



# Prebiotic synthesis and triphosphorylation of 3'-amino-TNA nucleosides

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**Nucleosides are essential to the emergence of life, and so their synthesis is a key challenge for prebiotic chemistry. Although amino-nucleosides have enhanced reactivity in water compared with ribonucleosides, they are assumed to be prebiotically irrelevant due to perceived difficulties with their selective formation. Here we demonstrate that 3'-amino-TNA nucleosides (TNA, threose nucleic acid) are formed diastereoselectively and regiospecifically from prebiotic feedstocks in four high-yielding steps. Phosphate provides an unexpected resolution, leading to spontaneous purification of the genetically relevant *threo*-isomer. Furthermore, 3'-amino-TNA nucleosides are shown to be phosphorylated directly in water, under mild conditions with cyclic trimetaphosphate, forming a nucleoside triphosphate (NTP) in a manner not feasible for canonical nucleosides. Our results suggest 3'-amino-TNA nucleosides may have been present on the early Earth, and the ease with which these NTPs form, alongside the inherent selectivity for the Watson-Crick base-pairing *threo*-monomer, warrants further study of the role they could play during the emergence of life.**

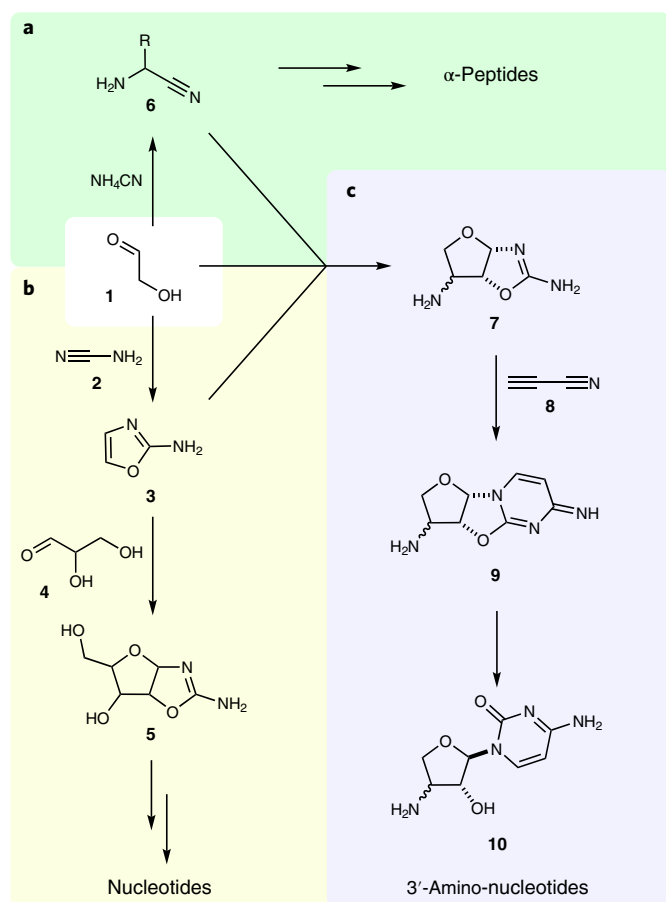
A substantial obstacle to understanding life's emergence on Earth is how the first nucleotides, which are essential for Darwinian evolution<sup>1</sup>, could have been synthesized. The cellular machinery currently used to enable replication of DNA is too complex to have emerged without invoking evolution, so must be the product of a simpler ancestral system<sup>2</sup>. The 'RNA world' hypothesis posits that this ancestral system was composed of self-replicating RNA that both encodes genetic information and can exhibit catalytic function. Although its capacity to act as both genotype and phenotype in extant biology makes RNA an attractive candidate for the first genetic material, it is plausible that a different—perhaps constitutionally simpler—nucleotide may have preceded it<sup>3</sup>, or that the first genetic material was chimaeric in nature, composed of several different classes of nucleotide<sup>4</sup>.

Non-canonical nucleotides have been considered as possible precursors to RNA (and DNA) for decades<sup>5,6</sup>. A variety of different nucleotide structures, with changes both to the sugar backbone and the nucleobases, have been investigated<sup>7,8</sup>. The key property of a potential genetic polymer is information transfer, that is, the capacity to pass on the information contained within its own sequence. Ultimately, for a genetic polymer to have preceded D/RNA it must be able to pass on genetic information to these polymers as well as to itself. Changes to the nucleobase are rarely heritable<sup>9</sup>, and changes to the sugar often prevent stable duplex formation (and so information transfer) with D/RNA<sup>10</sup>. However, almost 20 years ago, as part of a seminal study evaluating the potential of non-canonical nucleotides to have been the first genetic polymers of life, Eschenmoser evaluated RNA, threose nucleic acid (TNA)<sup>11</sup>, 2'-amino-TNA and 3'-amino-TNA<sup>3,12</sup>. Unusually for non-canonical nucleotides TNA and amino-TNA polymers are able to form stable duplexes with both RNA and DNA as well as with themselves, and can also form catalytic polymers akin to ribozymes<sup>13,14</sup>, setting the stage for a potential genetic takeover and for them to have played a role in the origins of life on Earth<sup>3,12</sup>.

Eschenmoser considered the differences in 'constitutional' versus 'generational' complexity of TNA and RNA nucleotides, comparing their structural complexity with how readily they could have

formed. He concluded that TNA's four-carbon sugar means that its monomers are 'constitutionally' simpler than RNA's monomers, but because the most difficult steps for the emergence of nucleotides seemed to be formation of the glycosidic bond and phosphorylation of the nucleosides, they were not necessarily 'generationally' simpler. On the other hand, he considered amino-TNA's monomers to be both generationally and constitutionally more complex than RNA's monomers due to the additional difficulty in regioselective incorporation of an amine into a sugar. Indeed, when Eschenmoser considered the effect that nitrogenous compounds would have on the aldol chemistry that, at the time, seemed the most likely source of sugars on the early Earth<sup>15</sup>, he concluded that 'huge chemical complications' would arise due to Amadori and Mannich chemistry occurring alongside the already unselective aldol reactions. He nevertheless recognized that these complications may 'harbour structural and transformational opportunities that may be relevant' to the origins of life. We therefore decided to re-examine the suggestion that amino-TNA's monomers are more generationally complex than RNA's monomers, in light of chemistry discovered since, with the aim of discovering a facile and selective amino-nucleoside synthesis. Our re-evaluation indicates that one such opportunity for monomer synthesis is the regioselective incorporation of an amine moiety at the 3' position of a sugar during aminooxazoline synthesis (Fig. 1).

