# A Sensitive Quantitative Analysis of Abiotically Synthesized Short Homopeptides using Ultraperformance Liquid Chromatography and Time-of-Flight Mass Spectrometry

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## ABSTRACT

In the origins of life field understanding the abiotic polymerization of simple organic monomers (*e.g.,* amino acids) into larger biomolecules (*e.g.,* oligopeptides), remains a seminal challenge. Recently, preliminary observations showed a limited set of peptides formed in the presence of the plausible prebiotic phosphorylating agent, diamidophosphate (DAP), highlighting the need for an analytical tool to critically evaluate the ability of DAP to induce oligomerization of simple organics under aqueous conditions. However, performing accurate and precise, targeted analyses of short oligopeptides remains a distinct challenge in the analytical chemistry field. Here, we developed a new technique to detect and quantitate amino acids and their homopeptides in a single run using ultraperformance liquid chromatography-fluorescence detection/time of flight mass spectrometry. Over an 8-minute retention time window, 18 target analytes were identified and quantitated, 16 of which were chromatographically separated at, or near baseline resolution. Compound identity was confirmed by accurate mass analysis using a 10 ppm mass tolerance window. This method featured limits of detection < 5 nM (< 1 fmol on column) and limits of quantitation (LOQs) <15 nM (< 3 fmol on column). The LODs and LOQs were upwards of ~28x and ~788x lower, respectively, than previous methods for the same analytes, highlighting the quantifiable advantages of this new method. Both detectors provided good quantitative linearity ($R^2 > 0.985$) for all analytes spanning concentration ranges ~3 - 4 orders of magnitude. We performed a series of laboratory experiments to investigate DAP-mediated oligomerization of amino acids and peptides and analyzed experimental products with the new method. DAP readily polymerized amino acids and peptides under a range of simulated environmental conditions. This research underscores the potential of DAP to have generated oligopeptides on the primordial Earth, enhancing prebiotic chemical diversity and complexity at or near the origin of life.

**KEYWORDS:** homopeptides, diamidophosphate, ultraperformance liquid chromatography, time-of-flight mass spectrometry, pre-column derivatization

## 1. Introduction

The primordial Earth likely contained a variety of simple organic compounds (*e.g.,* amino acids), the origins of which, could have included *in situ* synthesis [1-7] and exogenous delivery [8-11]. It has been hypothesized that once present, these monomers could have accumulated in localized environments (*e.g.,* tidal pools) [6] and subsequently undergone processing to generate oligomers [12], ultimately leading to the synthesis of primitive, functional biopolymers.

The prebiotic polymerization of amino acids and the analysis of such mixtures has been a decades-long challenge facing the origin of life field. Numerous previous efforts have been made to address such challenges, with varying degrees of success [13]. Possible prebiotic condensing reagents such as carbonyl sulfide [12], and cyanamide and dicyandiamide [14] have been investigated for their ability to induce the polymerization of amino acids into peptides. These studies have resulted in the successful formation of simple peptides composed of ~2-3 amino acid residues, but larger peptides were consistently difficult to generate. More recently, Forsythe and Yu et al. [15] explored the co-polymerization of α-amino acids and their chemical cousins, α-hydroxy acids, under wet-dry cycling conditions. This approach demonstrated that larger peptides composed of ~2 – 8 amide bonds could be synthesized. Identifying alternative and complementary prebiotically plausible polymerization pathways, capable of readily generating amino acid oligomers under mild conditions would increase the likelihood of the abiotic emergence of peptides on the early earth under diverse environments.

Diamidophosphate (DAP) is a prebiotically plausible phosphorylating agent that, in the presence of imidazole, can induce the polymerization of phosphates and generate ester bonds

between carboxylic acids and alcohols. [16-17] in water, at pH 6-8, and under thermally mild (room temperature) conditions. Furthermore, Gibard et al. [17] reported the tentative identification of higher order peptides upon exposing amino acids to imidazole and DAP. These detections were made using $^{1}$H NMR and electrospray ionization mass spectrometry (ESI-MS), but the presence of these higher order peptides in a solution comprised of amino acids, imidazole, and DAP affirmed that more rigorous, targeted analytical approaches were needed to comprehensively explore the capability of DAP to oligomerize amino acids.

Diamidophosphate is a very simple compound that could have readily formed on the early Earth via the reaction of prebiotic phosphorus [18] and aqueous ammonia [19-20]. Prebiotically accessible phosphorus could have been delivered by meteorites [21-22], or formed via *in situ* synthesis [23], and significant quantities of dissolved ammonia could have been released into the early Earth's oceans by hydrothermal vents [24]. Furthermore, the geochemical availability of DAP has recently been experimentally demonstrated [25]. Imidazole, which decelerates the condensation and hydrolysis of DAP [17], could have been prebiotically formed via irradiation of atmospheric water, nitrogen, and carbon monoxide or methane [26]. Thus, the reagents necessary to facilitate DAP-mediated polymerization are prebiotically plausible.

Based on the tentative identification of higher order peptides [17] stemming from a mixture of select amino acids, imidazole, and DAP, and given that DAP is a prebiotically plausible compound, we hypothesize that DAP will readily facilitate the formation of peptides composed of Gly, Ala, Asp, Glu, and also oligomerize short peptides (*e.g.,* glycylglycine) under thermally mild, aqueous conditions that are relevant to a prebiotic context. To evaluate this hypothesis, we performed a series of laboratory experiments to determine the effectiveness of DAP to oligomerize amino acids and simple peptides under mild, aqueous conditions. We

118 performed experiments designed to evaluate how changing the molar equivalents of DAP
119 relative to the amino acid in question effected the oligomerization products, using starting
120 reagent concentrations consistent with that which has been used previously in the literature [15,
121 27-28]. We also executed room temperature, mild heating (50 °C), and simulated environmental
122 cycling experiments using lower starting reagent concentrations to evaluate the change in
123 efficacy of the DAP polymerization chemistry with a change in reagent concentrations.
124 The analyses of the homopeptides generated from these experiments required a robust
125 analytical technique capable of accurately detecting and precisely quantitating oligomers
126 synthesized from the exposure of amino acids and simple peptides to imidazole and DAP.
127 However, the analysis of a mixture of short homopeptides is a formidable analytical challenge.
128 Currently available techniques reported in the literature that target peptides suffer from such
129 drawbacks as long run times [29-32], use of only one detection system [32, 33-36], lacking
130 accurate mass analysis [37-39], or detecting a limited number of analytes [40-42]. Forsythe et al.
131 [43] recently implemented a technique capable of detecting a wide range of depsipeptides (mixed
132 amide/ester linkages) based on retention time, drift time, accurate mass, and fragmentation
133 patterns. However, due to limited commercial availability of depsipeptide standards, absolute
134 quantitation was not reported. Ideally, an analytical technique would be developed that provides
135 rapid analysis of a wide range peptides using multiple detection systems, including accurate mass
136 analysis, and is capable of delivering absolute quantitation of target analytes. Therefore, to
137 facilitate the analysis of oligopeptides generated by laboratory experiments in this work, we
138 developed a new analytical method to detect and quantitate amino acids and associated
139 homopeptides in a single analytical run. The new technique developed here was optimized to
140 address the aforementioned analytical needs. The analytical approach employed here used a

combination of ultraperformance liquid chromatography, UV fluorescence detection, and time-of-flight mass spectrometry (UPLC-FD/ToF-MS). Furthermore, because linear oligopeptides were likely readily available to carry out further polymerization chemistry on the primitive Earth *en route* to the formation of the first functional biopolymers, as opposed to diketopiperazines, which are highly stable cyclic dipeptides that could have prevented amino acid residues from being available to engage in further polymerization chemistry [44-46], the method developed here was optimized to target linear homopeptides.

