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Prebiotic Chemistry ***Hot Paper***

A Nonenzymatic Analog of Pyrimidine Nucleobase Biosynthesis

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Abstract: Metabolic theories for the origin of life posit that inorganic catalysts enabled self-organized chemical precursors to the pathways of metabolism, including those that make genetic molecules. Recently, experiments showing nonenzymatic versions of a number of core metabolic pathways have started to support this idea. However, experimental demonstrations of nonenzymatic reaction sequences along the de novo ribonucleotide biosynthesis pathways are limited. Here we show that all three reactions of pyrimidine nucleobase biosynthesis that convert aspartate to orotate proceed at 60 °C without photochemistry under aqueous conditions in the presence of metals such as Cu^{2+} and Mn^{4+}. Combining reactions into one-pot variants is also possible. Life may not have invented pyrimidine nucleobase biosynthesis from scratch, but simply refined existing nonenzymatic reaction channels. This work is a first step towards uniting metabolic theories of life's origin with those centered around genetic molecules.

Thinking about the chemical origins of life is often divided along "genetics-first" and "metabolism-first" hypotheses. The "genetics-first" approach assumes that self-replicating genetic polymers, likely RNA, emerged directly from a prebiotic chemistry bearing little resemblance to life's biosynthetic pathways.[1] From that perspective, early prebiotic chemistry is simply a tool to furnish building blocks for a later self-organized process. A central challenge for this vision of prebiotic chemistry is the direct, robust and high-yielding synthesis of ribonucleotides or deoxyribonucleotides by any plausible means. Experimental work along these lines has been arguably the major thrust of the field for the past 50 years,[2] with recent experimental work from Sutherland,[3] Carell,[4] Powner,[5] Benner,[6] and Trapp.[7]

However, without overlooking the undeniable importance of ribonucleotides and RNA in the origin of life,[8] starting in the late 1980s conceptual difficulties with the "genetics-first" approach prompted "metabolism-first" proposals for the origin of life.[9–12] This approach considers the origin of life to have involved the spontaneous onset of a self-organized reaction network, which was driven into existence by a thermodynamic need to relax geochemical redox gradients. The initial catalysts enabling the self-organized reactivity would have been minerals, clays and metals, but certain organics produced by the network could additionally feed back as catalysts to reinforce the existing reactions and to enable new ones.[13–15] Such a reaction network would have been historically continuous with biological metabolism. Furthermore, because complex systems, such as metabolism, are difficult to disrupt and rewrite, certain energetic and chemical similarities should exist with metabolism today. From this alternative perspective, ribonucleotides and their oligomers would have been produced by the prebiotic reaction network, possibly initially in small quantities, but gained prominence within it because they offered a critical catalytic or regulatory function within the existing self-organized chemistry.[12] Viewed from this angle, a central challenge of prebiotic chemistry is to figure out what the conditions for such self-organized chemistry would have been. One way towards this goal is to search for conditions that recapitulate nonenzymatic variants of core metabolic reactions and processes,[16,17] including those for the synthesis of genetic molecules.[18] Multiple, possibly very different, conditions might be found to enable any particular reaction or pathway without enzymes, and some of these conditions may have no relevance to the origin of life. However, this strategy should eventually identify conditions that enable a larger system to self-organize.[17] Experimental efforts in this direction[19] have thus far focused on metal-catalyzed analogues of carbon fixation pathways,[20–24] amino acid[25–27] and cofactor[28,29] biosynthesis, and sugar metabolism.[30–33] Investigations into purely chemical conditions that trigger reactions of ribonucleotide biosynthesis in a prebiotic context have been limited, as we will discuss below.

