A Shared Prebiotic Formation of Neopterins and Guanine Nucleosides from Pyrimidine Bases

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Abstract: The prebiotic origins of biopolymers and metabolic co-factors are key questions in Origins of Life studies. In a simple warm-little-pond model, using a drying phase to produce a urea-enriched solution, we present a prebiotic synthetic path for the simultaneous formation of neopterins and tetrahydroneopterins, along with purine nucleosides. We show that, in the presence of ribose and in a formylating environment consisting of urea, ammonium formate, and water (UAFW), the formation of neopterins from pyrimidine precursors is robust, while the simultaneous formation of guanosine requires a significantly higher ribose concentration. Furthermore, these reactions provide a tetrahydropterin–pterin redox pair. This model suggests a prebiotic link in the origin of purine nucleosides and pterin cofactors that provides a possible deep prebiotic temporal connection for the emergence of nucleic acids and metabolic cofactors.

Introduction

The ubiquity of pteridines (pyrazino[2.3-d]pyrimidines) in biology and their biochemical connection with purines is well established;[1] the most important pteridines are the co-factor forming pterins (2-amino-4-hydroxypteridines), such as biopterin, folic acid, or Mo/W enzymes cofactor. Pterins are the core of one-carbon biochemistry, potentially acting in a similar role during the origin of autotrophic CO_2 fixation.[2,3] Surprisingly, despite their biochemical importance and relation with purine nucleosides, the pterins have received relatively little attention in the quest for understanding the origin of life's building blocks. However, the growing knowledge of chemistry which laid the foundation for the origin of life will remain incomplete unless the origin of nucleotide-like coenzymes is included. Proposed prebiotic syntheses of both pterins and purine nucleosides have used 5,6-diaminopyrimidine as a key intermediate,[4–6] with the prebiotic synthesis of the purine ribosides being based on the Traube reaction.[7] Recent work has demonstrated the viability of 5,6-diaminopyrimidines as intermediates in the prebiotic synthesis of purine nucleosides.[4,8]

These diaminopyrimidines are capable of forming pteridines through Isay reactions with α-β dicarbonyl compounds.[13] These reactions explain the occurrence of both heterocycles in cyanide-based prebiotic model experiments.[6]

Based upon these previous results, it is reasonable to hypothesize that the prebiotic source of purine nucleosides could have also provided pterins, allowing for the coevolution of nucleic acid components alongside related metabolic cofactors.

Inspired by this possibility, we explored the potential for 5,6-diaminopyrimidines to act a prebiotic starting point of both purine nucleosides and pteridines in an environment rich in urea-based solvents (Scheme 1). Urea would have been widely distributed on the surface of the prebiotic Earth, as urea is a major product of prebiotic reactions, such as the spark-discharge reactions pioneered by Miller.[14–16] The extreme water solubility of urea (> 18 M at $60\,^\circ$C) and its nonvolatility would have facilitated the collection and concentrating of urea in freshwater bodies of water on the early Earth. However, given the great diversity of organic molecules formed in model prebiotic reactions that also produce urea, it is unlikely that pure urea solutions would have formed on the prebiotic Earth. Moreover, stable urea eutectic solutions would have likely been formed upon the evaporation of urea together with other

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Scheme 1. Contextualization of the formation of purines, nucleosides and neopterins from tetrasubstituted pyrimidines. The primary building blocks (left) lead to canonical pyrimidines and 2,4,6-substituted noncanonical pyrimidines of the barbituric acid family. The 6-amino members of the family can form 5,6-diaminopyrimidines, which could be prebiotic precursors of purines, purine nucleosides and pteridines, particularly pterins. In this work, and in the context of this general model, we explored how a urea-rich solution can support the formation of nucleosides and pterins by the 5,6-diaminopyrimidine route. Model based on refs. [4], [5], [8–12] and present work (colored section).

plausible abundant and related prebiotic molecules (formate, acetate, and ammonia or hydantoins among others). For example, we previously proposed that a urea–ammonium formate–water (UAFW) eutectic, that remains in liquid state in a range of 50–100 °C, could be formed under prebiotic conditions through simple evaporation of a small water pond.[17,18] The UAFW solvent provides a fluid environment with reduced water activity, which can be favorable conditions for condensation and phosphorylation reactions,[17–19] providing stability to products against hydrolysis. Furthermore, the UAFW solvent is also an ideal formylation medium and reagent source by virtue of the formate present and the formamide generated by gentle heating and evaporation.[17] The presence of formylating agents would boost the purine production under mild, prebiotic conditions by the Traube reaction.

This scenario suggests that the prebiotic origin of both essential biochemicals were potentially connected through simple drying, urea-concentrating conditions – conditions that have been shown to drive the formation of nucleobases and organophosphates.[18,20] These results indicate that pterins are important accompanying products of purine nucleosides, maybe even the favored product when compared to nucleoside formation. Excitingly, these pteridines are formed in the same urea-rich prebiotic setting in which its pyrimidine precursors are synthesized,[19] conditions which favors the formation of pyrimidines in general,[9,21] as well as the organics phosphorylation,[17] leading to an environment rich in important prebiotic precursors. Regarding the prebiotic origin of the 5,6-diaminopirymidines, in this work we explore its prebiotic origin through the C5 nitrosylation and reductive formylation of 6-aminopyrimidines, which could be formed in prebiotic conditions from urea precursor.[19] The results presented here demonstrate, under conditions which are demonstrably good

for chemical evolution, a unified prebiotic context for the formation of pyrimidines, derived pterins, and nucleosides.