## 2. Materials and methods

### *2.1. Chemicals and reagents*

All sample handling tools, including glassware, were baked out overnight at 500 °C in air prior to use to remove organic contamination. Millipore Integral 10 ultrapure water (18.2 MΩ-cm, ≤3 ppb total organic carbon) was used for the experiments performed here. All commercially purchased reagents used were acquired from Sigma-Aldrich, Fisher Scientific, Acros Organics, Combi-Blocks, Bachem, Tokyo Chemical Industry, and Waters Corporation. The DAP used in this work was synthesized as described elsewhere [16, 47-48]. Stock amino acid and homopeptide solutions were prepared by dissolving individual analyte crystals in ultrapure water. The amino acids and homopeptides used to generate stock solutions were of purities in the 96% - 100% range, and the stock solutions were made to be between $10^{-2}$ M and 1 M. Once the individual standard solutions of each species were made, they were combined to facilitate the analysis of all target analytes in a single analytical run.

Four reagents were used to enable pre-column derivatization: 1) AccQ·Tag Ultra borate buffer, 2) AccQ·Tag Ultra reagent powder, 3) AccQ·Tag Ultra reagent diluent, and 4) AccQ·Tag derivatization agent**.** Four eluents were used to perform UPLC-FD/ToF-MS analysis: A)

AccQ·Tag A buffer, B) AccQ·Tag B buffer, C) strong wash, and D) weak wash. Daily calibrations of the ToF-MS and real-time lock mass corrections were performed using 2 separate solutions. The preparation and implementation of these reagents used for derivatization, analysis, and mass calibration are detailed in §1.1 of the supplementary material.

*2.2. Method development*

Amino acids and homopeptides were analyzed using a Waters Acquity H-Class UPLC, coupled to a Waters Acquity UPLC FD, and a Waters Xevo G2-XS mass spectrometer. Analyte identification was based on 3 analytical metrics: 1) chromatographic retention time, 2) optical fluorescence, and 3) accurate mass measurement, based on comparison to a mixed standard. The selection of amino acids and homopeptides to analyze in this study was based on a core set of four amino acids. The amino acids Gly, Ala, Asp, and Glu, and their respective homopeptides, were selected for study because these amino acids: 1) are commonly produced in prebiotic simulation experiments ([1, 6-7], 2) they have been detected in meteorites [49], and 3) they allow for the exploration of how homopeptide synthesis varies between those composed of structurally simple (*i.e.,* Gly and Ala) and more complex (*i.e.,* Asp and Glu) amino acids. In this study, we focused on analyzing the mixture of homopeptides from these amino acids as a demonstration of a proof of concept of analytical capabilities dealing with the first stage complexities at this 'simpler' homopeptide level, before we tackle the long-term issue of how to analyze more complex mixtures involving hetero-peptides . Consequently, the targeted homopeptides were diglycine ($Gly_2$), triglycine ($Gly_3$), tetraglycine ($Gly_4$), pentaglycine ($Gly_5$), hexaglycine ($Gly_6$), dialanine ($Ala_2$), trialanine ($Ala_3$), tetra-alanine ($Ala_4$), penta-alanine ($Ala_5$), diaspartic acid ($Asp_2$), triaspartic acid ($Asp_3$), tetra-aspartic acid ($Asp_4$), diglutamic acid ($Glu_2$), and triglutamic

186 acid ($Glu_3$). The lengths of homopeptides targeted in this study were dictated by aqueous
187 solubility limits of commercially available analytical standards.

188 Amino acids and homopeptides underwent pre-column derivatization using Waters
189 AccQ·Tag derivatization agent (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate), a
190 fluorophore that enhances analytical specificity by reacting with primary amino groups, and
191 select secondary amino groups [50]. A schematic representing the derivatization reaction is
192 provided in the supplementary material (Figure S1), which illustrates that only the N-terminal
193 primary amino groups of the homopeptides analyzed in this study were successfully derivatized
194 by AccQ·Tag. The AccQ·Tag derivatization agent has the added benefit of being effective in the
195 presence of different salts **[50]**, allowing for the mitigation of otherwise necessary desalting
196 procedures that are sources of sample contamination and loss. This is in contrast to alternative
197 derivatization approaches, such as 9-fluorenylmethyl chloroformate or phenylisothiocyanate [51]
198 and *o*-pthaldialdehyde/*N*-acetyl-L-cysteine [52], which are adversely impacted by the presence
199 of salts or interfering ions. Furthermore, derivatives of AccQ·Tag are stable for at least 1 week at
200 room temperature [50], whereas derivatives of other agents, such as *o*-pthaldialdehyde are often
201 unstable [53].Consequently, pre-column derivatization was executed by mixing 10 μL of the
202 sample or standard with 70 μL of AccQ·Tag Ultra borate buffer, then adding 20 μL of the
203 AccQ·Tag agent, prior to heating at 55 °C for 10 minutes. Following derivatization, samples and
204 standards were ready for analysis. Since the AccQ·Tag derivatization agent was the only reagent
205 responsible for derivatizing target analytes, only results from AccQ·Tag derivatization are
206 discussed here.

207 UPLC separations have been performed using a 2.1 x 150 mm, 1.7 μm Waters Acquity
208 UPLC BEH Phenyl column. Target analytes were eluted using the following gradient: 0 – 9.5

min, 100% eluent A, 9.5 – 16 min, 100 – 92 % eluent A, 16 – 20 min, 92 – 82 % eluent A, 20 – 21 min, 82 – 100 % eluent A, 21 – 25 min, 100 % eluent A. The autosampler was maintained at 25 °C, the injection volume was 2 μL, the eluent flow rate was held constant at 0.3 mL $min^{-1}$, and the column was maintained at 30 °C. The FD was operated with an excitation wavelength of 266 nm and an emission wavelength of 473 nm.

The ToF-MS was equipped with a dual ESI source arrangement, where both ESI sources were operated in positive ion mode. The primary ESI source was used under the following conditions: capillary voltage, 3.5 kV, sampling cone voltage, 40 V, source temperature, 120 °C, desolvation gas temperature, 500 °C, cone gas ($N_2$) flow, 50 L $hr^{-1}$, desolvation gas ($N_2$) flow, 1000 L $hr^{-1}$. When using the primary ESI source, the ToF-MS was calibrated over the 50 – 1200 *m/z* range. The purpose of implementing the secondary ESI source was to account for the possibility of small deviations in the *m/z* scale after daily calibrations were performed, and thus provide an independent leucine enkephalin standard signal at *m/z* 556.2771. The secondary ESI source was implemented using parameters identical to those when the primary ESI source was utilized, except the secondary ESI source used a capillary voltage of 2.8 kV and a reference cone voltage of 30 V. The ToF-MS analyzer was operated in V-optics mode, which implemented a reflectron to achieve a full width at half maximum resolution of 22,000 based on the *m/z* value of leucine enkephalin. The detector voltage setting was 2225 V, and the *m/z* range over which data were collected was 100 – 1000. A mass tolerance of 10 ppm was implemented for the purpose of accurate mass identification of target analytes by ToF-MS.