How the first nucleotides formed on Earth is still an open question<sup>16</sup>, but there is a strong growing consensus that the earliest life would have emerged from a system containing peptides and lipids as well as nucleotides<sup>17</sup>. Despite this, most proposed mechanisms by which nucleotides could have formed on the early Earth<sup>18</sup> are still based on two assumptions—first, that the sugar and base moieties formed separately and then combined at a late stage<sup>19</sup>, and second, that nucleotide synthesis and amino acid/peptide synthesis occurred separately. We have previously challenged the first assumption, discovering highly diastereoselective routes to ribonucleotides<sup>20,21</sup> in which the sugar and bases are constructed together in the same environment on the same scaffold. This sequence represents the only diastereoselective prebiotically plausible formation of



**Fig. 1 | Intersections of prebiotic peptide, sugar and nucleobase syntheses.** The three different classes of molecule presented (that is, peptides, nucleotides and amino-nucleotides) are for clarity demarcated by coloured boxes, and glycolaldehyde **1**, which is a precursor and synthetic node in all intersecting syntheses shown, is positioned centrally within a white box. **a–c**, Glycolaldehyde **1** is a prebiotic precursor to peptides via serine nitrile **6** ( $R = \text{CH}_2\text{OH}$ ) (**a**), to nucleotides via 2-aminooxazole **3** and its masked-aldol reaction with glyceraldehyde **4** (**b**) and to amino-nucleotides **10** via three-component Mannich reactivity (**c**).

ribonucleotides known so far, and proceeds via the coupling of glycolaldehyde **1** with cyanamide **2** to form 2-aminooxazole **3**, which then reacts with glyceraldehyde **4**, forming the key intermediate amino-oxazoline **5** (Fig. 1b).

The advantages delivered through unification of nitrile and sugar chemistry in our synthesis have led us to consider the relationship between peptides and nucleotides, and how their syntheses would intersect<sup>22</sup>. Aminonitriles **6** have long been considered to be precursors of amino acids<sup>23,24</sup> as their formation via the Strecker reaction is both facile and predisposed to form biogenic  $\alpha$ -aminonitriles. However, the intrinsic reactivity of these molecules has been overlooked, with a major focus on how to improve their relatively sluggish hydrolysis to amino amides and then acids. Unlike amino acids, aminonitriles are predisposed towards reactivity in water due to the kinetically inert but high-energy nitrile moiety and the suppressed  $pK_a$  of the amine rendering them nucleophilic at neutral pH. We recently demonstrated that this dual reactivity could be exploited to form peptide bonds in water, with high selectivity for biologically relevant (proteinogenic)  $\alpha$ -peptides over unnatural isomers<sup>25,26</sup>. But how then does this chemistry interact with nucleotide synthesis? And can it be used to overcome the perceived complexity of the

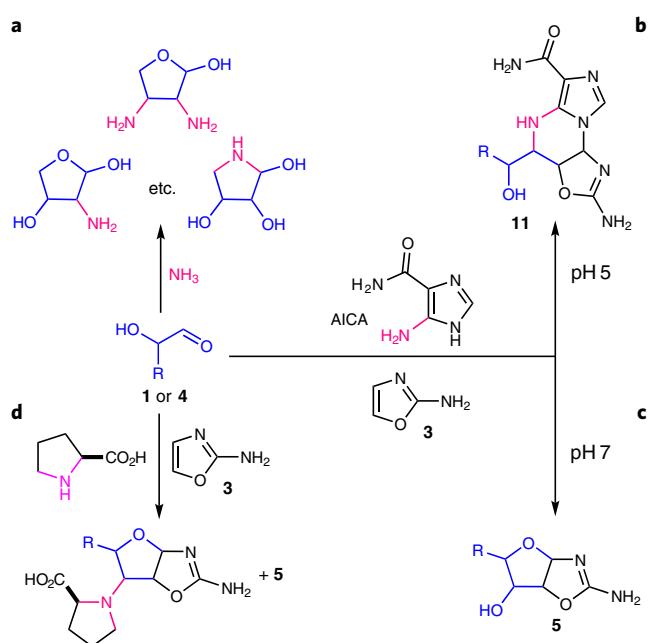
aldol, Mannich and Amadori reactions (Fig. 2a) expected in nitrogenous aldol chemistry?

## Results and discussions

**Amino-sugar synthesis.** We have previously reported that, at pH 5–6 in the presence of 5-aminoimidazole-4-carboxamide (AICA), the reaction between sugars and 2-aminooxazole **3** is out-competed by a three-component reaction. Imine formation between AICA and the sugar (for example, glycolaldehyde **1**) preceded reaction with **3**, forming an aminooxazoline product (**11**) regiospecifically functionalized by an amine at C3' (Fig. 2b)<sup>27</sup>. In a related reaction between amino acids, glyceraldehyde **4** and 2-aminooxazole **3**, Blackmond observed a mixture of both two- and three-component coupling products (Fig. 2d)<sup>28</sup>. The three-component reaction was diastereoselective with enantiopure proline, which, due to the low chemoselectivity, led to enantioenrichment of glyceraldehyde **4** and consequently enantioenrichment of the two-component amino-oxazoline products **5**. We recognized that if a variant of this reaction could be rendered selective for three-component coupling with an  $-\text{NH}_2$  source, a simple route to 2,3'-diaminooxazolines **7** would be uncovered. We envisaged that aminonitriles **6**, due to their low amine  $pK_{aH}$ , would be efficiently incorporated and that, due to the reversibility of the Strecker reaction, the free amine could then be liberated after coupling, and thus deliver a selective route to 3'-amino-nucleosides.

We envisaged that the reaction of glycolaldehyde **1**, 2-aminooxazole **3** and an aminonitrile **6** would form tetrose 3'-amino-aminooxazolines **7**. The nucleobase could then be constructed on this compound by reaction with cyanoacetylene **8**, and anomerization of the base would furnish the base-pairing  $\alpha$ -*threo*-nucleoside *threo*-**10** (Fig. 1c). We therefore began our investigation by looking at the three-component reaction between glycolaldehyde **1**, 2-aminooxazole **3** and glycine nitrile **6a** ( $R = \text{H}$ ) in water at neutral pH. Although the reaction was not selective, **12a** was clearly visible in the  $^1\text{H}$  NMR spectrum. Employing a slight excess of **6a** improved the chemoselectivity, with the yield increasing to 40% (Table 1, entry 1). Based on our previous work with AICA, we expected that three-component coupling would be favoured at low pH, and indeed at pH 5 we observed very efficient three-component coupling, forming **12a** in 94% yield after 16 h (Table 1, entry 2). The yield reached a maximum at pH 5. At lower pH, lower yields were observed—at pH 4 the reaction stalled after 4 h and 60% conversion, but all three starting materials were accounted for (Table 1, entry 3). When initiated at pH 4, the solution was observed to drop to pH 2 as the reaction proceeded, and at pH 2 coupling is observed to proceed only very slowly (Supplementary Fig. 1). **6a** ( $pK_{aH} = 5.6$ )<sup>21</sup> can act as the ideal buffer, as well as a coupling partner, keeping the pH close to the optimum value of pH 5. This buffering effect is highly apparent in reactions initiated at pD 4.5, where with 1 equiv. of **6a** the reaction stops at 55% conversion, but is then restarted if the solution is adjusted back from pD 2.5 to pD 4.5, reaching 75% conversion. However, when the reaction was initiated at pD 4.5 with 3 equiv. of **6a** it did not stall, the pH was maintained and **12a** was furnished in 90% yield. At pH 5, three-component coupling was seen to dominate even at concentrations as low as 10 mM, albeit more slowly, with **12a** formed in 70% yield after 10 days (Table 1, entry 4 and Supplementary Fig. 12).