Results and Discussion

To test the feasibility of urea-based solvent as medium for the synthesis of purines, we dried over the course of three days at 65 °C a solution of 2,5,6-triamino-4-hydroxypyrimidine **1 a** or tetraaminopyrimidine **1 b** in UAFW at different initial pH values. In the range 6 < pH < 9, the GC-MS analysis of the final mixture showed the formation of guanine **3 a** using **1 a** as a precursor, and a high yield (60 %) of 2,4-diaminopurine **3 b** using **1 b** as precursor (Figure 1a). ESI-MS analysis during the reaction confirmed the expected formylated pyrimidine. In the case of the **1 a**, the mass spectrum showed a prominent ion at m/z 170, corresponding to the 5-fapyguanine **2 b** (Figure 1b). The presence of a cation at m/z 152 (guanine **3 a**) confirms the observation by GC-MS of the trimethylsilyl derivatives (Figure S1 in the Supporting Information). It is interesting to note that, even in presence of significant urea, the main modification detected is formylation, with only minor persistence, based on GC-MS, of the expected carbamoylated precursor (m/z 184). The pH of the reaction was seen to be a key factor for these reactions with acidic solutions (pH < 5) not leading to the formation of purines, giving a complex mixture instead. The formation of **2 b** requires neutral or basic solutions; the nucleophilicity of the 5-amine group in the pyrimidine is higher and it thus favors the formylation.[22] In addition, the 5-amino is preferentially protonated at low pH, halting the formation of 5-fapyguanine under acidic conditions. Given the biochemical relevance of guanosine and pterins, and the easy formation of guanine in prebiotic model reactions along with pyrimidines,[10]

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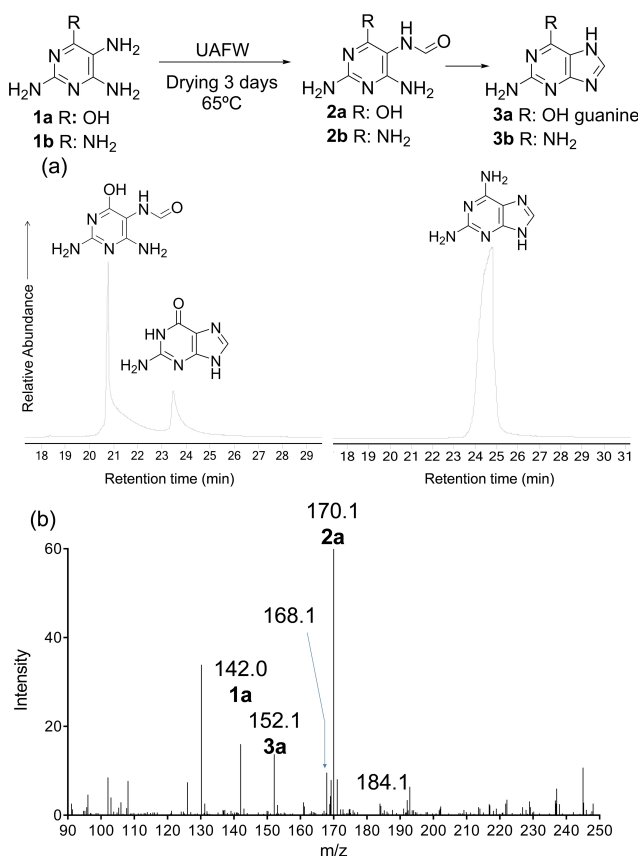**Chemistry Europe**
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Figure 1. a) GC-MS trace of the TMS-derivatized reaction products of drying a solution of 0.5 mmol of 2,5,6-triamino-4-hydroxypyrimidine **1a** (left) or tetraaminopyrimidine **1b** (right) in UAFW at 65 °C for 3 days at pH 9. b) ESI-MS spectrum in positive mode after drying a solution of 0.5 mmol of **1a** in UAFW at 65 °C. The identified ions corresponds to the expected *m/z* of the [*M*+H]⁺ ion of guanine **3a** (152), 5-fapyguanine **2a** (170). In spite of the presence of urea, formylation is the dominant modification of 2,5,6-triamino-4-hydroxypyrimidine.

which suggests that Traube reaction is possible in prebiotic conditions, we will focus on the pathway to canonical biochemicals in further experiments.

Pterins as main product in the prebiotic Traube's pathway to guanosine

It has been recently shown that the Traube reaction is possible in a prebiotic chemistry context as long as the formylation of the 5-amine group occurs before the glycosidation.[8] This work demonstrated that tetrosides and pentosides of 5-formamido-6-aminopyrimidines could be formed directly by a formose reaction. Further ring closure leads to the purine glycosides, including the canonical β-ribofuranoside anomers of adenosine and guanosine. A problem with this reaction is that it requires the previous formation of **2b** or other suitable 5-formylated pyrimidines. In a model prebiotic reaction, it is more likely that there is a competition for the nucleophilic positions in the pyrimidine molecule, which will tend to form other products than nucleosides even in presence of sugars, and we hypothe-

sized that the main products formed in a prebiotic setting would be pteridines.

To test the effect of the formylating, urea-rich, evaporating pond model on pterin formation and its impact on purine nucleoside formation, we subjected an equimolar solution of urea and the sulfate salt of **1a** (0.5 mmol mL⁻¹) to dry–wet cycles at 65 °C. This was carried out first after addition of 1 mmol of β-D-ribofuranose in slightly acidic conditions (pH 4) followed, after three days of drying cycles, by dilution and addition of Ba(OH)₂ until all sulfate is precipitated and pH 9 was reached. In a second experiment, the reaction started at basic pH after precipitation of sulfate. In both cases we observed the formation of neopterins (neopterin **4** and isoneopterin **5**), identified by their EI-MS fragmentation spectra[23] after gas chromatographic separation of the dried, TMS derivatized product (Figures 2b and S2).

