to the dimerization solution and incubated at 0 °C for another 48 h. For sample preparation, 1 µL of the prepared sample was mixed with 4 µL of water and 1 µL of Gel Loading Dye, Purple (NEB, #B7024S), and then loaded onto a pre-cast 10% TBE gel. Electrophoresis was performed in 0.5X TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.0) at 110 V for 2.5 h at 4 °C in a cold room. After electrophoresis, the gel was stained with 2.5 µL of SYBRTM Gold Nucleic Acid Gel Stain (Invitrogen, #S11494) in 50 mL of water for 10 min. The gel was then visualized using a Bio-Rad gel documentation system (GelDoc Go).

Preparation of EMSA native gel

Native gels for RNA dimerization or hybridization were prepared according to a previously published protocol (*Nucleic Acids Research*, 2022, Vol. 50, No. 4 1815–1828). In a 50 mL tube, 10 mL of 40% Acrylamide/Bis Solution (19:1) (Bio-Rad, #1610144) and 2 mL of 10X TBE buffer (Biosesang, #T2004) were mixed. Water was added to bring the total volume to 40 mL. After thorough mixing, 400 µL of 10% (wt/vol) APS and 40 µL of TEMED were added to the solution. The mixture was cast into a gel cassette using a serological pipette and allowed to polymerize overnight.

- On page 39 of SI, we now write:



Supplementary Figure 18. Dimerization of PTC analogues (P1c2^{UGGU}, P1c2, P2c^{UGGU}, and P2c) and their hybridization with tRNAs.

We monitored the ability of PTC analogues (P1c2^{UGGU}, P1c2, P2c^{UGGU}, and P2c; lanes 1-4) to self-dimerize and hybridize with tRNA. (A) Consistent with the previous results (*Nucleic Acids Research*, 2022, Vol. 50, No. 4 1815–1828), we observed that P1c2^{UGGU} and P1c2 dimerized (lanes 5-6, band marked with the blue arrow). In contrast, P2c^{UGGU} and P2c, which are inactive PTC analogues, did not form dimers (lanes 7 and 8), therefore, presumably preventing peptide bond and cyclic structure formation. (B) tRNA^{fMet} containing the 5'-ACCA-3' sequence (lane 9) was separately incubated with each PTC analogue. P1c2^{UGGU} produced additional bands (lane 10) likely corresponding to a monomer hybridized with one tRNA^{fMet} (red arrow) and a dimer hybridized with two tRNAs^{fMet} (purple arrow). P1c2 appeared to bind nonspecifically to tRNA^{fMet} as evidenced by smeared bands (lane 11). Although weak, P2c^{UGGU} produced a new band, likely corresponding to a hybridized form with tRNA (lane 12, marked with the orange arrow). P2c, which lacks a pairing sequence with tRNA did not produce this band (lane 13).

Reviewers #2

I am afraid the additional experiments have not clarified the issue regarding the purported RNA-catalyzed cyclic hydrazinedione formation.

First, the presented data on tRNA binding to their proposed dimer is unconvincing. The intensity of the rRNA fragment bands varies dramatically, as evidenced by the stark differences in signal strength (compare the bands marked by blue arrowheads in lanes 5 and 6 vs. 10 and 11). Given this inconsistency, how can we be certain that if the amount of the material in samples in lanes 5 and 6 was as high as those in samples in lanes 10 and 11, we would not observe the 'purple and red arrowhead' bands in lanes 5 and 6 as well?

Second, the experimental setup for the duplex formation and detection experiment is concerning. According to the newly added procedures section, the rRNA fragments were hybridized in the presence of 60 mM MgCl₂. However, the samples were then analyzed using what they call an "EMSA native gel," which lacks Mg²⁺ and instead contains 1 mM EDTA. This is particularly notable because the native gels referenced in the NAR paper contained 15 mM MgCl₂. Such a discrepancy raises questions about the comparability and reliability of the results.

This lack of consistency undermines the validity of their conclusions.

As a result, I remain as unconvinced about the reliability of the experiments involving the rRNA constructs as I was before the last revision.

