nature portfolio

Peer Review File

Simultaneous formation of peptides and RNA via prebiotic cross-catalytic enhancement

Corresponding Author: Professor Sudha Rajamani

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)
This manuscript investigates the simultaneous formation of oligopeptides and oligonucleotides from amino acids and ATP or 2′,3′-cAMP under wet–dry cycling conditions. The authors detect aminoacyl-AMP intermediates and propose that these species mediate cross-catalytic enhancement of peptide and nucleotide oligomerization. The topic is relevant to prebiotic chemistry, and the manuscript contains extensive analytical data (LC-MS/MS, HPLC) and thoughtful exploration across multiple amino acids.
However, despite the breadth of experiments, several aspects of the manuscript require significant clarification or reorganization. In particular, the novelty is not clearly articulated and the mechanistic interpretation appears underdeveloped. The manuscript would benefit from substantial revision to improve clarity, rigor, and contextual grounding within the existing literature.

Major Comments
The Introduction does not clearly state what conceptual or technical gap this study fills. There is extensive prior work on dry-state peptide formation at elevated temperatures, nucleotide oligomerization under dehydration, aminoacyl-nucleotide formation (e.g., phosphoramidates, mixed anhydrides), RNA–peptide co-polymerization and cross-activation.
The manuscript implies major conceptual advances, but it is unclear whether the key novelty lies in performing reactions at neutral pH, using ATP or cAMP specifically, obtaining longer peptides than in prior work, achieving simultaneous oligomer formation, or demonstrating activity across several amino acids.
These distinctions should be explicitly stated and discussed relative to the substantial existing literature.

The Discussion restates results but offers limited mechanistic interpretation. For example:
Why does cAMP produce significantly higher yields and longer oligomers than ATP?
Is the determining factor stability, intrinsic reactivity, or aminoacylation efficiency?
Why do certain amino acids (e.g., Asp) form AMP-aa in much larger amounts than others?
Is this driven by pKa, steric effects, or differential nucleophilicity?
How do bulky amino acids such as Trp yield similar peptide lengths to Gly under the same conditions?
How can Mg2+ simultaneously promote nucleotide oligomerization but inhibit peptide formation?
These opposing roles need reconciliation within a unified mechanistic framework.
A more analytical and comparative discussion would substantially strengthen the manuscript.

Several yield values reported in the manuscript appear unexpectedly high or defy typical polymerization behavior.
In some reactions, peptide yields approach or nearly match the initial amino acid concentration. This is unusually efficient for uncontrolled dry–down condensation at 130 °C and should be discussed mechanistically.
Also, the reported peptide yields show similar abundance across short and long oligomers (e.g., di- through decaglycine). This contradicts classical statistical growth models, which predict steeply declining abundance with length. The authors should explain whether this reflects real chemistry, MS detection biases, or normalization methods.
AMP-Asp is reported to accumulate in much higher amounts than AMP-Gly, AMP-Lys, or AMP-Trp. The manuscript does not analyze why Asp behaves differently, nor how this high degree of activation correlates with peptide formation.
It is unclear how the reported quantities of free amino acids, AMP-aa, oligopeptides, and hydrolysis products relate. Even approximate mass balance would help validate the quantitative approach.
Without clearer explanation, these quantitative trends are difficult to interpret.

Amino acids such as Asp and Lys contain multiple nucleophilic or electrophilic sites. The manuscript claims formation of AMP-Asp and AMP-Lys, but does not determine: whether AMP-Asp attaches via alpha-COOH or beta-COOH, whether AMP-Lys forms through alpha-COOH or epsilon-NH2, or whether peptide products contain branching or mixed linkages.
Given the centrality of mechanistic conclusions, such regioisomeric ambiguity should be addressed, or at minimum discussed explicitly.

The manuscript reports that β-Ala does not form AMP-β-Ala, but does not analyze why. Simple pKa arguments do not fully explain the outcome, especially in dehydrating/high-temperature regimes.
Likewise, experiments with Asp-methyl ester need more controls: esters are likely to hydrolyze under the high-temperature wet–dry cycles used here. Thus, the absence of AMP-Asp-OMe cannot be interpreted without verifying ester integrity.

The data show that Mg2+ promotes nucleotide oligomerization, inhibits peptide formation, and affects AMP-aa accumulation. The manuscript presents these observations but does not integrate them into a coherent picture. A systematic mechanistic explanation would greatly strengthen the claims about relevance to prebiotic scenarios.

Reviewer #2

(Remarks to the Author)
The manuscript by Roy et al. reports peptide formation catalyzed by activated RNA mononucleotides. The authors show that adenosine triphosphate and adenosine 3',5'-cyclic monophosphate can promote the formation of longer oligoglycine peptides. The work is valuable to the Origins of Life community, though its broader scientific impact is more incremental. I am not fully convinced that Communications Chemistry is the most appropriate venue for this manuscript. Most importantly, the analytical methodology does not, in its current form, support quantitative conclusions. I believe NMR is required for analysis (as previously performed). I would, however, support publication pending the revisions outlined below.

1. I do not think there is sufficient evidence (from the data provided) that the abstract should have "indicates the plausibility of the emergence of initial steps involved in a primordial translation system". This to me is overinflating the work and does not reflect the findings.
2. Line 57: many references are missing for this sentence. Seminal work by Orgel, Richert and Szostak need to be reference extensively here
3. Line 76: the authors need to make a more convincing argument for the prebiotic relevance of ATP and cAMP. Their presence has been extensively studied (e.g. PLOS Biol 2022 e3001437) would significantly strengthen the narrative. Also, Sutherland's work (and perhaps Powner's work) has shown the prebiotic synthesis of cyclic nucleosides.
4. Line 89: have the authors tried Mn instead of Mg. This is important for a number of reasons. Importantly, Mn drives polymerization in certain cases.
5. Line 106: perhaps the most significant issue I have with the work is the author's claim to a quantitative methodology. Mass Spectrometry quantitative analysis is highly dependent on ionization efficiency. To use a single amino acid, alanine, as the internal standard does not justify its use for an analytical internal standard for a dimer, trimer, tetramer, pentamer, etc. (unless a previously published reports state so). The authors cannot claim a quantitative analysis unless their methodology has been validated (in more than one context I might add). I believe this is essential for a study stating such conclusions.
6. The authors report concentrations (e.g. line 121). How have the authors assessed the significant figures on these values. That is, how do the authors know that there is a yield of "1.11 umoles", and not "1 umol" (note that umoles should be umols)
7. Fig 1A. There are many m/z signals that are unassigned, which is typical for MS spectra. My concern is that the authors assign importance to signals that are well below intensity to many other signals. For instance Gly4 is next to a towering peak. Gly 5/6/7/8/9 are well below many m/z (e.g the m/z=269.0846). How are the authors justifying this? In addition, have the authors performed a statistical analysis on the significance of a signal. This is paramount in such an analytical study.
8. The authors has consistently discussed physiological pH, but it is well-known that early earth could have succumb to pH fluctuations. The authors should consider repeating their reactions at pH 5, 6, 8, and 9 to better reflect these geological possibilities.
9. Fig 1A caption: the authors should explicitly state their internal standard (alanine if I am not mistaken)
10. Figure S1; the authors should specify the stereochemistry
11. For ion exchange studies, the gradient of 0-30% could have been changed to resolve so of the species that were unresolvable (AMP2 and ADP). Have the authors considered ion pairing agents like triethylammonium acetate?
12. It's ambiguous when the authors use AMP tetramers. Does this mean a tetranucleotide? If so, please define appropriately like on line 215
13. Fig 3A: "A" is not defined. Maybe it should be "Fig 3: (A)…"
14. Line 249: The AMP-aa which is presumably crucial to the study needs to be synthesized in lab and characterized by NMR. The authors can use this synthetic standards in their other studies and I believe this to be an essential addition to the study. This would also allow the authors to run spike experiments (e.g. countering the statement on line 269-270). Despite complex mixtures, spike experiments are normally very telling.
15. The AMP-Lys has no defined characterization. Although there is some information provided by the authors, its essential to provide proper NMR characterization at the very least. The MS is NOT convincing in my opinion. This goes for the other amino acids as well (Trp)
16. I am curious why the authors have chosen 130 oC. Is there any precedence to this actual values? If so, the authors should explicitly state this, or at least why they chose this.
17. Line 314. pKa should be pKa
18. Line 316. The authors look at the protected version of Asp. What about Lys? Or the other amino acids. This seems incomplete

19. Line 323-324: How do the authors know this is not occurring in the MS source? Is there any other evidence the authors can use like NMR? Again, I am not convinced by the MS analysis alone. I would strongly encourage the authors to use another analytical technique to validate their results.
20. Line 337: although this might be a logical conclusion, the authors have little evidence to state this. Not enough characterization in the reaction, in my opinion, has been performed to certifiably state this as a conclusion.
21. Fig 5A: I would encourage the authors to include an NMR panel with a spike text of the synthetic standard
22. Line 372: maybe place the decaglycine before the dodecaglycine?
23. The authors us ATP as a model compound but they should consider GTP as this is used in modern biology for various functions. I think this would increase generalizability as well.
24. Line 382: The authors overreach by saying that “amino with bulky R groups” as all amino acids other than Gly will have an R group. This is not reflective of prebiotic relevance or modern biology.
25. Line 389: methyl needs to be methylene
26. The paragraph starting at 389 is repetitive and needs to be revised.
27. Fig 6. The authors have not discussed regioselectivity which is very important in the context of nonenzymatic genome replication. Although this is beyond the scope of this paper. The authors should not assume one isomer over the other (e.g. 3’ 5’ 2’ 5’)
28. Line 431, the authors should include additional, more update, references on the topic. Many groups have “more-recently” reported data on this topic.
29. Line 465: a reference is needed here
30. Line 472 and onwards should be in a “conclusion section” in my opinion.
31. I believe the authors should discuss the RNA-peptide world to a larger extent (both the introduction and the discussion)

Reviewer #3

(Remarks to the Author)
The manuscript by Roy and colleagues investigates the co-catalytic role of amino acids and nucleotides in the formation of oligomerization products. Using wet–dry cycling under various conditions, they promoted oligomerization reactions yielding polypeptides and oligonucleotides. The authors report a significant co-catalytic effect that results in longer oligomerization products. This finding is highly intriguing, as it links the two fundamental chemistries of nucleic acid polymerization and peptide bond formation into a co-dependent mechanism, processes that are strictly separated in modern biology (polymerase vs. ribosome). The study thereby addresses one of the most fundamental questions in prebiotic chemistry: which came first, nucleic acids or proteins? The work aligns well with recent hypotheses suggesting a co-dependent evolution of these biopolymers.

There are a few points that need to be addressed:

1) I could not find any calibration curves for the different oligomerisation products. The authors claimed they used the integral of the mass spec signal but oligonucleotides or polypeptides of different length cannot be easily compared as they provide different signal intensities. The signal intensity and therefore the peak area, is dependent on many factors such as the mass of the molecule, the solvent system, presence of ions, etc. As the main finding fully depends on quantification, the authors need to show that their quantification is robust.

2) There are a large number of abbreviations in this manuscript. However, many of them are not clearly defined. Some definitions are found the caption of figure 2, even though such abbreviations have already been used in figure 1 and throughout the text without definition.

3) Figure 1, the mass spec data shows the different glycine polypeptides (mass difference for each monomer = 57 Da). It seems that there are other glycine species present as well, e.g. 212/269/326/383/440/497 Da (57 Da difference). The authors have any explanation for these species?

4) In the figures the authors usually provide cycle 0. Was this indeed measured? If not, could the authors measure those samples separately to ensure they are initially not containing any polymer specie of the amino acids or nucleotides?

5) P-N vs. P-O-C. The authors claim that the P-O-C bond is more reactive providing more easily the tetraglycine product. However, looking at figure 6 shows that the samples using the P-O-C bond the oligonucleotides are decreasing over time, while in the samples with the P-N bond the oligonucleotides seem to be increasing/stable. So, in this regard the P-N bond is much more favourable from an evolutionary perspective assuming a co-evolution of peptides and oligonucleotides. The authors might want to rephrase.

Reviewer #4

(Remarks to the Author)
Roy et al. report reactions in which peptides and RNA oligomerize in the presence of amino acids and either ATP or cAMP. Although the field has extensively examined RNA–amino-acid conjugates, expanding this line of research is a worthwhile goal. Nonetheless, in its present form, the manuscript does not provide sufficient experimental evidence to support the authors’ central claims.

Here are my main comments:
1. To begin with, the study's exclusive dependence on mass spectrometry for product quantification is concerning. MS-based quantification, while informative, can be misleading when used without proper calibration, yet no calibration curves for standards (peptides or nucleotides) are provided. The authors state that product yields are reported as relative values normalized to an internal standard (alanine), but without calibration data, it is difficult to assess whether these measurements are reliable or comparable. Moreover, the oligomer distribution presented in Figure 1B, where nearly all oligomers appear at similar abundances, is not typical and difficult to reconcile with expected oligomerization behavior.
2. Can the authors detect peptides via HPLC? It is also unclear why the authors did not employ HPLC for peptide detection and quantification. HPLC is a standard and widely used technique for identifying and quantifying peptides.
3. If not HPLC, NMR analysis could be used to substantiate the MS-based measurements of glycine consumption. Without such complementary evidence, the authors should clarify that their results are not quantitative.
4. GM130H – it is also unclear why the control reaction in Figure 1B shows almost no formation of glycine oligomers. Rodriguez-Garcia et al. (Nature communications 6, no. 1 (2015): 8385) demonstrated that simple dehydration of glycine, followed by heating between 90 °C and 130 °C, leads to substantial oligomerization even in the absence of any catalyst. Oligomers of up to 20 residues were observed after extended heating at these temperatures. The contrast between these established results and the negligible oligomer formation reported here warrants further explanation.
5. Furthermore, it is not clear why the authors did not quantify the nucleotide peaks using HPLC, especially given that their system is equipped with a DAD detector. As mentioned, HPLC-based quantification is generally more reliable and accurate than MS.
6. The authors also synthesized standards of suggested intermediates (AMP-Gly (P–N) and AMP-Gly (P–O–C)), which is very important. However, to support their mechanism, they need to conduct a kinetics analysis comparing the yield of products (oligonucleotides/peptides) when reactions start with these intermediates versus starting with ATP/AMP. If the reaction rate is faster with the intermediates compared in their absence, this is good support for what they are claiming. Without such a comparison, a conclusion cannot be drawn about the reaction mechanism.
7. AMP-Gly (P-O-C) doesn't look so pure (Figure S20b). What is the purity of the standards? HPLC of synthesized standards for AMP-Gly conjugates after purification is required.
8. Are the authors sure that the peaks in the HPLC are of AMP-G(P-N) or AMP-G(P-O-C) (e.g., in Figure S6?)? The authors need to spike the standards in the reaction products to verify that these are the speculated products in the chromatogram. Also, quantification of these products should be done via UV-HPLC based on the standards. The peaks in the HPLC chromatograms should be clearly labeled.
9. Many studies have explored reactions between amino acids and nucleotides. Some of which are not mentioned in this work. For example, the work of Suárez-Marina et al (Communications Chemistry 2.1 (2019): 28) and the work of Sawai and Orgel in the Journal of Molecular Evolution 6 (3) (1975): 165-184 (A different paper by these authors from 1975, which focuses on oligonucleotide formation, was mentioned, but not this paper).
10. What is the relevance of ATP as a prebiotic precursor? The abstract starts with "Nucleoside triphosphates, cyclic monophosphates and amino acids are hypothesized to have been present together in the prebiotic milieu" – yet, to my knowledge, nucleoside triphosphates are not considered plausible prebiotic molecules.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have responded to my comments, even though not all the points raised by me or others have made it into the revised manuscript. While I am still not entirely convinced about the novelty of this work, I believe the manuscript is now in a publishable form.

Reviewer #2

(Remarks to the Author)
This reviewer has submitted the confidential comments to editors only.

Reviewer #3

(Remarks to the Author)
The authors have revised the manuscript and addressed many of the previous comments. However, the calibration, which represents a key methodological aspect, is still not sufficiently clarified.

The authors present a single calibration curve for alanine; however, the axes are not labeled, which limits interpretability. In mass spectrometry–based quantification, two principal strategies are typically employed: (i) the use of an isotopically labeled internal standard corresponding to the exact analyte, or (ii) the use of a different internal standard, i.e., a spiked compound of known concentration.
In the present study, the authors appear to use alanine as an internal standard. However, they only provide a calibration curve for alanine itself. For accurate quantification, a separate calibration curve is required for each analyte of interest, constructed from the ratio of the analyte signal (across a dilution series) to a constant amount of internal standard. There is no clear evidence that such analyte-specific calibration curves were generated.
Consequently, significant concerns remain regarding the robustness of the quantification approach. As the study's results

and conclusions rely heavily on these quantitative measurements, I am currently unable to recommend the manuscript for publication.

Reviewer #4

(Remarks to the Author)
I appreciate the authors' efforts in revising the manuscript. However, my main concern remains unresolved, and I therefore still recommend major revision before the work can be considered for acceptance.

My main concern is the continued presentation of the MS-based measurements as quantitative yields. As mentioned in the original review, the study's exclusive dependence on mass spectrometry for product quantification is concerning. MS-based quantification, while informative, can be misleading. The rebuttal letter acknowledges the well-known limitations of LC–MS quantification in complex mixtures and explains that the authors use alanine as a single internal standard. However, no validation is provided showing that this approach permits reliable quantification across analytes with very different structures and ionization efficiencies, such as diglycine, triglycine, higher oligoglycines, nucleotide oligomers, and nucleotide–amino acid conjugates. In particular, the authors did not provide calibration using even a limited set of commercially available oligoglycine standards, despite the fact that such standards exist and could directly test whether the analyte/internal-standard ratio behaves sensibly in the relevant range.

Hence, I do not think the manuscript currently supports absolute claims such as cumulative nmol or µmol values for oligomer distributions. This is especially important because the product distributions shown in Figure 1B appear atypical, with several oligomers present at similar apparent abundances, which is difficult to reconcile with expected oligomerization behavior and may reflect analytical response bias rather than chemistry. This concern was not satisfactorily addressed. Notably, the authors also didn't refer to my comment about this atypical distribution of products, which was also raised by Reviewer 1.

I also note an inconsistency between the rebuttal and the manuscript text. In the rebuttal, the authors state that the measurements are "indicative rather than strictly quantitative" and that this has been clarified in the revised manuscript. I could not find such a clarification. Instead, the revised manuscript still refers to a "thorough quantitative analysis" and states that peak areas were used "to quantify the yields." The manuscript should be revised to match what the data can actually support.

More generally, it is very difficult to identify exactly what changes were made in response to the reviewers' comments.

I therefore see two acceptable paths forward:
1. Validate the quantitative method more rigorously, at minimum by showing calibration behavior for a limited number of relevant standards (for example several oligoglycine standards), clarifying the LC–MS acquisition mode and data extraction, and showing representative chromatograms.
2. Reframe the manuscript as qualitative, clearly stating the limitations of the analytical approach and removing or substantially toning down all absolute yield claims and any conclusions that depend on them. In that case, the conclusions should remain qualitative, e.g. enhancement of oligomer formation, appearance of longer products, detection of proposed intermediates, etc., rather than precise yield comparisons.

Regarding the yield, the reported "cumulative Gly-peptide yields" are difficult to interpret. The reactions are described as starting from 75 mM glycine, but the Results then switch to nmol/µmol product amounts without clearly stating the reaction volume. Since the reaction volume is 300 uL, 75 mM glycine corresponds to 22.5 µmol starting material. It would therefore be much clearer if the authors explicitly reported percent yields and defined exactly what quantity is being summed. For example, how do they get "cumulative Gly-peptide yields of ~21.3 µmoles"? Just by looking at Fig. 1B, I can do the following for cAG 130: ~1 µmole x 2 (diglycine) + ~2 µmole x 3 (triglycine)+ ~2 µmole x 4 (tetraglycine) + ~3 µmole x 5 (pentaglycine)... I count ~84 µmole when only summing up to octaglycine, and the authors only had 22.5 µ mole glycine. Do the authors count for the number of glycine units in each oligomer and how many moles of glycine they started with? This again goes to my comment about the results that cannot be interpreted quantitatively.

The methods statement that alanine (20 mg/mL) was injected with "150 nmoles of samples" is unclear. 150 nmoles of what? Does it refer to the initial amino acid concentration? What exactly was injected, and how were the reported nmol yields calculated from that measurement? Also, 20 mg/ml alanine is 225 mM alanine. This is a very high concentration for a standard for LCMS.

Additional points that still require clarification:
• The alanine calibration curve shown in the rebuttal is insufficient to validate the reported product yields. Please provide clear units and explain how this curve relates to the reported analyte amounts.
• Please provide representative LC–MS chromatograms/traces and clarify the acquisition method.
• Regarding peptide detection by UV (Comment #2), UV-based detection is widely used for peptide quantification. The paper cited by the authors (Campbell et al., Quantitative Analysis of Glycine Oligomerization by Ion-Pair Chromatography) used a C18 column to separate glycine oligomers, indicating that this type of analysis is feasible. Thus, it seems possible that peptide detection/quantification could also be performed on the column used here, or at least this point should be discussed more carefully.
• Regarding Comment #3, the authors' response regarding NMR and low glycine consumption should be reconciled with the large reported yields (even though the yield itself remains unclear, see comment above) in some experiments.
• Regarding comment #4 (comparison to Rodriguez-Garcia et al.), what is the final pH of the reactions studied here at the

end of the cycles? I could not find this information in the manuscript.
• Regarding point #7 (HPLC), the authors say: “HPLC of AMP-Gly (P-O-C) could not be obtained as the mixed anhydride linkage is highly susceptible to hydrolysis under aqueous conditions.” But the authors show HPLC data for this standard (Fig S6a) and 1H NMR in D2O, which is confusing. This should be clarified.
• Regarding point #9, while the paper by Suárez-Marina et al.it reports the simultaneous formation of glycosidic bonds between ribose, and purine and pyrimidine nucleobases, it also reported some glycine adducts (e.g., Gly-AMP) even though these were not the focus of that paper.
• If the manuscript continues to compare its results to prior peptide-formation literature, the comparison should be made carefully, including explicit discussion of pH conditions.

