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Prebiotic phosphorylation of 2-thiouridine provides either nucleotides or DNA building blocks via photoreduction

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Breakthroughs in the study of the origin of life have demonstrated how some of the building blocks essential to biology could have been formed under various primordial scenarios, and could therefore have contributed to the chemical evolution of life. Missing building blocks are then sometimes inferred to be products of primitive biosynthesis, which can stretch the limits of plausibility. Here, we demonstrate the synthesis of 2′-deoxy-2-thiouridine, and subsequently 2′-deoxyadenosine and 2-deoxyribose, under prebiotic conditions. 2′-Deoxy-2-thiouridine is produced by photoreduction of 2,2′-anhydro-2-thiouridine, which is in turn formed by phosphorylation of 2-thiouridine—an intermediate of prebiotic RNA synthesis. 2′-Deoxy-2-thiouridine is an effective deoxyribosylating agent and may have functioned as such in either abiotic or proto-enzyme-catalysed pathways to DNA, as demonstrated by its conversion to 2′-deoxyadenosine by reaction with adenine, and 2-deoxyribose by hydrolysis. An alternative prebiotic phosphorylation of 2-thiouridine leads to the formation of its 5′-phosphate, showing that hypotheses in which 2-thiouridine was a key component of early RNA sequences are within the bounds of synthetic credibility.

Non-canonical, sulfur-containing nucleobases are utilized by extant biology[1,2], and exert interesting effects on the stability, base-pairing properties and replication fidelity of nucleic acid polymers[3]. As such—and given the key role of sulfur in established prebiotic synthetic scenarios[4,5]—they are potential components of a genetic system that either complemented or perhaps predated, and ultimately evolved into, enzymatically replicated canonical RNA and DNA. We recently demonstrated a viable pathway to pyrimidine ribonucleosides and ribonucleotides via thioribonucleosides (Fig. 1)[5]. Highly crystalline ribo-aminooxazoline **1** was shown to react with cyanoacetylene **2** to give anhydronucleoside **3**, which on thiolysis furnished α-2-thioribocytidine **4**. **4** could be efficiently photoanomerized to β-2-thioribocytidine **5**, which serves as a precursor to the canonical pyrimidine ribonucleosides cytidine **6** and uridine **7**, β-2-thiouridine **8**, and oligomerizable[6] cyclic phosphates of cytidine and uridine (**9** and **10**, respectively).

Since we had demonstrated that thioribonucleosides **5** and **8** are probably formed in primordial pyrimidine synthesis, we set out to further explore prebiotic phosphorylation conditions that might yield oligomerizable thioribonucleotides. Herein, we demonstrate that phosphorylation of 2-thiouridine **8** can either lead to an oligomerizable nucleotide or initiate a prebiotic synthesis of a deoxythionucleoside, 2′-deoxy-2-thiouridine **11**, via hydrogen sulfide-mediated photoreduction as the key step. We show that **11** is a productive transglycosylating reagent in an abiotic or proto-enzymatic context; for example, in the supply of 2-deoxyribose **12** and the synthesis of a canonical 2′-deoxynucleoside, 2′-deoxyadenosine **13**. These results provide a chemical connection between RNA and DNA in a prebiotic context (that is, in the absence of biosynthesis).

Results and discussion

5′ Phosphorylation. First, we attempted the phosphorylation of 2-thiouridine **8** in hot formamide[7], but increased the concentrations of phosphate and nucleoside from 100 mM to 200 mM, compared with our previous phosphorylation of cytidine (full experimental details are included in the Supplementary Information). After heating at 100 °C for 20 h, we observed the initially formed phosphorylated product, thiouridine-5′-phosphate **14**, in 24% yield alongside the starting material **8** in 42% yield and some minor products (Fig. 2 and Supplementary Fig. 39). In hot urea, ribonucleosides generally undergo initial (kinetic) 5′ phosphorylation[8]; over time, the 2′,3′-cyclic phosphate dominates over the 5′-phosphate (thermodynamic control). The formation of phosphate monoesters in hot urea (and formamide) is reversible but slow, such that clear-cut kinetic or thermodynamic control is not shown by such systems. Szostak and coworkers[3,9] have already shown that non-enzymatic RNA replication using the 2-methyl- and 2-amino-imidazole-activated derivatives of **14** proceeds with a higher rate and fidelity than the corresponding activated uridine nucleotide, but until now a prebiotic provenance of **14** remained unestablished. These results show that the kinetic product of phosphorylation—the 5′ nucleotide **14**—is indeed accessible, and would become increasingly plausible if progressively milder prebiotically plausible phosphorylation procedures are uncovered. Our synthesis of **14** shows that it could have been available for non-enzymatic incorporation into primordial RNA sequences, and provides further impetus for the investigation of the potential role of 2-thiouridine in RNA.