Reviewers #3

In this latest revision, the authors worked to address critiques that:

(1) the P2c minimalist ribosome mutant does not dimerize and therefore can serve as a negative control for catalysis

(2) the minimalist ribosomes that have the complementary UGGU motif are able to bind to tRNAs with an acceptor stem encoding ACCA, but those without this motif cannot.

The gel shown in new Figure S18 does clearly demonstrate point #1 (compare lanes 5 and 6 vs. 7 and 8). However; I have a very hard time agreeing with the author's conclusions about point #2. The additional bands labeled with the red purple and orange arrows in lanes 10-12 that purport to demonstrate complex formation with the tRNAs are very faint, if not non-existent. Additional assays are needed to definitively show selective complex formation between the UCCU containing ribosome mimics and tRNAs.

- **We acknowledge that the band of the hybridized RNA construct in our previous revision was faint to conclusively demonstrate the ability of PTC analogues to selectively hybridize with tRNA. To address this, we repeated the experiment under newly optimized conditions.**
- **We found that low temperature is critical for visualizing the hybridized RNA construct on a native gel. A rough calculation of the melting temperature (T_m) for the ACCA-UGGU RNA duplex (4 complementary bases) is 12°C, based on the Wallace rule (T_m = 2°C × (A-U) + 4°C × (G-C)). To prevent dissociation of the hybrid construct, we performed electrophoresis in a -20°C freezer, minimizing heat generation from the electrophoresis apparatus.**

- To preserve the secondary structure of the hybrid construct, we added 15 mM MgCl_2 to Tris-borate gel-running buffer, as suggested by Reviewer 2 for native gel preparation. However, the addition MgCl_2 led to the formation of insoluble salts, likely magnesium borate ($\text{Mg}_2\text{B}_2\text{O}_5$). This precipitate adhered to the electrodes of the apparatus, interfering with the power supply and compromising gel integrity. To circumvent this issue, we switched to TAM buffer (Tris-Acetate- Mg^{2+}), which effectively maintained ionic conditions of Mg^{2+} without precipitation.
- In our second revision, dimerization was performed at a low concentration (5 μM), resulting in the formation of P1c2 (a catalytically active variant of the PTC analogue) as a dimeric construct. In this experiment, we increased the concentration of PTC analogues to 137 μM , the same concentration used for mass spectrometric analysis (Figure 6 and Supplementary Figures 19-20), and observed the formation of a dimeric construct of P2c (an inactive variant) as well, reproducing previous findings reported in *Nucleic Acids Research* (2022, Vol. 50, No. 4, pp. 1815-1828).
- Through these optimizations, we clearly demonstrated that PTC analogues can self-dimerize and that those containing an additional UGGU sequence selectively hybridize with tRNA.
- Further densitometric analysis estimated the concentration of the hybrid construct to be ~10 μM . Given a 20-50% tRNA acylation efficiency of the substrates, this is expected to yield at least ~100 ng of final cyclic products, an amount sufficient for detection using our LC-HRMS methods.
- On page 15 the main text, we now write:

To directly demonstrate that the cyclic hydrazinedione formation results from the ribosome's catalytic activity, we conducted an additional experiment using functional ribosome analogues that mimic the peptidyl transferase center (PTC) components of the bacterial 23S ribosomal RNA (Figure 6A). Specifically, we designed two PTC analogues, P1c2 (70-mer) and P2c (97-mer; Supplementary Information). P1c2 incorporates elements from the P1 region of the ribosome's domain V (Figure 6B),^{55, 56} forming a dimer that yields a symmetrical three-dimensional structure similar to the ribosome's active site and is capable of catalyzing peptide bond formation (Figure 6C). In contrast, P2c is an inactive variant with an extended sequence, despite forming a stable dimeric structure. Using in vitro transcription with T7 RNA polymerase, we synthesized four different PTC analogues (P1c2^{UGGU}, P1c2, P2c^{UGGU}, and P2c) and tRNA^{ACCA}. The 3'-UGGU sequence in the PTC analogues was specifically designed to pair with the complementary 3'-ACCA in tRNA. To assess the functionality of the RNA constructs, we incubated the P1c2^{UGGU} and P1c2 separately and observed that both formed dimeric constructs, regardless of the 3'-UGGU sequence (Supplementary Figure 18A and B), consistent with previous findings.⁵⁷ Additionally, when P1c2^{UGGU} was incubated with tRNA^{ACCA}, it produced a new construct (Supplementary Figure 18B), presumably reflecting the formation of a hybridized RNA product via UGGU-ACCA pairing. We hypothesized that this pairing would position the substrates in close proximity for peptide bond formation. Conversely, analogues lacking the complementary UGGU sequence would fail to interact with tRNA, preventing the formation of a cyclic structure.