Overall, I think the manuscript has improved, but the analytical basis for the quantitative claims remains insufficient. Unless the quantification is properly validated, the manuscript should be revised so that all claims are clearly presented as qualitative.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors did a good job in revising the manuscript by removing absolute product yield. In my opinion, the manuscript can be accepted for publication. I congratulate the authors for this very interesting study.

Reviewer #4

(Remarks to the Author)
While the revised manuscript has improved, I recommend that it undergo further revision before acceptance, as several major issues remain unresolved.

Major comments:
1. The revised version is improved in that the previous absolute mol yield plots have largely been removed, and the authors now acknowledge that the LC-MS data provide relative rather than absolute quantification. However, my main concern is only partially resolved. The manuscript and Supporting Information still describe the LC-MS-derived values as “percentage yields.” This terminology remains problematic. As currently described, the values are obtained from analyte peak areas normalized to an alanine internal standard, with the analyte/internal-standard ratio multiplied by 100. However, these values should not be described as yields, as mentioned in my former comments. These values are relative MS response values or relative signal intensities.

I also remain concerned that some interpretive statements are still stronger than the data justify. For example, claims that the chemistry “significantly improves the efficiency” of peptide formation, or that the work provides “concrete evidence” for RNA-peptide co-evolution, should be toned down. The data support detection of longer products and apparent changes in relative MS signal under certain reaction conditions, but they do not establish robust chemical yields or quantitative efficiencies.

Hence, the authors should replace “percentage yield” throughout the manuscript and Supporting Information with terminology such as “relative LC-MS response” or “relative abundance”. In addition, the authors should remove or substantially soften conclusions that depend on quantitative yield comparisons. The conclusions should be limited to qualitative or semi-quantitative observations, such as the appearance of longer oligomers, detection of proposed intermediates, and relative changes in MS signal under different reaction conditions.

2. I noted that the newly added heat-map presentation appears to contradict in some cases the results in the previous version. For example, in the revised Figure 1C, the cAG 130 condition appears to show lower peptide formation than AG 130 for several oligomer lengths, whereas the previous version of Figure 1, which presented the data as relative peptide yields, appeared to suggest the opposite trend. This discrepancy requires clarification. It is not clear whether the difference arises from a change in normalization or an error in plotting.

3. It is useful that the authors now show in the rebuttal letter UV-detected chromatograms for the available glycine oligomer standards, demonstrating that these standards can be separated and detected. Given this, the authors should now perform standard-spiking experiments with the oligoglycine standards they have available. Spiking the reaction mixtures with low amounts of authentic standards would help confirm the peak assignments. This would also strengthen the authors’ response to the concern about relying exclusively on MS-based analysis.

Minor comments:
- Figure 2b is cropped at the bottom.

- Figure 3A: The spiking experiment is not convincing as presented. The amount of spiked standard appears too high and seems to overload the original peak. For a meaningful spiking test, the spike should be much smaller, typically ~1–5% of the original peak area or height.

- Regarding comment #3 in the previous review. Thae authors state: “The Methods section has been revised to eliminate any ambiguity and now clearly describes the approach used for relative yield determination”. But the Methods section in the SI (pages 4-5) still contains the unclear statement: “An internal standard (alanine, 20mg/ml) was injected with 150 nmoles of samples to obtain the yields of resultant products, which were then summed up to get the overall oligomer yield.” - This was raised in my previous review but has not been revised. As mentioned, this is ambiguous: 150 nmoles of what was injected?

- Show chirality in fig. s1. Also fix the structure of Asp.

- There seems to be a recurring notation issue in the TOF-MS/MS assignments. The authors state that the MS data were acquired in positive mode; therefore, the precursor ions should be reported as protonated species (e.g. $[M+H]^+$). In several places, both the manuscript and SI appear to use “$(M–H)^+$”, which is incorrect. If the intended ion is the positive-mode protonated molecule, this notation should be corrected throughout.

- Regarding the comment in the rebuttal letter about 1H-NMR, can the authors provide a representative spectrum?

- In the rebuttal letter, the authors say: “The Methods section has been updated to explicitly state that rehydration was performed using pH-adjusted Milli-Q water (pH 7). Consequently, the pH was consistently maintained at 7 across all wet–dry cycles, ensuring uniform reaction conditions throughout the experiment.”. The statement remains ambiguous. Do the authors mean that the water used for rehydration was adjusted to pH 7 before addition, or that the reaction mixture was measured and readjusted to pH 7 after each rehydration step?

- The comparison with prior peptide-formation literature remains too brief and should be elaborated. Since the manuscript uses the neutral-pH conditions as an important point of distinction, the authors should compare their results more explicitly with prior work in the cited studies.

Version 3:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
The authors have addressed all of my concerns, and I recommend publication of the paper in its current form, subject to two minor changes: (1) replacing “percentage yield” with “relative abundance” in Figure 1C, and (2) adding a short explanation of the spiking with glycine oligomers to the Methods section of the SI, as I could not find this information there.

Title: Simultaneous formation of peptides and RNA via prebiotic cross-catalytic enhancement

We would like to thank all the reviewers for an in-depth evaluation of our manuscript, their constructive feedback and crucial comments. Accounting for them has allowed us to resubmit a version of the manuscript that is more comprehensive. In the below sections, we have addressed point-by-point all the questions and concerns that have been raised by the reviewers. The reviewers' comments are in green, while our responses are in black.

Reviewer #1 (Remarks to the Author):

This manuscript investigates the simultaneous formation of oligopeptides and oligonucleotides from amino acids and ATP or 2′,3′-cAMP under wet–dry cycling conditions. The authors detect aminoacyl-AMP intermediates and propose that these species mediate cross-catalytic enhancement of peptide and nucleotide oligomerization. The topic is relevant to prebiotic chemistry, and the manuscript contains extensive analytical data (LC-MS/MS, HPLC) and thoughtful exploration across multiple amino acids.
However, despite the breadth of experiments, several aspects of the manuscript require significant clarification or reorganization. In particular, the novelty is not clearly articulated and the mechanistic interpretation appears underdeveloped. The manuscript would benefit from substantial revision to improve clarity, rigor, and contextual grounding within the existing literature.

Major Comments
The Introduction does not clearly state what conceptual or technical gap this study fills. There is extensive prior work on dry-state peptide formation at elevated temperatures, nucleotide oligomerization under dehydration, aminoacyl-nucleotide formation (e.g., phosphoramidates, mixed anhydrides), RNA–peptide co-polymerization and cross-activation.
The manuscript implies major conceptual advances, but it is unclear whether the key novelty lies in performing reactions at neutral pH, using ATP or cAMP specifically, obtaining longer peptides than in prior work, achieving simultaneous oligomer formation, or demonstrating activity across several amino acids.
These distinctions should be explicitly stated and discussed relative to the substantial existing literature.

Prebiotic chemistry studies demonstrate plausible, nonenzymatic routes for the formation of prebiotic building blocks that would have led to the formation of peptides, oligonucleotides, etc. under early Earth–like conditions. Peptide formation has been shown to occurs via thermal dehydration, wet–dry cycling, mineral surfaces, or ester-mediated pathways, typically at elevated temperatures (60–130 °C) and at extreme pH conditions, yielding short oligomers from non-activated amino acids, with glycine-rich systems resulting in the best outcomes. The articles which have investigated the formation of peptides at neutral pH conditions mostly involved use/formation of depsipeptides. Similarly, oligonucleotide formation has been achieved through the use of activated monomers on mineral surfaces

(e.g., montmorillonite) or via intrinsically activated cyclic nucleotides at alkaline pH conditions. Typically, these result in short RNA oligomers (generally ≤10 units) under drying, heating, or UV exposure conditions (only exception where longer oligomers resulted involved the use of clay like montmorillonite).

Bridging the aforementioned chemistries, aminoacyl–nucleotide intermediates such as phosphoramidates and mixed anhydrides have been demonstrated to attach to short chain RNAs by chemical chemically synthesis intermediates that resulted in that then undergo templated-RNA replication or intra-strand amino acid transfer. Together, all these findings support a chemically coherent scenario in which RNA replication and peptide formation, individually and separately. Our reactions take a more organic approach to understand the formation of covalently linked intermediates, oligonucleotides and peptides at neutral settings, to give an understanding about how RNA and proteins could have co-emerged and co-evolved before the appearance of enzyme-driven biology.

Our one-pot reaction approach used in this study allows us to glean what messy prebiotic chemistry reactions could have occurred in a heterogenous soup, which could have readily occurred at more ambient conditions via *in situ* formation of active intermediates from otherwise 'not intrinsically activated' monomers. Notably, the *in situ* activation 'organically' happening in our studies involving the linear/cyclic nucleoside phosphates plus amino acids, resulted in the simultaneous formation of peptides and oligonucleotides via cross-catalysis that resulted in lengths and yields that have not been reported in previous studies. Importantly, the formation of peptides of up to tetramers using amino acids like tryptophan and aspartic acid, has not been shown in prior work.

To corroborate this, we have compiled the following two tables that details previous studies that demonstrated the formation of peptides and oligonucleotides, especially under drying/wet-dry cycling conditions.

Table 1: Studies that demonstrated the formation of oligonucleotides.

Paper (Citation)	**Reactants**	**pH Conditions**	**Temperature Conditions**	**Longest Peptide/Oligomer Observed**
Peptide formation in the prebiotic era: thermal condensation of glycine. Lahav, White & Chang, *Science* 1978	Glycine + clay minerals (e.g., bentonite/kaolin) with water	Not specifically pH-buffered (likely near neutral/ambient)	Cyclic variations in temperature & hydration (thermal fluctuations, dehydration/rehydration)	Oligoglycine formation upto pentamer is seen;
Effects of pH and temperature on	Glycine in aqueous solution	pH 3.1–10.9 explored; optimum around	Temperatures 120–180 °C examined	Primarily glycine dimer (Gly_2)

Paper (Citation)	Reactants	pH Conditions	Temperature Conditions	Longest Peptide/Oligomer Observed
dimerization rate of glycine. — Sakata et al., *Geochim. Cosmochim. Acta* 2010		pH ~9.8 for dimerization		formation & DKP kinetics;
Ester Formation & Hydrolysis during Wet–Dry Cycles — Mamajanov et al., *Macromolecules* 2014	α-Hydroxy acids (e.g., malic acid) forming model oligoesters; peptide formation not primary focus	Not explicitly controlled; generally reactions studied across broad conditions	60–95 °C wet/dry cycling conditions (model polymer system)	Oligoesters observed; *peptides not specifically quantified* in this polymer context
Formation of oligopeptides in high yield… — Rodriguez-Garcia et al., *Nat. Commun.* 2015	Glycine (± other AAs: Ala, Asp, Glu, His, Lys, Pro, Thr, Val)	pH conditions varied highest yield at pH ~9.75; low yield at ~pH 6.1-7.5	90–130 °C dehydration/rehydration cycles; typical ~130 °C	~Gly_{12} in a soluble fraction
Ester-Mediated Amide Bond Formation… — Forsythe et al., *Angew. Chem.* 2015	Mixture of α-hydroxy acids (e.g., lactic acid) + amino acids (glycine/alanine) → depsipeptides	Unadjusted mixtures start acidic (~pH ~3); pH adjusted experiments at pH 7 with base re-added each cycle	Wet phase ~65 °C (≈5.5 h), dry phase ~85 °C (≈18 h) across cycles	Depsipeptides with amino acids observed up to ~10 residues (mixed ester/amide) containing lactic acid
Prebiotic condensation through wet–dry cycling… — Campbell et al., *Nat. Commun.* 2019	Glycine + salts (KCl, NaCl, OH^- mixtures; phosphate salts)	Implicit pH changes via salt composition; no fixed bulk pH but hydration phase aqueous	Hot phases at 100–120 °C (dry) with cool ~40 °C (wet, varying %RH)	Primarily to Gly_3 and up to Gly_{13} upon complete dehydration oligomers observed by HPLC after 10 cycles
Prebiotic environments with Mg^2 (ACS	This article examines peptide stability but also	Typically near neutral to mildly	Often 60–90 °C in Earth-simulated settings	Glycine condensation outcomes for

Paper (Citation)	Reactants	pH Conditions	Temperature Conditions	Longest Peptide/Oligomer Observed
Earth & Space Chem. 2020)	reports on condensation conditions (glycine + environment)	basic (contextual studies)		small peptides (e.g., up to trimer/pentamer) depending on conditions
Proline Behavior in Model Prebiotic Peptides — ACS Earth & Space Chem. 2020	Mixtures of proline + glycine + alanine + valine in wet–dry cycling	Not strictly buffered; likely near neutral with reaction medium effect	Wet–dry cycles (specific T not in abstract; typical ~60–100 °C for such studies)	Linear oligomers including Pro and small cyclic species of dimeric or trimeric length was observed

Table 2: Studies that demonstrated the formation of oligonucleotides.

Paper (Citation)	Reactants	pH Conditions	Temperature Conditions	Longest Oligomer Observed (Reported)
Sawai & Orgel, JACS 1975	Activated nucleotides + Zn^{2+} catalyst	Slightly acidic to neutral	Ambient temperature	Dimers and trimers
Kawamura & Ferris, JACS 1994	5′-Phosphorimidazolide of adenosine (ImpA) + Na^{+}-montmorillonite	Near-neutral to mildly basic (≈ pH 7–8)	Ambient to ~40 °C	Up to 7-mer oligoadenylates (quantified kinetically; longer chains not reported in this study)
Prabahar, Cole & Ferris, JACS 1994	Activated adenosine phosphates + montmorillonite	Near-neutral	Ambient to moderate	Up to ~4-mer oligoadenylates, predominantly 3′,5′-linked
Crowe & Sutherland, ChemBioChem 2006	Cytidine nucleotides + cyanoacetylene	Mild aqueous conditions	Ambient to moderate	2′,3′-cyclic phosphate intermediates
Powner et al., ChemBioChem 2007	Cytosine nucleosides/nucleotides (photochemical synthesis)	Controlled lab pH	UV + low/moderate temperature	Monomers / activated nucleotides only (no oligomers reported)

Paper (Citation)	Reactants	pH Conditions	Temperature Conditions	Longest Oligomer Observed (Reported)
Costanzo et al., PLOS ONE 2016	3′,5′-cyclic AMP	Slightly acidic to neutral	~80–90 °C (drying)	Tetramer (4-mer) only for cAMP
Costanzo et al., ChemBioChem 2017	3′,5′-cyclic CMP + protons / UV	Acidic	UV irradiation with moderate heating (~60–80 °C)	Dimers and trimers (2–3-mers)
Dagar et al., RNA 2020	2′,3′- and 3′,5′-cyclic nucleotides (cAMP, cCMP) ± salts/minerals	Broad; effective under mildly alkaline conditions	~40–90 °C; wet–dry cycling	Up to ~4mers, condition-dependent
Braun et al., Preprint 2024	Ribonucleotides + amino acids (as catalysts)	Alkaline (≈ pH 9–10)	Ambient temperature	Up to ~6–10-mers for cGMP (~3 for other cNMPs)

1**. The Discussion restates results but offers limited mechanistic interpretation. For example: Why does cAMP produce significantly higher yields and longer oligomers than ATP? Is the determining factor stability, intrinsic reactivity, or aminoacylation efficiency?**

Answer: Cyclic nucleotides are predisposed for ring opening in the presence of anions (at alkaline pH) or in the presence of a suitable nucleophilic group (for example amino acids in our case), which is a thermodynamically favourable exothermic process which drives polymerization reactions forward [Ref a]. ATP has three phosphates, which undergoes hydrolysis in aqueous conditions releasing energy that does not result in formation of phosphodiester linkages via enzyme-free means, favouring breakdown rather than polymer synthesis [Ref b]. Additionally, cyclic nucleotides are relatively stable at neutral pH conditions over the observed time period (also observed in our study).

Therefore, stability at neutral pH- thus more availability of starting reactants, and the intrinsic reactivity in presence of amino acids leading to aminoacylated-nucleotide intermediates, could be contributing to higher yields in cAMP-based reactions when compared to ATP-based reactions.

a. Cassone, G., Šponer, J., Saija, F., Di Mauro, E., Saitta, A. M., & Šponer, J. E. (2017). Stability of 2′,3′ and 3′,5′ cyclic nucleotides in formamide and in water: a theoretical insight into the factors controlling the accumulation of nucleic acid building blocks in a prebiotic pool. Physical Chemistry Chemical Physics, 19, 1817–1825.

b. Gull, M., Mehta, C., Perez, M. J., Seeley, A., Rogers, K. L., & Pasek, M. A. (2025). Thermal decomposition and prebiotic formation of adenosine phosphates in simulated early-earth evaporative settings. *Molecules*, *30*(17), 3587.

2. Why do certain amino acids (e.g., Asp) form AMP-aa in much larger amounts than others? Is this driven by pKa, steric effects, or differential nucleophilicity? How do bulky amino acids such as Trp yield similar peptide lengths to Gly under the same conditions?

Despite having two potential reactive carboxylates, Asp undergoes predominantly α-selective aminoacylation due to favourable geometry and inherent α-activation, which results in higher yields of aminoacyl-nucleoside products (e.g., aspartyl-adenosine, Ref **c**). Therefore, Asp's enhanced formation of AMP-aa (or related aminoacyl-nucleotide species) is driven not just by pKa, but by a unique combination of structural steric effect, resistance to cyclization, favourable kinetics and strong intrinsic α-selectivity, explaining why it outperforms other amino acids in nonenzymatic aminoacylation chemistry.

c. Singh, J., Thoma, B., Whitaker, D., Satterly Webley, M., Yao, Y., & Powner, M. W. (2025). Thioester-mediated RNA aminoacylation and peptidyl-RNA synthesis in water. *Nature*, *644*(8078), 933–944.

The AWM (ATP+Trp+$MgCl_2$) reactions yielded up to trimers (Trp-Trp-Trp) trimers at both 90°C and 130° C reactions [Fig S29], which is similar to AGM (ATP+Trp+$MgCl_2$) reactions resulting in a yield of up to trimers at 90°C. However, this goes up to octaglycine at 130° C for the AGM reactions [Fig S5]. The similar length at 90°C, therefore, is more an outcome due to the sub-optimal temperature conditions for the formation of peptides at large. Nonetheless, there are no reports of yields of Trp trimers to our knowledge and this is something we have highlighted in our revised manuscript version.

3. How can Mg^{2+} simultaneously promote nucleotide oligomerization but inhibit peptide formation? These opposing roles need reconciliation within a unified mechanistic framework. A more analytical and comparative discussion would substantially strengthen the manuscript.

The reviewer correctly notes the apparent contradiction in Mg^{2+}'s dual role—promoting nucleotide oligomerization while inhibiting peptide formation, which is mainly due to its capacity to chelate amino acids in aqueous media [Ref 54]. Nevertheless, this paradox could have been readily resolved by the chemical heterogeneity of the prebiotic environments. Diverse microenvironments on early Earth, such as drying lagoons or mineral surfaces, could have spatially separated these reactions, with Mg^{2+} promoting nucleotide polymerization occurring in phosphate-rich pools, while peptide synthesis could have occurred in Mg^{2+}-poor or chelator-rich settings [Ref 18]. Temporally, nucleotide chemistry may have preceded peptide formation, allowing Mg^{2+} to assist in RNA-related processes like ribozyme function and cyclic phosphate activation, with peptides emerging later under more favorable

conditions. Furthermore, endogenous chelators such as citrate, aspartate, and phosphate can buffer Mg^{2+} concentrations, mitigating its inhibitory effects and enabling peptide bond formation even in its presence (Richter et al., 2017; Frenkel-Pinter et al., 2020). Lastly, selective catalysis by mineral surfaces like montmorillonite clay have been shown to facilitate RNA oligomerization, while could have permitted peptide synthesis by modulating local chemical reactivity. Together, spatial, temporal, and molecular strategies in prebiotic niches likely resolved the conflicting roles of Mg^{2+}, supporting the co-evolution of nucleotides and peptides.

4. Several yield values reported in the manuscript appear unexpectedly high or defy typical polymerization behavior. In some reactions, peptide yields approach or nearly match the initial amino acid concentration. This is unusually efficient for uncontrolled dry–down condensation at 130 °C and should be discussed mechanistically. Also, the reported peptide yields show similar abundance across short and long oligomers (e.g., di- through decaglycine). This contradicts classical statistical growth models, which predict steeply declining abundance with length. The authors should explain whether this reflects real chemistry, MS detection biases, or normalization methods.