Following the completion of analytical development, the qualitative and quantitative performance of the method was comprehensively evaluated by performing analytical figures of merit experiments. The details of these experiments can be found in §1.3 of the supplementary

material, and the results from the analytical figures of merit experiments can be found in §2.2 of the supplementary material.

*2.3. DAP/dry-down experiments*

Experimental reaction solutions composed of select amino acids, imidazole, and DAP were prepared for the DAP/dry-down experiments. The objectives of the DAP/dry-down experiments were two-fold: 1) to evaluate how homopeptide synthesis was affected by changing the molar equivalents of DAP relative to the amino acid, and 2) to determine how homopeptide synthesis was affected by either leaving the experimental reaction solutions in the liquid state at room temperature, or bringing the experimental reaction solutions to dryness.

The amino acids chosen for these experiments were Ala, Asp, and Glu. Each individual amino acid was combined with imidazole and DAP in an aqueous solution. In each solution, the amino acid and imidazole were both present at 0.1 M. Each amino acid was exposed to 3 different DAP concentrations in separate solutions, 0.05 M (*i.e.,* 0.5 equivalents), 0.1 M (*i.e.,* 1 equivalent), or 0.2 M (*i.e.,* 2 equivalents). Each solution was stirred at room temperature for 14 days. The pH of each solution was between 8.5 – 9.3 over the course of the 14-day reaction. At the end of 14 days, experimental reaction solutions were designated to either be brought to dryness in a Centrivap centrifugal vacuum dryer, or to be not dried. In addition to these experimental reaction solutions, experimental control solutions were prepared. The experimental control solutions were prepared in an identical fashion as the experimental reaction solutions, except after the 14-day reaction period, the experimental control samples were pH-adjusted between 1.0 – 1.35 and stirred for 16 hours to stop the reaction. Subsequently, experimental control solutions were designated to either be brought to dryness in a Centrivap, or to be not dried. The experimental solutions prepared for these experiments and the experimental

conditions used are detailed in Table 1. After generation, the experimental solutions underwent a 100x dilution. The purpose of this dilution step was to avoid potentially overwhelming the ToF-MS detector by otherwise exposing the detector to very large concentrations (≥ 1 mM) of the amino acids that existed in the experimental solutions. After dilution, experimental control solutions and experimental reaction solutions were analyzed identically.

*2.4. Room temperature/heating experiments*

The objectives of the room temperature/heating experiments were two-fold: 1) to evaluate homopeptide synthesis under room temperature (23 ± 3 °C), aqueous conditions, and 2) to determine how homopeptide synthesis was affected by exposing experimental reaction solutions to mild heating at 50 ± 0.6 °C. Individual experimental reaction solutions using all 4 target amino acids, imidazole, and DAP were prepared for these experiments. For each experimental reaction solution, the amino acid, imidazole, and DAP were all equimolar, at a concentration of 10 mM. Experimental reaction solutions were sampled for analysis according to the following frequency $t_i$ = 0 days, $t_1$ = 3 days, $t_2$ = 6 days, $t_3$ = 9 days, $t_4$ = 24 days, and $t_f$ = 106 days. Table 2 details the composition and treatment of each experimental reaction solution.

For each experimental reaction solution prepared, a corresponding experimental control solution was prepared. The experimental control solutions were prepared identically to the experimental reaction solutions, except the experimental control solution was composed of the amino acid and imidazole, but did not include DAP. The purpose of the experimental control solution was to verify that homopeptides did not form in the absence of DAP. After generation, and prior to analysis, the experimental solutions were sampled at their respective times, and underwent a 10x dilution. This dilution step served the purpose of avoiding potentially overwhelming the ToF-MS detector with very large concentrations of initial reactants.

278 *2.5. Simulated environmental wet-dry cycling experiments*

279 The objectives of the simulated environmental cycling experiments were three-fold: 1) to
280 evaluate homopeptide synthesis using starting reagent concentrations 10x less than those used in
281 the DAP/dry-down experiments, 2) to determine how homopeptide synthesis was affected by
282 exposing experimental solutions to multiple dry-down/rehydration cycles, and 3) to determine if
283 DAP is capable of oligomerizing short peptides, in addition to amino acids. Individual
284 experimental reaction solutions using all 4 target amino acids and $Gly_2$, imidazole, and DAP
285 were prepared for the simulated environmental cycling experiments. For each experimental
286 reaction solution, the amino acid or peptide, imidazole, and DAP were all equimolar, at a
287 concentration of 10 mM. The simulated environmental cycles were performed as described in
288 Table 3. After the completion of each simulated environmental cycle, samples were rehydrated
289 and prepared for analysis as described above prior to analysis by UPLC-FD/ToF-MS.
290 Experimental control solutions were prepared as described in the previous section. Experimental
291 reaction solutions and experimental control solutions were also diluted 10x prior to analysis, as
292 detailed previously. Analyses of samples generated from the simulated environmental wet-dry
293 cycling experiments were performed identically to the analyses of samples generated by the
294 other experiments performed in this work.

295

296 **3. Results and discussion**

297 *3.1. Method development*

298 The analytical technique developed here targeted a suite of 18 analytes composed of
299 amino acids and their respective homopeptides. The chromatographic approach implemented
300 achieved at, or near, baseline resolution of 16 of 18 species (Figure 1). The only 2 analytes that

were not well resolved were $Gly_3$ and $Gly_4$. While these compounds coeluted and therefore could not be differentiated by fluorescence, they were detected at different $[M+H]^+$ values and were therefore fully resolved by the mass spectrometer, within a mass accuracy window of 10 ppm. The AccQ·Tag derivatization agent facilitated reversed-phase UPLC separation of hydrophilic amino acids and peptides by covalently bonding a hydrophobic 6-aminoquinolyl carbamate moiety to their respective primary amino groups, which increased the overall hydrophobicity of the target analytes. Given the hydrophobic nature of the stationary phase used in the reversed-phase UPLC technique discussed here, the derivatized target analytes experienced increased interaction with the stationary phase compared to their non-derivatized counterparts. In turn, the AccQ·Tag derivatization agent enabled the enhanced chromatographic retention and separation of otherwise hydrophilic amino acids and peptides using a reversed-phase UPLC approach. Additional details pertaining to the observed chromatographic results are provided in §2.1 of the supplementary material. Furthermore, a breakdown of the detection parameters for each of the target analytes is provided in Table S1.

Reports of analytical methods used for the analysis of short oligopeptides, particularly those applied to prebiotic chemistry research, are unfortunately often not necessarily accompanied by an analytical figures of merit evaluation to rigorously constrain the quantitative performance of the methods [34, 54-55]. However, several reports of existing techniques have targeted select short oligopeptides also targeted in the current study, and concomitantly provided quantitative performance characteristic for these methods. For example, You et al. [56] used *N*-hydroxysuccinimidyl-α-(9-Phenanthrene)-acetate pre-column derivatization with high performance liquid chromatography (HPLC) and fluorescence spectrophotometry to obtain limit of detection (LOD) values for $Gly_2$ and $Gly_3$. Additionally, Zhu et al. [57] used fluorescamine

post-column derivatization with capillary electrophoresis and fluorescence detection to obtain LOD values for $Gly_2$ – $Gly_6$ oligomers, $Ala_2$, and $Ala_5$. Furthermore, Wang et al. [58] used a micro-fluidic chip and laser induced fluorescence detection to obtain an LOD value for $Gly_2$. Lastly, Campbell et al. [59] recently published limit of quantitation (LOQ) values for a method used to analyze $Gly_2$ – $Gly_6$ oligomers by ion-pair HPLC and UV-Vis detection. When comparing LOD values from existing methods to the LOD values (*e.g.,* Table S3) obtained from the method developed in this current work, the technique developed here provided upwards of ~3 – 12x lower LOD values for $Gly_2$ [56 -58], ~4 – 18x lower LOD values for $Gly_3$ [56–57], and ~4 – 10x lower LOD values for $Gly_4$ – $Gly_6$ and ~22 – 28x lower LOD values for $Ala_2$ and $Ala_5$ [57]. When comparing LOQ values from an existing method to the LOQ values (*e.g.,* Table S3) obtained from the method developed in the current work, the technique developed here provided upwards of ~119 – 788x lower LOQ values for $Gly_2$ – $Gly_6$ [59].