Ribonucleotides are biosynthesized in two general ways: by salvage pathways that regenerate ribonucleotides after they degrade, and by de novo pathways that build them up from scratch starting from amino acids, C1 compounds and phosphoribosylpyrophosphate (PRPP), an activated form of ribose-5-phosphate. The first three steps of de novo

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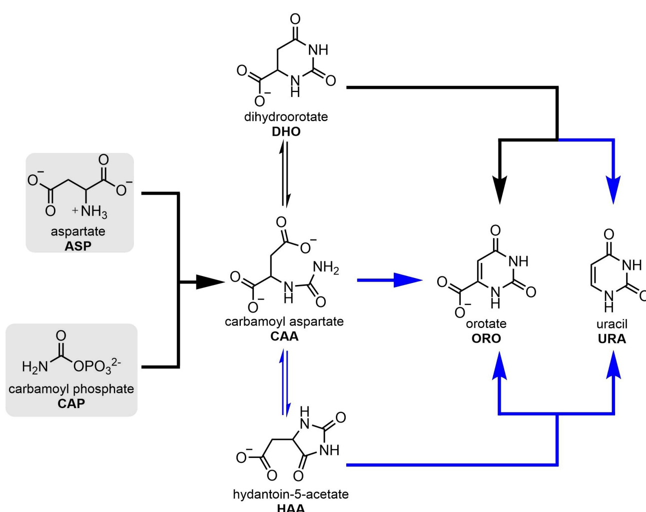
pyrimidine ribonucleotide biosynthesis are illustrated by the black arrows in Scheme 1. First, aspartate (**ASP**) reacts with carbamoyl phosphate (**CAP**) to give carbamoyl aspartate (**CAA**), catalyzed by carbamoyl phosphate synthetase.[34] **CAA** then undergoes a dehydrative cyclization to give dihydroorotate (**DHO**) catalyzed by dihydroorotase. **DHO** is oxidized to orotate (**ORO**) by various electron-accepting cofactors, catalyzed by dihydroorotate dehydrogenase. Later on in the pathway, not illustrated in Scheme 1, **ORO** couples with PRPP to give orotidine 5′-monophosphate, and, in subsequent steps, the canonical pyrimidine ribonucleotides uridine-5′-monophosphate and cytidine-5′-monophosphate. The conversion of **ASP** to **ORO** is therefore a key biosynthetic bottleneck in the formation of pyrimidine ribonucleotides.

Much prebiotic chemistry has been described that conceptually resembles ribonucleotide biosynthesis,[2] but little that involves precisely the same reactions and substrates. However, if metabolism is derived from self-organized chemistry, deviations from the reactions or substrates it uses, even if they lead to a biological product, might lack relevance to prebiotic chemistry. As for reports that specifically recapitulate ribonucleotide salvage pathways, Jaber, Georgelin and co-workers examined the non-enzymatic formation of adenosine monophosphate from adenine and phosphoribosylpyrophosphate (PRPP).[35] Chemistry that roughly resembles de novo pyrimidine nucleobase biosynthesis has also been reported, but not for the biological substrates along the entire path.[18,36–38] Yamagata used urea, rather than **CAP**, and UV light, rather than a chemical oxidant, at 80–90 °C.[38] In that study, the photooxidation of **DHO** to **ORO** was particularly challenging, reaching <1 % yield after 2.5 days and declining thereafter. It still remains unclear whether all the biological transformations of pyrimidine ribonucleobase biosynthesis can occur without enzymes and, crucially, without photochemistry, as none of the nonenzymatic analogues of the other pathways described above depend on light.[20–33] Here we examine these first three key steps experimentally and find that they all occur nonenzymatically in water at 60 °C without the need for UV light.



Scheme 1. The first three steps of de novo pyrimidine ribonucleotide biosynthesis convert aspartate (**ASP**) to orotate (**ORO**), as shown by the black arrows. Additional reactions observed in this report are described by blue arrows.