We appreciate the thoughtful comment regarding the reported yields. We have systematically revisited our calculations and confirm that they remain consistent with the data presented in our study. For example, 22.5 µmol of glycine was used as the initial concentration of the reactant. Of this, ~21.3 µmol of glycine-based oligopeptides was observed in cycle 24 of the reaction (Fig 1B). On the other hand, the relative yield of the AMP-Gly intermediate observed in cycle 24 was approximately 200 nmol (or 0.2 µmol, Fig 4C), which does account for the combined molecular contribution of AMP and glycine, (totalling to is 21.5 µmol). There is sufficient scope for the formation of other reactive transient intermediates, whose individual yields are significantly lower than that of the AMP-Gly species (totalling to ~0.05 µmoles), while leaving some initial starting reactant, Gly.

As for the trends observed in the peptide yield; this reaction does not seem to be following the route of linear sequential addition of monomers. For e.g., the formation of tetraglycine (Gly_4) might have resulted from multiple routes that could have contributed to the yield; Route1: the linear and multiple sequential addition of glycine monomers to the AMP-Gly adduct, Route 2: the addition of triglycine to an AMP-gly adduct and, Route 3: the addition of diglycine to a preformed AMP-di-gly adduct (which we have observed readily by mass spectrometry). These multiple possibilities could, in principle, result in the yield trends that we observe in our reactions.

5. AMP-Asp is reported to accumulate in much higher amounts than AMP-Gly, AMP-Lys, or AMP-Trp. The manuscript does not analyze why Asp behaves differently, nor how this high degree of activation correlates with peptide formation. It is unclear how the reported quantities of free amino acids, AMP-aa, oligopeptides, and hydrolysis products relate. Even approximate mass balance would help validate the quantitative approach. Without clearer explanation, these quantitative trends are difficult to interpret.

The reviewer raises a very valid question. The first part of the question regarding the yields of AMP-Asp has already been elaborated in the answer to question 2. Also, the correlation between activation and formation of peptides has been discussed in the results section under the subsection “Intermediate responsible for the cross-catalytic enhancement of oligopeptides and oligonucleotides” in Line 326“Finally, after confirming… have limited elongation”.

We agree that the mass-balance approach raised by the reviewers is a thoughtful and valid consideration. Because the molecular weights of the different compounds are not identical, back-calculating absolute masses from nmol quantities would inherently result in different corresponding weights. To enable an internally consistent comparison across all samples, we therefore, present the yields in terms of nmoles rather than mass as this better reflects the relative formation of each product independent of molecular weight differences.

6. Amino acids such as Asp and Lys contain multiple nucleophilic or electrophilic sites. The manuscript claims formation of AMP-Asp and AMP-Lys, but does not determine: whether AMP-Asp attaches via alpha-COOH or beta-COOH, whether AMP-Lys forms through alpha-COOH or epsilon-NH_2, or whether peptide products contain branching or mixed linkages. Given the centrality of mechanistic conclusions, such regioisomeric ambiguity should be addressed, or at minimum discussed explicitly.

The reviewer indeed correctly points out that multiple nucleophilic or electrophilic sites in Asp and Lys exist, which could lead to the formation of intermediates. However, the fragmentation spectra of AMP-aa intermediates [Fig S23] and ^{31}P NMR data that we obtained, confirms the peptide formation mainly via α-COOH group of the respective amino acids, thus, solidifying the argument of the trend of yields that we observed for these intermediates in case of the respective amino acids. Further, all the reactions in this study were conducted at pH 7.0; so a lower pKa of the α-COOH means greater ease of activation of amino acids by ATP [Ref 48].

7. The manuscript reports that β-Ala does not form AMP-β-Ala, but does not analyze why. Simple pKa arguments do not fully explain the outcome, especially in dehydrating/high-temperature regimes. Likewise, experiments with Asp-methyl ester need more controls: esters are likely to hydrolyze under the high-temperature wet–dry cycles used here. Thus, the absence of AMP-Asp-OMe cannot be interpreted without verifying ester integrity.

β-alanine differs from α-amino acids in that its amino group is separated from the carboxylate by an additional methylene residue. This increased distance of β-amino group in β-Ala makes it less geometrically constrained and thus, less favourable towards intramolecular activation lowering its ability to form a stable mixed anhydride or aminoacyl-adenylate intermediate.

We agree with the reviewer regarding the limited thermal stability of Asp–methyl ester at elevated temperatures and hence we did follow its yields over the course of the reaction. The below table shows the area-under-the-curve (AUC) values for Asp–methyl ester under

the two conditions that we tested (90 °C and 130 °C). As expected, the concentration of Asp–methyl ester decreases progressively with each cycle, with a more pronounced decline at 130 °C than at 90 °C. Nonetheless, during the initial cycles its concentration remains sufficiently high to support the formation of the intermediate. Due to the low signal intensity in later cycles, neither the AUC values nor the ppm error associated with the AMP–Asp methyl ester mass are reliable enough to report, which is why we did not include those details in the manuscript.

Table 3: Intensities of Asp–methyl ester observed in each reaction cycle

	90°C	130°C
ABA cycle 0	7.23E+04	6.59E+04
ABA cycle 0	7.27E+04	6.66E+04
ABA cycle 1	7.20E+04	5.72E+04
ABA cycle 1	7.25E+04	5.72E+04
ABA cycle 2	6.81E+04	4.77E+04
ABA cycle 2	6.81E+04	4.69E+04
ABA cycle 5	6.37E+04	6.85E+03
ABA cycle 5	6.37E+04	6.47E+03
ABA cycle 10	4.10E+04	5.63E+03
ABA cycle 10	4.40E+04	5.58E+03
ABA cycle 24	3.95E+04	1.06E+03
ABA cycle 24	3.83E+04	1.01E+03

d. Leman, L., Orgel, L., & Ghadiri, M. R. (2004). *Carbonyl sulfide–mediated prebiotic formation of peptides.* Science, 306, 283–286.

e. Krishnamurthy, R., Eschenmoser, A., & Lipscomb, W. N. (2007). *Formation of proteinogenic amino acid adenylates: implications for prebiotic chemistry.* Angewandte Chemie International Edition, 46, 2454–2457.

f. Danger, G., Plasson, R., & Pascal, R. (2012). *Pathways for the formation of aminoacyl adenylates in prebiotic chemistry.* Chemical Society Reviews, 41, 5416–5429.

8. The data show that Mg^{2+} promotes nucleotide oligomerization, inhibits peptide formation, and affects AMP-aa accumulation. The manuscript presents these observations but does not integrate them into a coherent picture. A systematic mechanistic explanation would greatly strengthen the claims about relevance to prebiotic scenarios.

Magnesium ions play a complex and often paradoxical role in prebiotic and biochemical reaction systems. In RNA chemistry, Mg^{2+} is frequently described as a “double-edged sword”: while it is indispensable for proper RNA folding and stabilization of secondary and tertiary structures, it can also promote phosphodiester backbone hydrolysis, ultimately

leading to RNA degradation. In our reaction system, a similar dual behavior of Mg^{2+} was observed. The presence of Mg^{2+} enhanced the formation and stability of the oligonucleotides generated in the reaction mixture, likely through charge shielding of the negatively charged phosphate backbone. At the same time, in control reactions designed to visualize peptide formation (GM-based reactions), magnesium alone resulted in a noticeable decline in peptide yield. Notably, when ATP was introduced into the reaction, a significant recovery in peptide formation was observed, suggesting that ATP may modulate or counterbalance the inhibitory effects of Mg^{2+}.

Furthermore, Mg^{2+} promoted the formation of nucleotide–amino acid adducts in higher yields, most likely by neutralizing the negative charges on phosphate groups and thereby facilitating nucleophilic attack by amino acids. Nevertheless, excessive Mg^{2+} may also chelate free amino acids, partially limiting their availability for peptide bond formation (Ref 55). Taken together, these observations highlight the nuanced role of magnesium in such reaction systems: although it can impose certain constraints, its overall contribution appears to create a chemically favorable environment that supports both nucleotide-based interactions and peptide formation, underscoring its potential importance in prebiotic chemical evolution.

Reviewer #2 (Remarks to the Author):

The manuscript by Roy et al. reports peptide formation catalyzed by activated RNA mononucleotides. The authors show that adenosine triphosphate and adenosine 3',5'-cyclic monophosphate can promote the formation of longer oligoglycine peptides. The work is valuable to the Origins of Life community, though its broader scientific impact is more incremental. I am not fully convinced that Communications Chemistry is the most appropriate venue for this manuscript. Most importantly, the analytical methodology does not, in its current form, support quantitative conclusions. I believe NMR is required for analysis (as previously performed). I would, however, support publication pending the revisions outlined below.

1. I do not think there is sufficient evidence (from the data provided) that the abstract should have "indicates the plausibility of the emergence of initial steps involved in a primordial translation system". This to me is overinflating the work and does not reflect the findings.

We thank the reviewer for highlighting the importance of precise framing. Our rationale for the original wording was based on the fact that, in extant biology, translation initiates with ATP-dependent activation of amino acids to form AMP-aa intermediates. In this study, we demonstrate that such AMP-aa species, under high-temperature selection pressures, can give rise to oligopeptide formation in the absence of ribosomal protein machinery. We, therefore, framed the statement to reflect the relevance of these findings to early, pre-ribosomal steps that may have preceded modern translation.

2. Line 57: many references are missing for this sentence. Seminal work by Orgel, Richert and Szostak need to be reference extensively here

The following are few of the pioneering work by Orgel, Richert and Szostak group.

The relevant articles directly linked to our study has been included in the citation index. We also found other findings from the group the reviewer has mentioned, which establish the principles of template-dependent ligation and replication of nucleic acids in the absence of enzymes. They show how complementary templates guide strand extension, ligation fidelity and backbone formation, highlighting fundamental physicochemical processes that could support information transfer and replication in early, prebiotic genetic systems. These studies primarily address nonenzymatic, template-dependent oligonucleotide ligation rather than *de novo* nonenzymatic formation of oligonucleotides as we show in this study. Given that these studies are not directly aligned with the focus of this section, we did not mention them in our article.

3. Line 76: the authors need to make a more convincing argument for the prebiotic relevance of ATP and cAMP. Their presence has been extensively studied (e.g. PLOS Biol 2022 e3001437) would significantly strengthen the narrative. Also, Sutherland's work (and perhaps Powner's work) has shown the prebiotic synthesis of cyclic nucleosides.

The article PLOS Biol 2022 e3001437 is already citated in the main manuscript [Ref 5]. Previous article by Powner and Sutherland has now been added in line 59.

4. Line 89: have the authors tried Mn instead of Mg. This is important for a number of reasons. Importantly, Mn drives polymerization in certain cases.

We agree with the reviewer alluding to the importance of Mn^{2+} cations in polymerization studies; divalent metal ions such as Mn^{2+} are widely recognized to influence nonenzymatic ligation and polymerization reactions in chemical and biochemical systems. We chose to focus in our current work on Mg^{2+} though for the following relevant aspects observed in extant biochemistry: Its essentiality for aminoacylation by tRNA ligases, stabilization of ATP and the aminoacyl-adenylate intermediate, facilitation of proper substrate positioning, and neutralization of negative charges on the phosphate backbone. These roles enable efficient catalysis and high fidelity during tRNA charging in all domains of life.

We would like to assure the reviewer that while this study focused on Mg^{2+}, exploring how other transition metal ions could affect reactions in a similar primordial setting, is a clear direction for future investigation. We intend to examine analogous reactions with Mn^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+} and other divalent cations to systematically assess their effects on peptide and nucleotide chain growth under prebiotic selection pressures. This is however beyond the scope of the current manuscript.

5. Line 106: perhaps the most significant issue I have with the work is the author's claim to

a quantitative methodology. Mass Spectrometry quantitative analysis is highly dependent on ionization efficiency. To use a single amino acid, alanine, as the internal standard does not justify its use for an analytical internal standard for a dimer, trimer, tetramer, pentamer, etc. (unless a previously published reports state so). The authors cannot claim a quantitative analysis unless their methodology has been validated (in more than one context I might add). I believe this is essential for a study stating such conclusions.

We are in agreement with the reviewer's point that the observed ion intensities for distinct molecular species in mass spectrometric analyses are influenced by multiple physicochemical and instrumental parameters, including—but not limited to—the molecular mass, intrinsic ionization efficiency, solvent composition, differential ion–molecule clustering, adduct formation, and matrix effects. Notably, even for a single compound, the absolute signal intensities can vary across analytical runs, instrument batches and day-to-day operating conditions due to fluctuations in ion source stability, spray characteristics, detector sensitivity and subtle changes in chromatographic behaviour. Nevertheless, it still is one of the analytical techniques that is used to characterize complex mixtures. Our reactions generates a highly heterogeneous molecular ensemble comprising unreacted starting materials, oligopeptides of diverse chain lengths, oligonucleotides spanning a broad distribution of degrees of polymerization, mixed nucleotide–peptide conjugates, transient reaction intermediates and various hydrolytic or degradation products. Given this compositional complexity, constructing calibration curves for every discrete molecular entity within the mixture is not experimentally feasible. Additionally, several of these species are not commercially available as purified standards, with many existing only transiently under the reaction conditions, precluding isolation in sufficient purity for quantitative calibration.

To address this limitation, we adopted an internal-standard–based quantification strategy. We selected alanine as it is a structurally simple, chemically inert and readily ionizable internal analyte. Prior to each analytical batch, we performed a short calibration sequence using alanine alone to ensure stable response characteristics. Subsequently, all reaction samples were spiked with alanine at a fixed, uniform concentration, maintaining identical internal standard levels across all vials and batches. Because the internal standard experiences the same ionization environment, solvent composition, chromatographic conditions, and instrumental fluctuations as all other analytes in a reaction mixture, its signal intensity remains highly reproducible across injections. Similarly, although different analytes possess distinct ionization efficiencies, each individual analyte exhibited consistent relative signal intensity across the various replicates that we performed from the same batch. Thus, quantification based on the analyte-to-internal-standard (analyte/IS) peak-area ratio does provides a robust, normalization-based metric that reflects the relative abundance of each analyte independent of absolute instrumental variability.

To substantiate this approach, we provide representative raw-data screenshots for several molecular species—including the AMP dimer, AMP trimer, diglycine, triglycine, and the AMP-G conjugate—measured across multiple dehydration–rehydration (DH–RH) cycles. As can be clearly observed, the **Analyte/IS ratios** remain consistent across technical replicates within each cycle, demonstrating that internal-standard normalization effectively compensates for

run-to-run variability and enables reliable comparative quantification within our complex reaction system. This is also an approach that has been used in several previous studies as detailed in the section below Table 4.

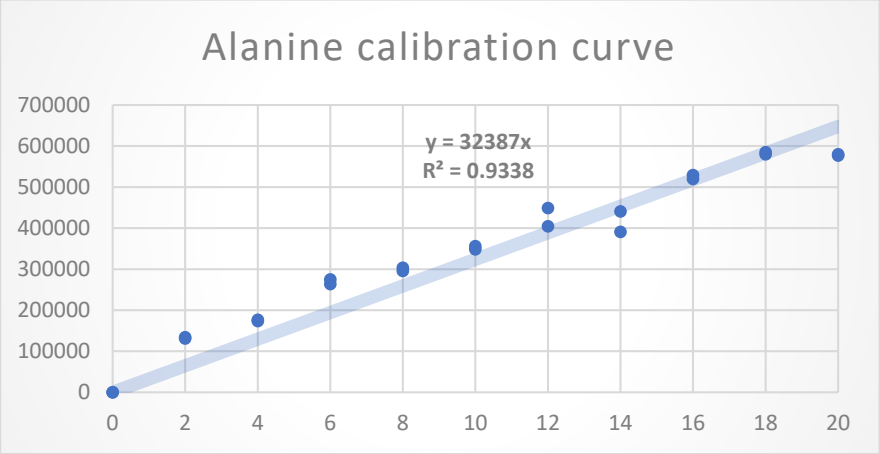
Table 4: Acquired screen shot of tabulated raw data obtained from LCMS runs of our AGM 130H reaction.

Sample Name	Component...	Expected RT	Area	Retent... Time	Adduct / C...	Formula	Precursor Mass	Reporta...	*Analy...	Isotope Confi...	Found At Mass	Mass Error (...	Isotope Ratio Difference
AGM (High)_130deg_cycle 0_R1_T1_positive...	AMP-AMP (+1)	10.26	N/A	N/A	[M+H]+	C20H26N...	677.1229	☑			N/A	N/A	N/A
AGM (High)_130deg_cycle 0_R1_T2_positive...	AMP-AMP (+1)	10.26	N/A	N/A	[M+H]+	C20H26N...	677.1229	☑			N/A	N/A	N/A
AGM (High)_130deg_cycle 1_R1_T2_positive...	AMP-AMP (+1)	10.26	7.448e4	10.25	[M+H]+	C20H26N...	677.1229	☑	0.451		677.1219	-1.4	13.9
AGM (High)_130deg_cycle 1_R2_T2_positive...	AMP-AMP (+1)	10.26	8.154e4	10.27	[M+H]+	C20H26N...	677.1229	☑	0.458		677.1219	-1.5	19.4
AGM (High)_130deg_cycle 2_R2_T1_positive...	AMP-AMP (+1)	10.26	1.252e5	10.32	[M+H]+	C20H26N...	677.1229	☑	0.630		677.1228	-0.1	6.2
AGM (High)_130deg_cycle 2_R2_T2_positive...	AMP-AMP (+1)	10.26	1.335e5	10.30	[M+H]+	C20H26N...	677.1229	☑	0.684		677.1231	0.4	5.5
AGM (High)_130deg_cycle 5_R3_T1_positive...	AMP-AMP (+1)	10.26	1.839e5	10.30	[M+H]+	C20H26N...	677.1229	☑	1.022		677.1231	0.4	3.8
AGM (High)_130deg_cycle 5_R3_T2_positive...	AMP-AMP (+1)	10.26	2.081e5	10.34	[M+H]+	C20H26N...	677.1229	☑	1.175		677.1224	-0.7	4.3
AGM (High)_130deg_cycle 10_R1_T1_positive...	AMP-AMP (+1)	10.26	1.734e5	10.30	[M+H]+	C20H26N...	677.1229	☑	1.063		677.1225	-0.6	8.9
AGM (High)_130deg_cycle 10_R1_T2_positive...	AMP-AMP (+1)	10.26	1.621e5	10.32	[M+H]+	C20H26N...	677.1229	☑	1.093		677.1235	0.9	0.5
AGM (High)_130deg_cycle 24_R1_T1_positive...	AMP-AMP (+1)	10.26	1.285e5	10.29	[M+H]+	C20H26N...	677.1229	☑	0.726		677.1225	-0.6	9.9
AGM (High)_130deg_cycle 24_R1_T2_positive...	AMP-AMP (+1)	10.26	1.288e5	10.30	[M+H]+	C20H26N...	677.1229	☑	0.746		677.1224	-0.7	1.7

Table 5: Analyte/IS data of a few other molecules are tabulated below:

Sample Name	**Analyte/IS**				
	AMP dimer	AMP trimer	Digylcine	Triglycine	AMP-G
AGM (High)_130deg_cycle 0	Not detected	Not detected	Not detected	Not detected	Not detected
AGM (High)_130deg_cycle 0	Not detected	Not detected	Not detected	Not detected	Not detected
AGM (High)_130deg_cycle 1	0.451	0.001	0.081	0.011	0.124
AGM (High)_130deg_cycle 1	0.458	0.001	0.085	0.011	0.125
AGM (High)_130deg_cycle 2	0.63	0.003	0.139	0.094	0.086
AGM (High)_130deg_cycle 2	0.684	0.003	0.135	0.093	0.08
AGM (High)_130deg_cycle 5	1.022	0.008	0.243	0.13	0.059
AGM (High)_130deg_cycle 5	1.175	0.01	0.244	0.137	0.059
AGM (High)_130deg_cycle 10	1.063	0.033	0.335	0.316	0.045
AGM (High)_130deg_cycle 10	1.093	0.036	0.338	0.312	0.045
AGM (High)_130deg_cycle 24	0.726	0.009	0.241	0.25	0.013
AGM (High)_130deg_cycle 24	0.746	0.01	0.241	0.245	0.014

The calibration curve for this batch:



<u>Similar approach of quantification has been used by the following articles</u>:

g. Van der Kloet, F. M., Bobeldijk, I., Verheij, E. R., & Jellema, R. H. (2009). Analytical error reduction using single point calibration for accurate and precise metabolomic phenotyping. *Journal of Proteome Research, 8*(11), 5132–5141.
h. Xiao, J. F., Zhou, B., & Ressom, H. W. (2012). *Metabolite identification and quantitation in LC-MS/MS–based metabolomics. TrAC — Trends in Analytical Chemistry*, *32*, 1–14.
i. Holman, J., et al. (2012). Internal standard strategies for relative and absolute quantitation of peptides in biological matrices by liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B, 893–894*, 1–14.
j. Lenco, J., Lan, R., Edwards, N., et al. (2012). MS/MS library–facilitated MRM quantification of native peptides prepared by denaturing ultrafiltration. Proteome *Science*, *10*, 7.
k. Wang, M., & Han, X. (2016). Selection of internal standards for accurate quantification of complex lipid species in biological extracts by electrospray ionization mass spectrometry – What, how and why? *Mass Spectrometry Reviews*, 36(6), 693–714. https://doi.org/10.1002/mas.21492
l. Zhang, J., Dasgupta, A., Ayala, R., Bonapace, C., Kassim, S., & Faustino, P. J. (2024). Internal standard variability: root cause investigation, parallelism for evaluating trackability and practical considerations. *Bioanalysis*, *16*(15), 771–776.
m. Jain, S. K., Bansal, S., Bansal, S., Singh, B., Klotzbier, W., Mehta, K. Y., & Cheema, A. K. (2024). An optimized method for LC–MS-based quantification of endogenous organic acids: metabolic perturbations in pancreatic cancer. *International Journal of Molecular Sciences*, *25*(11), 5901.
n. Mahmud, I., Wei, B., Veillon, L., Tan, L., Martinez, S., Tran, B., Raskind, A., de Jong, F., Liu, Y., Ding, J., Xiong, Y., Chan, W.-K., Akbani, R., Weinstein, J. N., & Lorenzi, P. L. (2025). Ion suppression correction and normalization for non-targeted metabolomics. *Nature Communications*, *16*, 1347.