If glycolyl aminooxazoline **12a** is heated at 60 °C, a slow retro-Strecker reaction occurs, liberating a free amino group to form **7** (Table 1, entry 5 and Supplementary Fig. 26). However, hydrolysis of the aminooxazoline moiety of **12a** to oxazolidinone **13** occurred concomitantly with glycolyl-retro-Strecker upon heating. Similar hydrolysis of pentose aminooxazolines (**5**) to their corresponding oxazolidinones has previously been reported to occur on heating in phosphate solution<sup>29</sup>. This C2-hydrolysis can be suppressed



**Fig. 2 | Formation of amino-sugar derivatives from C2 and C3 sugars.**

**a**, Uncontrolled aldol and Amadori reactions between ammonia and sugars as proposed by Eschenmoser to yield a complex mixture of amino-sugars<sup>15</sup>. **b**, Selective three-component coupling between 2-aminooxazole **3**, glycolaldehyde **1** and purine precursor AICA at pH 5 to form tricyclic product **11**<sup>27</sup>. **c**, Selective two-component coupling between 2-aminooxazole **3** and glycolaldehyde **1** in the presence of AICA to form aminooxazoline **5** at pH 7<sup>27</sup>. **d**, Unselective incorporation of amino acids into a mixture of aminooxazolines, including **5**, via coupling of glyceraldehyde **4** with 2-aminooxazole **3** and proline<sup>28</sup>. **1**, R = H; **4**, R = CH<sub>2</sub>OH.

with more sterically encumbered aminonitriles, which undergo a more rapid retro-Strecker reaction. For example,  $\alpha$ -alkylated aminonitriles **12b–e** (Table 1, entries 5–9) all undergo a retro-Strecker reaction at room temperature, proceeding efficiently over several days (Extended Data Fig. 1). The cyanohydrin formed in this process is not consumed and would be recycled into aminonitriles in the presence of a suitable source of ammonia<sup>30</sup>. Indeed, in the case of the hydrophobic R groups (for example, <sup>i</sup>Pr and <sup>t</sup>Bu), cyanohydrin crystallization was seen to occur spontaneously from the reaction mixture.

Although aminonitriles are known to be unstable compared to cyanohydrins and ammonium at low concentrations of free ammonia<sup>31</sup>, they are reasonably kinetically stable at neutral or acidic pH. Commeyras, however, has shown that  $\alpha,\alpha$ -disubstituted aminonitriles undergo retro-Strecker reactions easily even at acidic pH at room temperature, and that *N*-alkylation further increases the rate of the retro-Strecker reaction<sup>32</sup>. On the other hand, Commeyras reported that alanine nitrile (**6b**) was ~20 times more stable than glycine nitrile (**6a**)<sup>33</sup>, but our data suggest that  $\alpha$ -alkylation promotes more rapid retro-Strecker (**12b** > **12a**) and that **6a** and its derivatives are kinetically more stable to retro-Strecker reactions than the more substituted aminonitriles.

Aminonitriles **6** are more reactive coupling partners than either amino acids or ammonia under the optimal conditions for three-component coupling. For example, when 200 mM **1**, **3**, **6a** and glycine were mixed at pH 5, **12a** (80%) was seen to form as the predominant product after 24 h (Supplementary Fig. 20). Furthermore, if **1** and **3** were mixed in the presence of 600 mM **6a** in 1 M NH<sub>4</sub>Cl at pH 5, **12a** was seen to form in 95% yield after 24 h (Supplementary Fig. 16).

When the reactions of  $\alpha$ -substituted aminonitriles initiated at pH 5 were monitored for several weeks, the concentration of **7** was seen to fall slightly over time, without concomitant increases in either **12** or oxazolidinone **13**. The concentration of *threo*-**7** seemed to fall less than *erythro*-**7** (Supplementary Fig. 28). To explore this intriguing behaviour further, we isolated **7** as a mixture of diastereomers.

**threo-selective crystallization.** When **7** (3:2 *threo*:*erythro*; Fig. 3a) was mixed with 200 mM phosphate at near-neutral pH, a colourless precipitate was seen to form rapidly. <sup>1</sup>H NMR spectra indicated that the supernatant was substantially enriched in *erythro*-**7**, and *threo*-**7** had precipitated (Fig. 3b). Over time, *erythro*-**7** was then seen to transform into a new compound, assigned as the bridged bicyclic compound **14** on the basis of two-dimensional (2D) NMR and mass spectral data (Fig. 3c and Supplementary Figs. 42–44)<sup>3</sup>. Compound **14** was found to be unreactive in the subsequent synthetic steps. The high pK<sub>aH</sub> of the guanidine moiety (pK<sub>aH</sub> of guanidinium, 13.7) means that **14** is fully protonated, and so its nucleophilicity is quenched at neutral pH. The same rearrangement would also be expected to occur in 3'-amino-*ribo*-aminooxazolines, as *ribo* and *erythro* aminooxazolines have the same stereochemical orientation at their 2' and 3' carbons, which would preclude the formation of 3'-amino-RNA.

Analysis of the precipitate demonstrated that the phosphate salt of *threo*-**7** had indeed undergone a highly diastereoselective crystallization (Fig. 3d). This provides a natural route for separation and concentration of the genetically useful diastereomer, *threo*-**7**, while removing the potentially inhibitory *erythro*-isomer. This *threo*-selective precipitation is observed at concentrations of *threo*-**7** as low as 10 mM in 100 mM phosphate. In contrast, precipitation of *ribo*-aminooxazoline **5**, thought to provide a selection mechanism for RNA<sup>22,29,34</sup>, occurs efficiently only above 100 mM and does not co-accumulate **5** with phosphate (Supplementary Figs. 37–40).

The diastereoselectivity imparted in **7** by phosphate is therefore twofold: phosphate drives the co-precipitation, purification and concentration of the *threo*-isomer, while catalysing the conversion of the *erythro*-isomer into an unreactive by-product. That the chemistry is wholly predisposed to favour the genetically useful isomer is an interesting and unprecedented result.

**Nucleobase elaboration.** Phosphate-buffered addition of cyanoacetylene **8** to pentose aminooxazolines **5** in near-neutral water quantitatively converts them to 2,2'-anhydrocytidines<sup>11,20</sup>, building the nucleobase on the sugar scaffold. When *threo*-**7** was mixed with **8** at pH 7, competitive addition to both nucleophilic sites on the molecule was observed, forming *threo*-**9** and *threo*-**15** as the predominant products in 40% yield after 24 h (Supplementary Fig. 53). A portion of *threo*-**7** remains undissolved as its solid phosphate salt. If the salt is dissolved at pH 8.5 before addition of cyanoacetylene **8**, and the pH is then lowered to 7.0, a higher 74% combined yield is obtained (Fig. 4a and Supplementary Fig. 56). The NMR spectrum in D<sub>2</sub>O very clearly shows that only two species derived from *threo*-**7** are present after 24 h, *threo*-**9a** and *threo*-**15a** (Supplementary Fig. 62). However, addition of **8** to the 3'-amine does not inhibit addition to the aminooxazoline<sup>35</sup>, and once excess cyanoacetylene **8** has been consumed, *threo*-**15** slowly converts to *threo*-**9** and cyanoacetaldehyde **16**. If no adjustment to the reaction conditions are made, hydrolysis of *threo*-**15** to *threo*-**9** (33%) is complete after 28 days, but this sluggish hydrolysis is then accompanied by hydrolysis of *threo*-**17**, which forms in 22% yield after 28 days (Supplementary Fig. 57). Some precipitation of nucleoside-derived products is seen to occur over this time, alongside extensive precipitation of cyanoacetaldehyde oligomer products, which leads to partial enrichment of nucleosides in the supernatant. However, the sluggish hydrolysis of *threo*-**15** to *threo*-**9** can be greatly accelerated, for example by

