



# Local pH variability scenario: Prebiotic synthesis of cyclic dipeptides via prolinamide on the Hadean Earth

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## ABSTRACT

Small pools near early volcanoes are recognized as optimal field for the origin of life. However, the significance of pH variability in the polymerization of biomolecules has been undervalued. Within a simulated “warm little pond,” our study illustrates that a pH variability scenario provides an effective and innovative route for the generation of cyclic dipeptides from prolinamide and free amino acids, obviating the necessity for a catalyst. Subsequent investigations reveal the influential roles of activator (trimetaphosphate, abbreviated as P<sub>3</sub>m), clay minerals such as montmorillonite, calcite, magnetite, and metal ions including Ca<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, which might have been prevalent in the early Earth's environmental conditions, in facilitating this prebiotic synthetic process. In conclusion, this research provides valuable insights into the possible mechanisms of prebiotic cyclic peptide synthesis, which is a crucial step in understanding the origin of life. The proposed pH variability model offers a plausible explanation for how these peptides could have formed under early Earth conditions.

## 1. Introduction

The inquiry into the origin of life on Earth has long been entwined with debates surrounding the location and mechanisms of chemical evolution, as well as the environmental conditions prevailing during this transformative phase. The central question of whether life underwent chemical evolution underscores the importance of the emergence site meeting two fundamental requirements: the presence of liquid solvents, such as water, and a supply of energy to facilitate self-assembly. Therefore, hydrothermal environments emerge as potential cradles for the early evolution of life, with terrestrial hydrothermal fields representing a noteworthy example (Omran and Pasek, 2020). Dating back to the 19th century, Darwin introduced the concept of a “warm little pond” as a conceivable birthplace for life (Peretó et al., 2009). Subsequent discoveries of hydrothermal fields have bolstered support for the “primitive soup hypothesis” (Joyce, 2002). It is proposed that these hydrothermal fields, akin to the small pools near early volcanoes, witnessed the generation of numerous amino acids and inorganic salts. The intermittent drying caused by direct sunlight or volcanic eruptions led to thermal polymerization and dehydration of small molecular substances, resulting in the formation of polymers, including peptides. This narrative suggests that oxygen-deprived, arid continental environments

provided an ideal setting for dehydration (Barge et al., 2019). Throughout Earth's history, hydrothermal fields have cyclically varied between wet and dry conditions (Ross and Deamer, 2019), representing an efficient means of converting biomolecular monomers into polymers on a lifeless Earth (McKee et al., 2018).

The chemical evolution of life is intrinsically connected to the early evolution of non-living components. Clay minerals, metal ions, and environmental pH levels, especially during the Hadean and Archean eras, played pivotal roles in this process. Clay mineral surfaces, including montmorillonite (Cueto-Díaz et al., 2023), calcite (Hazen et al., 2001) and magnetite (Ozturk et al., 2023), have demonstrated the ability to adsorb, concentrate, and catalyze reactions of biomolecules. Notably, previous studies have confirmed the successful adsorption of amino acids on these surfaces in the form of anions and zwitterions. Investigations by Sakata et al. delved into the effects of metal ions (Ca<sup>2+</sup>, Mg<sup>2+</sup>, Zn<sup>2+</sup>, Fe<sup>2+</sup>, Mn<sup>2+</sup>, and Cu<sup>2+</sup>) and pH on the formation of linear and cyclic dipeptides, also known as diketopiperazines (DKPs), in aqueous solutions (Sakata et al., 2014). The early hydrosphere was characterized by acidity, a consequence of a higher concentration of acidic gases in the early atmosphere. However, the strong negative feedback between marine pH and authigenic clay formation likely stabilized Earth's climate (Isson and Planavsky, 2018), implying that early

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life existed within an environment with dynamically changing pH.

The synthesis of prebiotic peptides represents a key scientific challenge in the chemical evolution process. Under simulated prebiotic conditions, classical amino acids can form polypeptides through dehydration, but the efficiency of the reaction in water is low and heavily reliant on additives such as catalysts and activators (Muchowska and Moran, 2020). Proline, for instance, can generate cyclic dipeptides under the mediation of the activator trimetaphosphate ( $P_3m$ ). Cyclic dipeptides, crucial as precursors of prebiotic peptides, exhibit various chiral catalytic activities (Borthwick, 2012; Imai et al., 1999; Otsuka et al., 2019), and play an essential role in the origin of life. Additionally, non-classical amino acids, such as amino acid amides, detected during the synthesis of classical amino acids (The Strecker synthesis and cyanohydrin condensation processes generate nitriles that are then subjected to hydrolysis, resulting in the formation of amides.), are believed to have existed in primitive oceans and participated in the synthesis of linear dipeptides under primitive Earth conditions (Mullen and Sutherland, 2007; Nishizawa et al., 1983; Oro et al., 1959).

In this study, we simulated the wet-dry cycle of hydrothermal fields on dry land, an environment deemed ideal for the origin of life. Emphasizing often-overlooked pH variability in this setting, we explored the reactions between prolinamide, an amino acid amide, and 20 amino acids. Our findings reveal that prolinamide can independently generate proline cyclodipeptide or form heterocyclic dipeptides with 20 amino acids, all without the need for any catalyst during wet-dry cycling and pH variability. The inclusion of activator  $P_3m$  further enhances cyclic dipeptide production. Beyond this, we investigate other abiotic factors in the early Earth's environment, such as clay minerals (montmorillonite, calcite, magnetite) and metal ions ( $Ca^{2+}$ ,  $Mg^{2+}$ ,  $Mn^{2+}$ ), examining their influence on cyclic dipeptide production.

## 2. Materials and methods

### 2.1. Materials

The research group successfully synthesized the standard product of cycloPro-Pro. Prolinamide (Pro- $NH_2$ ) was purchased from Saen Chemical Technology Co., Ltd. (Shanghai, China). L-Methionine (L-Met), L-Histidine (L-His), L-Valine (L-Val), L-Tryptophan (L-Trp), L-Threonine (L-Thr), L-Proline (L-Pro), L-Arginine (L-Arg), L-Tyrosine (L-Tyr), L-Isoleucine (L-Ile), L-Asparagine (L-Asn), L-Glutamic acid (L-Glu), L-Lysine (L-Lys), L-Cysteine (L-Cys), L-Glutamine (L-Gln), L-Leucine (L-Leu), L-Serine (L-Ser), L-Aspartic acid (L-Asp), L-Alanine (L-Ala), L-Phenylalanine (L-Phe), L-Glycine (L-Gly),  $CaCl_2$  and  $MnCl_2$  were obtained from Shanghai Macklin Biochemical Co., Ltd. (China). Trimetaphosphate was purchased from Sigma Aldrich. (China).  $MgCl_2$  was purchased from Meilunbio. (China). Ultrapure water (18.2 M $\Omega$  cm) from a Milli-Q water purification system (Millipore, Bedford, MA) was used to prepare solutions and the mobile phase. Reagents were obtained and verified by MS spectra (Supplementary Figs. S6–23).