6. The authors report concentrations (e.g. line 121). How have the authors assessed the significant figures on these values. That is, how do the authors know that there is a yield of "1.11 umoles", and not "1 umol" (note that umoles should be umols).

The concentration of alanine in each individual run was determined using calibration curves generated for the corresponding batches. Quantification of each analyte was then performed using a unitary method, applying the formula: (area under the curve of the analyte / area under the curve of the internal standard) × moles of alanine obtained from the calibration curves. Yields were averaged across six replicates, and cumulative yields were calculated by summing contributions from each observed oligopeptide length. We have corrected for the notation of 'umols'.

7. Fig 1A. There are many m/z signals that are unassigned, which is typical for MS spectra. My concern is that the authors assign importance to signals that are well below intensity to many other signals. For instance, Gly4 is next to a towering peak. Gly 5/6/7/8/9 are well below many m/z (e.g the m/z=269.0846). How are the authors justifying this? In addition, have the authors performed a statistical analysis on the significance of a signal. This is paramount in such an analytical study.

The mass spectrometry (MS) method was designed to trigger collision-induced dissociation (CID), when the intensity of a specific analyte exceeded a predetermined threshold. When this threshold is reached, the instrument selectively fragments the parent ions of that analyte. Consequently, the resulting spectra contains signals corresponding not only to the intact parent ion masses but also to the masses of their characteristic fragment ions. This approach allows for simultaneous detection of the molecular ions and structural information from their fragmentation patterns, enhancing analyte identification and confirmation within a complex mixture. As the reviewer notes, certain masses are unassigned, for e.g. 269.0846. This and other unassigned masses correspond to a molecule with simultaneously fragmented NH_2 and OH groups, from the amino and carboxyl terminal, respectively. These masses that the reviewer mentioned are fragmentation masses of precursors peptides, which are tabulated in detail in the supplementary (Fig S3).

8. The authors has consistently discussed physiological pH, but it is well-known that early earth could have succumb to pH fluctuations. The authors should consider repeating their reactions at pH 5, 6, 8, and 9 to better reflect these geological possibilities.

While it is true that early Earth environments may have experienced pH fluctuations, our experimental focus on near-neutral pH is intentional and relevant. As highlighted in previous studies, peptide formation and oligonucleotide polymerization are highly sensitive to pH. For instance, wet-dry cycling at high temperatures is effective for peptide formation, but yields drop significantly under near-neutral conditions while being more efficient in extreme pH conditions [Fig 5b of "Formation of oligopeptides in high yield under simple programmable conditions" article of Lee Cronin group]. Similarly, nucleotide activation and polymerization using 3',5'/2',3'cyclic monophosphates favors nonenzymatic polymerization when cyclic

nucleotide monomers are subjected to wet–dry cycles at extreme pH, and at either ambient or elevated temperatures, while not resulting in oligomers in the acidic pH range. Described below are important considerations that stem from the relevant study:

o. Wunnava, S., Dirscherl, C. F., Výravský, J., Kovařík, A., Matyášek, R., Šponer, J., & Braun, D. (2021). *Acid-catalyzed RNA-oligomerization from 3',5'-cyclic GMP. Chemistry – A European Journal, 27*(70), 17581–17585. This study shows the dependence of 3',5'-cGMP polymerization on pH; polymerization is inhibited by base (higher pH) and occurs primarily when starting solutions are acidic (pH ~3) upon drying at 80 °C.

p. Vlassov, A. V., Kazakov, S., Guthrie, J., & Johnston, B. H. (2016). *Non-enzymatic oligonucleotide formation from 3',5' cyclic AMP. Journal of Molecular Evolution, 82*(3–4), 212–221.. This work reports that 3',5'-cAMP oligomerization requires neutral to alkaline conditions and elevated temperatures (≈80 °C) for any appreciable product; yields and chain lengths are lower than for cGMP, and polymerization is strongly reduced at low pH.

q. Šponer, J. E., Šponer, J., Kovařík, A., Šedo, O., Zdráhal, Z., Costanzo, G., & Di Mauro, E. (2021). *Questions and answers related to the prebiotic production of oligonucleotide sequences from 3',5' cyclic nucleotide precursors. Life, 11*(8), 800. This review notes that cyclic nucleotides are unstable under acidic conditions, undergoing rapid ring-opening that disfavors productive polymerization unless specific stacking/crystallization processes occur.

Thus, while exploring a broader pH range from for e.g. 5–9, as has been recommended, could provide additional information about prebiotic chemistry, previous studies have already characterized oligopeptide and oligonucleotide formation under similar pH conditions. Additionally, we also chose to intentionally focus on near-neutral pH where the formation of both oligopeptides and oligonucleotides is limited, as it allows us to isolate and highlight any mutual influences that stem from amino acids on oligonucleotide formation, and nucleotides on oligopeptide formation, respectively. By working under conditions that are intrinsically less favorable for spontaneous polymerization, we can more clearly demonstrate any synergistic or inhibitory effects arising from the coexistence of these biomolecular building blocks, without the confounding factor of high baseline yields.

9. Fig 1A caption: the authors should explicitly state their internal standard (alanine if I am not mistaken)

This has been corrected.

10. Figure S1; the authors should specify the stereochemistry

This has been corrected.

11. For ion exchange studies, the gradient of 0-30% could have been changed to resolve so of the species that were unresolvable (AMP2 and ADP). Have the authors considered ion pairing agents like triethylammonium acetate?

We attempted both strategies and systematically tested them using analytical HPLC. Decreasing the gradient slope and extending the run time, which is often effective for improving separation of closely eluting species, primarily resulted in pronounced peak broadening and loss of peak symmetry, without a corresponding improvement in chromatographic resolution. Additionally, we employed triethylammonium acetate (TEAA, pH 7.5) as an ion-pairing agent on a C18 reversed-phase column to enhance retention of the polar analytes. Although TEAA increased overall retention times, baseline separation of the target compounds was not achieved, indicating that their closely similar hydrophobicity and charge characteristics limit their effective resolution under conventional reversed-phase HPLC conditions.

Please refer to Fig 1 where we tried to resolve our DH-RH samples with a broader gradient but it only resulted with a peak broadening:

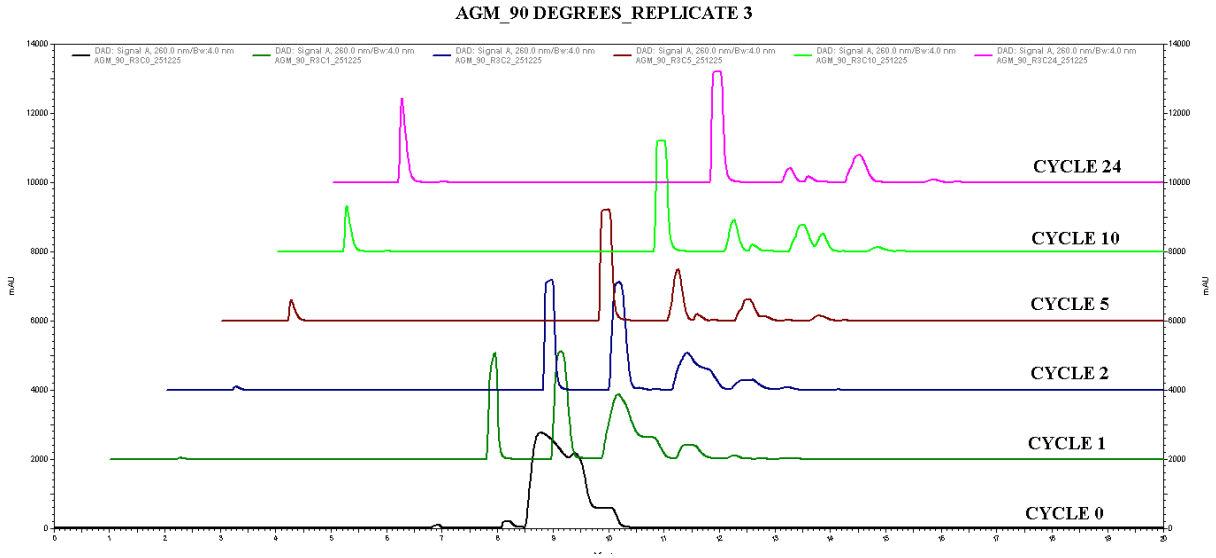
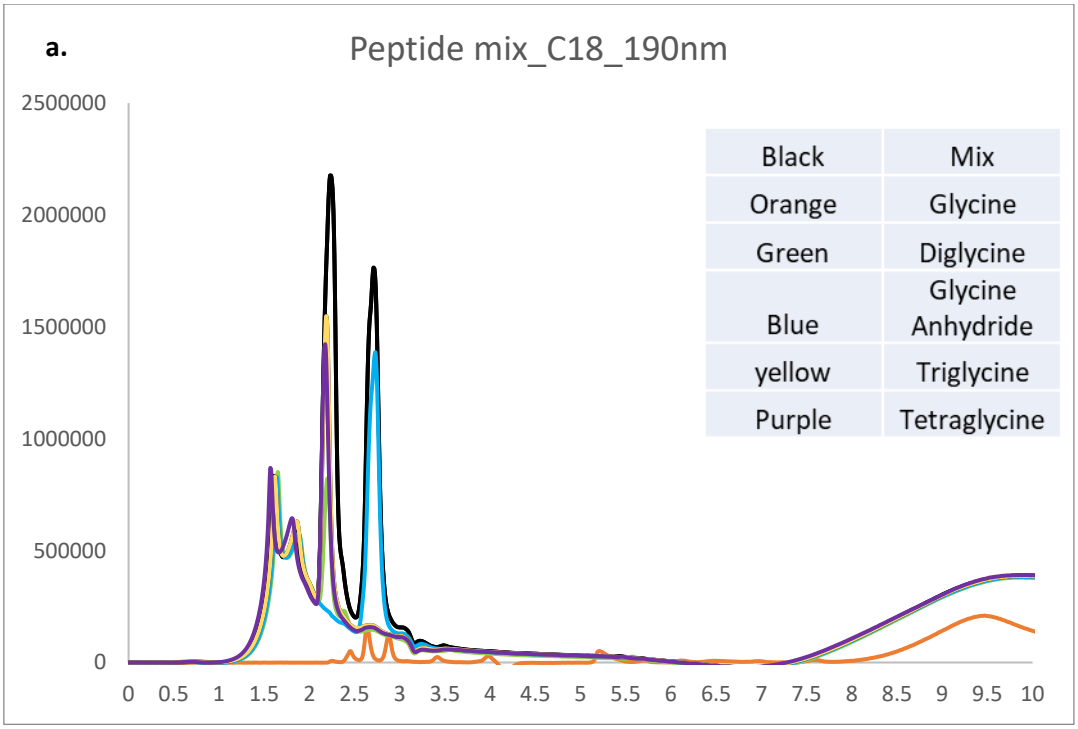


Fig 1: HPLC chromatograms of AGM 90H reaction at a lower percent of elution gradient (10%): The peaks obtained in this broad gradient showed peak broadening and thus, the resolution problem persisted. We also tried a similar approach with the 130H reaction timepoints but even that resulted in flattening of peaks.



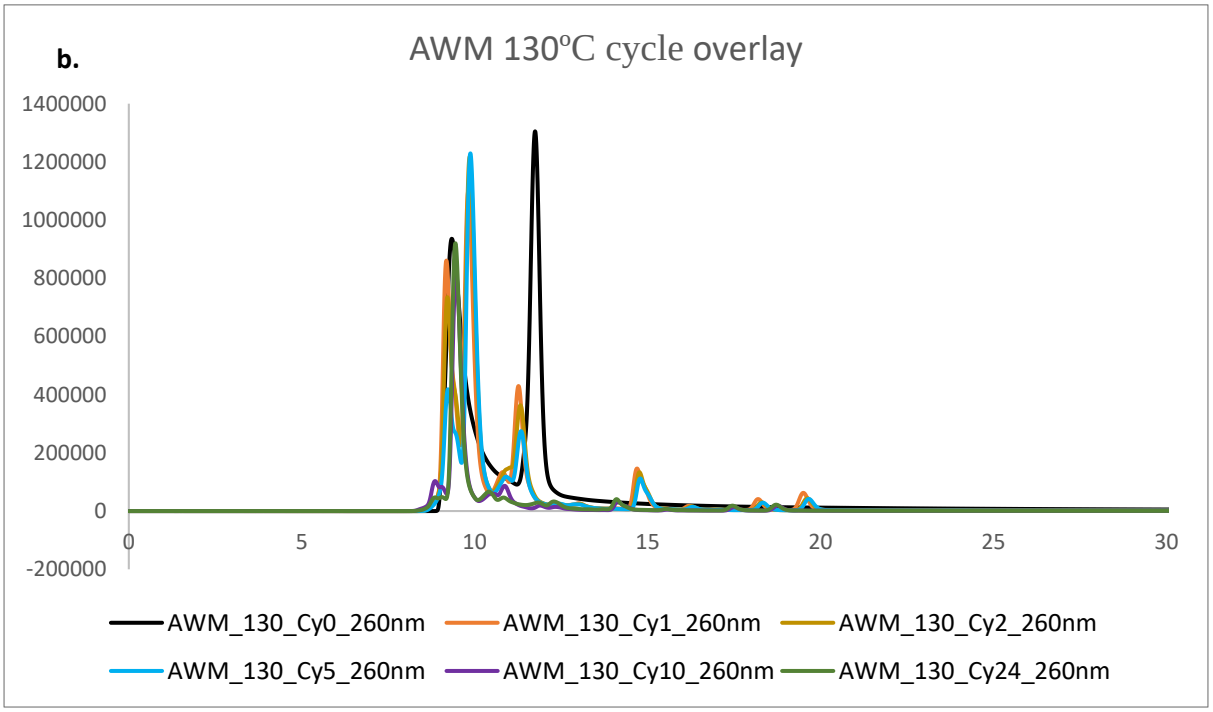


Fig 2: HPLC chromatograms with 50 mm TEAA as aqueous solvent and can as elution solvent. Fig 2a shows overlay of commercially bought peptides and Fig 2b shows AWM 130 reaction timepoints using the same gradient. As can be observed, the resolution between compounds with TEAA was not able to be achieved. (Mention trace colours to make your point precisely as even I am losing out on this info).

12. It's ambiguous when the authors use AMP tetramers. Does this mean a tetranucleotide? If so, please define appropriately like on line 215

This has been corrected.

13. Fig 3A: "A" is not defined. Maybe it should be "Fig 3: (A)…"

This has been corrected.

14. Line 249: The AMP-aa which is presumably crucial to the study needs to be synthesized in lab and characterized by NMR. The authors can use this synthetic standards in their other studies and I believe this to be an essential addition to the study. This would also allow the authors to run spike experiments (e.g. countering the statement on line 269-270). Despite complex mixtures, spike experiments are normally very telling.

We have now included Fig. 5b where the fragmentation spectra have been replaced with overlaid ^{31}P NMR profiles comparing the mixed anhydride peak of the chemically synthesized compounds with the mixed anhydride peak observed in our DH–RH run of AGM 130H for Cycle 1 and Cycle 5.

15. The AMP-Lys has no defined characterization. Although there is some information provided by the authors, its essential to provide proper NMR characterization at the very least. The MS is NOT convincing in my opinion. This goes for the other amino acids as well (Trp)

Fig S24a has been added, which is the ^{31}P NMR data obtained for AMP-Lys, AMP-Asp and AMP-Trp, all clearly demonstrating that the intermediates have a mixed anhydride linkage.

16. I am curious why the authors have chosen 130°C. Is there any precedence to this actual values? If so, the authors should explicitly state this, or at least why they chose this.

The two temperatures 90°C and 130°C has been deliberately chosen based on previous studies that evaluated for the efficient formation of oligomers that resulted over and above hydrolysis driven back reactions. They observed that the optimal temperature for formation and stabilization of oligonucleotides was about 90°C (please see reference below) while 130°C was chosen for facilitating the efficient formation of peptides [Fig 5a of "Formation of oligopeptides in high yield under simple programmable conditions" article from Lee Cronin's group]

Mungi, C. V., & Rajamani, S. (2015). Characterization of RNA-Like Oligomers from Lipid-Assisted Nonenzymatic Synthesis: Implications for Origin of Informational Molecules on Early Earth. *Life*, *5*(1), 65-84. (This reference is the same as SR3 in SI section.)

17. Line 314. pKa should be pKa

This has been corrected.

18. Line 316. The authors look at the protected version of Asp. What about Lys? Or the other amino acids. This seems incomplete.

The use of a protected form of Asp was intended to assess the reactivity of the side-chain (R-group) carboxylate while preventing participation of the α-COOH group in adduct formation. Under these conditions, no adduct formation was observed, suggesting that the α-carboxyl group is the primary functional group involved in the formation of the reactive intermediates. Furthermore, the newly added ^{31}P NMR profiles clearly indicate that the linkage between the nucleotide and these amino acids corresponds to a P–O–C mixed anhydride bond. Together, these results provide strong evidence for the specific functional group involved in the reaction, eliminating the necessity for using additional protected amino acid variants.

19. Line 323-324: How do the authors know this is not occurring in the MS source? Is there any other evidence the authors can use like NMR? Again, I am not convinced by the MS analysis alone. I would strongly encourage the authors to use another analytical technique to validate their results.

The tandem MS data elucidation of the mixed anhydride linkage of the adduct formed in cAG reactions has been corroborated with ^{31}P NMR of cAG data overlay in Fig S24b.

20. Line 337: although this might be a logical conclusion, the authors have little evidence to state this. Not enough characterization in the reaction, in my opinion, has been performed to certifiably state this as a conclusion.

We do partially agree with the reviewer when they say that the inference drawn in Line 337 could use additional characterization. However, the side chains of Asp, Lys and Trp have distinct chemical compositions and masses—Aspartic acid (Asp) with C_2H_4NO whose exact mass is 58.0293, Lysine (Lys) with $C_4H_{10}N$ whose exact mass is 72.0813, and Tryptophan (Trp) with C_9H_8N whose exact mass is 130.0657. In our experiments examining temperature-dependent oligopeptide formation, we observed that only oligomers of lengths up to trimers, tetramers and pentamers formed in reactions that involved Trp, Lys and Asp, respectively, under both the temperature conditions that were tested. This observation suggests that the size, polarity and the steric properties of the side chains are interfering with efficient molecular packing that is potentially necessary for the formation of longer homopeptides. In other words, the bulkiness or chemical functionality of these side chains likely limits chain elongation, providing a plausible mechanistic explanation for the restricted oligomer lengths observed in the experimental system.

21. Fig 5A: I would encourage the authors to include an NMR panel with a spike text of the synthetic standard

The previously reported yield of the synthetic standards was 40% for the mixed anhydride. After extensive standardization, we obtained the product in 66% yield but it still contains starting reactant AMP as the main impurity. Therefore, spiking of the synthetic standard could not be achieved in an effective manner. Therefore, we have included an overlay image (Fig 5B) of our reaction cycle with the synthetic standard, which clearly shows the formation of mixed anhydride intermediates in our reaction.

22. Line 372: maybe place the decaglycine before the dodecaglycine?

After also accounting for suggestions made by the other reviewers, this sentence has been removed from the discussion section to avoid redundancy.

23. The authors us ATP as a model compound but they should consider GTP as this is used in modern biology for various functions. I think this would increase generalizability as well.

Including other pyrimidine and purine nucleotides could enhance the generalizability of the observed reactions. However, time and finance-related logistics precludes us from including it within the scope of this manuscript. The focus on ATP in this work is deliberate: ATP serves as the universal energy currency in cellular systems and is the primary substrate for tRNA ligases, the enzymes responsible for the formation of aminoacylated AMP. Studying ATP allows us to directly connect our findings to biologically relevant processes, providing plausible mechanistic insight(s) into how early oligonucleotide–amino acid interactions could mirror extant biochemical pathways.

We are happy to share that we finally have just been able to initiate experiments in our laboratory using GTP- and CTP-based systems. The very preliminary results are indeed promising, suggesting that similar mechanistic trends may hold beyond ATP. However, these studies are far from complete and are part of future directions for our research program. We sure look forward to sharing them with the wider community when we have a comprehensive piece of work in place.

24. Line 382: The authors overreach by saying that "amino with bulky R groups" as all amino acids other than Gly will have an R group. This is not reflective of prebiotic relevance or modern biology.