We observed both 6-pterin **4** and 7-pterin **5** forms in all conditions tested, with regioselectivity for **5** (Figure 2c, d). This is expected as the C5 amine is more nucleophilic and the aldose reacts preferentially with it, yielding the 7-pterin. The relative proportions of **4** and **5** are less predictable. The pH during the glycosylation of the pyrimidine could be determinant, as at mildly acidic solution, ring N1 would be protonated, deactivating the C6 amine.[24] However, we did not observe a clear trend, being **5** the major product at both mild acid or neutral and basic pH. This is consistent with previous observations on the synthesis of biopterin[25,26] and the Isay reaction.[27]

These experiments show the dominance of the stable aromatic pterins, with only small amount of the corresponding dihydropterins **6** and **7**. To test the route from pyrimidine to pterin, we performed the same experiment in under a reducing environment using dithiothreitol (DTT) or sodium dithionite, monitoring the reaction using ESI-MS+ (Figure S3). The mass spectrum shows a prominent ion at *m/z* 256, assigned to the dihydroneopterins initially formed by the ring closure after intramolecular nucleophilic attack of the Amadori rearrangement product of the ribosylated pyrimidine to the free amine group (Figure 2a; Scheme S1). Consistently, neopterins, dihydroneopterins, and its exocyclic N-ribosylation products could be observed. The final mixture has an intense yellow color that could be caused by the oxidation of dihydroneopterin; specifically, the derivatives **8a** and **8b** were observed at *m/z* 238 and 370 (ribosyl derivative; Figure S3); the structure of these derivatives, formed by water elimination of **6** and **7**, have been proposed previously to explain the yellow color resulting from dihydroneopterins in an experimental model that could be considered prebiotically plausible, consisting in the reduction of neopterin with Fe.[1,28] The keto pterin derivatives **8a** and **b** are consistent with our experimental mass fragmentation spectra, and it is possible to confirm their structure based on expected pterin fragmentation[29,30] (Figure S4). We did not found pterins described for oxidation of dihydroneopterin,[28] consistently with our experimental setting, but there are formation of unidentified pterins which could be assigned to isomers of **8** (Figure S4).

The concentration of urea does not affect the formation of pterins. At high urea and ribose concentrations, an unidentified
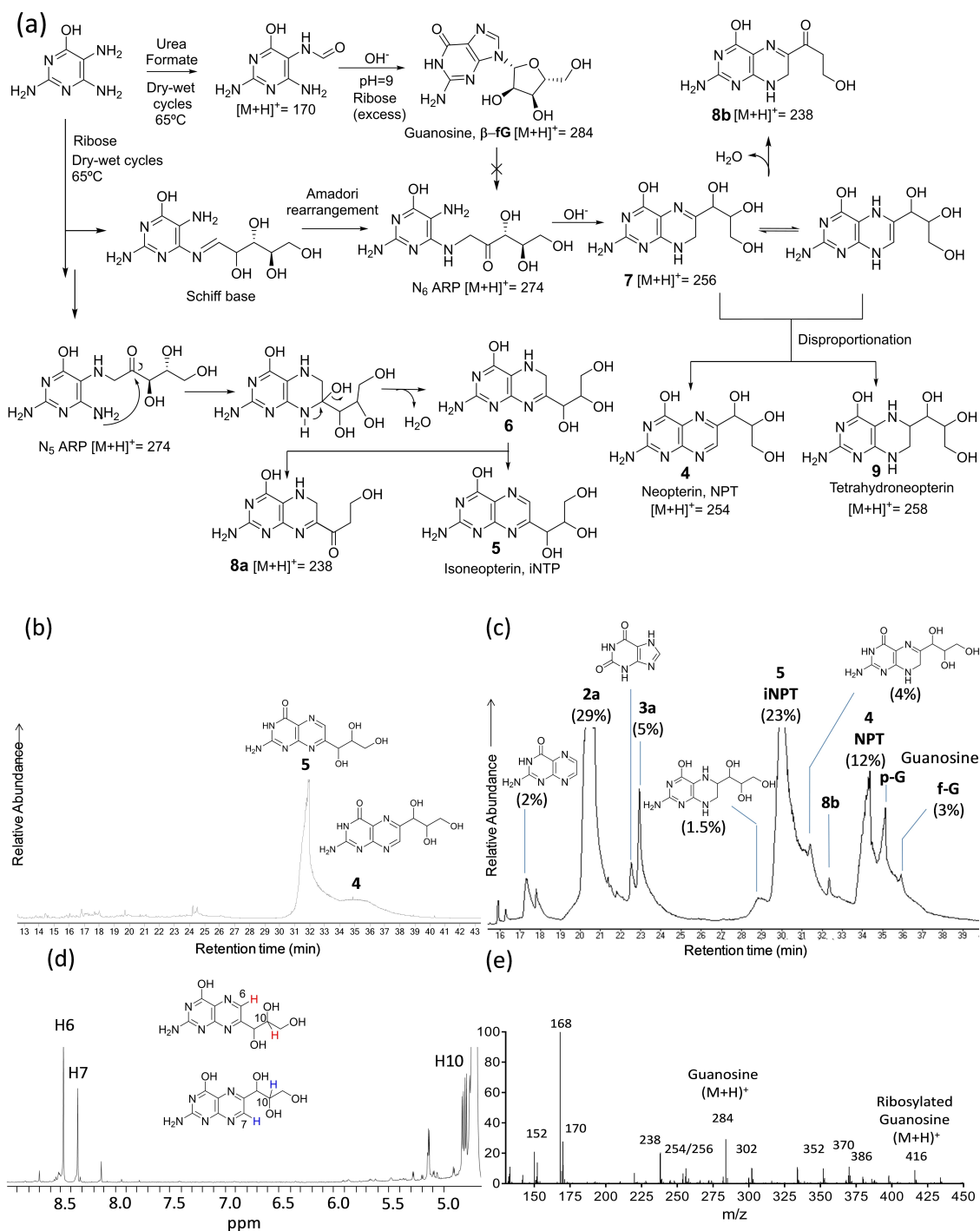
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Figure 2. a) Proposed scheme for the prebiotic synthesis of neopterins. Nucleophilic attack at the aldehyde by either C5 or C6 amine led to the corresponding Amadori rearrangement product (ARP), which undergoes ring closure to form the pyrazine ring of pterin. For simplicity, only the disproportionation of 7,8-dihydroneopterin and one Schiff base intermediate are shown. b) GC-MS trace of the TMS-derivatized products obtained after dry–wet cycles of a solution of 0.5 mmol of 2,5,6-triamino-4-hydroxypyrimidine **1 a**, 1 mmol of urea, and 1 mmol of ribose in 2 mL water, pH 9. c) GC-MS chromatogram of the TMS-derivatized product obtained after addition of 5 mmol ribose to a pH 9 solution made by dilution of a dried solution of 0.5 mmol of **1 a** in UAFW and heating at 85 °C for 5 days, with complete drying on the final day. The identified products are shown with estimated yields, calculated with respect to the pyrimidine precursor. The complex mixture contains neopterins, nucleosides and unidentified products. d) ^{1}H NMR spectrum of the reaction product in (b) diluted in D_2O, showing the assigned protons of neopterin isomers. e) ESI-MS in positive mode of the reaction product in (c); the consistent presence of a guanosine and exocyclic N-ribosylated guanosine confirms the mass fragmentation observations. The ions at m/z 168 and 170 suggest the formation of 8-oxoguanine (consistent with the urea-rich environment) and 5-fapyguanine respectively. Ions at m/z 300 (weak) and 302 are assigned to its ribosides 300; an ion observed at m/z 254 could be assigned to the neopterin; the two ion species at m/z 386 and 388 were assigned to the exocyclic N-ribosylation product of neopterin and dihydroneopterins; Ion species at m/z 284 and 416 were assigned to the $[M+H]^+$ ions of guanosine and exocyclic N-ribosylated guanosine.