These comparative results underscore the importance of conducting a rigorous analytical figures of merit assessment when reporting the development of a new method, or the application of an existing method. In addition to providing lower LOD and LOQ values for the aforementioned oligopeptides that were concomitantly targeted by existing methods [56–59], the method developed in the current work also detects a more diverse set of oligopeptides than reported in other works focused on the analysis of oligopeptides relevant to origins of life chemistry [60-62]. Overall, it can be concluded that the method developed here provides analytical chemists and prebiotic chemists with a fast, sensitive technique capable of analyzing a wide range of oligopeptides using multiple detectors, including an accurate mass analyzer, without needing to perform off-line desalting that may otherwise contribute to sample contamination and loss.

*3.2. DAP/dry-down experiments*

The results of the experimental reaction samples being subjected to the DAP/dry-down experiments are given in Figure 2. The formation of dimers and trimers of Ala (Figure 2A), and dimers of Asp (Figure 2B) and Glu (Figure 2C) were comparable between dried and non-dried samples. The experimental control samples (pH adjusted to 1.0 – 1.35 after the 14-day reaction) failed to match the homopeptide synthetic capabilities of the experimental reaction samples (not pH adjusted). This is consistent with the expectation that acidifying the experimental control samples would quench the polymerization reaction due to the hydrolysis of DAP (pKa ≈ 5) under these conditions [63].

Based on the results shown in Figure 2, it is clear that homopeptide elongation is greatest when solutions composed of an amino acid, imidazole, and DAP are dried down. This is consistent with previous publications, which have indicated that dehydration is a critical step for the polymerization of monomers [64-65]. What can also be deduced from Figure 2 is that dried experimental reaction solutions that contained 1 equivalent of DAP relative to the amino acid, generally produced higher abundances of longer homopeptides. This information was used to design the subsequent oligomerization experiments.

Subjecting a solution containing 0.1 M Ala + 0.1 M imidazole + 0.1 M DAP to a dry-down resulted in the formation of the following concentrations (% yields) of homopeptides: ~751 μM (0.75 %) $Ala_2$, ~84 μM (0.08 %) $Ala_3$, ~19 μM (0.02 %) $Ala_4$, and ~2 μM (0.002 %) $Ala_5$ (Figure 2A). Subjecting a solution containing 0.1 M Asp + 0.1 M imidazole + 0.1 M DAP to a dry-down resulted in the formation of the following concentrations (% yields) of homopeptides: ~1 mM (1.3 %) $Asp_2$, ~50 μM (0.05 %) $Asp_3$, and $Asp_4$ was below the instrumental LOD (Figure 2B). Likewise, bringing the experimental reaction solution composed

of 0.1 M Glu + 0.1 M imidazole + 0.1 M DAP to dryness resulted in the following concentrations (% yields) of homopeptides: ~654 μM (0.7 %) $Glu_2$ and ~7 μM (0.007 %) $Glu_3$ (Figure 2C). The % yields of homopeptides produced during the DAP/dry-down experiments are similar to those observed in other dehydration-based oligomerization chemistries [66-67].

*3.3. Room temperature/heating experiments*

The room temperature/heating experiments offer a glimpse into the DAP-mediated amino acid oligomerization chemistry at relatively low (room) temperature conditions, and also under thermally mild (50 °C) conditions. For the purpose of demonstrating the efficacy of DAP-mediated homopeptide synthesis, experimental data from the oligomerization of Gly to form $Gly_2$ is detailed here, while experimental data from the oligomerization of Gly to form $Gly_3$, and the oligomerizations of the other three amino acids considered (Ala, Asp, and Glu) are detailed in §2.3 of the supplementary material.

Maximum $Gly_2$ formation under room temperature and mild heating conditions was observed after 106 days (Table 4). By examining $Gly_2$ accurate mass chromatograms after 106 days, it can be seen that the magnitude of the $Gly_2$ signal is slightly greater in the 50 °C experimental reaction solution composed of Gly, imidazole, and DAP, than it is for the same experimental reaction solution kept at room temperature (Figure 3A). It can also be seen that in the absence of DAP, $Gly_2$ is not formed, demonstrating that the presence of DAP is essential to enable oligomerization. Experimental reaction solutions generated detectable quantities of $Gly_2$ by the first collection time point ($t_1$ = 3 days) when exposed to mild heating (50 °C), whereas experimental reaction solutions left at room temperature did not generate detectable quantities of $Gly_2$ until the second collection time point ($t_2$ = 6 days). At the end of the 106-day experiment,

392 the heated experimental reaction solutions contained ~45% more $Gly_2$ than did the experimental
393 reaction solutions left at room temperature (Figure 3B).

394 The homopeptide yields observed from the room temperature and heating experiments
395 are overviewed in Table 4, and are similar to those observed elsewhere [66-67]. It is worth
396 noting that during the room temperature experiments, the simple amino acids, Gly and Ala, were
397 able to form dimer and trimer homopeptides, however, the more complex amino acids, Asp and
398 Glu, could only form dimers. Furthermore, neither Gly nor Ala were able to facilitate the
399 synthesis of tetramers or longer species. This is in contrast to the solutions that were brought to
400 dryness during the DAP/dry-down experiments, which readily formed larger homopeptides,
401 illustrating the importance of dehydration to drive amino acid polymerization. It should be
402 pointed out that over the course of the room temperature/heating experiments, it was observed
403 that the application of mild heating only made a noteworthy impact to the oligomerization of
404 simple amino acids. Under both room temperature and mild heating conditions, the overall
405 amino acid polymerization efficiency, in terms of total homopeptide yields, was identical: Gly >
406 Asp > Ala >> Glu. Additionally, the overall amino acid polymerization efficiency, in terms of
407 homopeptide length is also identical for both room temperature and mild heating conditions: Gly
408 = Ala > Asp = Glu. The observed homopeptide synthetic discrepancies between simple and
409 complex amino acids may likely be due to the relative structural complexity of Asp and Glu
410 resulting in the formation of decreased homopeptide chain lengths in the absence of dehydration.

411 From the room temperature/heating experiments, three conclusions can be made: 1) DAP
412 is readily capable of inducing the oligomerization of amino acids under thermally mild, aqueous
413 conditions, thus indicating that elevated temperatures are not required to oligomerize amino
414 acids in the presence of DAP and imidazole, 2) DAP can oligomerize both simple and relatively

complex amino acids, and 3) application of mild heating does not necessarily result in an increased yield of oligomers for all amino acids tested.