**Table 1 | Three-component coupling of aldehyde 1, oxazole 3 and aminonitrile 6 to yield amino-nucleotide precursor 7 in water**

Entry	R	pH	t (days)	12 (%)	7 (%)	13 (%)	<i>threo:erythro</i>
1	H	7	1	40	0	0	1:3
2	H	5	1	94	0	0	1:3
3	H	4	1	60	0	0	1:3
4 <sup>a</sup>	H	5	10	70	0	0	1:3
5	H	5	15 <sup>b</sup>	19	37	38	1:3
6	Me	5	5	43	37	12	1.2:1
7	Me	4.5	5	22	52	18	1:1
8	<sup>i</sup> Pr	4.5	5	4	73	19	1.3:1
9	<sup>i</sup> Bu	4.5	5	8	57	21	1:1
10	<sup>n</sup> Bu	4.5	5	4	79	17	1.3:1

Optimization of three-component coupling of glycolaldehyde (**1**; 0.2 M) and 2-aminooxazole (**3**; 0.2 M) with aminonitrile (**6**; 0.6 M) at room temperature. <sup>a</sup>10 mM **1**, 10 mM **3** and 30 mM **6**. <sup>b</sup>Room temperature for one day, then 60 °C for 14 days. R' = H or CH<sub>2</sub>CH<sub>3</sub>

acid catalysis, and at pH 2 the conversion is rapid (<10 min) and quantitative.

These results demonstrate that the skeletal structure of 3'-amino-*threo*-nucleosides can be regioselectively established in only two steps from prebiotically plausible two- and three-carbon units. Formation of amino-nucleotides would then require anomerization of the nucleobase and phosphorylation. We set out to consider how these could have occurred on the early Earth. Anhydronucleoside hydrolysis seems to be the disposed pathway for anhydronucleosides in water (Fig. 4b). It is very sluggish, taking weeks to complete at neutral pH, but is accelerated at higher pH, occurring within 6 h at pH 8 or within 10 min at pH 11, to furnish *threo*-17 (quant.). However, *threo*-17 has not been shown to be an effective component of a proto-genetic material, and by comparison with the weaker duplex formation in  $\alpha$ -DNA<sup>36</sup>, would be expected to form less stable duplexes than polymers of *threo*-10. Therefore, if *threo*-17 were to be a precursor to a genetic polymer on the early Earth, anomerization of the 1' stereocentre would probably have been highly beneficial.

**Photochemical anomerization.** Irradiation at 254 nm is the most promising mechanism uncovered so far for anomerization of pyrimidine nucleosides. However, for canonical nucleobases this process is still inefficient, with their nucleotides undergoing condensation to form oxazolidinones as the major product upon irradiation, with anomerization only occurring in 4–6% yield<sup>37</sup>. In contrast, 2-thiocytosine ribonucleosides undergo extremely clean anomerization to preferentially form a *trans*-disposition between the 1' and 2' substituents<sup>38</sup>. We recently demonstrated that the same anomerization can occur on a *threo* skeleton<sup>11</sup>, and therefore decided to investigate amino-nucleoside photoanomerization. As expected, when *threo*-17 was irradiated at 254 nm in neutral water, it slowly formed oxazolidinone **13** along with base release to give cytosine **18** (Fig. 4b and Supplementary Figs. 78 and 79). We therefore considered how sulfur would have been incorporated into *threo*-9 to give *threo*-19.

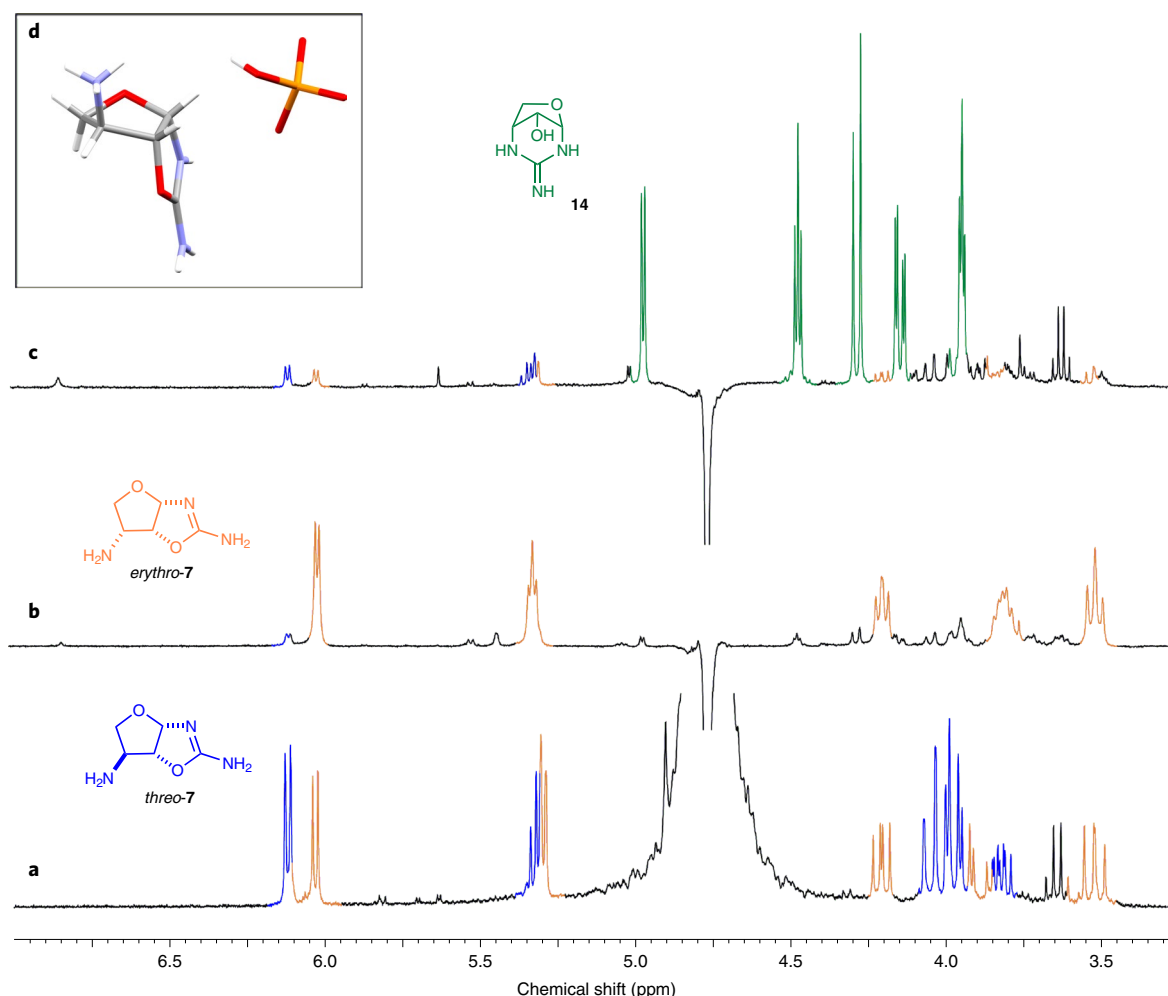
Thiolysis of  $\alpha$ -anhydrocytidines occurs in the presence of sodium hydrosulfide in formamide<sup>11,38</sup>. Although these conditions are not trivial to rationalize as prebiotically plausible, all the

chemical inputs are individually plausible, and the demonstrated efficiency and value of this chemical transformation outweighs speculative plausibility at this stage—it seems reasonable that there may be a different and more plausible route to *threo*-19, and it would be counterproductive to discount uniquely effective chemistry on unverifiable grounds of plausibility. We therefore tested the reaction of *threo*-9 and hydrosulfide in dimethylformamide (DMF) and observed quantitative conversion to *threo*-19 in the crude NMR (Supplementary Fig. 74). Similar, although less clean, thiolysis was observed in formamide (Supplementary Fig. 75).