Thioanhydride formation. Semi-molten urea is an alternative prebiotic phosphorylation medium to hot formamide[8], so we next used it to attempt phosphorylation of **8** (Fig. 3a). Surprisingly, no

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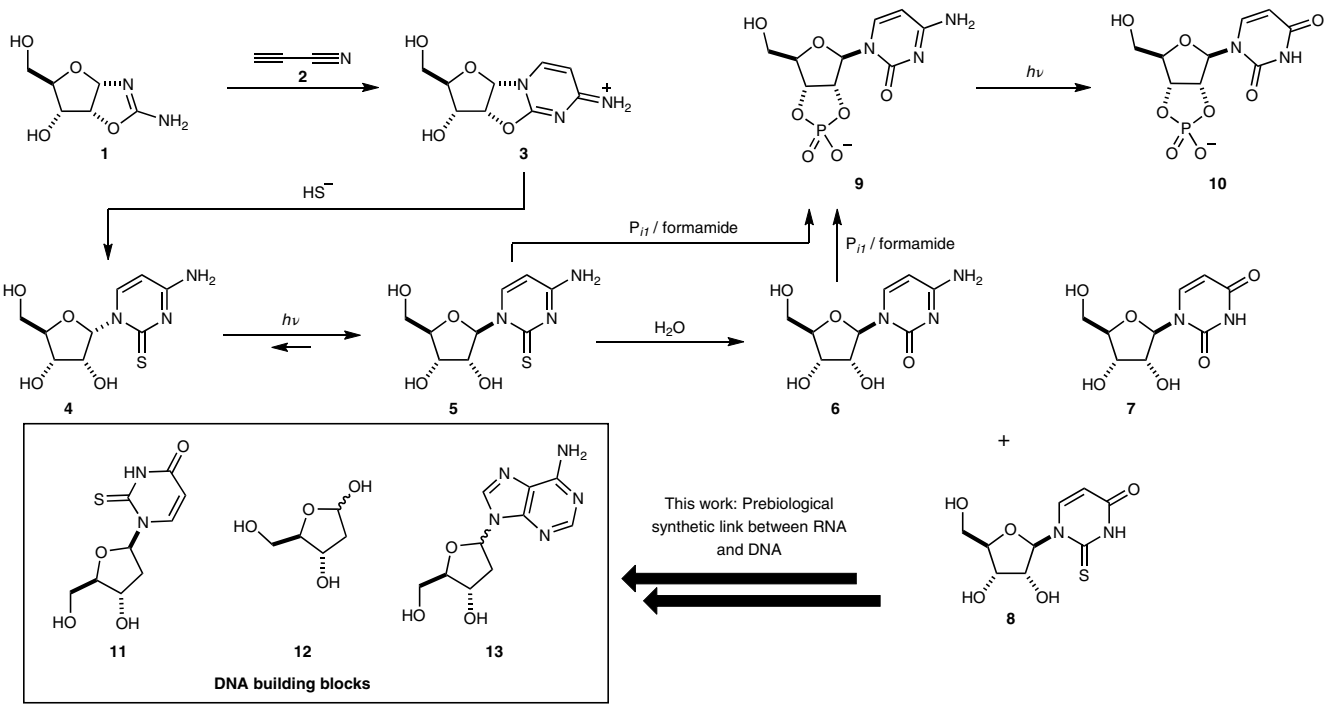

Fig. 1 | Summary of previous and present work. Previous work demonstrated the synthesis of the canonical pyrimidine RNA nucleotides **9** and **10** alongside other RNA building blocks, such as 2-thiouridine **8**. This work (boxed) demonstrates a link between **8** and a range of DNA building blocks, via prebiotic phosphorylation and photoreduction. hν, UV irradiation; P_{i1}, $NH_4H_2PO_4$/urea.