Alluding to tryptophan as having a bulky R group (when compared to the other amino acids) is not uncommon and has been done so in previous studies too. We have included references mentioning this.

r. **Barik, S. (2020).** *The uniqueness of tryptophan in biology: Properties, metabolism, interactions and localization in proteins. International Journal of Molecular Sciences, 21*(22), 8776.

s. **Bhaduri, S., & Motley, M. P. (2018).** Tryptophan-rich and proline-rich antimicrobial peptides. Molecules, 23(4), 815.
t. **Lee, J. et al. (Year).** *Tryptophan and other aromatic residues influence peptide packing due to large side-chain volume.*

We do concur that tryptophan (Trp) may not appear prebiotically plausible, as it is not produced, for e.g., in the classical Urey-Miller type experiments and is relatively rare in abiotic synthesis studies. However, according to the Frozen Accident Hypothesis proposed by Francis Crick in 1968, the assignment of codons to amino acids occurred very early in evolution, before the emergence of the last universal common ancestor (LUCA). This implies that Trp, despite its low prebiotic abundance, was potentially encoded early on in the genetic code, and therefore could have participated in biochemical reactions once primordial entities began using the code. In addition to its evolutionary relevance, we consciously included Trp in our experiments to increase the generalizability of our findings. This allowed us to evaluate whether amino acids with diverse side-chain structures, including bulky and aromatic residues, could influence oligonucleotide and oligopeptide formation under the conditions tested.

25. Line 389: methyl needs to be methylene

This has been corrected.

26. The paragraph starting at 389 is repetitive and needs to be revised.

This has been corrected.

27. Fig 6. The authors have not discussed regioselectivity which is very important in the context of nonenzymatic genome replication. Although this is beyond the scope of this paper. The authors should not assume one isomer over the other (e.g. 3'◊5' 2'◊5')

Thank you for pointing this out; we did consciously keep an eye out for this during analysis. We have now explicitly stated the isomeric variation that are possible in the figure legend.

28. Line 431, the authors should include additional, more update, references on the topic. Many groups have "more-recently" reported data on this topic.

We have included articles from Dieter Braun and Matthew Powners' groups to reflect this.

29. Line 465: a reference is needed here.

This has been corrected.

30. Line 472 and onwards should be in a "conclusion section" in my opinion.

The authors thank the reviewer on this input. Line 472 onwards has now been moved to the conclusion section.

31. I believe the authors should discuss the RNA-peptide world to a larger extent (both the introduction and the discussion)

The introduction and the discussion sections have been reworded to expand on the RNA-peptide world hypothesis as required.

Reviewer #3 (Remarks to the Author):

The manuscript by Roy and colleagues investigates the co-catalytic role of amino acids and nucleotides in the formation of oligomerization products. Using wet–dry cycling under various conditions, they promoted oligomerization reactions yielding polypeptides and oligonucleotides. The authors report a significant co-catalytic effect that results in longer oligomerization products. This finding is highly intriguing, as it links the two fundamental chemistries of nucleic acid polymerization and peptide bond formation into a co-dependent mechanism, processes that are strictly separated in modern biology (polymerase vs. ribosome). The study thereby addresses one of the most fundamental questions in prebiotic chemistry: which came first, nucleic acids or proteins? The work aligns well with recent hypotheses suggesting a co-dependent evolution of these biopolymers.

There are a few points that need to be addressed:

1) I could not find any calibration curves for the different oligomerisation products. The authors claimed they used the integral of the mass spec signal but oligonucleotides or polypeptides of different length cannot be easily compared as they provide different signal intensities. The signal intensity and therefore the peak area, is dependent on many factors such as the mass of the molecule, the solvent system, presence of ions, etc. As the main finding fully depends on quantification, the authors need to show that their quantification is robust.

We are in agreement with the reviewer's point that the observed ion intensities for distinct molecular species in mass spectrometric analyses are influenced by multiple physicochemical and instrumental parameters, including—but not limited to—the molecular mass, intrinsic ionization efficiency, solvent composition, differential ion–molecule clustering, adduct formation, and matrix effects. Notably, even for a single compound, the absolute signal intensities can vary across analytical runs, instrument batches and day-to-day operating conditions due to fluctuations in ion source stability, spray characteristics, detector sensitivity and subtle changes in chromatographic behaviour. Nevertheless, it still is one of the analytical techniques that is used to characterize complex mixtures.

Our reactions generate a highly heterogeneous molecular ensemble comprising unreacted starting materials, oligopeptides of diverse chain lengths, oligonucleotides spanning a broad distribution of degrees of polymerization, mixed nucleotide–peptide conjugates, transient reaction intermediates and various hydrolytic or degradation products. Given this compositional complexity, constructing calibration curves for every discrete molecular entity within the mixture is not experimentally feasible. Additionally, several of these species are not commercially available as purified standards, with many existing only transiently under the reaction conditions, precluding isolation in sufficient purity for quantitative calibration.

To address this limitation, we adopted an internal-standard–based quantification strategy. We selected alanine as it is a structurally simple, chemically inert and readily ionizable internal analyte. Prior to each analytical batch, we performed a short calibration sequence using alanine alone to ensure stable response characteristics. Subsequently, all reaction samples were spiked with alanine at a fixed, uniform concentration, maintaining identical internal standard levels across all vials and batches. Because the internal standard experiences the same ionization environment, solvent composition, chromatographic conditions, and instrumental fluctuations as all other analytes in a reaction mixture, its signal intensity remains highly reproducible across injections. Similarly, although different analytes possess distinct ionization efficiencies, each individual analyte exhibited consistent relative signal intensity across the various replicates that we performed from the same batch. Thus, quantification based on the analyte-to-internal-standard (analyte/IS) peak-area ratio does provides a robust, normalization-based metric that reflects the relative abundance of each analyte independent of absolute instrumental variability.

To substantiate this approach, we provide representative raw-data screenshots for several molecular species—including the AMP dimer, AMP trimer, diglycine, triglycine, and the AMP-G conjugate—measured across multiple dehydration–rehydration (DH–RH) cycles. As can be clearly observed, the **Analyte/IS ratios** remain consistent across technical replicates within each cycle, demonstrating that internal-standard normalization effectively compensates for run-to-run variability and enables reliable comparative quantification within our complex reaction system. This is also an approach that has been used in several previous studies as detailed in the section below Table 4.

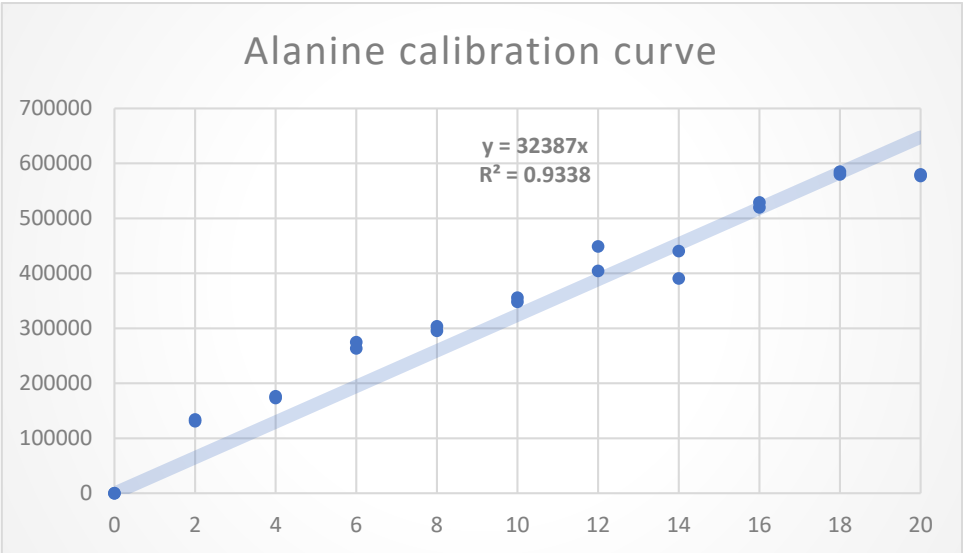
Table 4: Acquired screen shot of tabulated raw data obtained from LCMS runs of our AGM 130H reaction.

Sample Name	Component...	Expected RT	Area	Retent... Time	Adduct / C...	Formula	Precursor Mass	Reporta...	*Analy...	Isotope Confi...	Found At Mass	Mass Error (...	Isotope Ratio Difference
AGM (High)_130deg_cycle 0_R1_T1_positive...	AMP-AMP (+1)	10.26	N/A	N/A	[M+H]+	C20H26N...	677.1229	☑			N/A	N/A	N/A
AGM (High)_130deg_cycle 0_R1_T2_positive...	AMP-AMP (+1)	10.26	N/A	N/A	[M+H]+	C20H26N...	677.1229	☑			N/A	N/A	N/A
AGM (High)_130deg_cycle 1_R1_T2_positive...	AMP-AMP (+1)	10.26	7.448e4	10.25	[M+H]+	C20H26N...	677.1229	☑	0.451		677.1219	-1.4	13.9
AGM (High)_130deg_cycle 1_R2_T2_positive...	AMP-AMP (+1)	10.26	8.154e4	10.27	[M+H]+	C20H26N...	677.1229	☑	0.458		677.1219	-1.5	19.4
AGM (High)_130deg_cycle 2_R2_T1_positive...	AMP-AMP (+1)	10.26	1.252e5	10.32	[M+H]+	C20H26N...	677.1229	☑	0.630		677.1228	-0.1	6.2
AGM (High)_130deg_cycle 2_R2_T2_positive...	AMP-AMP (+1)	10.26	1.335e5	10.30	[M+H]+	C20H26N...	677.1229	☑	0.684		677.1231	0.4	5.5
AGM (High)_130deg_cycle 5_R3_T1_positive...	AMP-AMP (+1)	10.26	1.839e5	10.30	[M+H]+	C20H26N...	677.1229	☑	1.022		677.1231	0.4	3.8
AGM (High)_130deg_cycle 5_R3_T2_positive...	AMP-AMP (+1)	10.26	2.081e5	10.34	[M+H]+	C20H26N...	677.1229	☑	1.175		677.1224	-0.7	4.3
AGM (High)_130deg_cycle 10_R1_T1_positive...	AMP-AMP (+1)	10.26	1.734e5	10.30	[M+H]+	C20H26N...	677.1229	☑	1.063		677.1225	-0.6	8.9
AGM (High)_130deg_cycle 10_R1_T2_positive...	AMP-AMP (+1)	10.26	1.621e5	10.32	[M+H]+	C20H26N...	677.1229	☑	1.093		677.1235	0.9	0.5
AGM (High)_130deg_cycle 24_R1_T1_positive...	AMP-AMP (+1)	10.26	1.285e5	10.29	[M+H]+	C20H26N...	677.1229	☑	0.726		677.1225	-0.6	9.9
AGM (High)_130deg_cycle 24_R1_T2_positive...	AMP-AMP (+1)	10.26	1.288e5	10.30	[M+H]+	C20H26N...	677.1229	☑	0.746		677.1224	-0.7	1.7

Table 5: Analyte/IS data of a few other molecules are tabulated below.

Sample Name	Analyte/IS				
	AMP dimer	AMP trimer	Digylcine	Triglycine	AMP-G
AGM (High)_130deg_cycle 0	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
AGM (High)_130deg_cycle 0	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
AGM (High)_130deg_cycle 1	0.451	0.001	0.081	0.011	0.124
AGM (High)_130deg_cycle 1	0.458	0.001	0.085	0.011	0.125
AGM (High)_130deg_cycle 2	0.63	0.003	0.139	0.094	0.086
AGM (High)_130deg_cycle 2	0.684	0.003	0.135	0.093	0.08
AGM (High)_130deg_cycle 5	1.022	0.008	0.243	0.13	0.059
AGM (High)_130deg_cycle 5	1.175	0.01	0.244	0.137	0.059
AGM (High)_130deg_cycle 10	1.063	0.033	0.335	0.316	0.045
AGM (High)_130deg_cycle 10	1.093	0.036	0.338	0.312	0.045
AGM (High)_130deg_cycle 24	0.726	0.009	0.241	0.25	0.013
AGM (High)_130deg_cycle 24	0.746	0.01	0.241	0.245	0.014

The calibration curve for this batch:



Similar approach of quantification has been used by the following articles:

(Below are the same references as ref g-n that are as part of response to Rev 2)

u. Van der Kloet, F. M., Bobeldijk, I., Verheij, E. R., & Jellema, R. H. (2009). Analytical error reduction using single point calibration for accurate and precise metabolomic phenotyping. *Journal of Proteome Research, 8*(11), 5132–5141.

v. Xiao, J. F., Zhou, B., & Ressom, H. W. (2012). *Metabolite identification and quantitation in LC-MS/MS–based metabolomics. TrAC — Trends in Analytical Chemistry*, *32*, 1–14.
w. Wang, M., & Han, X. (2016). Selection of internal standards for accurate quantification of complex lipid species in biological extracts by electrospray ionization mass spectrometry – What, how and why? *Mass Spectrometry Reviews,* 36(6), 693–714. https://doi.org/10.1002/mas.21492
x. Holman, J., et al. (2012). Internal standard strategies for relative and absolute quantitation of peptides in biological matrices by liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B, 893–894*, 1–14.
y. Lenco, J., Lan, R., Edwards, N., et al. (2012). MS/MS library–facilitated MRM quantification of native peptides prepared by denaturing ultrafiltration. Proteome *Science, 10*, 7.
z. Zhang, J., Dasgupta, A., Ayala, R., Bonapace, C., Kassim, S., & Faustino, P. J. (2024). Internal standard variability: root cause investigation, parallelism for evaluating trackability and practical considerations. *Bioanalysis, 16*(15), 771–776.
aa. Mahmud, I., Wei, B., Veillon, L., Tan, L., Martinez, S., Tran, B., Raskind, A., de Jong, F., Liu, Y., Ding, J., Xiong, Y., Chan, W.-K., Akbani, R., Weinstein, J. N., & Lorenzi, P. L. (2025). Ion suppression correction and normalization for non-targeted metabolomics. *Nature Communications*, *16*, 1347.
bb. Jain, S. K., Bansal, S., Bansal, S., Singh, B., Klotzbier, W., Mehta, K. Y., & Cheema, A. K. (2024). An optimized method for LC–MS-based quantification of endogenous organic acids: metabolic perturbations in pancreatic cancer. *International Journal of Molecular Sciences, 25*(11), 5901.

2) There are a large number of abbreviations in this manuscript. However, many of them are not clearly defined. Some definitions are found the caption of figure 2, even though such abbreviations have already been used in figure 1 and throughout the text without definition.

The authors understand the concern of the reviewer; however, all the reaction abbreviations used in the article have been tabulated in Tables 1 and 2 in the SI document provided. Additionally, we have accounted for the change in Fig 2 as the reviewer has suggested.

3) Figure 1, the mass spec data shows the different glycine polypeptides (mass difference for each monomer = 57 Da). It seems that there are other glycine species present as well, e.g. 212/269/326/383/440/497 Da (57 Da difference). The authors have any explanation for these species?

These masses correspond to a simultaneous fragmented NH_2 and OH group from the amino and carboxyl terminal respectively. These masses that the reviewer mentioned are fragmentation masses of precursors peptides, which are tabulated in detail in the supplementary (Fig S3).

4) In the figures the authors usually provide cycle 0. Was this indeed measured? If not,

could the authors measure those samples separately to ensure they are initially not containing any polymer specie of the amino acids or nucleotides?

As can be observed in the screenshot provided in response to your first question, cycle 0 timepoint was indeed analyzed but the presence of oligomers or intermediates were not found.

5) P-N vs. P-O-C. The authors claim that the P-O-C bond is more reactive providing more easily the tetraglycine product. However, looking at figure 6 shows that the samples using the P-O-C bond the oligonucleotides are decreasing over time, while in the samples with the P-N bond the oligonucleotides seem to be increasing/stable. So, in this regard the P-N bond is much more favourable from an evolutionary perspective assuming a co-evolution of peptides and oligonucleotides. The authors might want to rephrase.

We agree with the reviewer that the importance of P-N bond in oligonucleotide formation is clear, however, the P-O-C is more reactive resulting in longer peptides. However, the oligonucleotides formed in both the linkage type reactions shows a drop in the yields of oligonucleotides. The probable cause for this could be the high temperature of 130°C being suboptimal for oligonucleotide/RNA stability. The authors point of view in stating "These findings highlight the high reactivity of the intermediates, particularly the P–O–C linkage species, which readily undergo self-condensation to form oligonucleotides and peptides" was because the P-O-C linkage showed longest oligopeptide, and that too without supplementing glycine in the reaction. Additionally, the P-O-C linkage showed the formation of up to AMP tetramer in Cycle 0 [Fig S21b] itself.

Reviewer #4 (Remarks to the Author):

Roy et al. report reactions in which peptides and RNA oligomerize in the presence of amino acids and either ATP or cAMP. Although the field has extensively examined RNA–amino-acid conjugates, expanding this line of research is a worthwhile goal. Nonetheless, in its present form, the manuscript does not provide sufficient experimental evidence to support the authors' central claims.

Here are my main comments:

1. **To begin with, the study's exclusive dependence on mass spectrometry for product quantification is concerning. MS-based quantification, while informative, can be misleading when used without proper calibration, yet no calibration curves for standards (peptides or nucleotides) are provided. The authors state that product yields are reported as relative values normalized to an internal standard (alanine), but without calibration data, it is difficult to assess whether these measurements are reliable or comparable. Moreover, the oligomer distribution presented in Figure 1B, where nearly all oligomers appear at similar abundances, is not typical and difficult to reconcile with expected oligomerization behavior.**

We are in agreement with the reviewer's point that the observed ion intensities for distinct molecular species in mass spectrometric analyses are influenced by multiple physicochemical and instrumental parameters, including—but not limited to—the molecular mass, intrinsic ionization efficiency, solvent composition, differential ion–molecule clustering, adduct formation, and matrix effects. Notably, even for a single compound, the absolute signal intensities can vary across analytical runs, instrument batches and day-to-day operating conditions due to fluctuations in ion source stability, spray characteristics, detector sensitivity and subtle changes in chromatographic behaviour. Nevertheless, it still is one of the analytical techniques that is used to characterize complex mixtures.

Our reactions generate a highly heterogeneous molecular ensemble comprising unreacted starting materials, oligopeptides of diverse chain lengths, oligonucleotides spanning a broad distribution of degrees of polymerization, mixed nucleotide–peptide conjugates, transient reaction intermediates and various hydrolytic or degradation products. Given this compositional complexity, constructing calibration curves for every discrete molecular entity within the mixture is not experimentally feasible. Additionally, several of these species are not commercially available as purified standards, with many existing only transiently under the reaction conditions, precluding isolation in sufficient purity for quantitative calibration.

To address this limitation, we adopted an internal-standard–based quantification strategy. We selected alanine as it is a structurally simple, chemically inert and readily ionizable internal analyte. Prior to each analytical batch, we performed a short calibration sequence using alanine alone to ensure stable response characteristics. Subsequently, all reaction samples were spiked with alanine at a fixed, uniform concentration, maintaining identical internal standard levels across all vials and batches. Because the internal standard experiences the same ionization environment, solvent composition, chromatographic conditions, and instrumental fluctuations as all other analytes in a reaction mixture, its signal intensity remains highly reproducible across injections. Similarly, although different analytes possess distinct ionization efficiencies, each individual analyte exhibited consistent relative signal intensity across the various replicates that we performed from the same batch. Thus, quantification based on the analyte-to-internal-standard (analyte/IS) peak-area ratio does provides a robust, normalization-based metric that reflects the relative abundance of each analyte independent of absolute instrumental variability.

To substantiate this approach, we provide representative raw-data screenshots for several molecular species—including the AMP dimer, AMP trimer, diglycine, triglycine, and the AMP-G conjugate—measured across multiple dehydration–rehydration (DH–RH) cycles. As can be clearly observed, the **Analyte/IS ratios** remain consistent across technical replicates within each cycle, demonstrating that internal-standard normalization effectively compensates for run-to-run variability and enables reliable comparative quantification within our complex reaction system. This is also an approach that has been used in several previous studies as detailed in the section below Table 4.

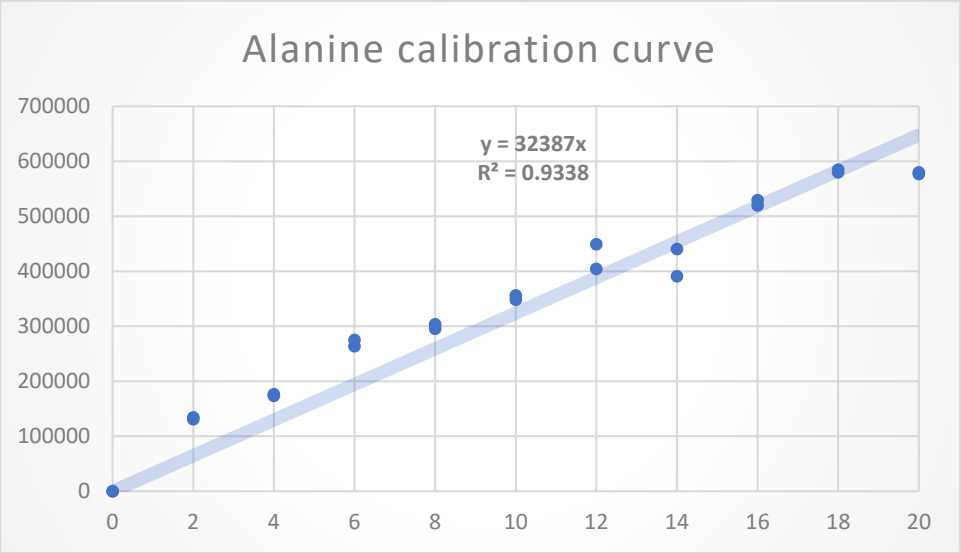
Table 4: Acquired screen shot of tabulated raw data obtained from LCMS runs of our AGM 130H reaction.