When **19** was irradiated at 254 nm in the presence of H<sub>2</sub>S in neutral water, clean anomerization was found to occur, providing *threo*-20 (65%) along with 7% 2-thiocytosine (Fig. 4c,d and Supplementary Figs. 76 and 77). The 2-thiopyrimidines are intriguing molecules from the point of view of Watson–Crick base pairing. For example, 2-thiouridine and 2-thiocytidine are found in transfer RNA (tRNA)<sup>39,40</sup> and the enhanced selectivity for U:A base pairing over the U:G wobble pair when uridine is replaced by 2-thiouridine<sup>41</sup> has been exploited to improve the fidelity of non-enzymatic primer extension<sup>42</sup> and ribozyme-catalysed copying of RNA<sup>43</sup>. Further to this, 5-methyl-2-thiocytidine has been shown to selectively bind with inosine<sup>44</sup>, which has been considered as a likely precursor to guanosine in early genetic materials due to its easier synthesis and greatly increased solubility<sup>45</sup>. 2-thioU:A and 2-thioC:I Watson–Crick pairing deserves further investigation from the point of view of duplex stability and copying efficiency, particularly in light of these compounds potentially being synthetic precursors to the oxygenic variants.

How the cytosine-nucleoside *threo*-10 could have formed from its 2-thiocytosine presents an interesting problem here due to the nucleophilicity of the 3'-amine. Activation of the sulfur, either by alkylation or oxidation, would render it a better leaving group and so greatly accelerate substitution at this position<sup>11</sup>, but in the case of *threo*-20, such activation leads to rapid cyclization to **24** (Fig. 5 and Supplementary Fig. 80), even at pH 5, where the amine would be highly protonated (we have not observed either cyclization or hydrolysis of *threo*-20 in the absence of an activating agent in rigorously deoxygenated water, even over several weeks, although we cannot rule out that substitution occurs extremely slowly).





**Fig. 3 | Stereochemical resolution and crystallization of *threo*-7.** The precipitation of *threo*-7 and rearrangement of *erythro*-7 to **14** in phosphate buffer are observed to result in stereoselective synthesis of *threo*-7. **a–c**,  $^1\text{H}$  NMR spectra to show the progression of stereochemical resolution upon addition of phosphate to *threo*-7 and *erythro*-7: (400 MHz,  $\text{D}_2\text{O}$ , zg30, 7.0–3.3 ppm) a mixture of *threo*-7 (blue) and *erythro*-7 (orange) (200 mM, 3:2) in  $\text{D}_2\text{O}$  at pD 6.5 without the addition of phosphate (**a**); (400 MHz,  $\text{H}_2\text{O}/\text{D}_2\text{O}$  9:1, noesygppr1d, 7.0–3.3 ppm) mixture of *threo*-7 (blue) and *erythro*-7 (orange) (200 mM, 3:2) in 200 mM phosphate at pH 6.5, soluble fraction after 1 h (**b**); (400 MHz,  $\text{H}_2\text{O}/\text{D}_2\text{O}$  9:1, noesygppr1d, 7.0–3.3 ppm) soluble fraction after 20 h showing formation of **14** (green) (**c**). **d**, Crystal structure of *threo*-7· $\text{H}_3\text{PO}_4$ ; grey, carbon; white, hydrogen; red, oxygen; blue, nitrogen; orange, phosphorus. CCDC 2087673. Note: all structures are racemates; L-enantiomers are shown for clarity.

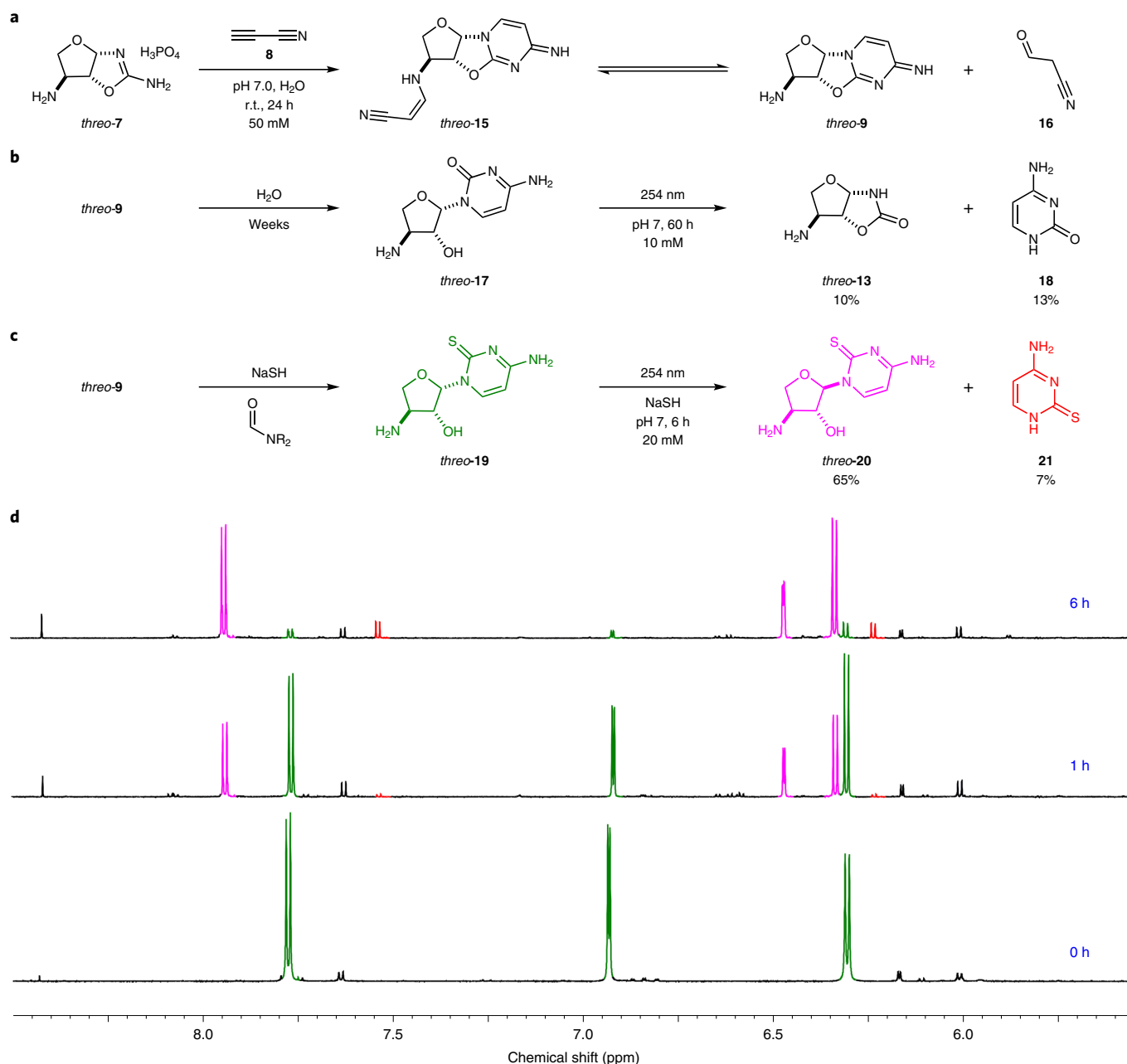
Considering the issue of cyclization, we next reflected on our target structure and realized it was the phosphorylated amino-TNA nucleotide, not the unphosphorylated nucleoside. Accordingly, we recognized that this cyclization problem may have arisen by assuming that the nucleoside should be an intermediate in the synthesis. The nucleophilicity of the amine would be suppressed by phosphorylation, and therefore the solution to monomer cyclization may lie in incorporation of the intermediate into a nucleotide—where we expected cyclization to be suppressed in favour of hydrolysis—before converting the pyrimidine to the canonical nucleobase.