- On page 16 the main text, we now write:

Importantly, the cyclic structure formation could potentially result from the proximity of the substrates on tRNA, rather than the catalytic activity of P1c2. To address this possibility, we performed additional experiments using P2c variants, known to be inactive in peptide bond formation, despite their ability to form a dimeric construct. Consistent with the previous findings,⁵⁷ we observed that P2c^{UGGU} and P2c formed a dimeric product (**Supplementary Figure 18C**), whereas P2c^{UGGU} produced only a hybridized product with tRNA. However, no peaks corresponding to the masses of 5- or 6-membered rings were detected in samples with the P2c^{UGGU} domain (**Figures 6D-6E**, purple), strongly suggesting that proximity alone is insufficient to drive peptide and ring formation.

- On page 6 of SI, we added the procedures for the new experiments.

Dimerization and hybridization assays of PTC analogues

- Dimerization of PTC analogues: 7.3 μ L of 137 μ M PTC analogue was mixed with 2 μ L of 500 mM HEPES (pH 7.5). The mixture was heated to 95 °C for 3 min, then cooled to 25 °C at a rate of 0.1 °C/sec. Once cooled, 4 μ L of 0.3 M MgCl₂ was added to reach a final concentration of 60 mM, and the solution was incubated on ice for 10 min to allow self-dimerization.

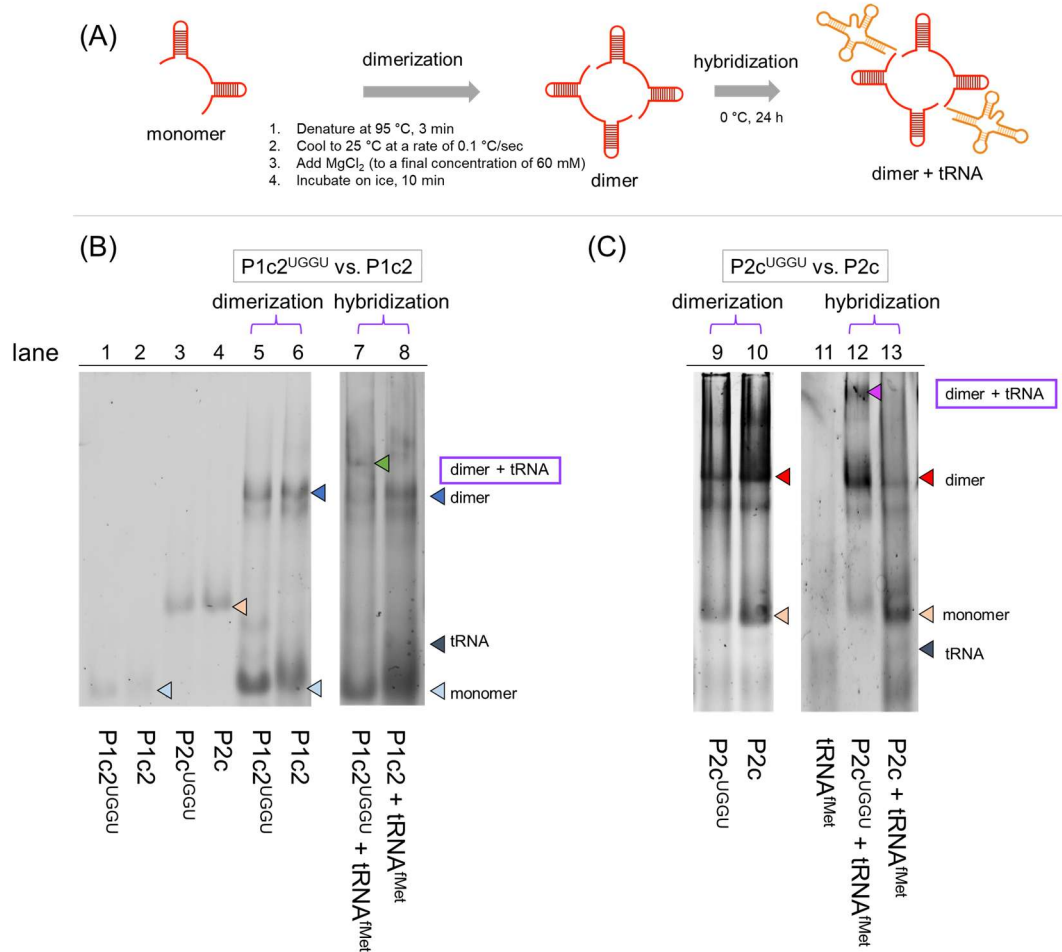
- Hybridization with tRNAs: To the dimerization solution, 6.7 μ L of 149 μ M tRNA^{fMet} (CAU) was added (resulting in a final concentration of 50 μ M) and the mixture was incubated at 0 °C for additional 24 h.