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product is formed, which undergoes derivatization with estimated five trimethylsilyl groups to yield a product at m/z 604 $[M^{\bullet+}]$ after derivatization with BSTFA. If the urea concentration is moderate relative to sugar, the higher nucleophilicity of the amine groups in the pyrimidine favors the synthesis of pterin.

Hence, given a source of suitable tetrasubstituted pyrimidines and a source of nucleoside-forming sugars, the main expected products can be pteridine derivatives, along with free purines and with their nucleosides restricted to very low yields.

The proposed prebiotic route (Figure 2a) is analogous to the biopterin and folate biosynthetic pathways. This does not suggest that biosynthesis reflects an evolutionary trait, but instead represents a case of chemical determinism, and it is consistent with the parallel roles of purines and pterins in extant biochemistry. The route leads to the formation of the dihydropterin, which could be oxidized in air to form neopterins and colored derivatives, or undergo disproportionation, yielding neopterin and tetrahydroneopterin **9**; fragmentation mass spectra for these products, after BSTFA derivatization, have been found in the GC-MS analysis (Figure 2c).

To check whether a formylating prebiotic environment could improve the nucleoside formation, we subjected an UAFW eutectic solution containing **1a** as precursor to drying at 65 °C, following the reaction with ESI-MS analysis (Figure S5). The use of UAFW gives two advantages: a good formylating environment and a fluid medium even in dry conditions. After one hour of reaction, the formation of formylated pyrimidine **2** was observed; a maximum yield was reached after 4–5 h. The solution was then adjusted to pH 9 and β-D-ribofuranose was added at a tenfold molar ratio to **2a** (along with 0.5 molar equivalent of sodium dithionite to avoid excessive oxidation of tetra- or dihydroneopterin), and the reaction was allowed to evaporate for 12 h, followed by dilution and constant heating at 85 °C and pH for 5 days. This reaction, which turned crimson red to purple, was freeze-dried and analyzed by GC-MS after derivatization with BSTFA. The complex mixture showed the formation of neopterins and guanine nucleosides (Figure 2c). The total yield of guanosine nucleoside was 10%, with an estimated 3.6% β-ribofuranoside (**f-G**, Figure 2c), calculated after separation by HPLC using a HILIC column (Figures S6 and S7) and GC-MS data. The fragmentation mass spectra also showed the formation of free guanine and tetrahydroneopterin (identification based on mass fragmentation spectra). The ESI-MS spectrum in positive ion mode is consistent with the formation of guanosine and exocyclic N-ribosylated guanosine (Figure 2e). The neopterin/dihydroneopterin ions (m/z 254/256) were also identified, together with the corresponding exocyclic N-ribosylated derivatives and the dihydroneopterin oxidation products. To further investigate the resulting composition and confirm the LC–MS and GC-MS observations, we performed ESI-Orbitrap-MS analysis of the reaction products (Table 1), which confirmed the described compounds.

Table 1. Proposed structures based on ESI-Orbitrap-MS analysis of the product obtained after addition of 5 mmol ribose to a pH 9 solution made by dilution of a dried solution of 0.5 mmol of 2,5,6-triamino-4-hydroxypyrimidine in UAFW.

m/z[a]	Ion	Proposed structure[b]	Error (ppm)[c]	Abundance (a.u.)
137.0458	$C_5H_5N_4O^+$		−0.09	5.12×10^5
168.0525	$C_5H_6N_5O_2^+$		−5.38	1.43×10^6
170.0678	$C_6H_7N_5O_2^+$		−3.25	7.77×10^7
238.0914	$C_9H_{12}N_5O_3^+$		−4.71	5.10×10^5
254.0889	$C_9H_{12}N_5O_4^+$		4.24	2.35×10^7
284.1003	$C_{10}H_{13}N_5O_5^+$		−4.79	3.94×10^7
256.1041	$C_9H_{14}N_5O_4^+$		−0.27	2.59×10^5
258.1181	$C_9H_{16}N_5O_4^+$		4.15	3.8×10^4
269.0883	$C_{10}H_{13}N_4O_5^+$		−0.95	1.59×10^4
274.1151	$C_9H_{15}N_5O_5^+$		−1.85	8.12×10^4
300.0938	$C_{10}H_{11}N_5O_6^+$		−0.80	8.20×10^5
416.1422	$C_{15}H_{22}N_5O_9^+$		−2.40	3.87×10^5

[a] Observed m/z, only listed structures confirmed with an error < 10 ppm.
[b] Only represented one isomer.

Guanine nucleosides formation is dependent on a high relative ribose concentration

In the urea-rich UAFW solvent, formylation of pyrimidine **1a** and formation of guanine is favored over 8-hydroxyguanine, which might be expected to be a major product of **1a** with urea (Figure S8) but is found only as a minor product (Table 1; Figure 2e). In a prebiotic setting in which 5,6-diaminopyrimidines are present as precursors, despite the favored formylation, the formation of guanosines is not the most robust route; instead, the formation of pteridines could be the preferred pathway. To test the robustness of this conclusion with a more controlled reaction, we formylated **1a** with pure formamide; then, the **2a** derivative formed was ribosylated with a 5 equiv. excess of ribose respect the pyrimidine, and the pH was raised

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to 9 with NH_4OH. Analysis of the final intense yellow mixture by GC-MS showed the formation of pterins, with minor formation of guanine nucleosides, obtaining the described composition observed with UAFW (Figure 2).