We began our investigations by exploring the non-enzymatic reaction between **ASP**, derived from the non-enzymatic reductive amination of oxaloacetate,[22] and **CAP** to give **CAA**. Previously, **CAP** and **CAA** have been obtained in a prebiotic context starting from cyanate or from urea (via cyanate),[38–41] however the use of **CAP** to prepare **CAA**, as in the biological pathway, has not yet been studied. The reaction between **ASP** (8.5 mM) and **CAP** (8.5 mM) was assayed over a pH range of 3–9 and a temperature range of 0–60 °C. Acidic conditions were found to be ineffective. The optimal conditions were found to be pH 8 and 60 °C, giving 43 % yield after 16 h, as determined by quantitative ^{1}H NMR by integrating against dimethyl sulfone (DMS) as an internal standard. The identity of the product was additionally confirmed by LC-QTOF-MS analysis of the reaction mixture through comparison with an authentic sample. The use of a two-fold excess of **CAP** relative to **ASP** gave **CAA** in 63 % yield after 16 h (Figure 1A). When the initial concentration of **ASP** was increased from 8.5 mM to 21 mM (still with a 2:1 ratio of **CAP** to **ASP**), the yield of **CAA** increased to 77 %. Full optimization details can be found in Table S1. From a mechanistic standpoint, cyanate is known to be produced, along with phosphate, by thermal fragmentation of **CAP** and is therefore a likely intermediate in the reaction.[42,43] In line with this proposal, the use of pH 8 phosphate buffer, rather than water adjusted to pH 8, decreased the yield of **CAA** to 10 % (Table S1, entry 10), which might be explained by a less favorable equilibrium between **CAP** and cyanate in the presence of excess phosphate.

Encouraged by this result, we next explored the non-enzymatic dehydrative cyclization reaction of **CAA** to **DHO**. It should be noted that this reaction is endergonic and at pH 7.4 at 37 °C the enzyme catalyzed process favors **CAA** over **DHO** by a factor of 16.6 at equilibrium.[44] Thus, low yields of **DHO** are to be expected under similar conditions. The reaction was assayed over a pH range of 1–8 and a temperature range of 20–60 °C. The products were quantified by ^{1}H NMR and confirmed by LC-QTOF-MS in the same way as described earlier. At pH values near the lower end of this range, the reaction did not produce any **DHO**, but instead gave rise to the 5-membered product, hydantoin-5-acetate (**HAA**). This result is similar to that observed in a non-prebiotic context during the total synthesis of orotic acid.[41] A new screen was therefore conducted across the same range of conditions but in the presence of a panel of metal salts and oxides. Zn^{2+}, Cu^{2+} and Fe^{3+} produced a small amount of **DHO** at the higher pH values, with Cu^{2+} being the most selective for **DHO** over **HAA**. For example, the reaction of **CAA** with $CuSO_4$ (40 mol %) at 60 °C, pH 8 after 16 h gave **DHO** in 1 % yield as the only observed cyclization product (Figure 1B). At lower pH values the **DHO** obtained increased, but **HAA** became the major product due to the acid-catalyzed back-