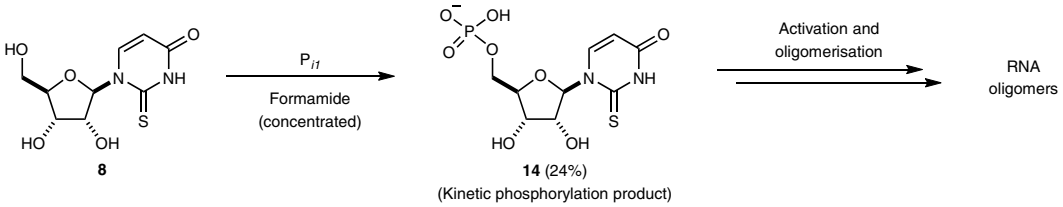

Fig. 2 | 5′ phosphorylation of 2-thiouridine 8 in concentrated formamide solution. Prebiotically plausible conversion of **8** into 2-thiouridine-5′-phosphate **14** in hot formamide potentially enables incorporation of **8** into primordial RNA sequences, by previously developed prebiotic activation of nucleoside phosphates and oligomerization chemistry[3,9].

2′,3′-cyclic nucleotide derivatives were observed. A major product had been formed, but a total of four monophosphate ^{31}P NMR resonances suggested that the hydroxyl groups of this product were phosphorylated to varying degrees, complexifying the NMR spectra. The crude mixture from the phosphorylation reaction was therefore treated with alkaline phosphatase, generating a single major nucleoside product. Extensive analysis of both one- and two-dimensional NMR spectra of the dephosphorylated product suggested the formation of 2′,2-thioanhydronucleoside **15a**[10]. Previously, Hampton and Nichol[11] synthesized a related derivative, 2′,2-anhydro-uridine **16**, when uridine **7** was treated with diphenylcarbonate in hot dimethylformamide (Fig. 3b). Under those conditions, intermediate uridine 2′,3′-cyclic carbonate[11] **17a** is a good substrate for ring opening at C2′ by 2-O to give cyclonucleoside **16**. Thus, under phosphorylation conditions, we propose cyclic phosphate **18** as the intermediate in the formation of **15** ($R_1 = R_2 = H$ or PO_3H^-), with rapid ring opening by the nucleophilic sulfur atom ensuing to convert **18** to **15**. To confirm the structure of the product and support our proposed mechanism, 2′,2-anhydro-2-thiouridine **15a** was unambiguously synthesized from 2-thiouridine **8** and diphenylcarbonate (Fig. 3b). The fully characterized synthetic standard was used for spiking experiments to confirm that the major product of phosphorylation of **8** (even before enzymatic dephosphorylation) was indeed **15a**. After the crude reaction products from the phosphorylation were subjected to alkaline phosphatase (Fig. 3a), addition of an NMR integration standard (sodium formate) revealed that 2′,2-anhydro-2-thiouridine **15a** had been formed in 50% yield, accompanied by some glycosidic bond cleavage (2-thiouracil **19** (15%) and isocytosine **20** (7%)). To obtain the ratio of the anhydronucleoside to its phosphorylated derivatives before treatment with alkaline phosphatase, the crude mixture from the phosphorylation of **8** was subjected to analytical high performance liquid chromatography, which showed that the ratio of **15a**: **15b: 15c** was 3.7:1:1 (Supplementary Figs. 57 and 58). The same phosphorylation conditions were also applied to 2-thiocytidine **5** (Fig. 3a), which afforded 2′,2-anhydro-2-thiocytidine **21**[12] (43% yield), cytidine **6** (8%), diaminopyrimidine **22** (16%), 2-thiocytosine **23** (8%) and cytosine **24** (8%). All structures were confirmed by spiking experiments, with synthetic standards made in house or purchased (Supplementary Figs. 32 and 35).