**Triphosphorylation.** Nucleoside phosphorylation is extremely challenging in water and has remained a long-standing problem, with reasonably efficient phosphorylation only being seen for vicinal diols in water<sup>46</sup>. Furthermore, mechanisms by which nucleoside triphosphates (NTPs), nature's chosen monomers and universal energy currency, could have formed on the early Earth are even more poorly understood than the formation of nucleoside monophosphates—the most promising methods all employ nucleoside monophosphates as a starting point<sup>46,47</sup>, but such processes compete

with formation of inorganic polyphosphates<sup>48</sup>, leaving the question of how nucleoside monophosphates could form in the first place an open question. Amino-nucleosides provide a potential way around this problem due to the increased nucleophilicity of the amine moiety relative to the solvent water<sup>49</sup>. We reasoned that if an amino-nucleoside were exposed to a condensed phosphate species, nucleoside polyphosphates could form directly and selectively in water.

We elected to study the interaction between *threo*-20 and cyclic trimetaphosphate,  $(\text{PO}_3\text{Na})_3$  (ref. 50). In neutral water at room temperature, no reaction was seen to occur over five days, so we investigated the effect of pH. If the phosphorylation is initiated in alkaline solution (pD 12) with 6 equiv.  $(\text{PO}_3\text{Na})_3$ , 50% NTP *threo*-22 is observed to form over 25 days (Supplementary Fig. 85). Over this period the solution also fell to pD 9, so we next initiated the phosphorylation at pD 9 in the presence of a divalent metal ion ( $\text{Mg}^{2+}$ ) to accelerate the reaction, and we observed 60% *threo*-22 form after only 5 days (Fig. 5c and Supplementary Fig. 82).

We next exposed *threo*-22 to sulfur-activating agents (such as  $\text{H}_2\text{O}_2$ ) and found that hydrolysis to *threo*-23 and *threo*-10 occurs,

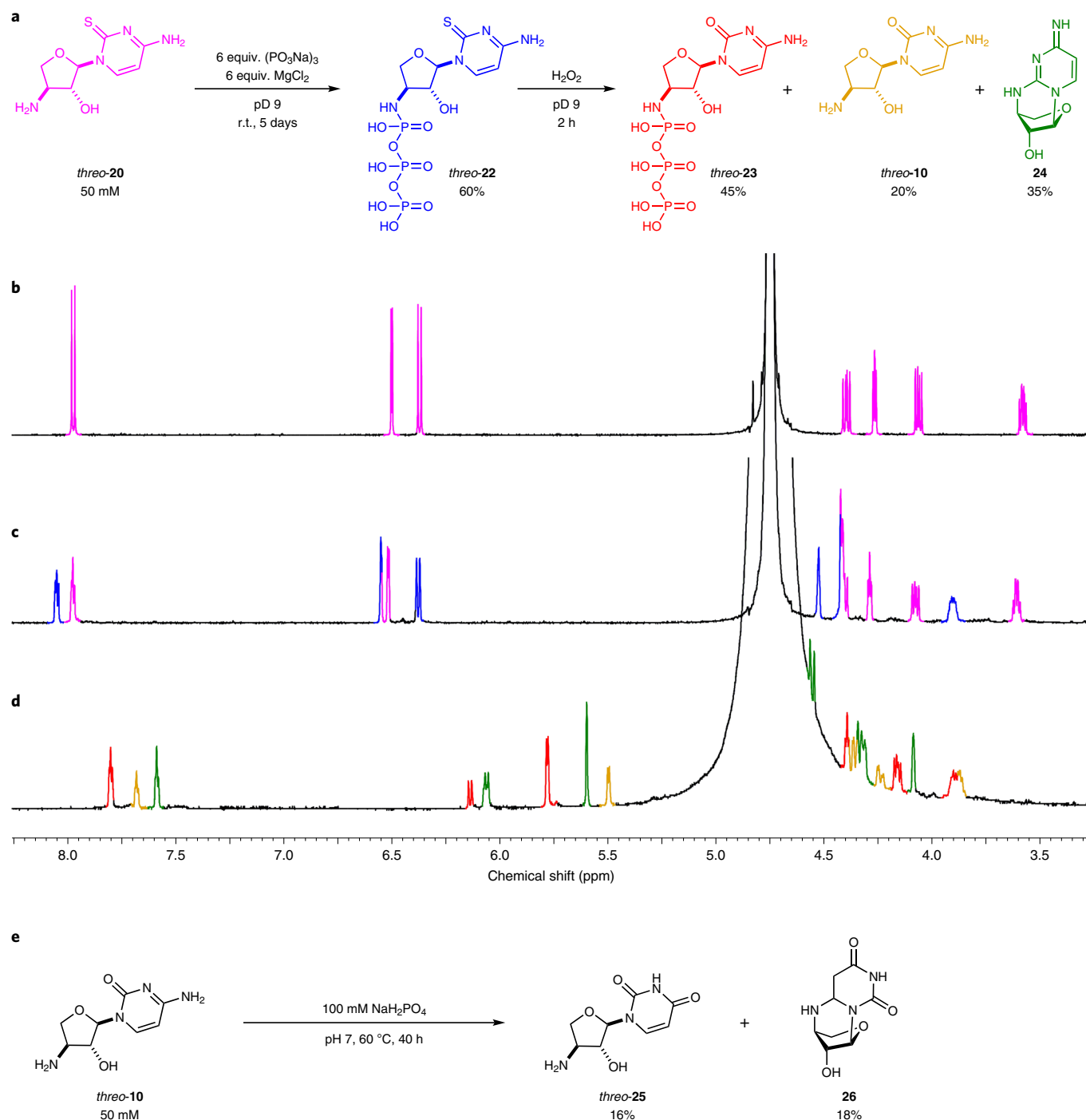


**Fig. 4 | Formation of anhydrocytidine *threo*-9 and its photochemical products.** **a**, Reaction of *threo*-7 with cyanoacetylene **8** yields anhydronucleoside *threo*-9 and aldehyde **16**. **b**, Hydrolysis of *threo*-9 to *threo*-17 and subsequent photochemical formation of *threo*-13 and cytosine **18**. **c**, Thiolysis of *threo*-9 to *threo*-19 and subsequent photochemical anomerization of *threo*-19 to *threo*-20, along with thiocytosine **21**. R = Me or H. **d**,  $^1\text{H NMR}$  (700 MHz,  $\text{H}_2\text{O}/\text{D}_2\text{O}$  9:1, noesygppr1d, 5.5–8.5 ppm) spectra showing the photoanomerization of *threo*-19 to yield *threo*-20 and **21** on irradiation at 245 nm in water. Note: all structures are racemates; L-enantiomers are shown for clarity, and intermediate *threo*-15 is observed to be a mixture of *E*- and *Z*-cyanovinyl isomers. r.t., room temperature.