*3.4. Simulated environmental wet-dry cycling experiments*

For the purpose of evaluating DAP-mediated homopeptide synthesis when subjected to multiple simulated environmental wet-dry cycles, experimental data from the oligomerizations of all 4 amino acids are detailed here. Experimental data from the oligomerizations of short peptides ($Gly_2$) are detailed in §2.4 of the supplementary material.

Amino acid-based homopeptide syntheses observed in the experimental reaction solutions is demonstrated in Figure 4. Homopeptides were not detectable in experimental reaction solutions that had not undergone simulated environmental cycling. Experimental control solutions failed to produce homopeptides, while experimental reaction solutions readily synthesized homopeptides. Gly-based homopeptides, up to $Gly_6$, were promptly generated by wet-dry cycling; however, Gly-based homopeptide abundances were not significantly impacted by exposure to multiple simulated environmental wet-dry cycles, except for $Gly_4$ abundance, which dropped noticeably after exposure to a second cycle (Figure 4A). Ala-based homopeptides, up to $Ala_5$, were generated after 1 wet-dry cycle, and $Ala_5$ abundances gradually increased with increased cycling, while other Ala-based homopeptide abundances varied to a lesser extent (Figure 4B). Asp- and Glu-based homopeptides were generated less readily than Gly- or Ala-based homopeptides. $Asp_2$ and $Asp_3$ homopeptides were produced after 1 simulated environmental wet-dry cycle; however, $Asp_4$ did not occur until 3 simulated environmental wet-dry cycles had been performed. (Figure 4C) Once formed, Asp-based homopeptide abundances did not vary significantly when subjected to additional simulated environmental wet-dry cycles. $Glu_2$ was synthesized after 1 simulated environmental wet-dry cycle (Figure 4D), albeit at

relatively low abundances (~1 μM) (Table 5). $Glu_2$ was consistently present in the experimental reaction solutions with each additional simulated environmental cycle; however, $Glu_3$ was not synthesized at detectable quantities after 4 simulated environmental wet-dry cycles.

The homopeptide yields observed from simulated environmental cycling experiments are overviewed in Table 5. The order of combined homopeptide yields from their respective starting reagents during the simulated environmental wet-dry cycling experiments was $Gly_2$ > Gly > Ala > Asp >> Glu. Interestingly, subjecting a 10 mM equimolar solution of $Gly_2$, imidazole, and DAP to simulated environmental cycling resulted in a combined $Gly_2$ oligomer yield of >18 %, including a 15% yield of $Gly_4$. This is in contrast to a combined Gly oligomer yield of ~15 %, including a <2 % yield of $Gly_4$ from a solution containing 10 mM each of Gly, imidazole, and DAP. The observed greater efficiency of oligomerizing a small peptide is consistent with the report that amino acid monomers are more difficult to condense than two small peptides of at least dipeptide size [64]. Furthermore, this result indicates that DAP shows promise as an agent capable of oligomerizing polymerized species, with one implication being that DAP may be able to convert linear dipeptides into linear tetrapeptides, thereby helping to limit the formation of highly stable diketopiperazines [68-70] that would otherwise act as an amino acid thermodynamic sink, which hinders further polymerization chemistry [44-46].

The yields of homopeptides synthesized from amino acids in the simulated environmental wet-dry cycling experiments all exceed those observed in the room temperature/heating experiments, except for Glu-based homopeptides. In the simulated environmental wet-dry cycling experiments, the Glu-based homopeptide yield (0.01%) was comparable to that observed in the room temperature experiments (0.02%). These results affirm that for most amino acids, dehydration instituted by simulated environmental wet-dry cycling is critical to generating longer

chain lengths, and abundances, of homopeptides. The caveat in this case being Glu, which generally struggles to oligomerize with itself, possibly due to its relatively complex structure. It is also possible that Glu oligomerization yields pyroglutamic acid, a 5-membered gamma-lactam structure [71] by the attack of the alpha-amino group onto the gamma-carboxylic acid that has been activated by DAP. Alternatively, Glu oligomerization could be hindered by the formation of Glu diketopiperazine, the thermodynamic end-product of Glu oligomerization [72-74].

When comparing the results of the simulated environmental wet-dry cycling experiments to those of the DAP/dry-down experiments (Figure 2), two stark contrasts exist: 1) the difference in Asp-based homopeptide synthesis and 2) the difference in Glu-based homopeptide synthesis. The DAP/dry-down experiments demonstrated that $Asp_4$ was not synthesized after 1 dry-down, yet the simulated environmental wet-dry cycling experiments showed that $Asp_4$ was generated, but multiple wet-dry cycles were required to do so (Figure 4C). The need for additional cycling to form larger Asp-based homopeptides, may be due to the structural complexity of Asp hindering oligomerization. Additionally, the DAP/dry-down experiments indicated that $Glu_3$ was formed after 1 dry-down event when 0.1 M Glu was exposed to equimolar quantities of imidazole and DAP (Figure 2). However, the simulated environmental wet-dry cycling experiments indicated that when the starting reagent concentrations were dropped by 1 order of magnitude, $Glu_3$ could not be formed, even after exposure to 4 wet-dry cycles. It is likely this observed lack of oligomerization efficiency was a direct result of the reduction in starting Glu concentration used in the simulated environmental wet-dry cycling experiments, compared to those used in the DAP/dry-down experiments.

The impact that the starting reagent concentration has on the efficacy of a given polymerization chemistry being studied is critical to constraining the prebiotic plausibility of the

484 oligomerization reaction in question. In the case of possible primitive amino acid polymerization
485 reactions, the current prebiotic chemistry literature does not provide experimental evidence
486 demonstrating that relatively large concentrations of amino acid starting reagents were likely to
487 have accumulated under simulated primordial Earth environments. In contrast, the prebiotic
488 chemistry literature suggests that considering the world's oceans travel through the hydrothermal
489 vents every 10 million years [75], amino acid concentrations in the primitive oceans likely did
490 not surpass 300 μM [76]. However, localized environments, such as tidal lagoons or eutectic
491 ponds are thought to have possibly been more impactful for primordial chemical evolution [77-
492 78] because organic species may have accumulated to larger concentrations in these types of
493 environments [79]. Yet, prebiotically plausible concentrations that amino acids could have
494 accumulated to in these localized environments remain poorly understood.
495 One potential way to help improve the understanding of the accumulation of larger
496 concentrations of amino acids in localized environments on the early Earth is to attempt to
497 quantitatively constrain amino acid precursor concentrations in these types of environments.
498 Toner and Catling [80] recently examined possible prebiotic concentration mechanisms for
499 cyanide, which is an integral reagent in the Strecker synthesis of amino acids. In this work,
500 Toner and Catling [80] used an aqueous model based on experimental data to provide a
501 quantitative estimate of cyanide concentrations reached in sodium bicarbonate-rich, closed-basin
502 lakes, which may have served as a prebiotic environment in which cyanides could have
503 accumulated in the presence of evaporation and inflowing water. It was determined that in such a
504 closed-lake basin, cyanide, in the form of ferrocyanide could reach concentrations as high as 700
505 mM when atmospheric $CO_2$ partial pressures were relatively low and atmospheric HCN partial
506 pressures were relatively high, but that cyanide abundances would dip to the micromolar

concentration range if the converse atmospheric conditions were present [80]. While these results are pertinent to prebiotic amino acid concentrations, it remains uncertain to what extent these cyanide concentrations would have necessarily contributed to the synthesis of amino acids in such an environment, and how subsequent peptide syntheses would be affected. This points to an important limitation in the current origin of life literature, which is that the possible primordial concentrations of amino acids in localized environments are poorly understood because these concentration estimates depend on the abundances of precursors and the volumes of solvents in such microenvironments, both of which are also not necessarily well-constrained. Thus, further work is needed to better understand if wet-dry cycle-induced peptide synthesis experiments reported in the literature that use relatively high starting concentrations of organic monomers, constitute a geochemically plausible prebiotic polypeptide synthetic pathway.