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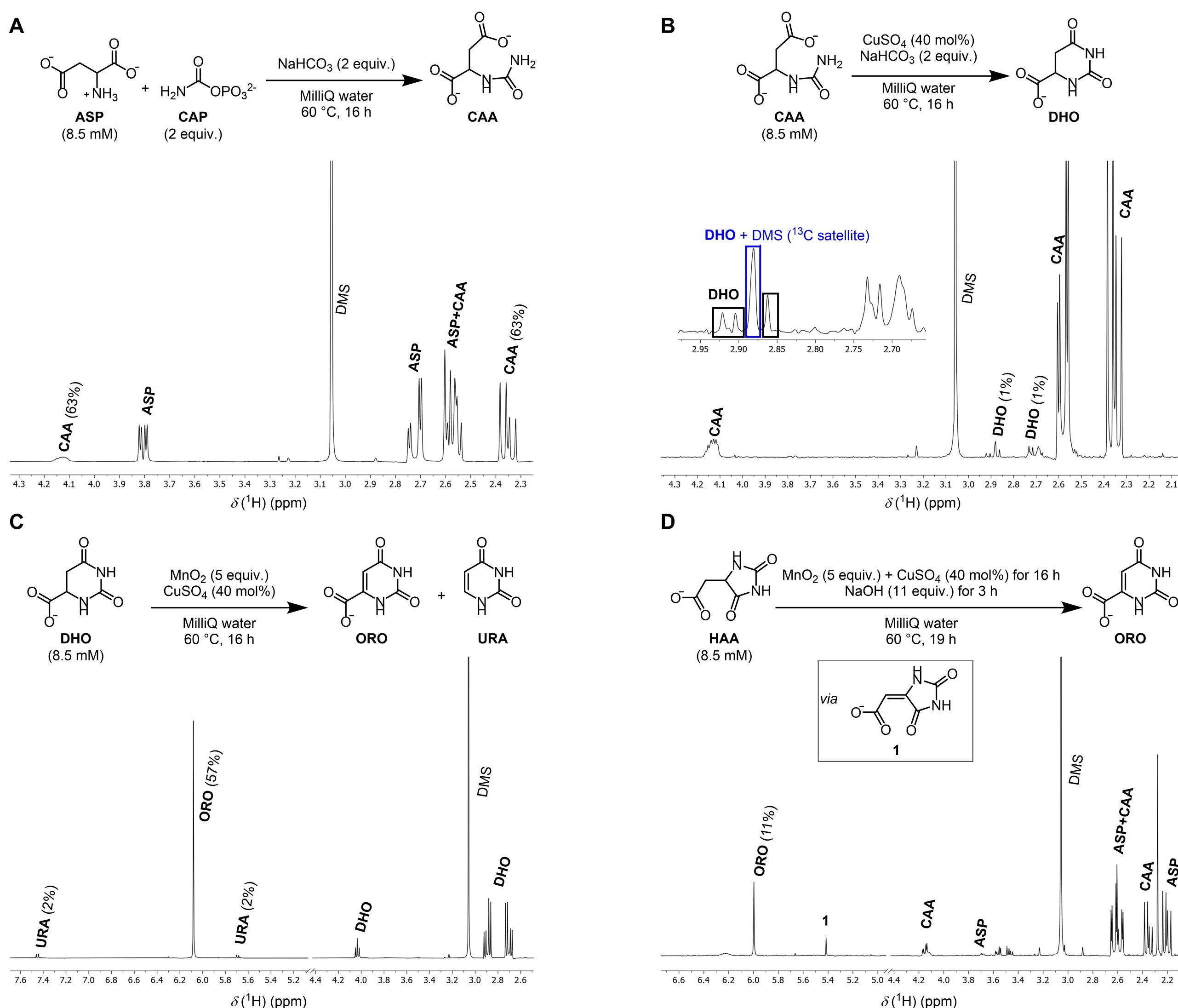

Figure 1. Study of single reaction steps. A) ^{1}H NMR spectrum of the products of reaction of aspartate **ASP** and carbamoyl phosphate **CAP**. δ, chemical shift. B) ^{1}H NMR of the products of cyclization reaction of carbamoyl aspartate **CAA**. C) ^{1}H NMR of the products of oxidative reaction of dihydroorotate **DHO**. D) ^{1}H NMR of the products of oxidative reaction of hydantoin-5-acetate (**HAA**). Yields were determined by quantitative ^{1}H NMR with water suppression techniques, integrating against dimethyl sulfone as an internal standard.

ground reaction (Table S2). For example, at pH 2 in the presence of Cu^{2+}, **DHO** was obtained in 3 % yield together with 25 % of **HAA**. Increasing the initial concentration of the starting material to 21 mM improved the yield of both products, giving 4 % **DHO** and 40 % **HAA**.[45] We note that Cu^{2+} has proven an intriguing additive in a number of different prebiotic contexts.[27,46]