### 2.2. General procedure for synthesizing cyclic dipeptides

Proline cyclodipeptide (Cyclo-Pro-Pro): A 0.1 M solution of Pro- $NH_2$  was mixed with or without a 0.1 M solution of sodium  $P_3m$  in a 1 mL neutral (pH 7) aqueous solution. The reactants were exposed to an open environment at a temperature of 80 °C for one day. Subsequently, 1 mL of water was added, and the pH was adjusted to 11 using a 2 M NaOH solution, followed by incubation at a temperature of 80 °C for another day.

Heterocyclic dipeptides (Cyclo-Pro-AAs): A solution containing 0.05 M Pro- $NH_2$  was combined with a solution containing 0.3 M AAs in a neutral aqueous medium (pH 7, 1 mL). The reaction mixture was exposed to an open environment at a temperature of 80 °C for one day. Subsequently, additional 1 mL of water was added, and the pH was adjusted to 11 using a concentrated NaOH solution (2 M), followed by

incubation at a temperature of 80 °C for another day.

The general process of adding metal ions or clay minerals: In the general procedure of cycloPro-Pro synthesis, a solution containing metal ions at a concentration of 0.05 M or clay minerals at a concentration of 10 mg/mL was introduced. Subsequently, the minerals were eluted with 0.5 M HCl upon completion of the reaction.

Note: The “standard sample” refers to the standard substance, while the “actual sample” refers to the experimental sample.

### 2.3. HPLC-HRMS analysis method

Mass spectrometry (MS) was performed in positive mode on the Q-Exactive Plus system. HRMS instrument parameters were set as follows: The capillary voltage was set at 3800 V, dry gas at 3 L $\cdot$ min $^{-1}$  and dry temperature at 320 °C. Mass spectra were recorded in the scan range from  $m/z$  = 50 to 750. For ESI-HRMS, approximately 1/3 of the LC eluent was introduced through a splitting T valve. To enable online detection of reaction products in HPLC-HRMS, a divert valve on the MS instrument was configured to switch between HPLC flows. HPLC was performed using UltiMate 3000 RSLCnano system equipped with an Agilent TC-C18, 5  $\mu$ m, 4.6 mm  $\times$  150 mm column. The column temperature was kept at room temperature. A binary mobile phase (solvent A: water with 0.1% formic acid; solvent B: acetonitrile) was used with a flow rate of 0.8 mL $\cdot$ min $^{-1}$ . The linear gradient elution program was as follows: 0 ~ 4 min, 5% B; 4 ~ 19 min, 5%–70% B; 19 ~ 21 min, 70% B; 21 ~ 22 min, 70 ~ 5% B; 22 ~ 30 min, 5% B. The detection wavelength was 210 nm and the sample size was 5  $\mu$ L. For HPLC-HRMS online detection of reaction products, the diverter T valve was used to cleverly switch the HPLC eluent to achieve online desalting and detection. The specific setting was: from 0 to 2 min, the diverter valve switched the HPLC eluent to the waste liquid; After 2 min, the diverter valve switches the HPLC eluent to HRMS.

In this paper, the quantitative/semi-quantitative analysis of cyclic peptide yield is based on the peak area of ion flow or UV peak area of HPLC-HRMS. The maximum peak area of the product was considered as 100%, and the percentage of the peak area of the product of other products represented the relative concentration of cyclic dipeptide.

### 2.4. FTIR analysis method

The IR data was acquired using a Thermo Nicolet 4700 FTIR spectrometer. Prior to analysis, the samples were placed on Durapore® hydrophobic PVDF membranes with a pore size of 0.22  $\mu$ m and allowed to be examined using Total Reflectance (ATR) sample chamber. Spectra were background-subtracted within the range of 500 to 2500  $cm^{-1}$  and signal-averaged by performing 16 scans per spectrum. The sample was prepared by pressing tablet method: The sample was frozen at a temperature of 80 °C for thirty minutes followed by freeze-drying to obtain solid powder using a freeze-dryer. Subsequently, KBr and the solid sample were meticulously mixed and ground into fine powder of micrometer grade at a ratio of 100:1. The resulting mixture was ultimately compressed into a transparent sheet to facilitate subsequent detection and analysis.

## 3. Results

### 3.1. Peptide synthesis on early Earth

The present study focuses on the synthesis of unique cyclic dipeptides within a confined geological environment. The previous experiments have demonstrated that linear proline dipeptide readily undergoes cyclization to form cyclic dipeptide in an alkaline aqueous solution, but they encounter challenges in neutral or acidic conditions (Ying et al., 2018). Based on the aforementioned research, we conducted a preliminary investigation into the potential impact of pH on the formation of cyclic dipeptides in the novel system.

The selection of Pro-NH<sub>2</sub> as the starting material was followed by its dissolution in an aqueous medium. Subsequent to this, the pH was meticulously calibrated to three distinct values: 3, 7, and 11. The experimental setup included two cycles of alternating dry and wet conditions, conducted at a temperature of 80 °C over a period of two days. Throughout the duration of the experiment reaction, no further adjustments were made to the pH. All experimental data presented in this paper were obtained through triplicate experiments, and samples were analyzed using high-performance liquid chromatography coupled with high resolution mass spectrometry (HPLC-HRMS). Notably, a retention time of 10.34 min was observed in the extracted ion chromatogram (EIC) at  $m/z$  195.1120, corresponding to the cycloPro-Pro molecular ion  $[M + H]^+$  (Fig. 1a, b; Supplementary Fig. S1). Comparing the production of cycloPro-Pro at different pH levels by the peak area of ion flow revealed the highest production at pH 7 (Fig. 1c), deviating from previous findings (Ying et al., 2018). The formation of cycloPro-Pro from Pro-NH<sub>2</sub> involves two sequential reactions, namely condensation and cyclization. Specifically, under these conditions, Pro-NH<sub>2</sub> undergoes polymerization to form linear-Pro-Pro (condensation) before cyclizing into cycloPro-Pro (cyclization). As anticipated, the by-product linear-Pro-Pro was also detected in the spectrum (Supplementary Fig. S2). Is this due to a difference in pH requirements between the two reaction steps?