To further support a cyclic phosphate intermediate, we sought milder conditions for phosphorylation of **8**. We chose diamidophosphate (DAP)—a reagent known to generate not only the

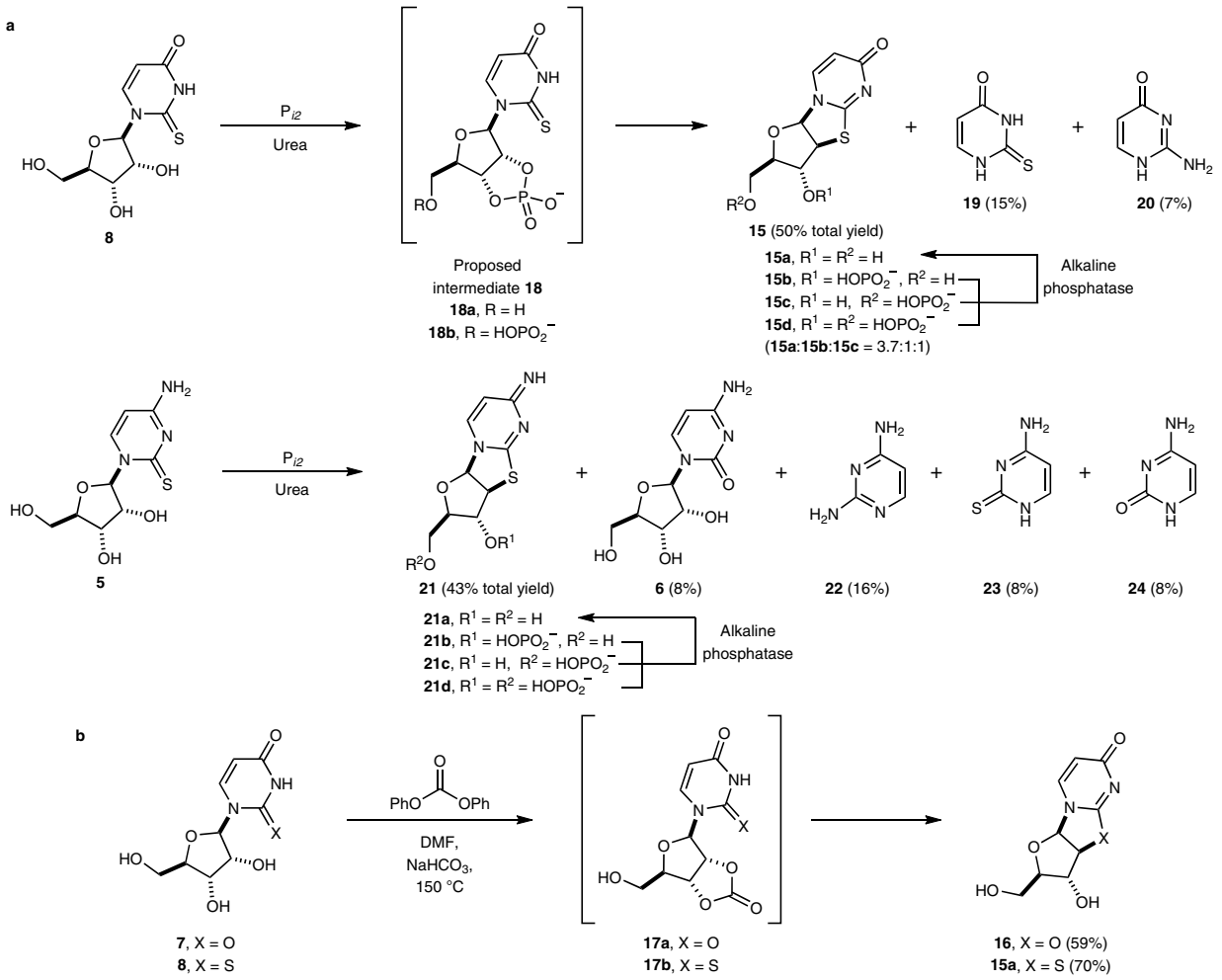

Fig. 3 | Prebiotic and synthetic routes to (thio)anhydronucleosides. a, Synthesis of the thioanyhydronucleosides of uridine **15** and cytidine **21** under prebiotic phosphorylation conditions. 2-Thiouridine **8** and 2-thiocytidine **5** both undergo phosphorylation to form the cyclophosphate intermediate **18** shown for the uridine variant. These intermediates undergo rearrangement to give structures of type **15** and **21**, respectively. **15d** was not directly observed, but is inferred from the mechanism. In addition to rearrangement, the nucleosides undergo some cleavage of the glycosidic C–N bond to yield free nucleobases **19** and **23**, as well as their hydrolysis/ammonolysis products **20**, **22** and **24**, respectively. **b**, Hampton and Nichol's synthesis of 2′,2-anhydrouridine **16** and our use of the same conditions to synthesize the sulfur analogue **15a** as a standard. **17a** and **17b** are the proposed intermediates leading to the formation of **16** and **15a**, respectively. P_{i2}, $Na_2HPO_4/NH_4Cl/NH_4HCO_3$.