We then addressed the possibility that the formation of purine nucleosides, which is clearly not as favored as pterin formation, is dependent on the relative proportion of ribose in the reaction. Unlike guanine/guanosine formation, the formation of neopterins does not require the previous formylation of the pyrimidine **1a**. Nevertheless, even in a formylating environment and following a stepwise process of formylation and subsequent ribosylation, which should be more favorable for guanosine formation than the reactions reported above, the formation of neopterins is still the preferred route. We wondered if, given the low solubility of pterins, an equilibrium in the formylation of **1a** to **2a** would be displaced, reducing gradually the availability of soluble pyrimidine; in that case, an excess or ribose would favor the formation of 5-ribosyl derivative of **2a**, favoring the route to the purine nucleoside.

To understand the effect of relative proportion of ribose, we synthesized 5-fapyguanine **2a** (Figure S9) and performed two experiments (Figure 3a): first, we incubated it overnight with 2 equiv. ribose in solution at 85 °C. Then, the pH was raised with aqueous ammonia until pH 9 and the solution were left at 85 °C during one to two weeks, until a noticeable precipitate was formed. In the second experiment, we melted **2a** with 10 equivalents of ribose overnight at 95 °C. Then, the mixture was dissolved in water, and the solution treated as in the first experiment. Next, the solutions were dried, and the mixtures analyzed by GC-MS after BSTFA derivatization. In the first experiment, the main products were the pterins **5** and **6**, guanine and neopterin oxidation products (Figure 3c). No guanine nucleosides were detected. In contrast, in the 10x ribose-excess melting experiment, guanosine nucleosides were formed along pterins, including canonical β-ribofuranoside (Figure 3b). Fragmentation spectra indicates the formation of the guanine pyranoside as main product, accompanied by six guanine nucleopentosides, possibly resulting from the epimerization of ribose.

These results suggest that in a prebiotic system in which formation of guanosine is expected through Traube reaction from pyrimidine 1a, the formation of pterins should be expected as well, predating the nucleoside formation, and following a synthetic route that resembles the extant biochemical formation of neopterin. To form the purine nucleoside, a large relative excess of ribose could be required, a result consistent with previous works upon the prebiotic formation of guanosine.[4] We wondered if guanine nucleosides could undergo a retro-Traube route to neopterins, in a non-enzymatic chemomimetic of the biochemical pathway. To test this possibility, we heated **β-f-G** at 85 °C in UAFW under drying-wetting cycles, as well as in aqueous solution at pH 9 with either ammonia or TEA for 2 weeks. The result showed that guanosine is stable in our experimental conditions, without significant depurination or formation of pterins (Figure S10)

The incorporation of that large excess of nucleoside-forming sugars leads to another question. Prebiotic sugar synthesis
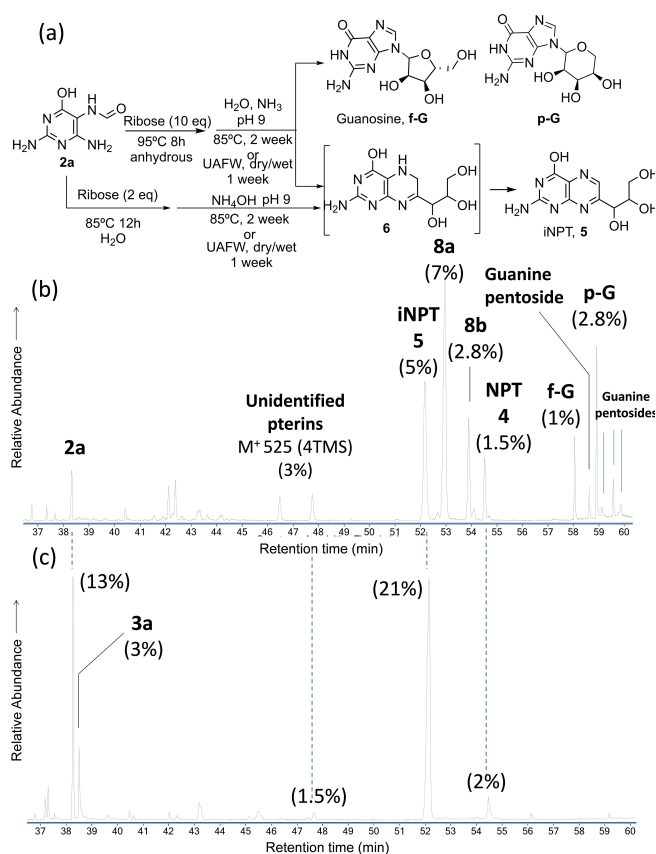

Figure 3. a) Summation of results showing that the formation of guanine nucleosides from 5-fapyguanine **2a** is dependent upon the relative proportion of ribose. b) GC-MS trace of the product resulting from the ribosylation of **2a** in dry conditions with 10 equiv of ribose, followed by heating at pH 9 for 2 weeks. Even starting with the formylated pyrimidine, the final mixture is dominated by pterins. Unidentified pterins showed a similar mass fragmentation spectrum to **8a,b**, being possible isomers. c) GC-MS trace of the product resulting from ribosylation with 2 equiv. of ribose in water or a solution of **2a** in UAFW, followed by incubation at pH 9 for 1 (UAFW) or 2 (water) weeks. No guanine nucleosides were detected. Estimated yields are indicated in brackets.

would likely occur in a completely different setting from pyrimidine synthesis, as the synthesis of sugars from highly reactive aldehydes would be incompatible with a setting enriched in urea. Assuming that the problem of prebiotic sugar availability is provided by its synthesis in alternative or extraterrestrial icy environments,[31,32] the formation of nucleosides from pyrimidines in a plausible prebiotic environment would require a strict timing in the formylation and sugar concentration for ribosylation, together with pH control, to divert the preferential formation of pteridines to nucleosides. An efficient nucleoside formation through Traube synthesis would require the incorporation of a high relative amount of sugar exactly between the formylation and the ring closure to form the free nucleobase. Such limitations in the purine nucleoside formation reinforces the importance of chemical evolution processes as formation of self-assemblies that select and concentrate key molecules.[33–35] Furthermore, the formation of pterins in different redox states, is interesting as it could

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supply a prebiotic 'cofactor' for redox reactions, as tetrahydro-neopterin could act as a hydride transfer agent.