To investigate, we conducted a similar reaction using Ala-NH<sub>2</sub> to validate the optimal pH for the condensation reaction. As the dominant dimeric product produced by this reaction is the linear dipeptide, HPLC-HRMS was employed to analyze the experimental samples, revealing a retention time of 3.03 min with  $m/z$  161.0916, corresponding to the molecular ion  $[M + H]^+$  of linear-Ala-Ala (Fig. 2a, b). By comparing the peak area of ion flow at different pH levels, optimal conditions for the reaction were observed at pH 7 (Fig. 2c). Furthermore, the yield of linear-Ala-Ala was determined to be 1.4% under conditions with a pH of 7, utilizing the standard curve (Supplementary Fig. S3). We hypothesize that variability environmental conditions, especially in terms of pH, during prebiotic periods were more favorable for generating cycloPro-Pro through single-component reactions using Pro-NH<sub>2</sub> as building block. To test this hypothesis, we designed the following experiment.

### 3.2. Cyclic peptide synthesis in pH variability environments

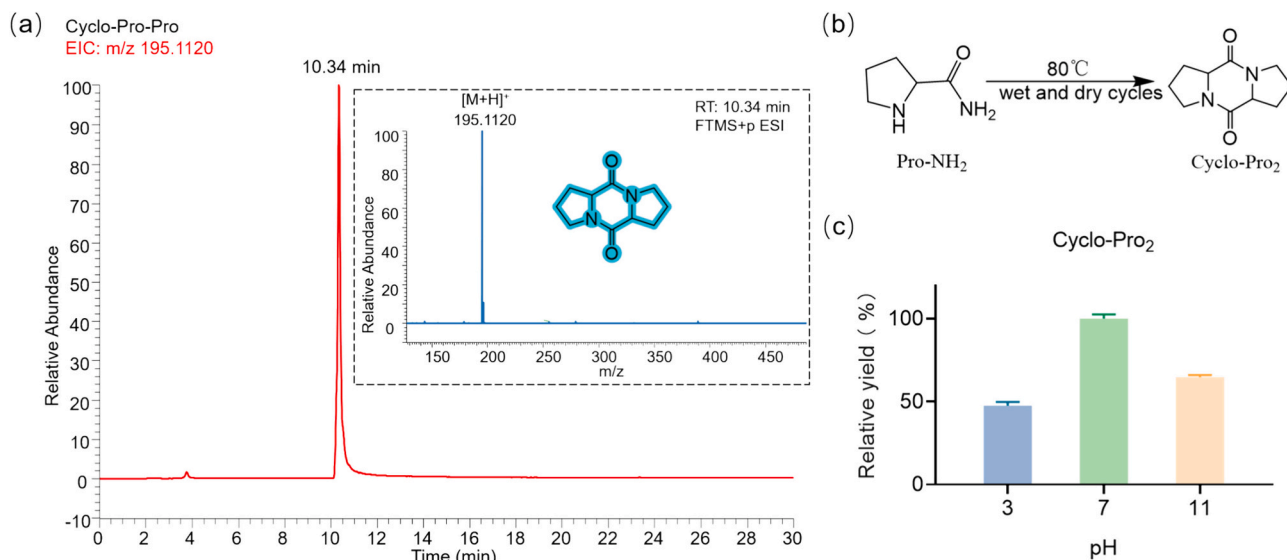
Expanding upon the preliminary experiments, we devised a study to

explore pH variability. The synthesis of cycloPro-Pro from Pro-NH<sub>2</sub> was initiated at pH 7. The reactants were exposed to an open environment at a temperature of 80 °C for a duration of 24 h. Subsequently, 1 mL of water was introduced, and the pH was adjusted to 11 using a solution of 2 M NaOH solution. The mixture was then placed in a temperature-controlled block set at a temperature of 80 °C for an additional day. Analysis using HPLC-HRMS revealed that the optimal yield was achieved during pH variability between 7 and 11 (Fig. 3a). The experimental results are in accordance with our expectations, confirming that the reaction of Pro-NH<sub>2</sub> to generate cycloPro-Pro indeed proceeds through a two-step mechanism.

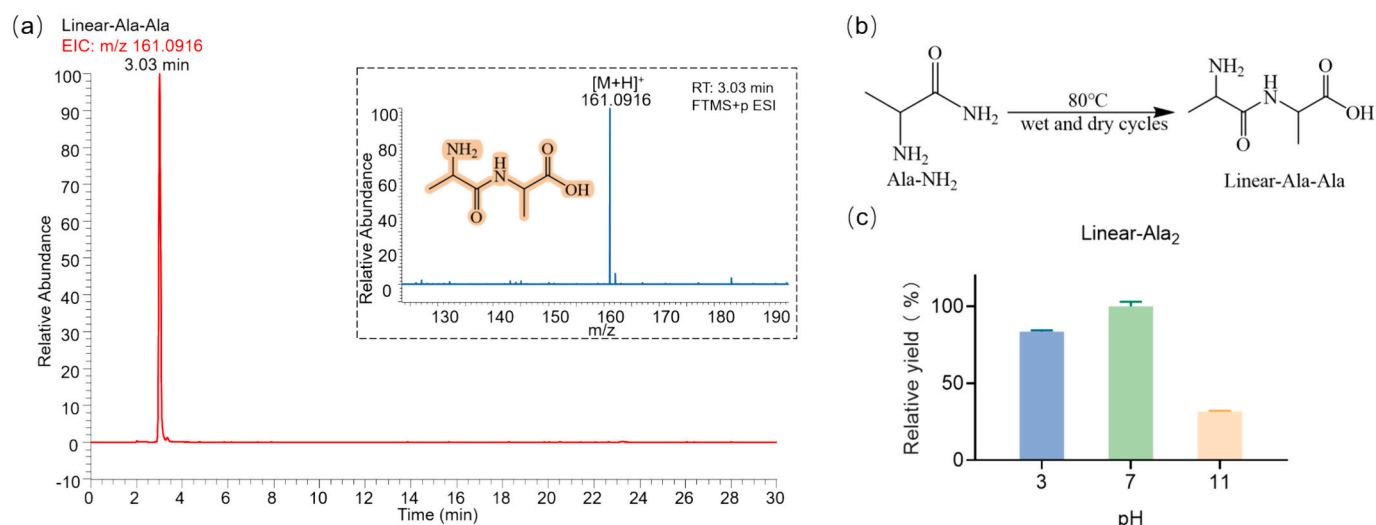
Moreover, we systematically organized and summarized the fragmented products generated by the cycloPro-Pro ions in MS<sup>2</sup>. As depicted in Fig. 3b, ionic fragment 1 corresponds to 3,6-dimethylpiperazine-2,5-dione, representing the characteristic peak of  $[M-C_4H_4 + H]^+$ . Additionally, a distinct peak arises from the loss of C<sub>5</sub>H<sub>4</sub>O resulting in 2,5-dimethylimidazolidin-4-one (ionic fragment 2). Analogously, N-ethyl propionamide (ionic fragment 3), 3, 4-dimethylazet-2 (1H) -one (ionic fragment 4), 2-methylcyclobut-2-en-1-one (ionic fragment 5), 3-methylaziridin-2-one (ionic fragment 6), and finally, acetamide (ionic fragment 7) are formed successively. Fragments 1, 2, 4, 5 and 6 exhibit cyclic structures that require further investigation to understand the phenomenon of maternal ion remaining structures undergoing cyclization upon fragmentation.