The comparative results in this work shed light on how reducing the starting concentration of an amino acid reduces the efficiency of the studied polymerization chemistry. In turn, such a reduction in polymerization efficiency can impact the prebiotic plausibility of the polymerization chemistry in question. Therefore, it can be concluded that future explorations pertaining to possible prebiotic polypeptide synthetic chemistries should be evaluated using lower starting reagent concentrations that are more likely to be prebiotically plausible.

## 4. Conclusions

The research presented here entailed the development of a new analytical technique necessary to evaluate the capability of a plausible prebiotic phosphorylating agent, DAP, to induce the oligomerization of amino acids and simple peptides into homopeptides that could have helped set the stage for the chemistries important for life on the early Earth. The new UPLC-FD/ToF-MS method was optimized to be fast, sensitive, and selective, capable of

detecting and quantitating a suite of amino acids and associated homopeptides. At, or near, baseline resolution was achieved for 16 of the 18 target analytes by employing AccQ·Tag pre-column derivatization to increase analytical specificity for primary amino groups. Target analyte identification was confirmed by accurate mass analysis using a mass tolerance of 10 ppm. This new method provided quantitative advantages over existing methods. When compared to previous techniques that targeted identical short homopeptides to those in this study, the new analytical capability developed here was found to provide LOD values upwards of >1 order of magnitude lower [56-58] and LOQ values upwards of 2 – 3 orders of magnitude lower [59]. Both the FD and ToF-MS responded very linearly ($R^2 > 0.985$) to all analytes over a concentration range of ~3 - 4 orders of magnitude.

A series of laboratory experiments were performed to ascertain the effectiveness of DAP at inducing oligomerization, and the experimental samples were analyzed with the newly developed technique. The results of these laboratory experiments demonstrated that DAP readily facilitated the oligomerization of amino acids and simple peptides under mild thermal conditions, in aqueous solutions. The polymerization chemistry also worked at reduced starting reagent concentrations, but the efficiency of the reaction was decreased. This underscores a potential limitation of the oligomerization reaction at even lower starting reagent abundances, similar to those reported for possible geochemical scenarios [76, 81]. The results of the laboratory experiments performed here were products of single executions of each experiment, followed by replicate measurements of the samples generated by these experiments. Uncertainty estimates associated with these replicate measurements were calculated as the standard errors of the means, and provide quantitative constraints on the accuracy and precision of the method developed here.

552 The primary implications of this work are: 1) DAP is a prebiotically plausible
553 phosphorylating agent that could have helped facilitate the chemical evolution necessary to
554 bridge the gap between simple organic molecules and more complex biomolecules with greater
555 biological functionality at, or near, the time of the origin of life, and 2) the new analytical
556 technique developed here is broadly applicable to a wide variety of disciplines that need an
557 analytical capability to detect and quantitate short peptides. Examples of research fields and
558 topics that fall within this category include pharmaceutical drug discovery [82-83],
559 metabolomics of liver disease [84-85], antioxidant properties of food chemistries [86-89], and
560 various agricultural disciplines [36-37]. Perhaps the most exciting implication of this newly
561 developed method for the origin of life field, is its potential for application to investigate
562 peptides in complex, natural samples, including carbonaceous meteorites. Peptides remain a
563 vastly understudied class of soluble organic compounds in meteorites [90], largely due to
564 analytical limitations that previously precluded effectively targeting these species.
565 The results of this study highlight the need for further exploration into the ability of DAP
566 to facilitate the oligomerization of a mixture of amino acids that would generate heteropeptides.
567 Given the chemical diversity observed in meteorites [91], it is likely that if peptides were formed
568 on meteorite parent bodies, heteropeptides were among those synthesized. The method
569 developed in this current work was optimized for the analysis of homopeptides and therefore the
570 chromatographic gradient of this method may not be able to sufficiently chromatographically
571 resolve a wide array of heteropeptides in its current form. However, the method developed here
572 could be adapted and modified to facilitate the chromatographic resolution and analysis of
573 heteropeptides generated from a mixture of meteoritic amino acids. Additionally, the findings of
574 this exploration underscore the importance of investigating the ability of DAP to enable the

synthesis of homochiral peptides form a suite of chiral amino acids with small L-enantiomeric excesses, which could hold significant implications for understanding the origin of homochirality. Lastly, given the presence of the phosphorus-bearing mineral schreibersite in meteorites [21], and the detection of meteoritic ammonia [92], the necessary precursors for DAP formation likely could have existed on meteorite parent bodies to facilitate the formation of DAP. Therefore, DAP should be searched for in meteorites to further evaluate the plausibility of DAP-mediated amino acid oligomerization in extraterrestrial environments.

**Acknowledgements**

Funding: This work was supported by the Simons Collaboration on the Origin of Life (SCOL) [grant number 302497 issued to Dr. Jason P. Dworkin, and grant number 327124 issued to Prof. Ramanarayanan Krishnamurthy].

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**Figures**

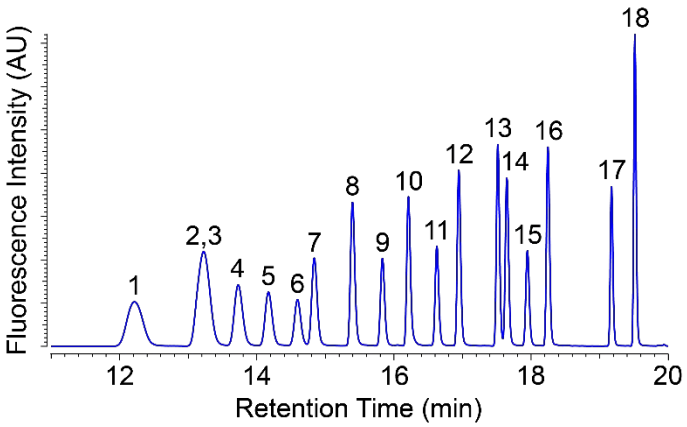


***Figure 1. At, or near, baseline separation was achieved for 16 of the 18 target analytes.*** The 11 – 20-minute region of a fluorescence chromatogram for a combined standard containing the amino acids and homopeptides targeted in this study, demonstrating the efficacy of the chromatographic technique developed. Peak identification: 1) $Gly_2$, 2) $Gly_3$, 3) $Gly_4$, 4) $Gly_5$, 5) $Gly_6$, 6) Gly, 7) $Asp_2$, 8) $Asp_3$, 9) Asp, 10) $Asp_4$, 11) Glu, 12) $Glu_2$, 13) $Glu_3$, 14) $Ala_2$, 15) Ala, 16) $Ala_3$, 17) $Ala_4$, 18) $Ala_5$. Here, AU = arbitrary units and min = minutes. Please see Table S1 for a summary of the detection metrics observed for the method developed here.