From 2,6-diamino-4-hydroxypyrimidine to pterins/purines

Our results suggest that the 5,6-diaminopyrimidines are efficient precursors of pteridines, and, conditioned by sugar availability, purine nucleotides in a prebiotic drying pond scenario.

Overall, a potential limitation of this experimental model for the formation of pterins and purine nucleoside is the low or lack of availability of 5,6-diaminopyrimidines as prebiotic precursor; it is still an open question, and we are working on the urea-rich solutions as its potential sources. A possible prebiotic pathway to the 2,5,6-triaminopyrimidines starts from the HCN trimer aminomalononitrile, by reaction with guanidine to yield tetraaminopyrimidine **1b** or by its hydrolysis in presence of formaldehyde to produce aminocyanoacetamide, which further reacts with guanidine to produce **1a**.[8,36,37] Although the detection of 2,5,6-diaminopurines in undirected prebiotic model reactions like cyanide irradiation and polymerization is not reported, the formation of pteridines in such reactions suggests that 2,5,6-triaminopurines are formed at least as intermediates.[6,38,39] The formation of 6-aminopyrimidines in urea drying pond could overcome the possible limitations, if the prebiotic environment provides a source of NO, nitrites or nitrous acid;[4,40] the prebiotic geochemical or atmospheric availability of nitrite is a topic still under study and discussion,[41] but, as long as it remains a prebiotic possibility, it is worth study as a potential path for prebiotic purines formation.

The nitrosylation in the C5 of 2,4,6-triaminopyrimidine (TAP) or 4-hydroxy-2,6-diaminopyrimidine **10**, followed by reduction to the corresponding 5,6-diaminopyrimidines[7,42–44] could be a prebiotic source of the purine/pterin precursor (Figure 4). Recently, the nitrosylation route to formamidopyrimidines in a prebiotic dry–wet cycle model have been tested in a remarkable series of experiments, demonstrating that native iron/nickel, arguably very abundant in the prebiotic era, would be a good reducing agent for the reduction of nitrosopyrimidines to the aminopyrimidines.[4] We must then ask, is this nitrosylation pathway compatible with our prebiotic experimental model?[19] To answer this question we set to dry an UAFW solution containing either TAP or **10**, and sodium nitrite in equimolar concentration. In both cases, an abundant pink precipitate of the corresponding 5-nitrosopyrimidine was formed and its identity verified (Figure S11). The use of UAFW solvent provides both a suitable medium for the synthesis of pyrimidines of the barbituric acid family, including 4,6-diaminopyrimines; also, as we have shown, UAFW is compatible with nitrosylation pathway. It has been observed previously[43] that the combination of formate and formamide lead to the best yields of purine base synthesis through Traube synthesis. In UAFW, formamide produced after the urea–ammonium formate solution was mixed, possibly converts the eutectic solvent to a medium also suitable for purine formation. In his original synthesis of
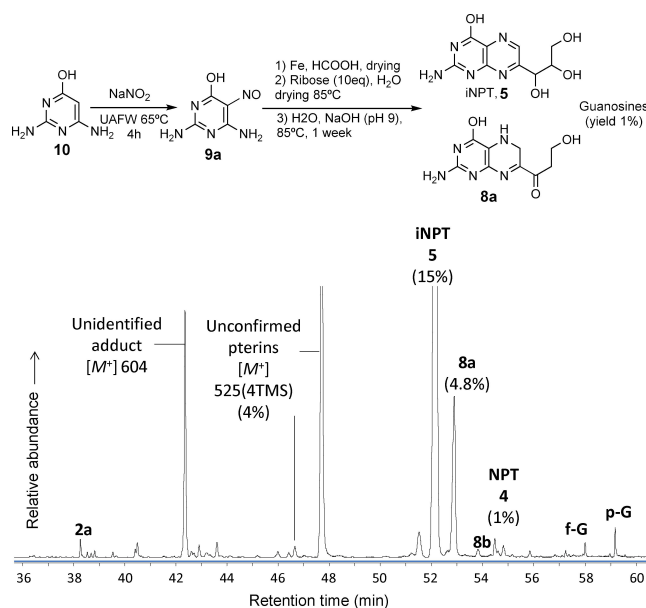

Figure 4. GC-MS trace of the product resulting from the synthesis of nitrosopyrimidine in UAFW, followed by reduction of 5-fapyguanine by iron powder, ribosylation with a 10 equiv. excess of ribose, and heating at basic pH. The very small yield of nucleosides and consistent formation of neopterins suggest that pterin formation would be favored over guanosine formation under a variety of prebiotic conditions.

purines,[7] Traube used ammonium sulfide as the reducing agent to form **1b**, in conditions that arguably could be considered prebiotic, especially if we consider that SH_2 and sulfides could have been present on prebiotic Earth. Nevertheless, we tested the one-pot synthesis by adding iron powder to the UAFW mixture in which the 5-nitrosopyrimidine was formed, keeping the pH 5 with HCOOH, followed by heating and drying, until disappearance of the brilliant reddish-pink color and formation of iron formate crystals (Figure S12). The dry mixture was redissolved in water with excess of ribose, heated and dried. The mixture was then redissolved in water, alkalinized to pH 9–10, and heated at 85 °C for one week. The analysis of the mixture showed the formation of the pterin **5** as the main derivative, with neopterin, pterin **8a** and other unidentified pterins, with very low yield (< 1% total) of guanine nucleosides (Figure 4). An unidentified adduct of M + 604 (TMS derivative) was always found at significant yield in experiments involving ribose with presence of urea. It is worth to note that, even in presence of urea, which could be detrimental in reactions involving ribose, the UAFW solvent promotes the reductive formylation of 5-nitrosopyrimidines in moderate conditions, with very low formation of carbamoyl derivatives and 8-hydroxyguanine, formed significantly in Traube reaction driven by urea alone (Figure S8).