Fourier transform infrared spectroscopy (FTIR) played a pivotal role in scrutinizing the samples both pre- and post-reaction, uncovering noteworthy modifications in the amide region (Fig. 3c). These alterations furnish compelling evidence of peptide formation. Over the course of the experiment, Pro-NH<sub>2</sub> encountered fluctuations in environmental conditions, including dryness, moisture, and pH variability. Consequently, the substance underwent structural and compositional changes, leading to discernible modifications in the infrared spectrum. The amide I band (1650–1750 cm<sup>-1</sup>) predominantly reflects the stretching vibrations of the C=O functional group within the amide bond (Kaczmarczyk, 1998). Additionally, an additional characteristic peak associated with acyl group vibration (Amide II) was observed at 1250–1350 cm<sup>-1</sup> (Guillén et al., 2008). Essentially, characteristic peaks of amide bonds undergo alterations during the reaction process.

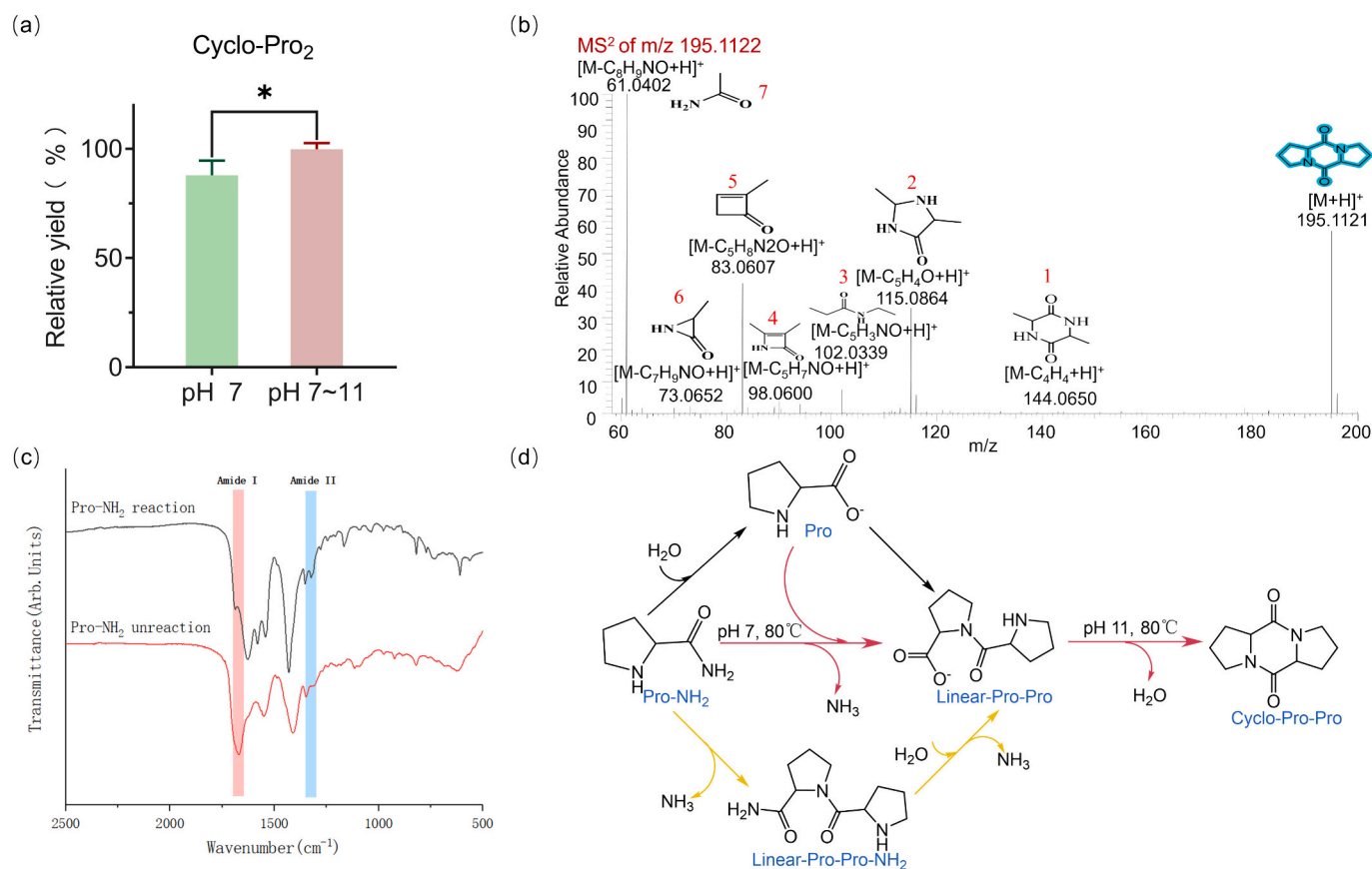
A plausible reaction scheme is illustrated in Fig. 3d. The initial step of the condensation reaction is more favorable under pH 7 conditions. In the subsequent cyclization reaction, alkaline aqueous solutions facilitate



**Fig. 1.** Reaction pathway and HPLC-HRMS analysis of the cycloPro-Pro product. The calculated average mass value of cycloPro-Pro  $[M + H]^+$  is 195.1120. (a) The EIC-MS profile illustrates the product cycloPro-Pro obtained from Pro-NH<sub>2</sub>. (b) The reaction pathway of cycloPro-Pro is depicted. (c) The bar graph illustrates the percentage of relative yield EIC-MS peak area for cycloPro-Pro under different pH conditions.



**Fig. 2.** Reaction pathway and HPLC-HRMS analysis of the linear-Ala-Ala product. The calculated average mass value for  $[M + H]^+$  of linear-Ala-Ala is 161.0916. (a) EIC-MS profile illustrates the obtained product, linear-Ala-Ala, derived from Ala-NH<sub>2</sub>. (b) The reaction pathway elucidates the formation mechanism of linear-Ala-Ala. (c) The bar graph illustrates the relative yield of linear-Ala-Ala under varying pH conditions.