- Gel assay: 0.5 μ L of the prepared sample was combined with 4.5 μ L of water and 5 μ L of 2X RNA loading dye, (Thermo Scientific, #R0641), and loaded onto a 12% polyacrylamide gel. The gel was run in 0.5X TAM buffer (44.5 mM Tris, 44.5 mM sodium acetate, 15 mM MgCl₂, pH 7.5) at 100 V for 7 h in a -20 °C freezer. Afterward, the gel was stained with 2.5 μ L of SYBRTM Gold Nucleic Acid Gel Stain (Invitrogen, #S11494) in 50 mL of water for 10 min, then visualized using a Bio-Rad GelDoc Go system.

Preparation of native gels for electrophoretic mobility shift assay (EMSA)

EMSA native gels for RNA dimerization or hybridization were prepared according to a previously published protocol (*Nucleic Acids Research*, 2022, Vol. 50, No. 4 1815–1828). In a 50 mL tube, 12 mL of 40 % Acrylamide/Bis Solution (19:1) (Bio-Rad, #1610144) and 4 mL of 10X TAM buffer (890 mM Tris, 890 mM sodium acetate, and 150 mM MgCl₂, pH 7.5) were mixed. Water was added to bring the total volume to 40 mL. After thorough mixing, 400 μ L of 10 % (wt/vol) APS and 40 μ L of TEMED were added to the solution. The mixture was cast into a gel cassette using a serological pipette and allowed to polymerize overnight.

- On page 39 of SI, we now write:



Supplementary Figure 18. Dimerization of PTC analogues (P1c2^{UGGU}, P1c2, P2c^{UGGU}, and Pc2) and their hybridization with tRNAs.

(A) Schematic representation of PTC analogue dimerization and subsequent hybridization with tRNA. We examined the ability of four PTC analogues [P1c2^{UGGU} and P1c2 (marked with a light blue arrow on lanes 1-2) and P2c^{UGGU} and Pc2 (marked with a light orange; lanes 3-4)] to self-dimerize and hybridize with tRNA. (B-C) Consistent with previous findings (*Nucleic Acids Research*, 2022, Vol. 50, No. 4 1815-1828), each monomer produced a new construct likely indicating a dimeric product (lanes 5-6: dark blue; lanes 9-10: red) following the self-dimerization reaction. After subsequent incubation of this dimeric product with tRNA containing the 3'-ACCA sequence (lane 11; dark gray), we observed that P1c2^{UGGU} and P2c^{UGGU} each produced an additional band (lane 7; green, lane 12; pink, respectively), likely indicating a dimer hybridized with tRNA. In contrast, P1c2 and P2c (lanes 8 and 13), which lack the pairing sequence for tRNA hybridization did not produce this band. The faint tRNA band in lanes 7 and 12 suggests that most of the tRNA was utilized for hybridization. Densitometric analysis of the hybridized bands (green and pink) indicates that ~8 and 12 μM of the PTC:tRNA hybridized constructs were used in the reaction for cyclic product production, respectively.