corresponding 2′,3′-cyclophosphates from nucleosides, but also short oligonucleotides after prolonged reaction[13]. Indeed, reaction of 2-thionucleoside **8** with DAP and imidazole under moist-paste conditions at room temperature resulted in formation of the cyclic phosphate **18a** after one week (Fig. 4a). Monitoring the phosphorylation reaction by ^{31}P, ^{1}H and ^{13}C NMR, we observed the transformation of cyclophosphate **18a** to a new species that showed characteristic NMR, electrospray ionization high-resolution mass spectrometry and ultraviolet spectral signatures of anhydronucleotide **15b** (Supplementary Figs. 60–63). Furthermore, the DAP reaction of **8** does not stop at **15b**; the 3′ phosphate of **15b** reacts again with DAP, leading to the corresponding 3′-amidodiphosphate of the 2′,2-thioanhydronucleoside **25**. The total conversion (measured by ^{1}H NMR) of **8** to **15b/25** was 78% after one month at room temperature. When 4-thiouridine **26** was subjected to the same DAP phosphorylation reaction as a control, the expected cyclophosphate **27** was formed after a few days at room temperature, but no further transformation to the corresponding 2′,2-anhydride was observed (Fig. 4a and Supplementary Figs. 66 and 67). In this case, cyclization was presumably prevented due to the lower nucleophilicity of the oxygen on C2 compared with sulfur.

We also investigated the reaction of DAP (moist paste) with 2-thiocytidine **5**, which barely proceeded under the conditions employed for **8**. By lowering the pH of the reaction paste from 7 to 4 and raising the reaction temperature to 60 °C, we were able to detect cyclic phosphate **28**, and later we were able to identify the corresponding 3′-phosphate and 3′-amidopyrophosphate of thioanhydrocytidine (**21b** and **29**) by ^{31}P NMR (Fig. 4b and Supplementary Figs. 64 and 65). An accurate conversion was difficult to determine by ^{1}H NMR, but the combined yield for **28**, **21b** and **29** was estimated at slightly greater than 10%. The greater reactivity of 2-thiouridine **8** towards DAP compared with 2-thiocytidine **5** probably stems from its greater solubility and pK_a near neutrality (8.0, N3–H)[2].

C2′ reduction. Our syntheses of thioanhydronucleosides **15** and **21** from 2-thionucleosides **8** and **5** are the first prebiotically plausible routes to nucleoside derivatives containing a sulfur atom connected to C2′. Such compounds are of significant interest in the origins-of-life field because the potential conversion of a C–S to a C–H bond (a chemically facile process[14,15]) would result in the synthesis of 2′-deoxynucleosides—components of DNA. In mod-