**Fig. 3.** Reaction pathway and HPLC-HRMS analysis of cycloPro-Pro product. (a) The bar graph presents the relative yield of cycloPro-Pro at different pH conditions, expressed as a percentage of UV peak areas relative to overall yield. The “\*” denotes that a  $p$ -value of less than 0.05, as determined by the  $t$ -test, signifies a statistically significant difference. (b) The MS/MS( $MS^2$ ) profile of  $m/z$  195.1122 confirms the successful formation of Cyclo-Pro-Pro. (c) The samples were subjected to FTIR verification. The graphical representation features a red curve, which corresponds to the absorbance measurements of the Pro-NH<sub>2</sub> starting material. In contrast, the black curve delineates the absorbance of the sample post the completion of the Pro-NH<sub>2</sub> reaction. Fourier transform infrared spectroscopy (FTIR) analysis of mixtures before and after the reaction revealed the emergence of characteristic peaks corresponding to Amide I (N—H stretching vibration) and Amide II (C=O stretching vibration), indicating the formation of amide bonds during the Pro-NH<sub>2</sub> reaction and providing support for peptide synthesis. (d) The reaction scheme illustrates the formation from Pro-NH<sub>2</sub>, with emphasis on the red arrow indicating the main pathway. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



the formation of cyclic compounds from linear-Pro-Pro (Fig. 3a). The percentage of cycloPro-Pro generated at pH 7–11 was calculated to be approximately 10.8% (Supplementary Fig. S4). Therefore, the reaction demonstrates enhanced efficiency under pH variability, which are considered novel geochemical scenarios for investigating the origin of life (Guo et al., 2021a).

### 3.3. Synthesis of heterocyclic dipeptides under simulated early Earth conditions

Previous studies have established that Pro can engage with amino acids (AAs) to produce cyclic dipeptides containing Pro (Guo et al., 2021b; Guo et al., 2022; Ying et al., 2018). To investigate whether prolinamide (Pro-NH<sub>2</sub>) could generate corresponding cyclic dipeptides with other AAs in our reaction system without the addition of other activators, we initially fine-tuned the dosing ratio for the reaction (Fig. 4). Notably, when the dosing ratio of Pro-NH<sub>2</sub> and Ala was set at 1:6, a higher yield was observed, and further increasing the amount of Ala did not significantly enhance the yield increase. Utilizing the standard curve, the concentration of Cyclo-Pro-Ala was determined to be 0.33 mM, which corresponds to a yield of 0.7% (Supplementary Fig. S5). It is well-established that DKPs containing proline are often components of various natural products (Naman et al., 2017; Nsengiyumva et al., 2015). The question arises: Can proline-containing cyclic dipeptides be formed under potentially prebiotic conditions? To address this query, we investigated the reactions of Pro-NH<sub>2</sub> with other AAs based on the optimal reaction conditions established for cycloPro-Ala.

The experiments comprehensively covered all 20 naturally occurring amino acids (Met, His, Val, Trp, Thr, Pro, Arg, Tyr, Ile, Asn, Glu, Lys, Cys, Gln, Leu, Ser, Asp, Ala, Phe, Gly) in an aqueous solution under pH variability conditions ranging from pH 7 to 11 at a temperature of 80 °C for a duration of 2 days (Fig. 5a). The products containing proline-derived cyclic dipeptides were subjected to analysis using HPLC-HRMS. Gratifyingly, cycloPro-AA products of 20 amino acids were successfully identified (Supplementary Table 1). The HPLC-HRMS spectra related to the experiment can be found in the supplementary information (Supplementary Figs. S6–23). Remarkably, cycloPro-Ser emerged as the predominant product in the reaction of Pro-NH<sub>2</sub> and Ser, suggesting that the hydroxyl group of Ser exerted an accelerating effect on the reaction (Fig. 5b). In contrast, Thr exhibited significantly lower reaction efficiency, indicating the high sensitivity of Pro-NH<sub>2</sub> toward subtle structural alterations. Similar observations were also made

in other analog pairs, such as Leu and Ile, Gly and Ala. The emergence of cycloPro-AAs and the distinct recognition between Pro-NH<sub>2</sub> and AAs carry profound implications for the understanding of the evolution of life.

### 3.4. Catalytic factor in small water pools near volcanoes

Moreover, to augment the yield of cyclic dipeptides, we considered incorporating trimetaphosphate (P<sub>3</sub>m), a prebiotic source known to act as a potential activator. P<sub>3</sub>m, discovered in volcanic products (Yamagata et al., 1991), has been reported as an effective water-soluble reagent for promoting the condensation of small biological molecules, such as amino acids and nucleotides (Gan et al., 2022). The conjecture was validated by the inclusion of P<sub>3</sub>m in the reaction system (Fig. 6). Consistent with previous investigations (Dolan et al., 2015; Rabinowitz, 1970; Sibilska et al., 2018; Sibilska et al., 2017), our findings demonstrated that P<sub>3</sub>m effectively enhanced the synthesis of cyclic dipeptides. Specifically, the addition of P<sub>3</sub>m catalyst resulted in a threefold increase in cycloPro-Pro yield compared to its absence (Fig. 6b). The proposed mechanistic hypothesis elucidating the role of P<sub>3</sub>m in facilitating the synthesis of cyclic dipeptide peptides is delineated within the Supplementary Fig. S24.

In the context of the chemical evolution of early Earth, the non-living processes played a crucial role. To gain a deeper understanding of this process, it is imperative to explore the clay minerals that potentially existed on Earth's surface during the Hadean and Archean periods, encompassing montmorillonite, calcite, and magnetite. Moreover, metal ions such as Ca<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, etc., are also significant factors that cannot be overlooked. As depicted in Table 1, within the reaction system of Pro-NH<sub>2</sub> and P<sub>3</sub>m, minerals and metals exhibit potential for promoting polymerization of prebiotic molecules. Specifically, calcite demonstrated a remarkable enhancing effect on chemical polymerization of cycloPro-Pro. The discovery reveals a fundamental link in the origin of early life on Earth, particularly the process of polymerization of prebiotic molecules. However, we must not disregard the influence exerted by the environment in this process. When investigating the polymerization of prebiotic biogenic molecules, it is crucial to consider environmental conditions to accurately reconstruct and understand the processes that occurred on early Earth. The exploration of life's origins on early Earth necessitates comprehensive investigation into minerals, metal ions, and even environmental conditions themselves, all of which constitute integral components in unraveling this intriguing puzzle.

## 4. Discussion

The formation of cyclic dipeptides induced by Pro-NH<sub>2</sub> represents a prebiotic pathway for peptide chemical synthesis, occurring in the early Earth's environment without the need for any catalyst. This perspective is grounded in the presence of warm pools near volcanoes during the Hadean and Archean periods, providing plausible environments for the origin of life (Deamer et al., 2019). These small pools exhibit an unparalleled energy output (Barge et al., 2019), resembling cradles of life that foster endless possibilities. Therefore, it is postulated that the alternation between dry and wet cycles constitutes one of the most efficient mechanisms for generating monomer polymers of essential molecules on a lifeless Earth (Becker et al., 2019; Rajamani et al., 2008). Furthermore, these warm pools not only provide liquid solvents but also serve as energy sources owing to their distinctive geographical conditions. Concurrently, the frequent geological activities in proximity to volcanoes supply abundant building blocks for various chemical reactions (Damer and Deamer, 2020; Washington, 2000).