We next examined the oxidation of **DHO** to **ORO**. Previously, a nonenzymatic version of this reaction had only been observed in <1 % yield under direct UV irradiation.[38] A screen was devised to assay oxidants over a pH range of 4–13 and a temperature range of 20–60 °C. At 60 °C, two optimal oxidants were found to be H_2O_2 and MnO_2, the prebiotic plausibility of which was recently discussed.[47,48] In the case of H_2O_2 as oxidant, the reaction worked best at pH 9, giving 34 % yield of **ORO** after 16 h. In the case of MnO_2 as oxidant, at pH 4, **ORO** was obtained in 18 % yield, together with 2 % of uracil (**URA**) after 16 h. Next, we studied the influence of metal additives on the oxidation. The presence of metal ions inhibited the H_2O_2 promoted oxidation to **ORO**. For example, the presence of Cu^{2+} reduced the yield to <3 % **ORO**, together with 4 % **URA**. However, in the case of the MnO_2 promoted oxidation, a screen of metal ion additives (Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}) revealed that the presence of Cu^{2+} (40 mol %) enhanced the outcome, giving **ORO** in 51 % yield together with 2 % **URA** (Figure 1C). The other metal co-catalysts assayed did not display any enhancing effects (Table S3). Cu^{2+} is thus capable of influencing both the cyclization and oxidation steps on the nonenzymatic version of the biological path to **ORO**.

Given that the cyclization of **CAA** to **DHO** occurred in highest yield at lower pH, where it is accompanied by **HAA** as major product (Table S2), we wondered whether **HAA**

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might also be converted to **ORO** under similar oxidative conditions. Could **HAA** be oxidized to **1** under plausibly prebiotic conditions, which could then undergo a known rearrangement under alkaline aqueous conditions to give **ORO**?[41] Exposing **HAA** to H_2O_2 did not result in its oxidation under the conditions we tested. In contrast, exposing **HAA** to MnO_2 in the presence of Cu^{2+} (40 mol %) at 60 °C for 16 h indeed resulted in its oxidation to **1** (characteristic singlet at 5.4 ppm in ^{1}H NMR) and to **URA** (5 %) (Table S4). Basifying the reaction mixture with NaOH and continued heating for a further 3 h resulted in a decrease in concentration of **1** due to its conversion to **ORO** (11 %) along with hydrolysis to **CAA** (Figure 1D). Thus, the adventitious conversion of **CAA** to **HAA** is not necessarily a dead end, as it can be funneled towards the same end-product, **ORO** (Scheme 1, blue arrows).

In order to assess the feasibility of a non-photochemical one-pot process mimicking pyrimidine nucleobase biosynthesis, we first attempted to merge the dehydrative cyclization and oxidation steps. Heating **CAA** in the presence of Cu^{2+} at 60 °C, followed by the addition of MnO_2 at 16 h and NaOH at 32 h gave **ORO** in 2 % yield at 35 h. It was later found that the yield could be improved to 13 % NMR yield (24 % by LC-QTOF-MS) of **ORO** by lowering the initial reaction pH, along with a 1 % yield of **URA** and 13 % of unreacted **CAA** (Figure 2A). This outcome is in agreement with the aforementioned influence of pH on the cyclization of **CAA** to **DHO** and **HAA**. Next, we examined a one-pot process for the conversion of **ASP** and **CAP** to **ORO**. Heating **ASP** and **CAA** (1:2) at 60 °C at pH 8, followed by the addition of Cu^{2+} and HCl at $t=16$ h, MnO_2 at $t=32$ h, and NaOH at $t=48$ h gave **ORO** in 1 % NMR yield (4 % by LC-QTOF-MS) along with 14 % **CAA** at $t=54$ h. Intermediate **1** is notably observed by ^{1}H NMR in both of the one-pot experiments described in Figure 2, indicating that the pathways proceeding through **DHO** and through **HAA** are both occurring. It was also possible to obtain **ORO** from **ASP** in one pot by adding all the reagents from the beginning apart from the basification of the reaction at $t=16$ h with NaOH, however, the yield decreased to <1 % (Table S6, entry 2). NaOH must not be added from the beginning as **DHO** rapidly decomposes in its presence. Control experiments indicate that the decrease in yield observed when all reagents (other than NaOH) are present from the beginning is due to the decomposition of **CAP** by Cu^{2+} (Table S1, entry 6).