Sample Name	Component...	Expected RT	Area	Retent... Time	Adduct / C...	Formula	Precursor Mass	Reporta...	*Analy...	Isotope Confi...	Found At Mass	Mass Error (...	Isotope Ratio Difference
AGM (High)_130deg_cycle 0_R1_T1_positive...	AMP-AMP (+1)	10.26	N/A	N/A	[M+H]+	C20H26N...	677.1229	☑			N/A	N/A	N/A
AGM (High)_130deg_cycle 0_R1_T2_positive...	AMP-AMP (+1)	10.26	N/A	N/A	[M+H]+	C20H26N...	677.1229	☑			N/A	N/A	N/A
AGM (High)_130deg_cycle 1_R1_T2_positive...	AMP-AMP (+1)	10.26	7.448e4	10.25	[M+H]+	C20H26N...	677.1229	☑	0.451		677.1219	-1.4	13.9
AGM (High)_130deg_cycle 1_R2_T2_positive...	AMP-AMP (+1)	10.26	8.154e4	10.27	[M+H]+	C20H26N...	677.1229	☑	0.458		677.1219	-1.5	19.4
AGM (High)_130deg_cycle 2_R2_T1_positive...	AMP-AMP (+1)	10.26	1.252e5	10.32	[M+H]+	C20H26N...	677.1229	☑	0.630		677.1228	-0.1	6.2
AGM (High)_130deg_cycle 2_R2_T2_positive...	AMP-AMP (+1)	10.26	1.335e5	10.30	[M+H]+	C20H26N...	677.1229	☑	0.684		677.1231	0.4	5.5
AGM (High)_130deg_cycle 5_R3_T1_positive...	AMP-AMP (+1)	10.26	1.839e5	10.30	[M+H]+	C20H26N...	677.1229	☑	1.022		677.1231	0.4	3.8
AGM (High)_130deg_cycle 5_R3_T2_positive...	AMP-AMP (+1)	10.26	2.081e5	10.34	[M+H]+	C20H26N...	677.1229	☑	1.175		677.1224	-0.7	4.3
AGM (High)_130deg_cycle 10_R1_T1_positive...	AMP-AMP (+1)	10.26	1.734e5	10.30	[M+H]+	C20H26N...	677.1229	☑	1.063		677.1225	-0.6	8.9
AGM (High)_130deg_cycle 10_R1_T2_positive...	AMP-AMP (+1)	10.26	1.621e5	10.32	[M+H]+	C20H26N...	677.1229	☑	1.093		677.1235	0.9	0.5
AGM (High)_130deg_cycle 24_R1_T1_positive...	AMP-AMP (+1)	10.26	1.285e5	10.29	[M+H]+	C20H26N...	677.1229	☑	0.726		677.1225	-0.6	9.9
AGM (High)_130deg_cycle 24_R1_T2_positive...	AMP-AMP (+1)	10.26	1.288e5	10.30	[M+H]+	C20H26N...	677.1229	☑	0.746		677.1224	-0.7	1.7

Table 5: Analyte/IS data of a few other molecules are tabulated below.

Sample Name	Analyte/IS				
	AMP dimer	AMP trimer	Digylcine	Triglycine	AMP-G
AGM (High)_130deg_cycle 0	Not Found	Not Found	Not Found	Not Found	Not Found
AGM (High)_130deg_cycle 0	Not Found	Not Found	Not Found	Not Found	Not Found
AGM (High)_130deg_cycle 1	0.451	0.001	0.081	0.011	0.124
AGM (High)_130deg_cycle 1	0.458	0.001	0.085	0.011	0.125
AGM (High)_130deg_cycle 2	0.63	0.003	0.139	0.094	0.086
AGM (High)_130deg_cycle 2	0.684	0.003	0.135	0.093	0.08
AGM (High)_130deg_cycle 5	1.022	0.008	0.243	0.13	0.059
AGM (High)_130deg_cycle 5	1.175	0.01	0.244	0.137	0.059
AGM (High)_130deg_cycle 10	1.063	0.033	0.335	0.316	0.045
AGM (High)_130deg_cycle 10	1.093	0.036	0.338	0.312	0.045
AGM (High)_130deg_cycle 24	0.726	0.009	0.241	0.25	0.013
AGM (High)_130deg_cycle 24	0.746	0.01	0.241	0.245	0.014

The calibration curve for this batch:



Similar approach of quantification has been used by the following articles:

(Below are the same references as ref g-n that are as part of response to Rev 2)

a. Van der Kloet, F. M., Bobeldijk, I., Verheij, E. R., & Jellema, R. H. (2009). Analytical error reduction using single point calibration for accurate and precise metabolomic phenotyping. *Journal of Proteome Research, 8*(11), 5132–5141.
b. Xiao, J. F., Zhou, B., & Ressom, H. W. (2012). *Metabolite identification and quantitation in LC-MS/MS–based metabolomics. TrAC — Trends in Analytical Chemistry*, *32*, 1–14.
c. Wang, M., & Han, X. (2016). Selection of internal standards for accurate quantification of complex lipid species in biological extracts by electrospray ionization mass spectrometry – What, how and why? *Mass Spectrometry Reviews,* 36(6), 693–714. https://doi.org/10.1002/mas.21492
d. Holman, J., et al. (2012). Internal standard strategies for relative and absolute quantitation of peptides in biological matrices by liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B, 893–894*, 1–14.
e. Lenco, J., Lan, R., Edwards, N., et al. (2012). MS/MS library–facilitated MRM quantification of native peptides prepared by denaturing ultrafiltration. Proteome *Science*, *10*, 7.
f. Zhang, J., Dasgupta, A., Ayala, R., Bonapace, C., Kassim, S., & Faustino, P. J. (2024). Internal standard variability: root cause investigation, parallelism for evaluating trackability and practical considerations. *Bioanalysis*, *16*(15), 771–776.
g. Mahmud, I., Wei, B., Veillon, L., Tan, L., Martinez, S., Tran, B., Raskind, A., de Jong, F., Liu, Y., Ding, J., Xiong, Y., Chan, W.-K., Akbani, R., Weinstein, J. N., & Lorenzi, P. L. (2025). Ion suppression correction and normalization for non-targeted metabolomics. *Nature Communications*, *16*, 1347.
h. Jain, S. K., Bansal, S., Bansal, S., Singh, B., Klotzbier, W., Mehta, K. Y., & Cheema, A. K. (2024). An optimized method for LC–MS-based quantification of endogenous organic acids: metabolic perturbations in pancreatic cancer. *International Journal of Molecular Sciences*, *25*(11), 5901.

2. Can the authors detect peptides via HPLC? It is also unclear why the authors did not employ HPLC for peptide detection and quantification. HPLC is a standard and widely used technique for identifying and quantifying peptides.

We did not rely on HPLC for peptide detection as the detection of non-aromatic and, thus, non-UV absorbent peptides is difficult due to the following reasons:

i. The quantitative detection of non-aromatic peptides via UV-based HPLC detectors remains analytically challenging for several reasons. First, although peptide bonds do absorb in the ultraviolet range, their molar absorptivity at around 214 nm is inherently low, meaning that non-aromatic sequences—lacking strong chromophores such as tryptophan or tyrosine—produce weak absorbance signals that are often near the absorbance of organic solvent such as acetonitrile. This limitation has been noted in peptide and amino acid analyses, where UV detection

often requires derivatization or alternative chromatographic approaches to improve sensitivity for non-chromophoric compounds.

ii. Small peptides such as glycine oligomers are known to exhibit poor retention on conventional reversed-phase (RP) HPLC columns, particularly C18 phases. The separation mechanism in RP-HPLC relies on hydrophobic interactions between the stationary phase and the analyte. Peptides composed predominantly of glycine residues have minimal hydrophobic character and, as a result, often elute very early, sometimes within the void volume, with little to no resolution between homologous oligomers. Without adequate chromatographic separation, overlapping peaks further reduce the accuracy of UV-based quantification.

iii. Campbell et al., in their article "Quantitative Analysis of Glycine Oligomerization by Ion-Pair Chromatography" have mentioned articles where compounds such as hexanesulfonate have been used as an ion-pairing agent, or the peptides were pre-derivatized, to measure oligomers up to Gly_6 by UV detection. Although, these articles do demonstrate ways to quantify peptides, these methods use isocratic gradient where retention time over multiple runs is problematic, in addition to the resolution amongst peptides being lost altogether. Rodriguez et al., have used the IP detection method in conjunction with MS analysis. Additionally, upon trying the IP detection of molecules has given us very low yet broad peaks (ranging ~ 2 mins) for nucleotide compounds.

3. If not HPLC, NMR analysis could be used to substantiate the MS-based measurements of glycine consumption. Without such complementary evidence, the authors should clarify that their results are not quantitative.

We appreciate the reviewer's suggestion to substantiate the MS-based measurements with complementary techniques such as HPLC or NMR. However, in our experimental setup, the reaction mixture contains a complex combination of nucleotides, amino acids, and their intermediates, which makes reliable quantification of glycine consumption by HPLC challenging due to significant peak overlap/ co-elution. Similarly, direct monitoring by NMR is limited by the relatively low concentration of glycine consumed during the reaction and signal overlap with other components in the mixture. Therefore, MS analysis was used primarily to qualitatively monitor trends in glycine utilization. We have clarified in the revised manuscript that the measurements presented are indicative rather than strictly quantitative. Nonetheless, the ^{31}P NMR profiles have now been included, demonstrating the gradual formation of AMP–G in the AGM 130L reactions. The comparison of intermediate peaks between Cycle 1 and Cycle 5 closely reflects the yields observed in Fig. 6B. Similarly, consistent peak identification is observed in the comparison between Cycle 5 and Cycle 24 of the cAG 130 reaction, where the ^{31}P NMR profile (Fig. S24B) corresponds well with the relative yields depicted in Fig. 6C.

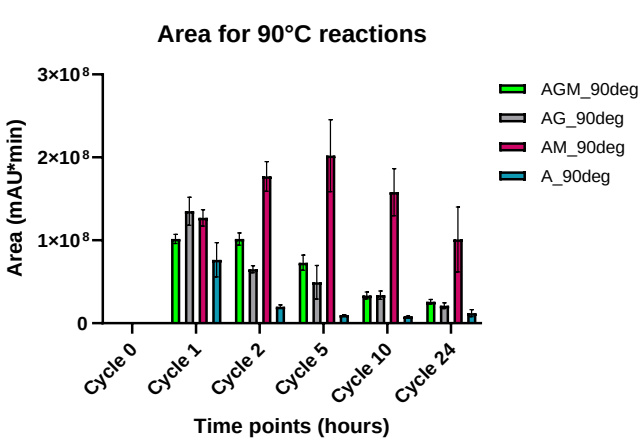
4. GM130H – it is also unclear why the control reaction in Figure 1B shows almost no formation of glycine oligomers. Rodriguez-Garcia et al. (Nature communications 6, no. 1 (2015): 8385) demonstrated that simple dehydration of glycine, followed by heating between 90 °C and 130 °C, leads to substantial oligomerization even in the absence of any

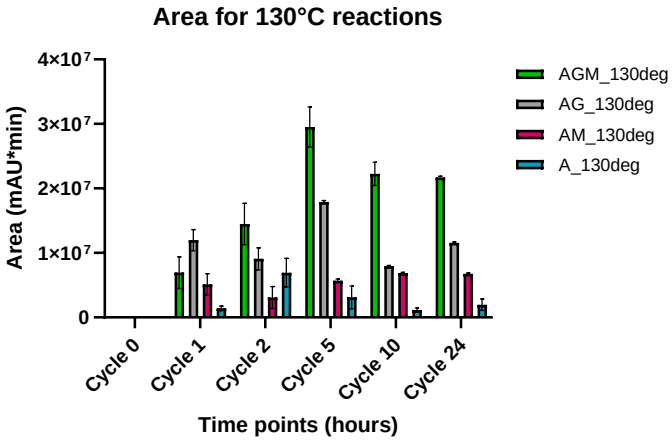
catalyst. Oligomers of up to 20 residues were observed after extended heating at these temperatures. The contrast between these established results and the negligible oligomer formation reported here warrants further explanation.

The article that the reviewer mentions- "Formation of oligopeptides in high yield under simple programmable conditions"- demonstrates that wet-dry cycling at high temperatures is effective for peptide formation. However, the authors of this same article show that the peptide yields drop significantly under near-neutral conditions [**Fig 5b** of the article shows the curve almost touching zero (in the y-axis) at near neutral pH conditions]. Thus, our results do not necessarily contradict their result. Rather, our results show that oligomerization of Gly peptides is very low at near neutral pH in the absence of ATP. ATP needs to be present in the reaction for the yields to go up at this ambient pH.

5. Furthermore, it is not clear why the authors did not quantify the nucleotide peaks using HPLC, especially given that their system is equipped with a DAD detector. As mentioned, HPLC-based quantification is generally more reliable and accurate than MS.

The authors would like to take this opportunity to state that we indeed quantify our results for nucleotide formation. These results are provided below for the peak 4 from HPLC chormatograms. The Peak 4 is the time at which ADP and AMP dimer elutes. Similarly, we have the area under the curve (AUC) from HPLC chromatograms for all the peaks obtained in our reaction mixtures. However, a significant limitation in this analysis arises from the insufficient chromatographic resolution between certain species present in the reaction mixture. In particular, compounds such as ADP and the AMP dimer, as well as ATP and the AMP trimer, exhibit overlapping signals and cannot be reliably separated even using different gradient (Solvent A:Solvent B 30:70, 20:80, 10:90 tested) conditions [Please see Fig 1 & 2 of Reviewer 2's section where we have detailed this]. Due to this lack of resolution, it becomes difficult to accurately distinguish and quantify hydrolyzed products relative to oligomerized species. Consequently, precise quantification of the extent of hydrolysis versus oligomerization remains challenging within this dataset. Additionally, the intermediates and hydrolysed products of ATP such as adenosine and adenine elute in the void volume, adding to problems with quantification. Below is the quantification of HPLC chromatogram of the peaks observed at 90°C and 130°C showing greater stability of potential oligomerized peak at higher temperatures in the presence of glycine.





6. The authors also synthesized standards of suggested intermediates (AMP-Gly (P–N) and AMP-Gly (P–O–C)), which is very important. However, to support their mechanism, they need to conduct a kinetics analysis comparing the yield of products (oligonucleotides/peptides) when reactions start with these intermediates versus starting with ATP/AMP. If the reaction rate is faster with the intermediates compared in their absence, this is good support for what they are claiming. Without such a comparison, a conclusion cannot be drawn about the reaction mechanism.

The yields of the products and the intermediates formed in the reaction mixtures with ATP as the starting reactant are shown in the main manuscript figures (Fig 5C inset). It can be clearly observed that the product formation is much faster in the reactions where we started with the intermediates than when we began with ATP and Gly as the starting reactants. Particularly, the P-O-C linkage showed the formation of AMP tetramer in Cycle 0 itself [Fig S21b], suggesting the intermediates could undergo self-condensation to give oligopeptides and oligonucleotides.

7. AMP-Gly (P-O-C) doesn't look so pure (Figure S20b). What is the purity of the standards? HPLC of synthesized standards for AMP-Gly conjugates after purification is required.

It is almost impossible to obtain AMP-Gly (P-O-C) with high purity. Previously reported yields were 40%. We have obtained a 66% product yield after much standardization. The main impurity is of the unreacted or hydrolysed AMP. This compound is highly unstable in aqueous conditions and undergoes hydrolysis to AMP very rapidly. The other impurities that could be found is that of octylamine and butylamine that are used in the reaction to obtain the product. **Fig S6a** demonstrates the overlay of HPLC chromatograms of purified controls with other nucleotides. HPLC of AMP-Gly (P-O-C) could not be obtained as the mixed anhydride linkage is highly susceptible to hydrolysis under aqueous conditions.

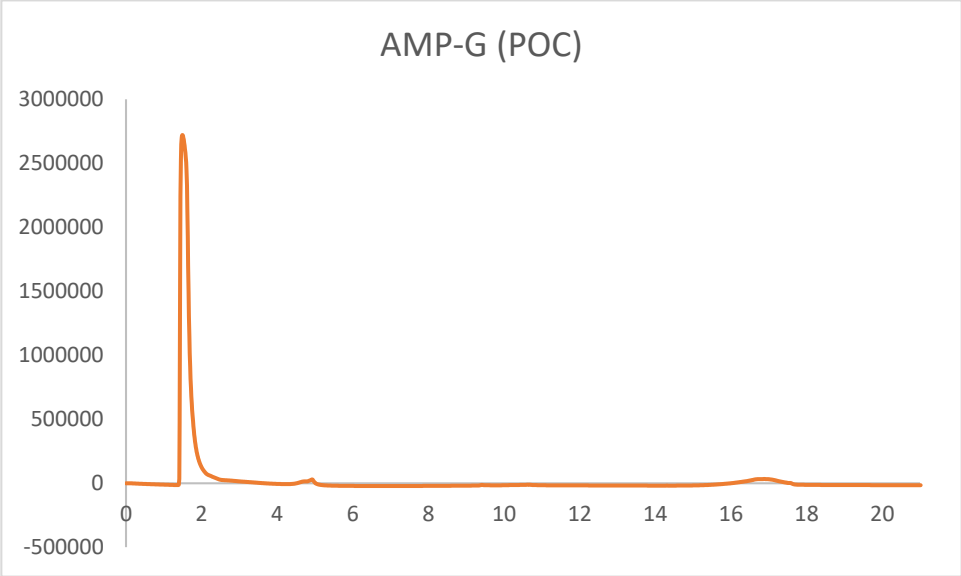
8. Are the authors sure that the peaks in the HPLC are of AMP-G(P-N) or AMP-G(P-O-C) (e.g., in Figure S6?)? The authors need to spike the standards in the reaction products to verify that these are the speculated products in the chromatogram. Also, quantification of

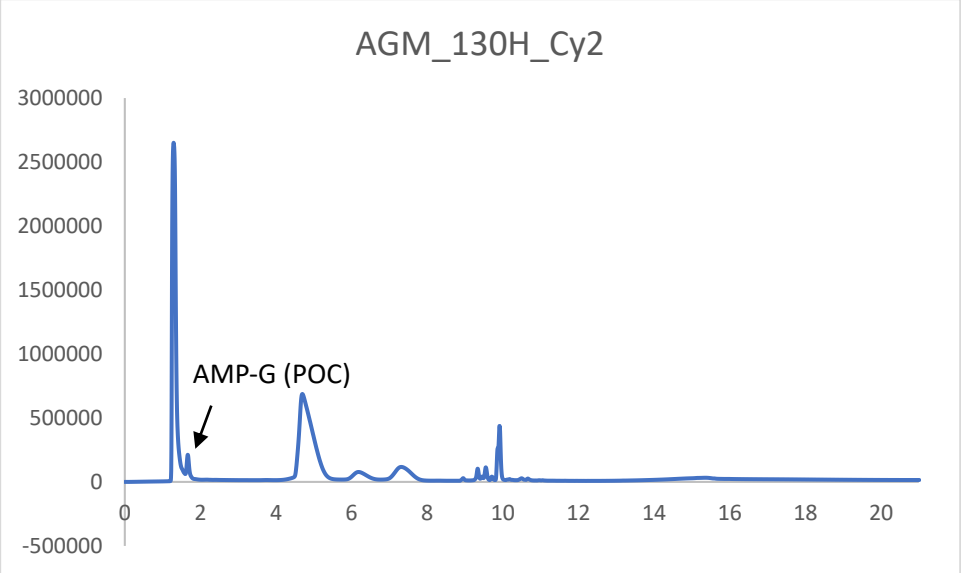
these products should be done via UV-HPLC based on the standards. The peaks in the HPLC chromatograms should be clearly labeled.

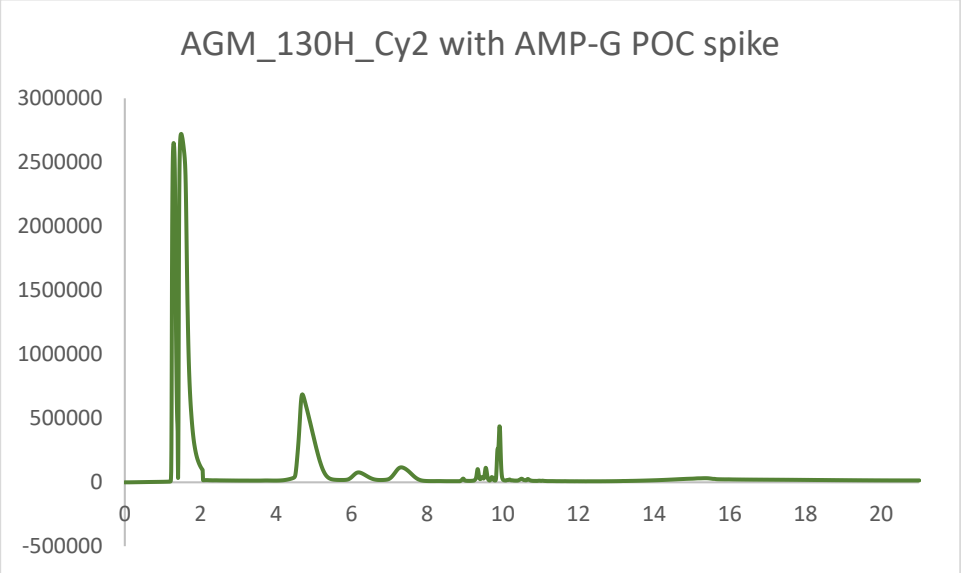
The chromatograms presented below include those of the AMP–G (POC) intermediate (RT ≈ 1.8 min), followed by AGM_Cy2_130H, and AGM_Cy2_130H spiked with AMP–G (POC). In the second chromatogram, the AMP–G (POC) signal appears as a minor peak (indicated by a black arrow), which becomes significantly more pronounced at the same retention time (RT ≈ 1.8 min) upon spiking in the third chromatogram. This observation supports the assignment of this peak to the AMP–G (POC) intermediate formed during the reaction.