An important effect of the 4-amine group of tetraaminopyrimidine **1b** is that this amine increases the reactivity of the 5-amine, facilitating formation of the 5-formylated pyrimidine and, subsequently, the formation of 2,6-diaminopurine **3b**. Under the same reaction conditions the formation of guanine **3a** from **1a**, which lacks the 4-amine, is less favorable (Figure 1).

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In the case of using TAP as starting material in the nitrosylation pathway, the analysis by ESI$^+$-MS/MS of the resulting mixture, after precipitation of dissolved iron at alkaline pH, showed a dominant ion in positive mode at m/z 169; its fragmentation confirms the formation of **2b** by the transition 169→141, corresponding to the 5-formamido derivative (Figure S12). Despite this result, we were unable to identify the corresponding pteridines or 2,4-diaminopurine nucleosides using the same experimental strategy, leading all the experiments involving TAP as precursor to complex, strongly colored mixtures.

Overall, in a prebiotic setting consisting of solutions containing urea in a drying pond model, 5,6-diaminopyrimidines could be formed by nitrosylation of 6-aminopyrimidines (assuming the prebiotic availability of a nitrous acid source) followed by nitrosyl reduction. The urea solution could be both a precursor of pyrimidines, or, in the form of UAFW solvent, a suitable medium for formylation and formation of purines. Assuming a source of nucleoside-forming sugars, the same scenario would promote both the formation of nucleosides and pteridines with polyol side chains (Scheme 2).

A unified prebiotic scenario: phosphorylation of neopterin in urea eutectic solution

Assuming the availability of ribose, we showed the robust formation of neopterins by reaction of the corresponding 5,6-diaminopyrimidine with ribose in a *drying pond* model, using urea-based solvent, even in formylating conditions. The prebiotic synthesis of purine nucleosides is possible in the proposed model, but at limited yields, requiring high relative ribose concentration at the ribosylation steps. In prebiotic

models based upon the origin of purine nucleic acid components, using the formation of active pyrimidine precursors, the formation of pteridines as major products should be expected, suggesting that pterins and purines could have been temporally and spatially linked during chemical evolution. The potential formation of pterins with polyol side chains, usually overlooked in prebiotic model reactions involving pentoses, could divert a significant amount of prebiotically available free sugars in a pyrimidine-rich setting. Neopterins and other pteridines have low solubility, allowing them to precipitate and concentrate in aqueous environment, but limiting its interaction with other molecular species. Hence, to consider pteridines as participants in the chemical evolution, either as cofactor or component in pre-RNA structures, their mobilization is desiderable. One of the possible ways to mobilize/solubilize a pterin with polyol side chain is by phosphorylation.[46] Specifically, the possibility of prebiotic phosphorylation catalyzed by urea in urea-enriched systems[47] is suggestive, as an unique scenario could be proposed for both synthesis and phosphorylation of nucleosides and related molecules. Given its similarity to nucleosides and the biochemical importance of neopterin phosphate, we decided to test the phosphorylation of neopterin in the UAFW phosphorylation experimental model we tested previously,[18] unifying the scenario for both syntheses of purines/pterins and phosphorylation. The neopterin is easily phosphorylated by drying in UAFW at 65 °C in presence of NaH_2PO_4. After 72 h of heating, the neopterin is solubilized, and the formation of neopterin phosphate and stable dimers could be observed (Figure 5). The neopterin phosphate dimer formation in this scenario is easier compared to ribonucleotides, for which the formation of dinucleotides is usually very low or undetectable.[17,18] This could be explained by the geometry of
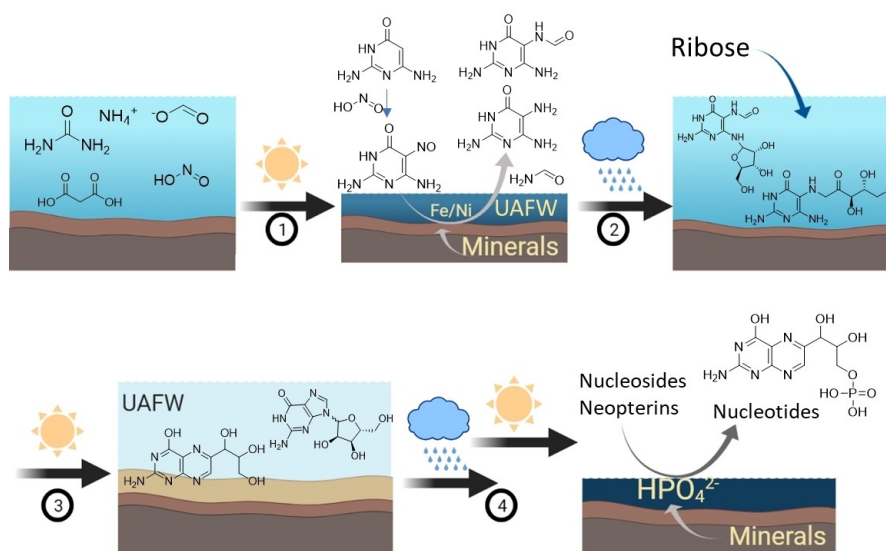
Scheme 2. A model for the prebiotic formation of nucleotides and phosphorylated pterins in a warm drying pond environment. ① formation of 5,6-diaminopyrimidines by nitrosylation and reduction of the corresponding 6-aminopyrimidines, formed in urea-rich systems;[19] ② Incorporation of formate and evaporation converts the urea solution in the UAFW solvent, which is a good formylating agent; ③ the incorporation of ribose to the system, together with a rise in pH, leads to the formation of neopterins and, in less proportion, nucleosides; ④ dry–wet cycles on UAFW promotes the mobilization of phosphate from minerals, and the phosphorylation of the polyol chain in neopterins and nucleosides. Mineral sources of phosphate for phosphorylation could be free phosphate, hydroxyapatite,[18] or reduced phosphorus forms, as schreibersite;[45] which could accompany the Fe,Ni used in the NO reduction step.
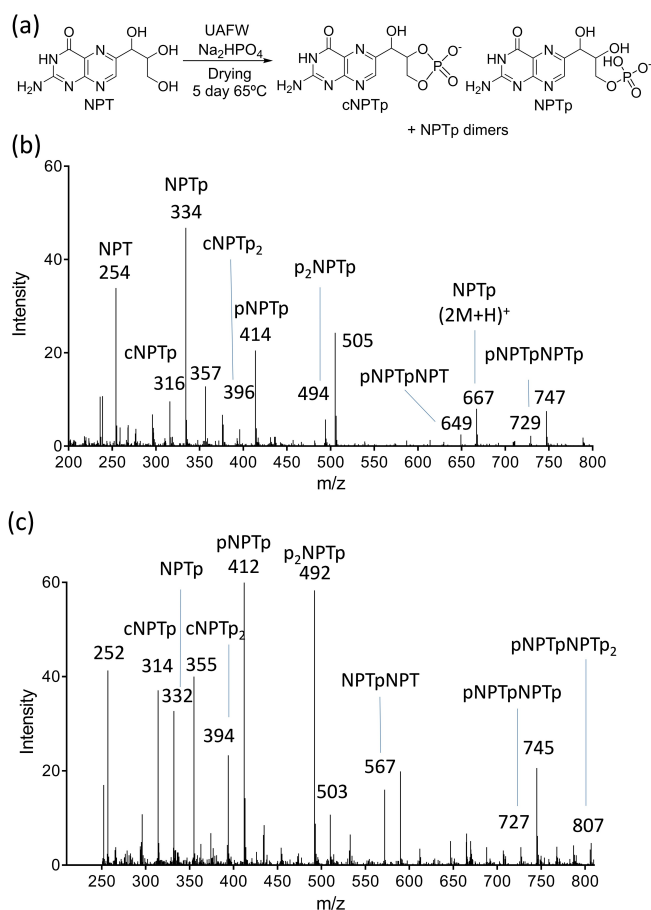
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Figure 5. ESI-MS spectra of the mixture formed after drying 0.5 mmol of neopterin (NPT) and 1 mmol Na₂HPO₄ in 500 μL UAFW, at 65 °C for 72 h. Sample final pH 5.5 and mobile phase buffered at pH 5 to favor the formation of protonated cations. Ions assigned to phosphorylated neopterin (NPTp) derivatives, cyclic neopterin phosphate (cNPTp) and neopterin phosphate dimers (pNPTpNPT and pNPTpNPTp) are indicated. a) General scheme and possible structures formed by phosphorylation; ESI-MS spectra in b) positive and c) negative modes.