In conclusion, we have uncovered a non-photochemical nonenzymatic analog of pyrimidine nucleobase biosynthesis in which some steps are promoted by metals. Some of the steps are inhibited by metals or by unfavorable pH values, which explains why the one-pot version of this chemistry was helped by timed addition of metals or timed changes in pH. Although pH gradients do exist in certain natural environments, such as hydrothermal vents,[49] we feel it is premature to place this chemistry in a specific geological location. Whether or not the particular set of conditions described here are directly relevant for the origin of life, they nonetheless constitute a proof of principle that this key biological process on the path to genetic molecules could have existed even before enzymes had evolved to catalyze it. In addition to the specific reactions of the biosynthetic pathway shown here, parallel nonenzymatic reactions not found in biology also occur but still eventually arrive at the same biological endpoint: orotate. The conversion of **ASP** to **ORO** does not necessarily require metals, especially if a potentially prebiotic alternative oxidant could be found. However, at least at 60 °C in aqueous solution,[45] only the metal-mediated reaction sequence follows the de novo biosynthetic pathway, passing through **DHO**. It is this path that carries greater explanatory value when viewed in the framework of the hypothesis that nonenzymatic reactions templated the evolution of the biological pathway.[9–14,16–19] Evolutionary refinement of the redundant branched non-enzymatic reaction network shown in Scheme 1 (both black and blue arrows) would logically result in a streamlined linear pathway like the one found in biology today (black arrows only).[50] In line with this idea, some enzymes still catalyze an equilibrium between **CAA**, **HAA** and **DHO**,[51] even though there appears to be no biological use for **HAA**. The present work can be viewed as a first experimental step toward incorporating genetic molecules into a self-consistent metabolic framework for the origin of life. Preliminary experiments indicate that the *N*-carbamoylation of **ASP** with **CAP** is not selective over other amino acids (Figure S7). However, the potential implications of this observation to a self-organized prebiotic chemistry depends on many factors which are at present difficult to constrain. Future work should investigate to what extent a one-pot nonenzymatic version of this pathway can occur without controlled changes to reaction conditions, and whether the nonenzymatic assembly of pyrimidine ribonucleotides can occur in ways paralleling their de novo biosynthesis.[52]

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Conflict of Interest

The authors declare no conflict of interest.