To improve stability during analysis, we employed a buffered HPLC system at pH 6.5, as alkaline conditions (pH 8) are known to destabilize POC intermediates. However, comparable resolution for the oligonucleotides generated in the reaction mixture could not be achieved. This is likely due to their similar charge states—for instance, an AMP trimer and ATP both carry a net charge of −3—rendering spiking experiments ineffective for their differentiation.

As shown in Fig. S6a, the HPLC chromatograms display substantial overlap among the labelled peaks corresponding to nucleotides, oligonucleotides, and hydrolysed species; we could not label the peaks with certainty in the chromatograms of our analysed reaction mixture.







9. Many studies have explored reactions between amino acids and nucleotides. Some of which are not mentioned in this work. For example, the work of Suárez-Marina et al (Communications Chemistry 2.1 (2019): 28) and the work of Sawai and Orgel in the Journal of Molecular Evolution 6 (3) (1975): 165-184 (A different paper by these authors from 1975, which focuses on oligonucleotide formation, was mentioned, but not this paper).

The articles from Sawai and Orgel on oligonucleotide formation have now been added to the references, thanks for pointing this out. As for the study by Suárez-Marina et al., it reports the simultaneous formation of glycosidic bonds between ribose, and purine and pyrimidine nucleobases. While this work provides valuable insights into nucleoside formation chemistry, it does not directly relate to the specific reactions or questions that we examined in our study.

10. What is the relevance of ATP as a prebiotic precursor? The abstract starts with "Nucleoside triphosphates, cyclic monophosphates and amino acids are hypothesized to

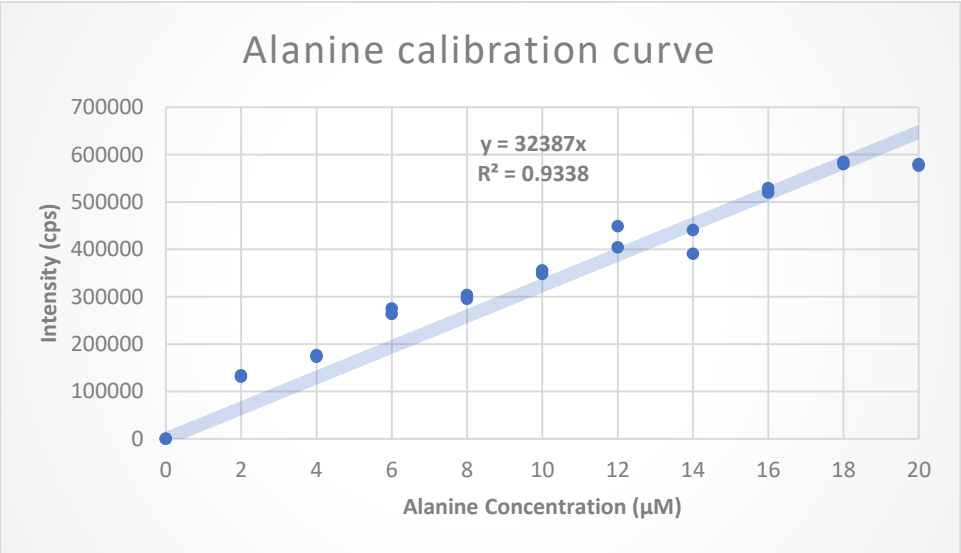
have been present together in the prebiotic milieu" – yet, to my knowledge, nucleoside triphosphates are not considered plausible prebiotic molecules.

Please note that references 15 to 19 demonstrate the formation of ATP under prebiotic conditions. Few articles are from highly regarded researchers in the OoL/astrobiology community such as Brenner, Krishnamurthy and Yamagata. We have reworded this sentence to reflect the same clearly.

Reviewer 3

The authors have revised the manuscript and addressed many of the previous comments. However, the calibration, which represents a key methodological aspect, is still not sufficiently clarified. The authors present a single calibration curve for alanine; however, the axes are not labeled, which limits interpretability.

The authors thank the reviewer for this observation and sincerely apologize for the unintended oversight regarding the missing axis labels in the alanine calibration curve. This has been corrected now.



In mass spectrometry–based quantification, two principal strategies are typically employed: (i) the use of an isotopically labeled internal standard corresponding to the exact analyte, or (ii) the use of a different internal standard, i.e., a spiked compound of known concentration.
In the present study, the authors appear to use alanine as an internal standard. However, they only provide a calibration curve for alanine itself. For accurate quantification, a separate calibration curve is required for each analyte of interest, constructed from the ratio of the analyte signal (across a dilution series) to a constant amount of internal standard. There is no clear evidence that such analyte-specific calibration curves were generated.
Consequently, significant concerns remain regarding the robustness of the quantification approach. As the study's results and conclusions rely heavily on these quantitative measurements, I am currently unable to recommend the manuscript for publication.

Our reaction set up contains products such as peptides, oligonucleotides, intermediates, starting reactants and hydrolyzed products making it a complex mixture. Given this and the concerns articulated regarding quantification, we have systematically revised the manuscript to remove claims pertaining to absolute yield quantification. Instead, the product formation is now reported using a relative quantification approach, wherein percentage yields are derived from the ratio of analyte signal intensity to that of the alanine internal standard. We sincerely believe that this revision ensures an honest representation of the data, which is also consistent with the limitations of the analytical methodology employed.

The main manuscript has undergone the following revisions in the figure panels:

Figure No.:	Previous	Replaced by
Figure 1	A. LC-MS spectra observed for different length peptides B. Relative yields of different length peptides in µmoles C. The table denoting the theoretical m/z, the observed m/z from the spectra and the ppm error of peptides.	A. LC-MS spectra observed for different length peptides. B. Table denoting the theoretical m/z, the observed m/z from the spectra and the ppm error of peptides. C. A heat map illustrating the relative percentage yield of glycine oligomers formed.
Figure 2.	A. Relative yields of **A.** AMP dimers, **B.** AMP trimers and **C.** AMP tetramers formed (in µmoles). D. Comparison of TOF MS spectra of AMP tetramer $(AMP)_4$ formed in the AGM reactions with AMP tetramer $(AMP)_4$ control	A. Comparison of TOF MS spectra of AMP tetramer $(AMP)_4$ formed in the AGM reactions with AMP tetramer $(AMP)_4$ control. B. Heat map showing the longest oligonucleotide length observed across various wet–dry cycling reaction conditions. C. LC-MS spectra obtained for different length and types (linear and cyclic-ending) of oligonucleotides that formed in the 2',3' cAMP + Gly (cAG 130) reaction at 130°C.
Figure 3.	A. LC-MS spectra obtained for different length and types (linear and cyclic-ending) of oligonucleotides that formed in the 2',3' cAMP + Gly (cAG 130) reaction at 130°C. B. Relative yields of different length oligonucleotides (panel **B –** cyclic, panel **C-** linear) in µmoles.	Validation of AMP–Gly (P–O–C) intermediate formation via HPLC co-elution, NMR and LC–MS analysis.
Figure 4.	A. Chemical structure of AMP-Gly. B. ^{31}P NMR comparison of AMP-Gly formed in reaction, with synthetic control. C. TOF MS/MS spectra comparison of AMP-Gly formed in reaction, with synthetic control.	Relative abundance of products obtained in LC–MS analysis of reactions with chemically synthesized AMP–Gly intermediates under wet–dry cycling conditions.
Figure 5.	The relative yields of compounds found in reactions undertaken with chemically synthesized AMP-Gly (P-N) over a cycle of 5 hours at 130°C in nmoles.	Plausible reaction mechanism of product formation in the reaction mixtures containing ATP and amino acid

Figure 6.	Chemical structure of AMP-aa containing a mixed anhydride (P-O-C) linkage between phosphate and carboxylate group of the AMP and amino acid, respectively, and relative yields of AMP-aa compounds formed with different amino acids in different reactions.	Removed.
Figure 7.	Plausible reaction mechanism of product formation in the reaction mixtures containing ATP and amino acid	Moved to Figure 5.

Reviewer #4 (Remarks to the Author):

I appreciate the authors' efforts in revising the manuscript. However, my main concern remains unresolved, and I therefore still recommend major revision before the work can be considered for acceptance.

My main concern is the continued presentation of the MS-based measurements as quantitative yields. As mentioned in the original review, the study's exclusive dependence on mass spectrometry for product quantification is concerning. MS-based quantification, while informative, can be misleading. The rebuttal letter acknowledges the well-known limitations of LC–MS quantification in complex mixtures and explains that the authors use alanine as a single internal standard. However, no validation is provided showing that this approach permits reliable quantification across analytes with very different structures and ionization efficiencies, such as diglycine, triglycine, higher oligoglycines, nucleotide oligomers, and nucleotide–amino acid conjugates. In particular, the authors did not provide calibration using even a limited set of commercially available oligoglycine standards, despite the fact that such standards exist and could directly test whether the analyte/internal-standard ratio behaves sensibly in the relevant range.

Hence, I do not think the manuscript currently supports absolute claims such as cumulative nmol or µmol values for oligomer distributions. This is especially important because the product distributions shown in Figure 1B appear atypical, with several oligomers present at similar apparent abundances, which is difficult to reconcile with expected oligomerization behavior and may reflect analytical response bias rather than chemistry. This concern was not satisfactorily addressed. Notably, the authors also didn't refer to my comment about this atypical distribution of products, which was also raised by Reviewer 1.

I also note an inconsistency between the rebuttal and the manuscript text. In the rebuttal, the authors state that the measurements are "indicative rather than strictly quantitative" and that this has been clarified in the revised manuscript. I could not find such a clarification. Instead, the revised manuscript still refers to a "thorough quantitative analysis" and states that peak areas were used "to quantify the yields." The manuscript should be revised to match what the data can actually support.

More generally, it is very difficult to identify exactly what changes were made in response to the reviewers' comments.

I therefore see two acceptable paths forward:
1. Validate the quantitative method more rigorously, at minimum by showing calibration behavior for a limited number of relevant standards (for example several oligoglycine standards), clarifying the LC–MS acquisition mode and data extraction, and showing representative chromatograms.
2. Reframe the manuscript as qualitative, clearly stating the limitations of the analytical approach and removing or substantially toning down all absolute yield claims and any conclusions that depend on them. In that case, the conclusions should remain qualitative, e.g. enhancement of oligomer formation, appearance of longer products, detection of proposed intermediates, etc., rather than precise yield comparisons.

The authors thank the reviewer for their critical inputs. In response to this, we have systematically revised the manuscript to remove all claims pertaining to absolute yield quantification. Instead, product formation is now reported using a relative quantification approach, wherein percentage yields are derived from the ratio of analyte signal intensity to that of the alanine internal standard. This revision ensures a more accurate representation of the data, consistent with the limitations of the analytical methodology employed.

The main manuscript has undergone the following revisions on the figure panels:

Figure No.:	Previous	Replaced by
Figure 1	A. LC-MS spectra observed for different length peptides B. Relative yields of different length peptides in µmoles C. The table denoting the theoretical m/z, the observed m/z from the spectra and the ppm error of peptides.	A. LC-MS spectra observed for different length peptides. B. Table denoting the theoretical m/z, the observed m/z from the spectra and the ppm error of peptides. C. A heat map illustrating the relative percentage yield of glycine oligomers formed.
Figure 2.	A. Relative yields of **A.** AMP dimers, **B.** AMP trimers and **C.** AMP tetramers formed (in µmoles). B. Comparison of TOF MS spectra of AMP tetramer $(AMP)_4$ formed in the AGM reactions with AMP tetramer $(AMP)_4$ control	A. Comparison of TOF MS spectra of AMP tetramer $(AMP)_4$ formed in the AGM reactions with AMP tetramer $(AMP)_4$ control. B. Heat map showing the longest oligonucleotide length observed across various wet–dry cycling reaction conditions. C. LC-MS spectra obtained for different length and types (linear and cyclic-ending) of oligonucleotides that formed in the 2',3' cAMP + Gly (cAG 130) reaction at 130°C.
Figure 3.	A. LC-MS spectra obtained for different length and types (linear and cyclic-ending) of oligonucleotides that formed in the 2',3' cAMP + Gly (cAG 130) reaction at 130°C. B. Relative yields of different length oligonucleotides (panel	Validation of AMP–Gly (P–O–C) intermediate formation via HPLC co-elution, NMR and LC–MS analysis.

	B – cyclic, panel **C-** linear) in µmoles.	
Figure 4.	A. Chemical structure of AMP-Gly. B. ^{31}P NMR comparison of AMP-Gly formed in reaction, with synthetic control. C. TOF MS/MS spectra comparison of AMP-Gly formed in reaction, with synthetic control.	Relative abundance of products obtained in LC–MS analysis of reactions with chemically synthesized AMP–Gly intermediates under wet–dry cycling conditions.
Figure 5.	The relative yields of compounds found in reactions undertaken with chemically synthesized AMP-Gly (P-N) over a cycle of 5 hours at 130°C in nmoles.	Plausible reaction mechanism of product formation in the reaction mixtures containing ATP and amino acid
Figure 6.	Chemical structure of AMP-aa containing a mixed anhydride (P-O-C) linkage between phosphate and carboxylate group of the AMP and amino acid, respectively, and relative yields of AMP-aa compounds formed with different amino acids in different reactions.	Removed.
Figure 7.	Plausible reaction mechanism of product formation in the reaction mixtures containing ATP and amino acid	Moved to Figure 5.

2. Regarding the yield, the reported "cumulative Gly-peptide yields" are difficult to interpret. The reactions are described as starting from 75 mM glycine, but the Results then switch to nmol/µmol product amounts without clearly stating the reaction volume. Since the reaction volume is 300 uL, 75 mM glycine corresponds to 22.5 µmol starting material. It would therefore be much clearer if the authors explicitly reported percent yields and defined exactly what quantity is being summed. For example, how do they get "cumulative Gly-peptide yields of ~21.3 µmoles"? Just by looking at Fig. 1B, I can do the following for cAG 130: ~1 µmole x 2 (diglycine) + ~2 µmole x 3 (triglycine)+ ~2 µmole x 4 (tetraglycine) + ~3 µmole x 5 (pentaglycine)... I count ~84 µmole when only summing up to octaglycine, and the authors only had 22.5 µ mole glycine. Do the authors count for the number of glycine units in each oligomer and how many moles of glycine they started with? This again goes to my comment about the results that cannot be interpreted quantitatively.

We have carefully revised the manuscript to remove all claims pertaining to absolute yield quantification.

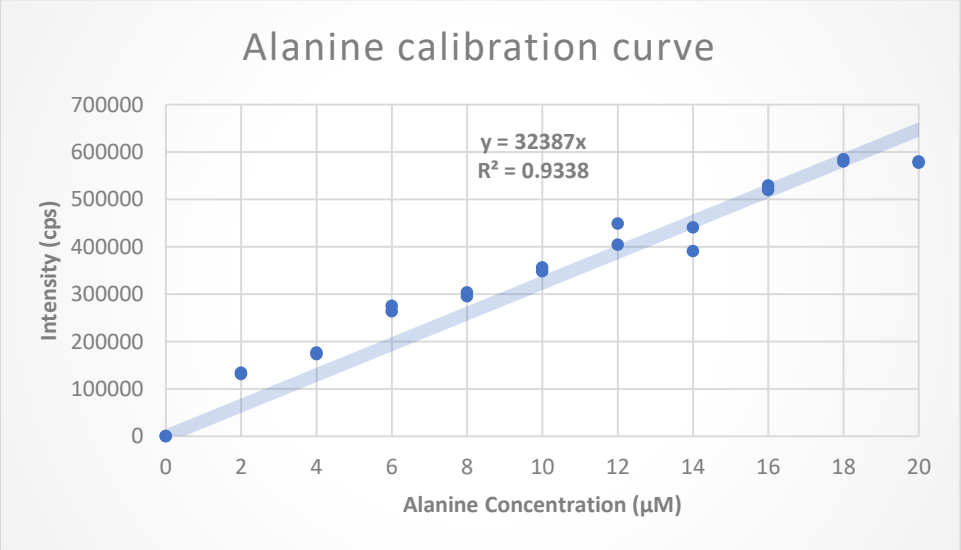
3. The methods statement that alanine (20 mg/mL) was injected with "150 nmoles of samples" is unclear. 150 nmoles of what? Does it refer to the initial amino acid concentration? What exactly was injected, and how were the reported nmol yields calculated from that measurement? Also, 20 mg/ml alanine is 225 mM alanine. This is a very high concentration for a standard for LCMS.

The Methods section has been revised to eliminate any ambiguity and now clearly describes the approach used for relative yield determination. Specifically, percentage yields were calculated based on the ratio of the observed signal intensities of the products, relative to that of the alanine internal standard. This clarification ensures methodological transparency and aligns the reported quantification with the analytical framework employed.

Additional points that still require clarification:

• The alanine calibration curve shown in the rebuttal is insufficient to validate the reported product yields. Please provide clear units and explain how this curve relates to the reported analyte amounts.

We sincerely apologize for the oversight regarding the missing axis labels in the alanine calibration curve.



Sample Name	Analyte/IS ratio				
	AMP dimer	AMP trimer	Digylcine	Triglycine	AMP-G
AGM (High)_130deg_cycle 0	Not detected	Not detected	Not detected	Not detected	Not detected
AGM (High)_130deg_cycle 0	Not detected	Not detected	Not detected	Not detected	Not detected
AGM (High)_130deg_cycle 1	0.451	0.001	0.081	0.011	0.124
AGM (High)_130deg_cycle 1	0.458	0.001	0.085	0.011	0.125
AGM (High)_130deg_cycle 2	0.63	0.003	0.139	0.094	0.086
AGM (High)_130deg_cycle 2	0.684	0.003	0.135	0.093	0.08
AGM (High)_130deg_cycle 5	1.022	0.008	0.243	0.13	0.059
AGM (High)_130deg_cycle 5	1.175	0.01	0.244	0.137	0.059
AGM (High)_130deg_cycle 10	1.063	0.033	0.335	0.316	0.045
AGM (High)_130deg_cycle 10	1.093	0.036	0.338	0.312	0.045

AGM (High)_130deg_cycle 24	0.726	0.009	0.241	0.25	0.013
AGM (High)_130deg_cycle 24	0.746	0.01	0.241	0.245	0.014

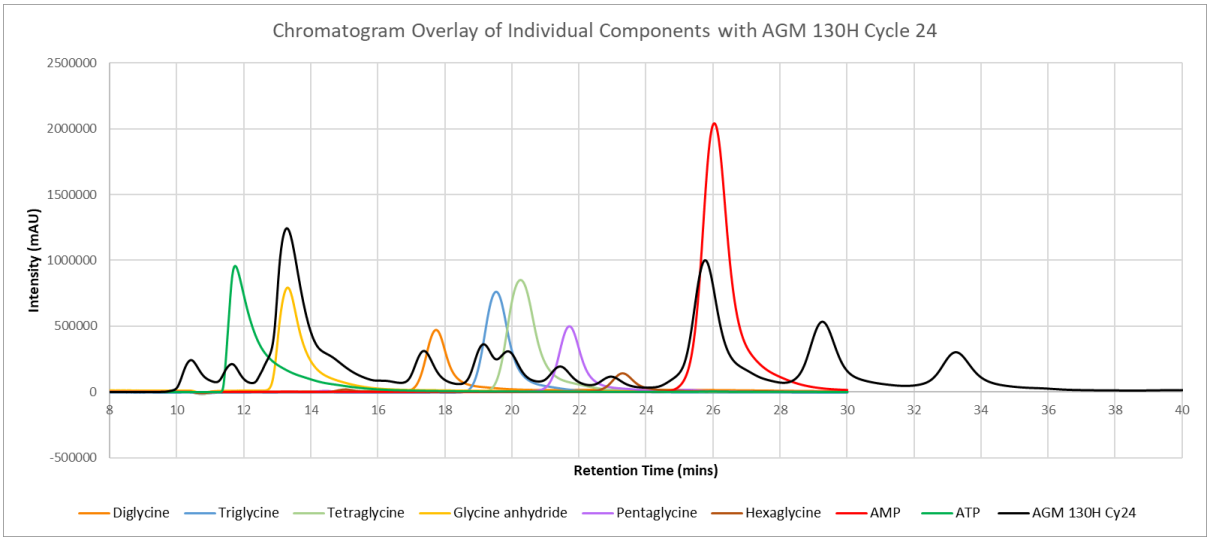
Further, as the analyte-to-internal standard (Analyte/IS) intensity ratio was found to be consistent across replicates, we proceeded to calculate percentage yields based on these datasets. This approach leverages the reproducibility of the relative signal intensities to enable reliable relative estimation of product formation.

• Please provide representative LC–MS chromatograms/traces and clarify the acquisition method.

Representative LC–MS chromatograms for all datasets have been provided in the revised version (Fig 1 &2) of the main manuscript as well as in the revised (S2-S4, S10-S11, S14-S15, S17,S21-S23,S25-S26,S30-S31) SI.