demonstrating that phosphorylation of the 3'-NH<sub>2</sub> enables selective hydrolysis to occur in place of the cyclization that is observed without phosphorylation (Fig. 5d and Supplementary Fig. 86). Further hydrolysis of the cytosine base to uracil can also occur to give both canonical pyrimidine bases, a process that is accelerated by heating in phosphate buffer (Fig. 5e and Supplementary Fig. 88). *threo*-10 undergoes hydrolysis (34% after 40 h) to furnish *threo*-25 at 40 °C in 100 mM phosphate at neutral pH. Uridine *threo*-25 is observed to also undergo reversible addition of its 3'-amine to the C6-carbon of the uracil base when heated to yield **26**.

### Summary and outlook

We have found that regio- and stereoselective incorporation of an amine into a nucleotide sugar moiety can be achieved with prebiotically plausible reactions. The 3'-amino-TNA NTPs are formed readily from two- and three-carbon building blocks. The phosphate-mediated diastereoselectivities of these reactions, for *threo*-nucleotides, are unparalleled in prebiotically plausible nucleotide syntheses, and the 3'-amino-isomer that is exclusively formed has been shown to form more stable duplexes with both itself and with RNA than the 2'-amino-isomer<sup>12</sup>. The ease and selectivity for the formation of 3'-amino-TNA monomers suggests that they could



**Fig. 5 | Aqueous phosphorylation of amino-nucleoside *threo*-20 and hydrolysis to canonical nucleobases.** **a**, Phosphorylation of *threo*-20 with cyclic trimetaphosphate ( $\text{PO}_3\text{Na}$ )<sub>3</sub> in water, catalysed by  $\text{MgCl}_2$ , and subsequent oxidative hydrolysis to cytosine-containing nucleotide *threo*-23. **b–d**,  $^1\text{H}$  NMR (700 MHz,  $\text{D}_2\text{O}$ , 3.25–8.25 ppm) spectra showing the reaction of 50 mM *threo*-20, 6 equiv. ( $\text{PO}_3\text{Na}$ )<sub>3</sub>, 6 equiv.  $\text{MgCl}_2$ , pH 9 after 1 h (**b**), 5 days (**c**) and subsequent reaction of the solution shown in spectrum **c** with 3 equiv.  $\text{H}_2\text{O}_2$ , resulting in the formation of *threo*-23, *threo*-10 and **24** (**d**). Note that the apparent change in multiplicity at C6–H is due to partial deuteration at C5–H. **e**, Phosphate-catalysed hydrolysis of cytidine *threo*-10 to uridine *threo*-25.

have been present on the early Earth. Indeed, our results suggest that 3'-amino-TNA nucleosides are no more 'generationally' complex than RNA nucleosides, in stark contrast to the predictions made by Eschenmoser<sup>3,15</sup>. Our initial expectation, at the outset of our work, was that selective 2'-alcohol phosphorylation would be required for 3'-amino-TNA synthesis. However, guided by the reactivity of the molecules, we have discovered that these amino-nucleosides react selectively with ( $\text{PO}_3\text{Na}$ )<sub>3</sub> in water to yield NTPs.

Amino-nucleotides have been employed experimentally to overcome the issues plaguing non-enzymatic replication of RNA. Despite decades of excellent work<sup>1,51</sup>, RNA-templated primer extensions using all four nucleobases and chemically activated ribonucleotides have reached a copying limit of about seven nucleotides<sup>52</sup>. The increased nucleophilicity of amine moieties compared with hydroxyls enables superior templated ligations<sup>53–59</sup>, but, as non-biological molecules, amino-nucleotides have so far been discussed explicitly

as model substrates with no prebiotic relevance. Our results challenge this view. The 3'-amino-TNA NTPs we have discovered do not possess a free amine group, and so would not necessarily be expected to be more nucleophilic than ribonucleotides, but, unlike ribonucleosides, they are readily and selectively phosphorylated in water.

How ribonucleoside triphosphates (or other activated ribonucleoside-5'-phosphates) could have formed under aqueous conditions is an unsolved question. Phosphorylation of ribonucleotides with  $(\text{PO}_3\text{Na})_3$  in water not only requires extremely alkaline conditions ( $\text{pH} > 12$ ), but also results in a mixture of deactivated 2'- and 3'-monophosphates<sup>60</sup>. In comparison, 3'-amino-TNA nucleosides can be selectively triphosphorylated in water. It is worth noting that although chemical (phosphorus-anhydride) activation towards ligation<sup>61,62</sup> is retained, polymerization of these amido-triphosphates has not yet been studied, and although triphosphates are universally used as activated nucleotide monomers in biology, as well as being substrates for (ribozyme) polymerases, NTPs have been less well studied as substrates in non-enzymatic replication than the experimentally more convenient phosphorimidazolides<sup>52,59</sup>. This is due to their relatively slow (albeit more selective) ligation<sup>63</sup>, as well as the apparent difficulties of their synthesis on the early Earth. We hope that our discovery of a selective NTP synthesis will promote further exploration of this space.

Overall, our results suggest 3'-amino-TNA triphosphates appear to be generationally simpler than the triphosphates of RNA or DNA. When combined with the known performance of 3'-amino-TNA in self and interspecies Watson–Crick base pairing with D/RNA, this may suggest that these molecules played a role in the origins of life. Important gaps remain in our understanding of how best to drive and control prebiotic (non-enzymatic) nucleotide ligation and replication for all nucleic acids. The implications of 3'-amino-threo-cytidine and uridine synthesis, and the scenario for continuous (in situ) monomer phosphorylation and activation, opened by their enhanced reactivity in water, will be the object of further studies and future comparisons of the generational simplicity of RNA with 3'-amino-TNA.

## Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41557-022-00982-5>.

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## Methods

**Caution statement.** Cyanide and (hydro)sulfide are highly toxic poisons by inhalation, contact and ingestion. They generate poisonous hydrogen cyanide ( $pK_a = 9.2$ ) and hydrogen sulfide ( $pK_a = 7.1$ ) gas at neutral or acidic pH. Solutions containing cyanide or (hydro)sulfide, or compounds that may generate these, must be handled in a well-ventilated fumehood equipped with appropriate chemical quenchers, such as sodium hypochlorite (bleach) or iron(II) sulfate solution. Read and follow the material safety data sheet (MSDS) instructions for personnel handling, exposure and disposal information.