In this process, pH variability triggered by volatile acid-base substances within these small pools plays a crucial role in determining experimental outcomes (Guo et al., 2021a). Our study reveals that pH exerts a significant influence on the polymerization of cyclic dipeptides. Specifically, neutral conditions promote the polymerization of linear

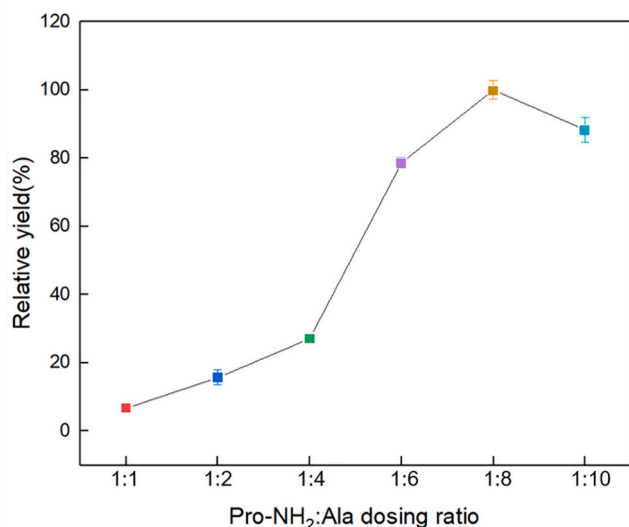
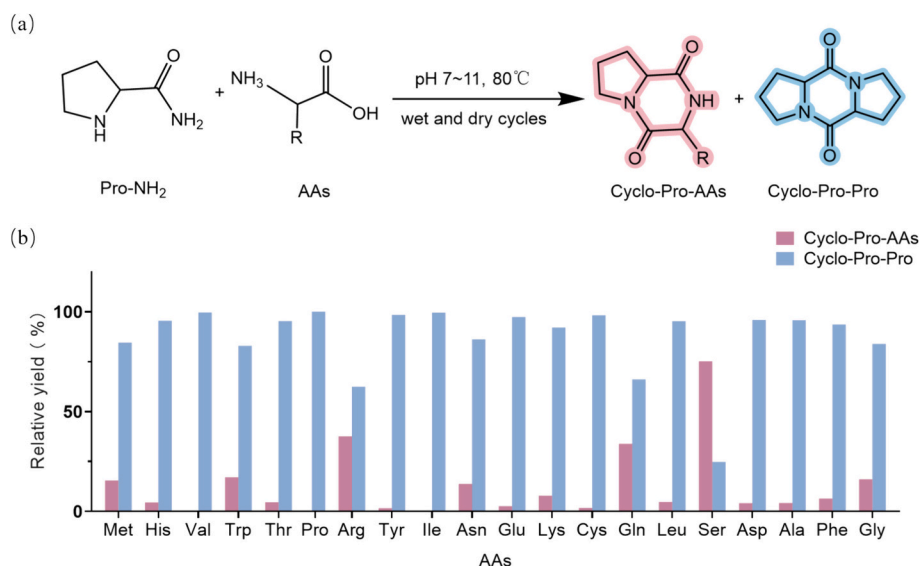
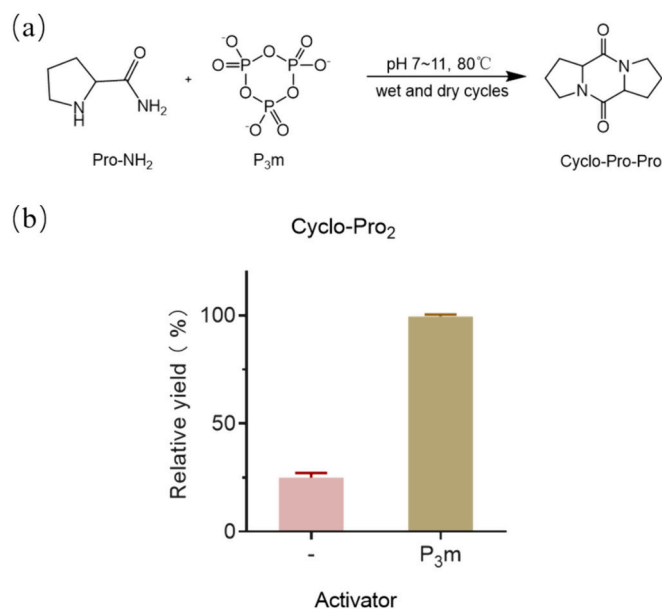


Fig. 4. The line chart depicts the relative yield variation at various dosing ratios of Pro-NH<sub>2</sub> to Ala.



**Fig. 5.** The reaction between Pro-NH<sub>2</sub> and amino acids (AAs). (a) Reaction pathway of Pro-NH<sub>2</sub> and AAs. (b) The bar graph illustrates the relative yields of Cyclo-pro-AAs compared to cycloPro-Pro.



**Fig. 6.** Comparison of cycloPro-Pro product generated in the presence or absence of P<sub>3</sub>m. (a) Reaction scheme depicts the formation of cycloPro-Pro from Pro-NH<sub>2</sub> and P<sub>3</sub>m. (b) The bar graph presents the relative yield (UV peak areas) of cycloPro-Pro in the presence or absence of P<sub>3</sub>m.

**Table 1**

Impact of metal ions and clay minerals on the production of cycloPro-Pro.

Number	Ions	Clay minerals	Cyclo-Pro <sub>2</sub>
1	–	–	1*
2	Ca <sup>2+</sup>	–	1.66
3	Mg <sup>2+</sup>	–	1.48
4	Mn <sup>2+</sup>	–	2.09
5	–	Calcite	2.23
6	–	Montmorillonite	1.76
7	–	Magnetite	2.05

1\*: The peak area of the extracted ion flow in the control group measured 1.

dipeptides, as such an environment facilitates molecular interactions and accelerates the dipeptide polymerization processes. Conversely, under alkaline conditions, we observed a more pronounced tendency toward dipeptide cyclization, attributed to the alkaline environment favoring the formation of cyclic dipeptides (Fig. 3). Hence, the selection of appropriate acid-base conditions becomes highly significant when optimizing cyclic dipeptide cyclization processes (Guo et al., 2019). Understanding these effects enables us to better regulate reaction conditions and provides valuable insights into comprehending the chemical evolution process underlying origin of life (Martín et al., 2009).