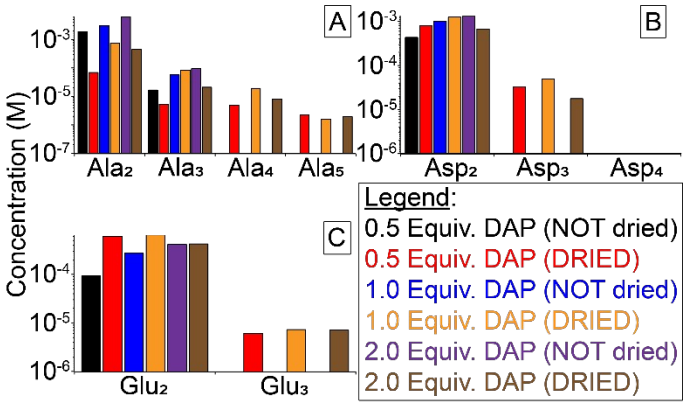


***Figure 2. Results of DAP/dry-down experiments.*** These experiments were performed using Ala (A), Asp (B), and Glu (C) as the amino acids. The findings demonstrate that longer homopeptides formed when mixtures were dried down as opposed to left as a solution.

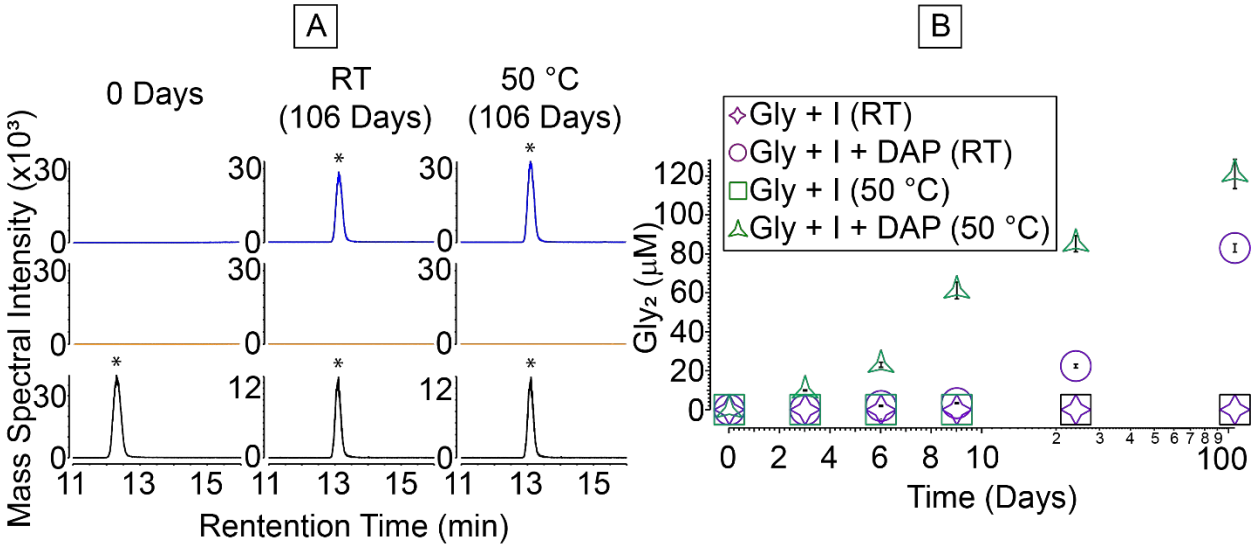


***Figure 3. $Gly_2$ production under room temperature and mild heating conditions.*** A) Accurate mass chromatograms of a $Gly_2$ standard (bottom, black), reaction mixture of a solution of Gly + imidazole (middle, orange), and reaction mixture of a solution of Gly + imidazole + DAP (top, blue). Data are collected after 0 days ($t_i$) and after 106 days ($t_f$), when maximum $Gly_2$ concentrations were reached for both room temperature and mild heating (50 °C) experimental reaction solutions. Accurate mass chromatograms were extracted from *m/z* 303.1093. Asterisks denote peaks that represent $Gly_2$. B) $Gly_2$ is not synthesized in the absence of DAP, and $Gly_2$ synthesis is consistently enhanced when mild heating is applied to the experimental reaction solutions, compared to room temperature conditions. Samples portrayed here were analyzed in triplicate. Uncertainties ¿) were determined as the standard error ($\delta_x = \sigma_x \cdot (n)^{\frac{-1}{2}}$), whereby the uncertainties were based on the standard deviation ($\sigma_x$ ¿of the average value of 3 separate measurements ($n$). Here, I = imidazole, min = minutes, and RT = room temperature.

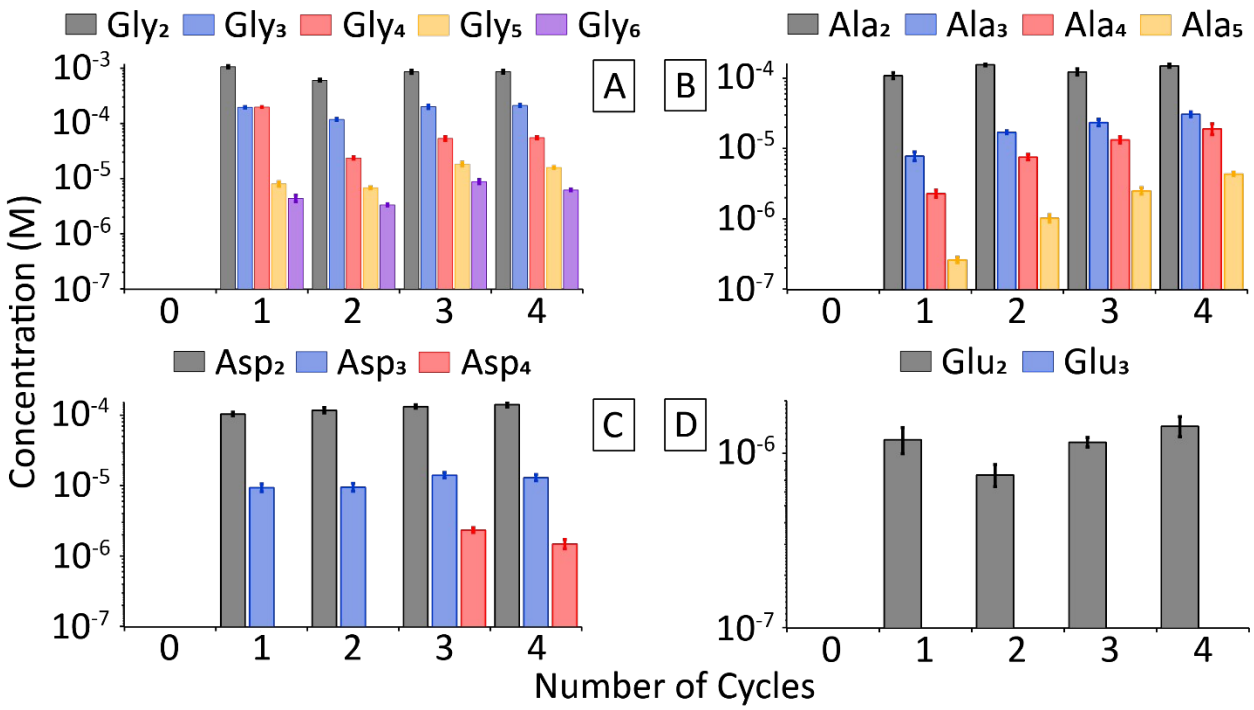


***Figure 4. Amino acid-based homopeptide production as a function of simulated environmental wet-dry cycling.*** $Gly_2$ – $Gly_6$ homopeptides were synthesized after just 1 cycle, and were consistently present with each additional cycle (A). $Ala_2$ – $Ala_5$ homopeptides were synthesized after just 1 cycle and were consistently present with each additional cycle (B). $Asp_2$ and $Asp_3$ homopeptides were synthesized after 1 cycle; however, it was not until a third simulated environmental cycle was performed that $Asp_4$ was synthesized (C). $Glu_2$ was synthesized after 1 cycle; however, $Glu_3$ was not synthesized over the course of the simulated environmental wet-dry cycling experiments (D). Samples portrayed here were analyzed in triplicate. Uncertainties ¿) were determined as the standard error ($\delta_x = \sigma_x \cdot (n)^{\frac{-1}{2}}$), whereby the uncertainties were based on the standard deviation ($\sigma_x$ ¿of the average value of 3 separate measurements ($n$).