### General procedure for three-component coupling to form diaminooxazolines.

Glycolaldehyde **1** (12 mg, 0.2 mmol), 2-aminooxazole **3** (17 mg, 0.2 mmol) and aminonitrile hydrochloride **6.HCl** (0.6 mmol) were dissolved in  $H_2O$  (800  $\mu$ l), and the solution was adjusted to pH 5 with 4 M NaOH and 4 M HCl. Methylsulfonylmethane (internal standard; 100  $\mu$ l of a 0.083 M solution in  $D_2O$ ) was added, then the solution volume was increased to 1 ml with  $H_2O$ .  $^1H$  NMR spectra were then obtained periodically.

**General procedure for the reaction of *threo*-7 with cyanoacetylene **8**.** *threo*-7.  $H_3PO_4$  (0.05 mmol) was suspended in  $H_2O$  (400  $\mu$ l). The solution was adjusted to pH 8.5 to ensure full dissolution of the salt, then the solution was adjusted to the desired pH with 4 M NaOH or 4 M HCl. Cyanoacetylene **8** (0.5 ml of a 1 M solution in  $H_2O$ , 10 equiv.) was added, followed by methylsulfonyl methane (internal standard; 100  $\mu$ l of a 0.083 M solution in  $D_2O$ , 0.0083 mmol). The volume of the resultant solution was increased to 1 ml with  $H_2O$ , and NMR spectra were obtained periodically.

**General procedure for the thiolysis of *threo*-9 in formamide.** *threo*-9. 2HCl (50 mg, 0.2 mmol) and  $NaSH \cdot xH_2O$  (110 mg, 1 mmol) were sealed under argon in a flame-dried flask. Anhydrous formamide (2 ml) was added and the yellow suspension was stirred at room temperature for three days. The reaction was quenched with HCl (0.04 M, 10 ml), and argon was bubbled through the suspension into a bleach trap for 30 min to remove excess  $H_2S$ . The suspension was concentrated under reduced pressure, and co-evaporated with  $D_2O$  ( $3 \times 3$  ml—to partially deuterate the formamide  $NH_2$ ) then mixed with  $D_2O$  (3 ml) and analysed by NMR spectroscopy.

**General procedure for the photochemical anomerization of *threo*-19.** *threo*-19 (2.4 mg, 0.01 mmol) and  $NaSH \cdot xH_2O$  (2.6 mg, 0.02 mmol) were dissolved in  $H_2O$  (0.45 ml), and the solution was adjusted to pH 7 with 4 M HCl. Methylsulfonylmethane (internal standard; 40  $\mu$ l of a 0.083 M solution in  $D_2O$ , 0.003 mmol) was added then the solution was transferred to a quartz NMR tube

and irradiated with UV light (principal emission at 254 nm). NMR spectra were obtained periodically.

**General procedure for the aqueous triphosphorylation of *threo*-20.** *threo*-20 (0.02 mmol),  $(PO_3Na)_3$  (36 mg, 0.12 mmol, 6 equiv.) and  $MgCl_2$  (6 mg, 0.06 mmol) were suspended in  $D_2O$  (0.4 ml), the solution was adjusted to pD 9 with 4 M NaOD, and then NMR spectra were obtained periodically. After 5 days the solution had fallen to pD 7.8. The solution was adjusted to pD 9 with 4 M NaOD, and then NMR spectra were again acquired periodically.

## Data availability

All data (experimental procedures and characterization data) supporting the findings of this study are available within the article and its Supplementary Information. Crystallographic data for the *threo*-7- $H_3PO_4$  reported in this article have been deposited at the Cambridge Crystallographic Data Centre (CCDC) under deposition number 2087673. Copies of the data can be obtained free of charge from CCDC via <https://www.ccdc.cam.ac.uk/structures/>.

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## Author contributions

M.W.P. conceived the research. M.W.P. and D.W. designed and analysed the experiments and wrote the manuscript. D.W. conducted the experiments.

## Competing interests

The authors declare no competing interests.

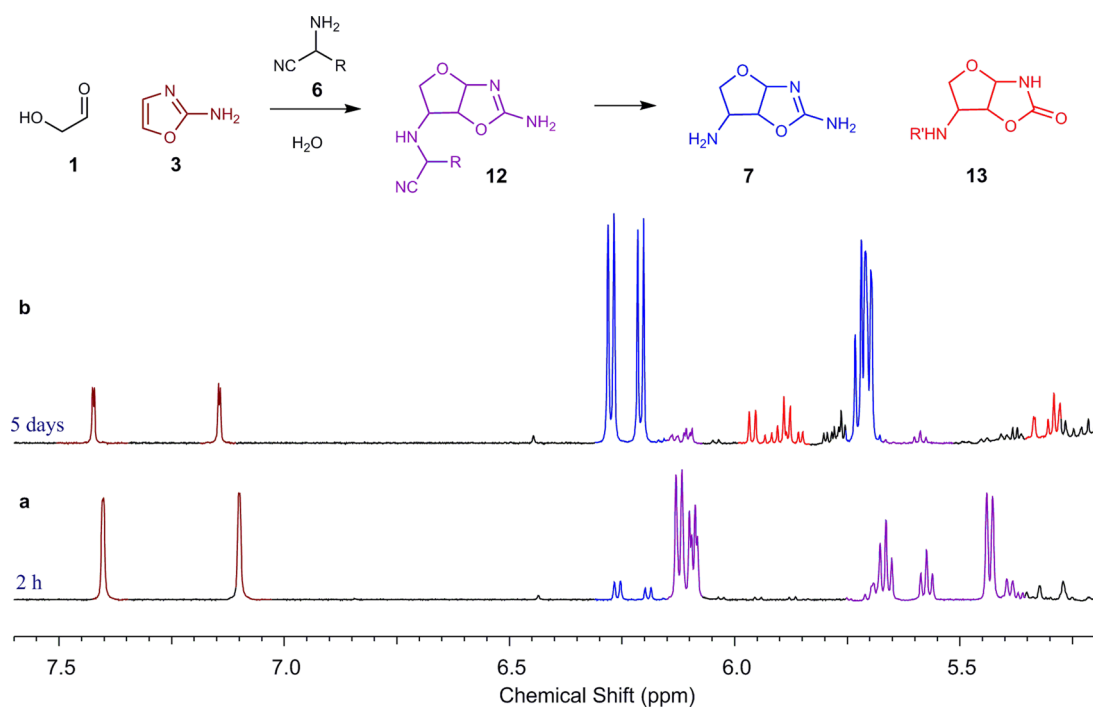
## Additional information

**Extended data** is available for this paper at <https://doi.org/10.1038/s41557-022-00982-5>.

**Supplementary information** The online version contains supplementary material available at <https://doi.org/10.1038/s41557-022-00982-5>.

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**Extended Data Fig. 1 | Three-component coupling of aldehyde **1**, oxazole **3** and aminonitrile **6e** to yield amino-nucleotide precursor **7** in water.** <sup>1</sup>H NMR spectra [400 MHz, H<sub>2</sub>O/D<sub>2</sub>O (9:1), 7.6–5.2 ppm] to show the: **a**, formation of oxazoline **12e** (R = <sup>i</sup>Bu) after 2 h at room temperature and pH 4.5 and **b**, retro-Strecker of **12e** at room temperature and pH 4.5 to form **7** after 5 days.