During the prebiotic Earth, it is hypothesized that warm pools harbored AA-NH<sub>2</sub> molecules, which served as the fundamental building blocks of primitive life chemistry (Parker et al., 2014). Certain research literature proposes that mechanochemical activation can convert ferrocyanide complexes into aminonitrile through chemical reaction pathways associated with the early Earth environment. This process consequently leads to the formation of AA-NH<sub>2</sub> acid molecules, enhancing energy storage efficiency and partially overcoming thermodynamic limitations in peptide bond formation (Bolm et al., 2018). Our study further illustrates that in the presence of Pro-NH<sub>2</sub> alongside 20 amino acids, 20 cyclic dipeptides can be synthesized abiotically, significantly enhancing the diversity of the peptide library. Moreover, Iyer et al. have affirmed that cyclic dipeptides exhibit asymmetric catalytic effects on Strecker amino acid synthesis (Iyer et al., 1996). Given their varied chiral catalytic activities, cyclic peptides may act as precursors for prebiotic peptides during the chemical evolution catalyzed by biological enzyme processes, gradually contributing to the establishment of our current homochirality (L-amino acids and D-nucleosides).

Furthermore, our study has demonstrated the effective promotion of dipeptide production by P<sub>3</sub>m (Fig. 6), providing further evidence for the crucial regulatory role of phosphorus in prebiotic chemical evolution (Handschuh and Orgel, 1973; Lohrmann and Orgel, 1971; Westheimer and F., 1987). Additionally, we observed significant promoting effects on cyclic dipeptide formation from clay minerals and metal ions (Table 1), indicating a close relationship between chemical evolution of early Earth and abiotic environmental conditions (Franchi and Gallori, 2005; Muchowska et al., 2020; Rasmussen et al., 2021). Given that clay minerals and metal ions were present during Earth's early formation, they may have played an important catalytic role in the emergence of life on Earth. Therefore, future investigations into the chemical evolution of early Earth should consider the evolutionary processes of non-

living components to comprehensively understand the origin and development of life.

## 5. Conclusion

In conclusion, this study significantly enhances our understanding of the formation of cyclic dipeptides on prebiotic Earth. Notably, we have successfully addressed the challenge of abiotic synthesis of cyclic dipeptides without the need for a catalyst. This groundbreaking discovery not only satisfies the prerequisites for monomer polymerization in the emergence of life but also provides a partial solution to the energy supply challenge. Furthermore, it implies that variations in environmental pH may have played a pivotal role in the origin of life. The exploration of the Pro-NH<sub>2</sub> induced cyclic dipeptide-generating reaction system reveals a potential pathway for life's emergence in early Earth environments. The plausibility of such a pathway provides novel insights, advancing our understanding and exploration of the origin of life.

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## Data and materials availability

All data is available in the main text or the Supplementary Information.

## CRediT authorship contribution statement

**Xiaofan Guo:** Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Li Zhang:** Writing – review & editing. **Min Zhang:** Writing – review & editing, Supervision, Conceptualization. **Yufen Zhao:** Writing – review & editing, Resources, Funding acquisition. **Jianxi Ying:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization.

## Declaration of competing interest

There are no conflicts to declare.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.palaeo.2024.112402>.

## References

Barge, L.M., Flores, E., Baum, M.M., VanderVelde, D.G., Russell, M.J., 2019. Redox and pH gradients drive amino acid synthesis in iron oxyhydroxide mineral systems. *Proc. Natl. Acad. Sci. USA* 116, 4828–4833.

- Becker, S., Feldmann, J., Wiedemann, S., Okamura, H., Schneider, C., Iwan, K., Crisp, A., Rossa, M., Amatov, T., Carell, T., 2019. Unified prebiotically plausible synthesis of pyrimidine and purine RNA ribonucleotides. *Science* 366, 76–82.
- Bolm, C., Mocci, R., Schumacher, C., Turberg, M., Puccetti, F., Hernández, J.G., 2018. Mechanochemical activation of iron cyano complexes: a prebiotic impact scenario for the synthesis of  $\alpha$ -amino acid derivatives. *Angew. Chem. Int. Ed. Eng.* 57, 2423–2426.
- Borthwick, A.D., 2012. 2,5-Diketopiperazines: synthesis, reactions, medicinal chemistry, and bioactive natural products. *Chem. Rev.* 112, 3641–3716.
- Cueto-Díaz, E.J., Gálvez-Martínez, S., Colín-García, M., Mateo-Martí, E., 2023. A new approach in prebiotic chemistry studies: proline sorption triggered by mineral surfaces analysed using XPS. *Life* 13, 908.
- Damer, B., Deamer, D., 2020. The hot spring hypothesis for an origin of life. *Astrobiology* 20, 429–452.
- Deamer, D., Damer, B., Kompanichenko, V., 2019. Hydrothermal chemistry and the origin of cellular life. *Astrobiology* 19, 1523–1537.
- Dolan, G.F., Akoopie, A., Muller, U.F., 2015. A faster triphosphorylation ribozyme. *PLoS One* 10, e0142559.
- Franchi, M., Gallori, E., 2005. A surface-mediated origin of the RNA world: biogenic activities of clay-adsorbed RNA molecules. *Gene* 346, 205–214.
- Gan, D., Ying, J., Zhao, Y., 2022. Prebiotic chemistry: the role of trimetaphosphate in prebiotic chemical evolution. *Front. Chem.* 10, 941228.
- Guillén, M.D., Carton, I., Goicoechea, E., Uriarte, P.S., 2008. Characterization of cod liver oil by spectroscopic techniques.: New approaches for the determination of compositional parameters, acyl groups, and cholesterol from <sup>1</sup>H nuclear magnetic resonance and Fourier transform infrared spectral data. *J. Agric. Food Chem.* 56, 9072–9079.
- Guo, J., Poros-Tarcali, E., Perez-Mercader, J., 2019. Evolving polymersomes autonomously generated in and regulated by a semibatch pH oscillator. *Front. Chem.* 55, 9383–9386.
- Guo, J.S., Poros-Tarcali, E., Perez-Mercader, J., 2021a. Periodic polymerization and the generation of polymer giant vesicles autonomously driven by pH oscillatory chemistry. *Front. Chem.* 9, 576349.
- Guo, Y., Ying, J., Sun, D., Zhang, Y., Zheng, M., Ding, R., Liu, Y., Zhao, Y., 2021b. Cyclic dipeptides formation from linear dipeptides under potentially prebiotic earth conditions. *Front. Chem.* 9, 675821.
- Guo, Y.T., Zhang, Y.M., Ying, J.X., Liu, Y., Zhang, G.Y., Zhao, Y.F., 2022. Selection of amino acid chirality induced by cyclic dipeptide synthesis in plausible prebiotic conditions. *Front. Astron. Space Sci.* 9, 794932.
- Handschuh, G.J., Orgel, L.E., 1973. Struvite and prebiotic phosphorylation. *Science* 179, 483–484.
- Hazen, R.M., Filley, T.R., Goodfriend, G.A., 2001. Selective adsorption of L- and D-amino acids on calcite: Implications for biochemical homochirality. *Proc. Natl. Acad. Sci. USA* 98, 5487–5490.
- Imai, E., Honda, H., Hatori, K., Brack, A., Matsuno, K., 1999. Elongation of oligopeptides in a simulated submarine hydrothermal system. *Science* 283, 831–833.
- Isson, T.T., Planavsky, N.J., 2018. Reverse weathering as a long-term stabilizer of marine pH and planetary climate. *Nature* 560, 471–475.
- Iyer, M.S., Gigstad, K.M., Namdev, N.D., Lipton, M., 1996. Asymmetric catalysis of the Strecker amino acid synthesis by a cyclic dipeptide. *Amino Acids* 11, 259–268.
- Joyce, G.F., 2002. The antiquity of RNA-based evolution. *Nature* 418, 214–221.
- Kaczmarczyk, B., 1998. FTIR study of hydrogen bonds in aliphatic polyestaramides. *Polymer* 39, 5853–5860.
- Lohrmann, R., Orgel, L.E., 1971. Urea-inorganic phosphate mixtures as prebiotic phosphorylating agents. *Science* 171, 490–494.
- Martin, O., Peñate, L., Alvaré, A., Cárdenas, R., Horvath, J.E., 2009. Some possible dynamical constraints for life's origin. *Orig. Life Evol. Biosph.* 39, 533–544.
- McKee, A.D., Solano, M., Saydjari, A., Bennett, C.J., Hud, N.V., Orlando, T.M., 2018. A possible path to prebiotic peptides involving silica and hydroxy acid-mediated amide bond formation. *ChemBiochem* 19, 1913–1917.
- Muchowska, K.B., Moran, J., 2020. Chemistry peptide synthesis at the origin of life. *Science* 370, 767–768.
- Muchowska, K.B., Varma, S.J., Moran, J., 2020. Nonenzymatic metabolic reactions and life's origins. *Chem. Rev.* 120, 7708–7744.
- Mullen, L.B., Sutherland, J.D., 2007. Simultaneous nucleotide activation and synthesis of amino acid amides by a potentially prebiotic multi-component reaction. *Angew. Chem. Int. Ed. Eng.* 46, 8063–8066.
- Naman, C.B., Rattan, R., Nikoulina, S.E., Lee, J., Miller, B.W., Moss, N.A., Armstrong, L., Boudreau, P.D., Deboni, H.M., Valeriote, F.A., Dorrestein, P.C., Gerwick, W.H., 2017. Integrating molecular networking and biological assays to target the isolation of a cytotoxic cyclic octapeptide, samoamide A, from an American Samoan Marine Cyanobacterium. *J. Nat. Prod.* 80, 625–633.
- Nishizawa, M., Makino, Y., Egami, F., 1983. A probable prebiotic peptide formation from glycineamide and related compounds in a neutral aqueous medium participation of nucleoside and 5'-mononucleotide. *J. Mol. Evol.* 19, 179–183.
- Nsengiyumva, O., Hamedzadeh, S., McDaniel, J., Macho, J., Simpson, G., Panda, S.S., Ha, K., Lebedeva, I., Faidallah, H.M., Al-Mohammadi, M.M., Hall, C.D., Katritzky, A.R., 2015. A benzotriazole-mediated route to protected marine-derived hetero-2,5-diketopiperazines containing proline. *Org. Biomol. Chem.* 13, 4399–4403.
- Omran, A., Pasek, M., 2020. A constructive way to think about different hydrothermal environments for the origins of life. *Life* 10, 36.
- Oro, J., Kimball, A., Fritz, R., Master, F., 1959. Amino acid synthesis from formaldehyde and hydroxylamine. *Arch. Biochem. Biophys.* 85, 115–130.