the polyol moiety in the neopterin molecule, which facilitates the formation of intermolecular phosphodiester bonds; ribonucleosides, instead, yield the most stable intramolecular 2′–3′ phosphodiester. The neopterin cyclic phosphate could also be observed at m/z 316.

The facile prebiotic synthesis of pyrimidines, pterins and phospho-pterins in the same environment (Scheme 2), along with the favorable formation of intermolecular phosphodiesters, could suggest that the origin of cofactors was coincident with a prebiotic origin of purine nucleotides. The presence in the prebiotic environment of mobile phospho-pterins could have a potential influence in the chemical evolution of nucleic acids. Furthermore, the formation of 'proto-cofactors' based on pterins at different oxidation state, activated by phosphorylation and forming mixed pterin-purine dinucleotides, could have driven redox reactions during the chemical evolution to proto-metabolic systems.

Conclusions

The prebiotic formation of purine nucleosides is a challenging topic in prebiotic chemical evolution. This work demonstrates that the formylation of 4-hydroxy-2.5,6-triaminopyrimidine combined with ribose addition in a drying-pond model generates neopterins as the favored products. These results suggest that, although the formation of pterins is very robust, the formation of purine nucleosides is very sensitive to the specific conditions and, of particular note, is dependent on the presence of a significant excess of ribose in the system. These specialized conditions potentially undermine models advocating for the prebiotic origin of canonical purine nucleosides, especially those based on the Traube reaction for the origin of purine rings.

The formation of the corresponding 5-formamidopyrimidine starting from the 2,4,6-trisubstituted pyrimidine by C5 nitrosylation and reduction in the presence of formate/formamide is favored in the UAFW warm-little-pond model. Depending on the geochemical or atmospheric availability of nitrogen oxides/nitrous acid in a prebiotic planetary environment, this could be a favored pathway for the formation of purines under prebiotic conditions. The formation of neopterins as the main product in the prebiotic synthesis of guanosine suggests that the formation of pterins and purine nucleosides could be a related process in chemical evolution. Furthermore, the pterin route of formation through the three redox states of the pterin ring (tetrahydropterin, dihydropterin, and pterin) could have implications regarding the prebiotic origin of pterin redox cofactors. Hence, the biochemical relationship between guanosine and the pterin cofactors could have its roots in the prebiotic epoch, through its shared synthesis in a warm little pond under simple drying. The urea eutectic solvent provides a single scenario for the formation of the pyrimidine precursors, the synthesis of the nucleosides and pterins, and for their phosphorylation in the presence of inorganic phosphates. The concomitant prebiotic formation of phosphorylated polyol pterins and nucleotides could have been relevant for the evolution of nucleic acids.

Experimental Section

Nucleobases, nucleosides and pterins were analyzed by GC-MS after derivatization to their corresponding trimethylsilyl ethers, by electrospray mass spectrometry, ¹H NMR, and by high-resolution electrospray ionization Orbitrap mass spectrometry. Compounds were identified in GC-MS by comparison of electron ionization mass spectra against authentic standards and spectral databases. Identification in HRMS was carried out by comparison of accurate masses obtained experimentally against theoretical m/z values of target compounds, followed by tandem MS experiments. Methods and experimental details are given in the Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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