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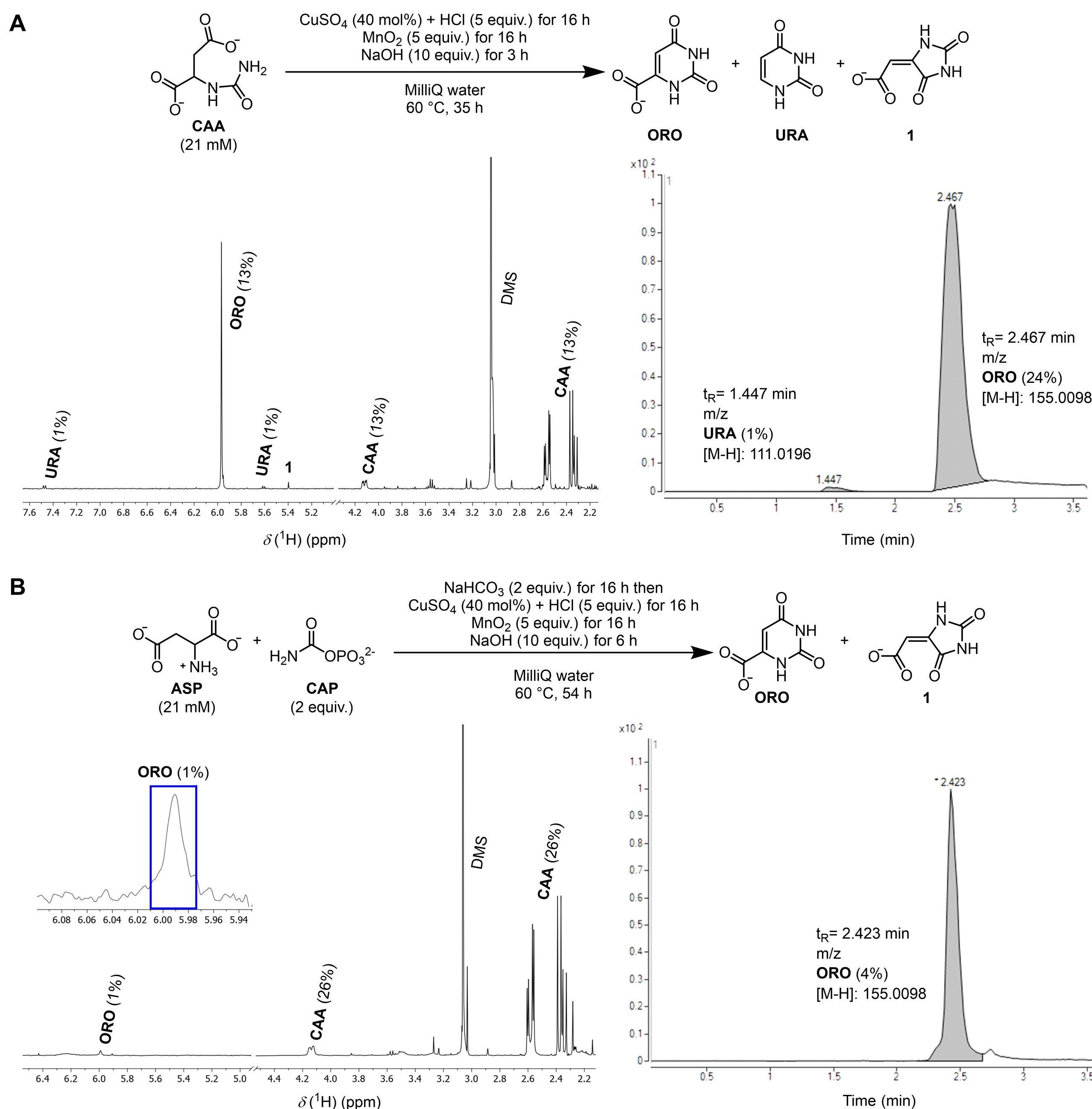

Figure 2. One-pot formation of pyrimidine bases. A) ¹H NMR and LC- QTOF-MS of the products of multi-step reaction of carbamoyl aspartate (**CAA**). B) ¹H NMR and LC-QTOF-MS of the products of multi-step reaction of aspartate, (**ASP**) and carbamoyl phosphate (**CAP**). $[M-H]$ at $m/z = 155.0098$ was chosen as characteristic fragment for **ORO**, $[M-H]$ at $m/z = 111.0196$ was chosen as characteristic fragment for **URA**.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

Keywords: Prebiotic Chemistry · Metabolism · Nucleobase · Orotate · Pyrimidines

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[50] Following submission of this work, a prebiotic synthesis of orotate was reported starting from hydantoin and glyoxylate, which also proceeds through **1**. Interestingly, the reaction occurs under conditions similar to the ones reported here (60–80 °C). As the reaction of glycine with **CAP** followed by dehydrative cyclization would form hydantoin, just as **ASP** reacts with **CAP** to eventually form **HAA**, that pathway and the two reported here would all be expected to arrive at **ORO** starting from the amino acids of core metabolism. It remains unclear why only the path passing through **DHO** was retained in biology. A. P. Clay, R. E. Cooke, R. Kumar, M. Yadav, R. Krishnamurthy, G. Springsteen, *Angew. Chem. Int. Ed.* **2022**, *61*, e202112572; *Angew. Chem.* **2022**, *134*, e202112572.
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