- Otsuka, Y., Arita, H., Sakaji, M., Yamamoto, K., Kashiwagi, T., Shimamura, T., Ukeda, H., 2019. Investigation of the formation mechanism of proline-containing cyclic dipeptide from the linear peptide. *Biosci. Biotechnol. Biochem.* 83, 2355–2363.
- Ozturk, S.F., Liu, Z.W., Sutherland, J.D., Sasselov, D.D., 2023. Origin of biological homochirality by crystallization of an RNA precursor on a magnetic surface. *Sci. Adv.* 9 eadg8274.
- Parker, E.T., Zhou, M., Burton, A.S., Glavin, D.P., Dworkin, J.P., Krishnamurthy, R., Fernández, F.M., Bada, J.L., 2014. A plausible simultaneous synthesis of amino acids and simple peptides on the primordial. *Earth Angew. Chem. Int. Ed. Engl.* 53, 8132–8136.
- Peretó, J., Bada, J.L., Lazcano, A., 2009. Charles Darwin and the origin of life. *Origins Life Evol. B* 39, 395–406.
- Rabinowitz, J., 1970. Peptide and amide bond formation in aqueous solutions of cyclic or linear polyphosphates as a possible prebiotic process. *Helv. Chim. Acta* 53, 1350–1355.
- Rajamani, S., Vlassov, A., Benner, S., Coombs, A., Olasagasti, F., Deamer, D., 2008. Lipid-assisted synthesis of RNA-like polymers from mononucleotides. *Origins Life Evol. B* 38, 57–74.
- Rasmussen, B., Muhling, J.R., Fischer, W.W., 2021. Greenalite nanoparticles in alkaline vent plumes as templates for the origin of life. *Astrobiology* 21, 246–259.
- Ross, D., Deamer, D., 2019. Prebiotic oligomer assembly: what was the energy source? *Astrobiology* 19, 517–521.
- Sakata, K., Yabuta, H., Kondo, T., 2014. Effects of metal ions and pH on the formation and decomposition rates of di- and tri-peptides in aqueous solution. *Geochem. J.* 48, 219–230.
- Sibilska, I.K., Chen, B., Li, L., Yin, J., 2017. Effects of trimetaphosphate on abiotic formation and hydrolysis of peptides. *Life* 7, 50.
- Sibilska, I., Feng, Y., Li, L., Yin, J., 2018. Trimetaphosphate activates prebiotic peptide synthesis across a wide range of temperature and pH. *Origins Life Evol. B* 48, 277–287.
- Washington, J., 2000. The possible role of volcanic aquifers in prebiologic genesis of organic compounds and RNA. *Origins Life Evol. B* 30, 53–79.
- Westheimer and F., 1987. Why nature chose phosphates. *Science* 235, 1173–1178.
- Yamagata, Y., Watanabe, H., Saitoh, M., Namba, T., 1991. Volcanic production of polyphosphates and its relevance to prebiotic evolution. *Nature* 352, 516–519.
- Ying, J., Lin, R., Xu, P., Wu, Y., Liu, Y., Zhao, Y., 2018. Prebiotic formation of cyclic dipeptides under potentially early Earth conditions. *Sci. Rep.* 8, 936.