## Tables

***Table 1.*** Composition of experimental reaction solutions and experimental conditions used during the execution of the DAP/dry-down experiments.

| Amino Acid (M) | DAP (M) | Imidazole (M) | Temperature | pH | Time (days) | Processing |
|---|---|---|---|---|---|---|
| Ala (0.10)<br>Asp (0.10)<br>Glu (0.10) | 0.05,<br>0.10,<br>0.20 | 0.10 | Room Temperature | 8.5 – 9.3 | 14 | Dried, or Not Dried |

***Table 2.*** Composition of experimental reaction solutions and experimental protocol implemented during the execution of the room temperature/heating experiments.

| Amino Acid (M) | DAP (M) | Imidazole (M) | Temperature | Sample Collection Frequency |
|---|---|---|---|---|
| Gly (0.01)<br>Ala (0.01)<br>Asp (0.01)<br>Glu (0.01) | 0.01 | 0.01 | Room Temperature, or 50 °C | ti = 0 days<br>$t_1$ = 3 days<br>$t_2$ = 6 days<br>$t_3$ = 9 days<br>$t_4$ = 24 days<br>$t_f$ = 106 days |

***Table 3.*** Composition of experimental reaction solutions and experimental protocol implemented during the execution of the simulated environmental wet-dry cycling experiments. In this table, DD = dry-down, hrs = hours, No. = number, and RT = room temperature.

| Species (M) | DAP (M) | Imidazole (M) | No. of Cycles | Cycle Descriptions |
|---|---|---|---|---|
| Gly (0.01)<br>Ala (0.01)<br>Asp (0.01)<br>Glu (0.01)<br>$Gly_2$ (0.01) | 0.01 | 0.01 | 0 - 4 | **0 Cycles:** no DD<br>**1 Cycle:** DD at 50 °C for 24 hrs<br>**2 Cycles:** 1 cycle + rehydrate at RT for 24 hrs + DD at 50 °C for 24 hrs<br>**3 & 4 Cycles:** similar to 2 cycles |

***Table 4. Homopeptide yields from room temperature/heating experiments.*** This table summarizes 1) the maximum homopeptide syntheses that occurred within each solution subjected to room temperature, or mild heating (50 °C) conditions, 2) the respective yields of these homopeptides synthesized based on the $t_i$ concentration (10 mM) of the amino acids being evaluated, and 3) the time point at which the maximum homopeptide syntheses were observed. In this table, I = imidazole and RT = room temperature. Uncertainties ¿) were determined as the standard error ($\delta_x = \sigma_x \cdot (n)^{\frac{-1}{2}}$), whereby the uncertainties were based on the standard deviation ( $\sigma_x$ ¿of the average value of triplicate measurements ($n$).

| Solution (Temperature) | Product | Concentration (μM) | Yield (%) | Time (Days) |
|---|---|---|---|---|
| Gly + I + DAP (RT) | $Gly_2$ | 83.0 ± 2.1 | 0.83 | 106 |
| | $Gly_3$ | + | ◊ | 106 |
| Gly + I + DAP (50 °C) | $Gly_2$ | 120.7 ± 7.5 | 1.21 | 106 |
| | $Gly_3$ | + | ◊ | 106 |
| Ala + I + DAP (RT) | $Ala_2$ | 76.7 ± 3.4 | 0.77 | 106 |
| | $Ala_3$ | + | ◊ | 106 |
| Ala + I + DAP (50 °C) | $Ala_2$ | 39.6 ± 1.2 | 0.40 | 106 |
| | $Ala_3$ | + | ◊ | 106 |
| Asp + I + DAP (RT) | $Asp_2$ | 77.5 ± 2.7 | 0.78 | 24 |
| Asp + I + DAP (50 °C) | $Asp_2$ | 50.2 ± 5.1 | 0.50 | 9 |
| Glu + I + DAP (RT) | $Glu_2$ | 2.1 ± 0.4 | 0.02 | 106 |
| Glu + I + DAP (50 °C) | $Glu_2$ | + | ◊ | 24 |

[+]Analyte was detected in the 10x diluted experimental reaction solutions that were analyzed, but concentrations did not exceed the LOQ.

[◊]Yields are not reported because the analyte concentrations in the analyzed 10x diluted experimental reaction solutions, were below the LOQ.

***Table 5. Homopeptide yields from simulated environmental cycling experiments.*** This table summarizes 1) the maximum homopeptide syntheses that occurred within each solution exposed to simulated environmental cycling experiments, 2) the respective yields of these homopeptides synthesized based on the $t_i$ concentration (10 mM) of the amino-based compound being evaluated, and 3) the cycle number at which the maximum homopeptide syntheses were observed. In this table, I = imidazole, RT = room temperature, N.D. = not detected, and N/A = not applicable. Uncertainties ¿) were determined as the standard error ($\delta_x = \sigma_x \cdot (n)^{\frac{-1}{2}}$), whereby the uncertainties were based on the standard deviation ($\sigma_x$ ¿of the average value of triplicate measurements ($n$).

| Solution | Product | Concentration (μM) | Yield (%) | Cycle No. |
|---|---|---|---|---|
| Gly + I + DAP | $Gly_2$ | 1,065.1 ± 48.3 | 10.7 | 1 |
| | $Gly_3$ | 212.0 ± 10.1 | 2.12 | 4 |
| | $Gly_4$ | 197.6 ± 3.5 | 1.98 | 1 |
| | $Gly_5$ | 18.3 ± 1.4 | 0.18 | 3 |
| | $Gly_6$ | 8.8 ± 0.7 | 0.09 | 3 |
| Ala + I + DAP | $Ala_2$ | 155.8 ± 6.6 | 1.56 | 2 |
| | $Ala_3$ | 30.7 ± 2.5 | 0.31 | 4 |
| | $Ala_4$ | 19.1 ± 3.4 | 0.19 | 4 |
| | $Ala_5$ | 4.3 ± 0.3 | 0.04 | 4 |
| Asp + I + DAP | $Asp_2$ | 142.0 ± 11.1 | 1.42 | 4 |
| | $Asp_3$ | 14.1 ± 1.2 | 0.14 | 3 |
| | $Asp_4$ | 2.3 ± 0.2 | 0.02 | 3 |
| Glu + I + DAP | $Glu_2$ | 1.4 ± 0.2 | 0.01 | 4 |
| | $Glu_3$ | <0.02 | N/A | N/A |
| $Gly_2$ + I + DAP | $Gly_4$ | 1,496.0 ± 379.4 | 15.0 | 4 |
| | $Gly_6$ | 362.5 ± 38.8 | 3.63 | 4 |