• Regarding peptide detection by UV (Comment #2), UV-based detection is widely used for peptide quantification. The paper cited by the authors (Campbell et al., Quantitative Analysis of Glycine Oligomerization by Ion-Pair Chromatography) used a C18 column to separate glycine oligomers, indicating that this type of analysis is feasible. Thus, it seems possible that peptide detection/quantification could also be performed on the column used here, or at least this point should be discussed more carefully.

We thank the reviewer for this suggestion and apologize for the omission of the corresponding HPLC chromatogram in the previous submission. We applied the SHS method, following Rodriguez-García *et al.*, to analyze glycine-containing peptides on a C18 column, and the chromatogram has been included below for clarity.



It is important to note that the system studied by Rodriguez-García *et al.* involved only glycine peptides, which likely facilitated clearer peak resolution. In contrast, our reaction mixtures comprise a complex distribution of both peptide and oligonucleotide products. This increased chemical complexity leads to partial co-elution and overlap in signal intensities, making baseline separation of individual components more challenging under comparable chromatographic conditions.

• Regarding Comment #3, the authors' response regarding NMR and low glycine consumption should be reconciled with the large reported yields (even though the yield itself remains unclear, see comment above) in some experiments.

We would like to clarify that glycine can be reliably detected only in ^{1}H NMR. However, in our experimental system, the reaction mixture comprises a complex ensemble of nucleotides, amino acids, and their corresponding intermediates. This compositional complexity leads to significant spectral overlap, making it challenging to unambiguously monitor only the glycine signal across successive reaction cycles.

Also, we have now removed absolute yield claims and also have toned down our yield-based assumptions and inferences in the discussion section.

• Regarding comment #4 (comparison to Rodriguez-Garcia et al.), what is the final pH of the reactions studied here at the end of the cycles? I could not find this information in the manuscript.

The Methods section has been updated to explicitly state that rehydration was performed using pH-adjusted Milli-Q water (pH 7). Consequently, the pH was consistently maintained at 7 across all wet–dry cycles, ensuring uniform reaction conditions throughout the experiment.

• Regarding point #7 (HPLC), the authors say: "HPLC of AMP-Gly (P-O-C) could not be obtained as the mixed anhydride linkage is highly susceptible to hydrolysis under aqueous conditions." But the authors show HPLC data for this standard (Fig S6a) and 1H NMR in D2O, which is confusing. This should be clarified.

The HPLC trace in Fig. S6a clearly indicates partial hydrolysis of AMP–Gly to AMP under the analytical conditions. As for NMR characterization, a substantial quantity of highly pure compound is required; which was straightforward to obtain when we were dealing with just the standard sample that we synthesized and spiking chromatograms. However, as detailed in the Methods section, the chromatographic buffer system (20 mM Tris, 400 mM sodium perchlorate, pH 8) in itself adds to the hydrolytic instability of the AMP–Gly linkage. Consequently, although the chromatographic runs from relevant reaction mixtures readily revealed the presence of the intermediate, repeated fraction collection of these samples were impractical. The problem is further accentuated as the isolated fractions also end up containing high salt concentrations and only minimal amounts of the desired product when this is done. This serious challenge of obtaining high amounts of pure AMP-Gly (P-O-C) from our synthetic reactions via HPLC purification, hence, added severe complications for downstream purification and concentration for NMR analysis.

• Regarding point #9, while the paper by Suárez-Marina et al. It reports the simultaneous formation of glycosidic bonds between ribose, and purine and pyrimidine nucleobases, it also reported some glycine adducts (e.g., Gly-AMP) even though these were not the focus of that paper.

The reference has now been added to our manuscript.

• If the manuscript continues to compare its results to prior peptide-formation literature, the comparison should be made carefully, including explicit discussion of pH conditions.

As mentioned previously, our experimental set-up was maintained at pH 7 throughout all cycles.

• Overall, I think the manuscript has improved, but the analytical basis for the quantitative claims remains insufficient. Unless the quantification is properly validated, the manuscript should be revised so that all claims are clearly presented as qualitative.

We would like to reiterate that we have now removed all absolute yield claims and have toned down our yield-based assumptions and inferences in the discussion section.

Manuscript title: Simultaneous formation of peptides and RNA via prebiotic cross-catalytic enhancement

Please note that the reviewer's comments are mentioned in green below, while our responses have been indicated in black.

Reviewer #4 (Remarks to the Author):

While the revised manuscript has improved, I recommend that it undergo further revision before acceptance, as several major issues remain unresolved.

Major comments:

1. The revised version is improved in that the previous absolute mol yield plots have largely been removed, and the authors now acknowledge that the LC-MS data provide relative rather than absolute quantification. However, my main concern is only partially resolved. The manuscript and Supporting Information still describe the LC-MS-derived values as "percentage yields." This terminology remains problematic. As currently described, the values are obtained from analyte peak areas normalized to an alanine internal standard, with the analyte/internal-standard ratio multiplied by 100. However, these values should not be described as yields, as mentioned in my former comments. These values are relative MS response values or relative signal intensities.

I also remain concerned that some interpretive statements are still stronger than the data justify. For example, claims that the chemistry "significantly improves the efficiency" of peptide formation, or that the work provides "concrete evidence" for RNA-peptide co-evolution, should be toned down. The data support detection of longer products and apparent changes in relative MS signal under certain reaction conditions, but they do not establish robust chemical yields or quantitative efficiencies.

Hence, the authors should replace "percentage yield" throughout the manuscript and Supporting Information with terminology such as "relative LC-MS response" or "relative abundance". In addition, the authors should remove or substantially soften conclusions that depend on quantitative yield comparisons. The conclusions should be limited to qualitative or semi-quantitative observations, such as the appearance of longer oligomers, detection of proposed intermediates, and relative changes in MS signal under different reaction conditions.

Response: We wish to state that the sentence using the phrase "concrete evidence" has now been replaced with: "Such simultaneous characterization of oligopeptides and oligonucleotides in a one pot reaction has not been explored before and strengthens the hypothesis of the co-evolution of RNA and peptides, underscoring the feasibility and importance of the RNA-peptide world hypothesis."

Further, all instances of the term "percentage yields" have now been replaced with 'relative abundance'. We have also toned down the phrase, "significantly improves the efficiency", to

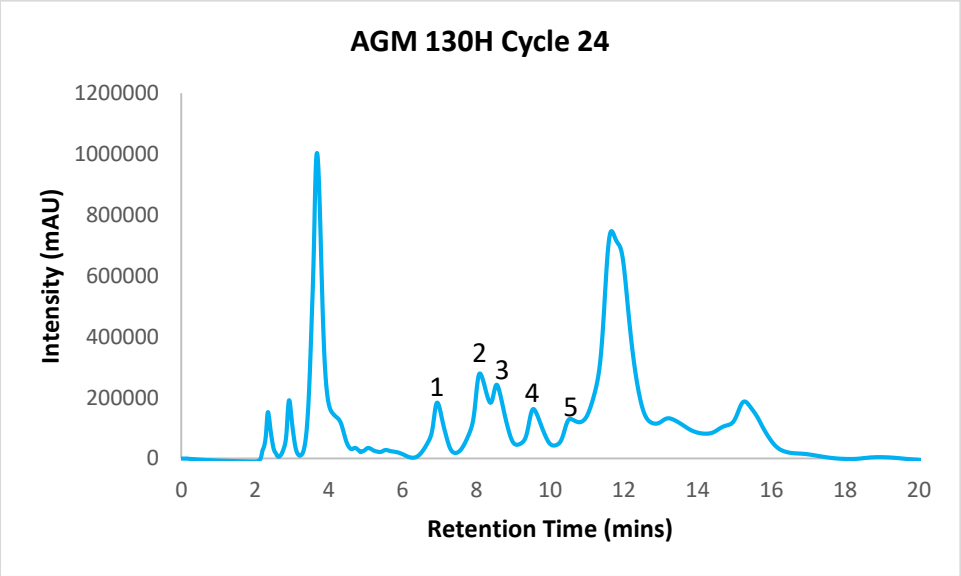
“resulted in an improved relative abundance of peptides", which still highlights the outcome that resulted in our reactions upon the addition of nucleotides.

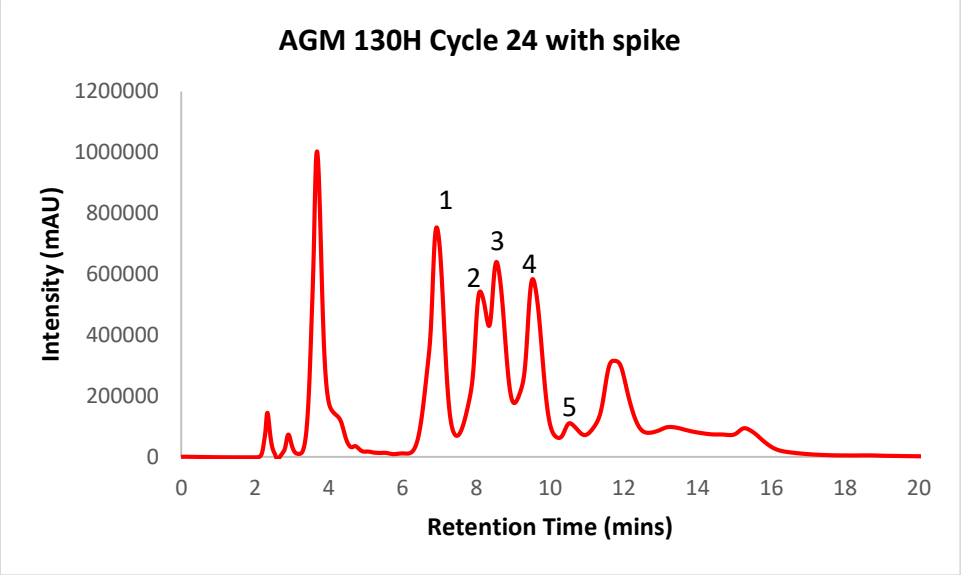
2. I noted that the newly added heat-map presentation appears to contradict in some cases the results in the previous version. For example, in the revised Figure 1C, the cAG 130 condition appears to show lower peptide formation than AG 130 for several oligomer lengths, whereas the previous version of Figure 1, which presented the data as relative peptide yields, appeared to suggest the opposite trend. This discrepancy requires clarification. It is not clear whether the difference arises from a change in normalization or an error in plotting.

Response: The difference arose from a change in normalization. Also, we are no longer comparing yields or any quantitative values between the ATP- and cAMP-based reactions.

3. It is useful that the authors now show in the rebuttal letter UV-detected chromatograms for the available glycine oligomer standards, demonstrating that these standards can be separated and detected. Given this, the authors should now perform standard-spiking experiments with the oligoglycine standards they have available. Spiking the reaction mixtures with low amounts of authentic standards would help confirm the peak assignments. This would also strengthen the authors' response to the concern about relying exclusively on MS-based analysis.

Response: We did a standard spiking for the available glycine oligomers, which includes diglycine to hexaglycine and the chromatograms have been included below.





The chromatogram showing the blue HPLC trace is for the reaction AGM 130H Cyc 24 sample, and the one with the red HPLC trace is for the same reaction that was spiked with the following glycine oligomers to characterize the identity of the peptides in this reaction beyond doubt: diglycine (1), triglycine (2), tetraglycine (3), pentaglycine (4), and hexaglycine (5). Notably, as has already been discussed in the previous response-to-reviewers document, even hexaglycine resulted in partial co-elution and overlapping signal intensities. This is evident in the chromatogram with the blue HPLC trace, where the hexaglycine peak (denoted as 5), nearly co-eluted with the AMP peak (observed at ~12 minutes). Given this, it was clear that heptaglycine and any other higher glycine oligomers would have readily co-eluted with AMP, adenosine, and other compounds that typically eluted on this gradient at retention times greater than ~11 minutes, making baseline separation of individual components beyond hexaglycine not achievable.

These HPLC traces, and the corresponding explanation, have now been added to SI (Figures 6i-ii). The methodology for acquiring these chromatograms has also been added to the materials and methods section.

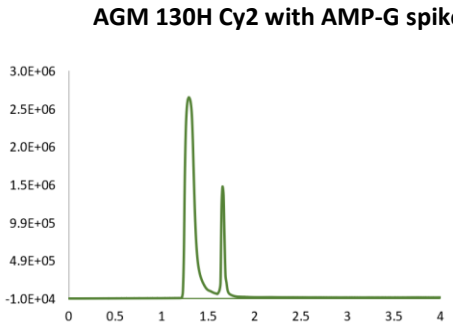
Minor comments:

- Figure 2b is cropped at the bottom.

Response: This has been corrected

- Figure 3A: The spiking experiment is not convincing as presented. The amount of spiked standard appears too high and seems to overload the original peak. For a meaningful spiking test, the spike should be much smaller, typically ~1–5% of the original peak area or height.

Response: To improve clarity, the above chromatogram was obtained with a diluted purified AMP-G to avoid overloading concerns as suggested by the reviewer. This figure has now been added to main manuscript figure number 3.



- Regarding comment #3 in the previous review. The authors state: "The Methods section has been revised to eliminate any ambiguity and now clearly describes the approach used for relative yield determination". But the Methods section in the SI (pages 4-5) still contains the unclear statement: "An internal standard (alanine, 20mg/ml) was injected with 150 nmoles of samples to obtain the yields of resultant products, which were then summed up to get the overall oligomer yield." - This was raised in my previous review but has not been revised. As mentioned, this is ambiguous: 150 nmoles of what was injected?

Response: Sorry about missing this. The nmoles value has been removed. 20mg/ml was the stock concentration of alanine out of which 1.4 µl was injected along with 6 µl of sample. It has now been reworded to reflect this in the methods section as well.

- Show chirality in fig. s1. Also fix the structure of Asp.

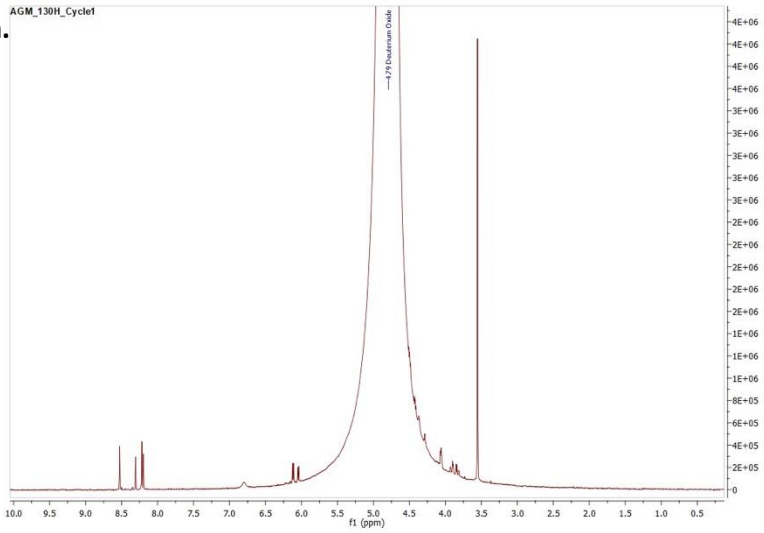
Response: The figure depicting Asp has been corrected. As the manuscript does not address stereochemistry, we have deliberately omitted any indication of chirality from the figure to maintain consistency with the scope of the article.

- There seems to be a recurring notation issue in the TOF-MS/MS assignments. The authors state that the MS data were acquired in positive mode; therefore, the precursor ions should be reported as protonated species (e.g. $[M+H]^+$). In several places, both the manuscript and SI appear to use "$(M–H)^+$", which is incorrect. If the intended ion is the positive-mode protonated molecule, this notation should be corrected throughout.

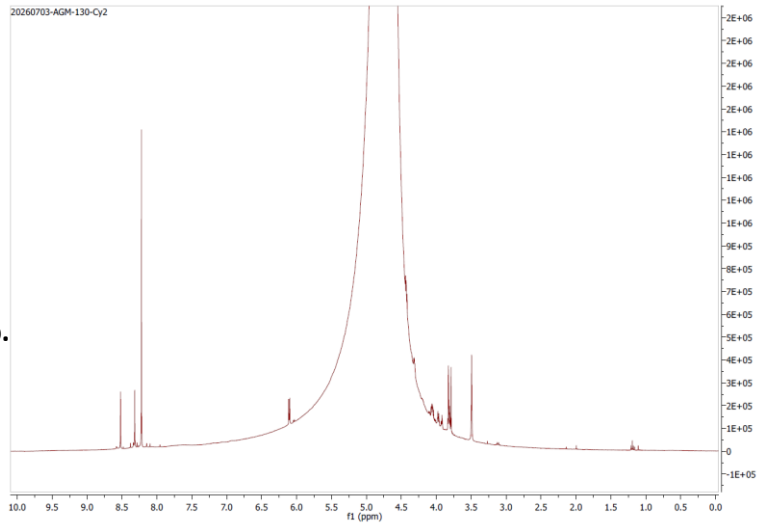
Response: Thank you for pointing this; it has been corrected in both the main manuscript and supplementary.

- Regarding the comment in the rebuttal letter about 1H-NMR, can the authors provide a representative spectrum?

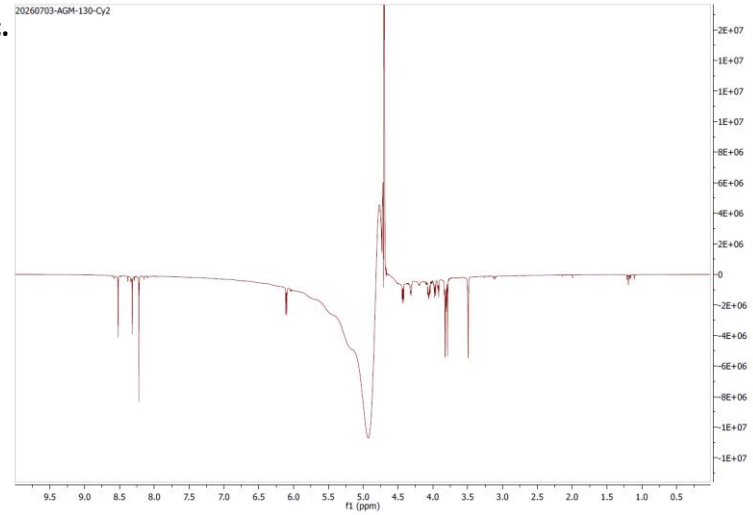
a.



b.



c.



Response: The representative ^{1}H NMR spectra of the AGM 130H reaction at Cycle 1 (Figure a) and Cycle 2 (Figure b) are shown above. As discussed previously, the glycine methylene proton resonance could not be unambiguously assigned because it substantially overlapped with the intense residual D_2O signal. Consequently, changes in the glycine proton intensity could not be monitored reliably, thus, quantitative assessment of glycine consumption during the course of the reaction through ^{1}H NMR could not be performed.

To overcome this limitation, water suppression experiments were performed (Figure c). However, suppression of the residual water signal did not significantly improve the resolution of the glycine resonance, as the overlap between the two signals were inverted below the axis. Therefore, the ^{1}H NMR spectra did not provide a reliable means to monitor glycine depletion or the progression of the reaction.

Given these limitations, subsequent analyses focused on techniques that directly probe the formation of reaction intermediates. ^{31}P NMR was employed to monitor phosphorus-containing activated intermediates, including phosphoramidate and mixed anhydride species, while LC–MS was used to detect and characterize these intermediates based on their accurate masses and fragmentation patterns. Together, these complementary analytical approaches provided a more robust and unambiguous assessment of intermediate formation than what could be achieved using ^{1}H NMR alone.

- In the rebuttal letter, the authors say: "The Methods section has been updated to explicitly state that rehydration was performed using pH-adjusted Milli-Q water (pH 7). Consequently, the pH was consistently maintained at 7 across all wet–dry cycles, ensuring uniform reaction conditions throughout the experiment.". The statement remains ambiguous. Do the authors mean that the water used for rehydration was adjusted to pH 7 before addition, or that the reaction mixture was measured and readjusted to pH 7 after each rehydration step?

Response: The MiliQ used for rehydration was set at pH7 and the resultant solution mixture was checked to be sure that it was at pH 7. Deviation from it, if observed, was again addressed with adding diluted HCl or NaOH.

- The comparison with prior peptide-formation literature remains too brief and should be elaborated. Since the manuscript uses the neutral-pH conditions as an important point of distinction, the authors should compare their results more explicitly with prior work in the cited studies.

Response: A comprehensive table summarizing all experiments conducted to date in the field of peptide and oligonucleotide oligomerization, along with the corresponding conditions tested, was provided in our previous rebuttal letter. We have now included this as part of the supplementary material (Tables 3 and 4). In the main article, we have discussed and cited the studies that are directly relevant to contextualizing the present work.

Simultaneous formation of peptides and RNA via prebiotic cross-catalytic enhancement

Reviewer No. 4:

The authors have addressed all of my concerns, and I recommend publication of the paper in its current form, subject to two minor changes: (1) replacing "percentage yield" with "relative abundance" in Figure 1C, and (2) adding a short explanation of the spiking with glycine oligomers to the Methods section of the SI, as I could not find this information there.

The authors thank the reviewer for their constructive criticism and persistence in making the article better. All percentage yield mentions have been changed to 'relative abundance' (including in Fig 1C) in the main article. The protocol from Rodriguez-Garcia, M. *et. al.*, was used for separation of glycine peptides and this has been mention in Methods Section C of Supplementary Information file.