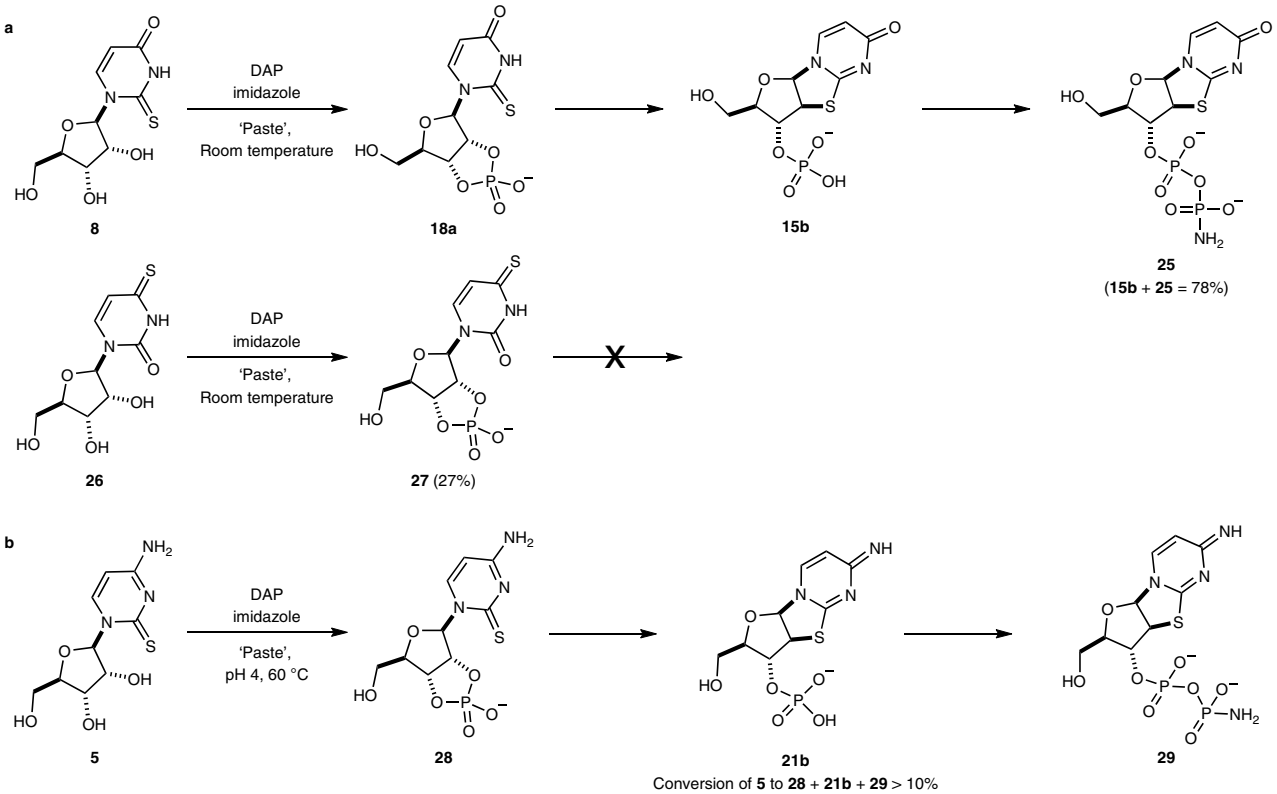

Fig. 4 | Phosphorylation of thionucleosides under mild conditions with DAP. a, Phosphorylation of 2-thiouridine **8** with DAP leads to the formation of thioanhydrouridine-3′-phosphate **15b** and corresponding 3′-amidodiphosphate **25** via the intermediate cyclophosphate **18a**. The same phosphorylation with 4-thiouridine **26** results in the cyclophosphate **27**, which can undergo no further transformation. **b**, Phosphorylation of 2-thiocytidine **5** with DAP under slightly more forcing conditions affords thioanhydrocytidine-3′-phosphate **21b** and corresponding 3′-amidodiphosphate **29**, again via an intermediate, cyclophosphate **28**.

ern biology, ribonucleotide reductases convert ribonucleotides to deoxyribonucleotides[16]. This enzymatic reduction proceeds via a radical mechanism with a dithiol as the stoichiometric reductant. We wondered if a mechanism involving sulfur-based reductants might have led to the formation of 2′-deoxyribonucleosides/2′-deoxyribonucleotides on early Earth, in the absence of complex enzymes, from 2′-thio-functionalized ribonucleosides. Nagyvary and coworkers[17–19] first experimentally investigated this idea by synthesizing 2′-thiocytidine (using an enzyme) and studying a variety of reduction procedures, including photoreduction. However, the limited published characterization data for the supposed 2′-thiocytidine substrate have since been pointed out as apparently inconsistent with the structure[20]. Recently, Powner and coworkers[21] revisited the idea with a proposal to modify the prebiotically plausible pyrimidine synthesis[5,22] by including a sulfur atom attached to C2′ from the outset. This route proved unsuccessful because the sulfur-containing building blocks exhibited different reactivity to analogues from the pyrimidine synthesis. Indeed, while a route from pure ribose or arabinose to deoxyribose has recently been established[23], a plausible abiotic synthesis of 2′-deoxynucleosides themselves—whether by desulfurization or other means—has not yet been reported. 2′,2-thioanhydronucleosides **15a** and **21a** were therefore subjected to ultraviolet irradiation at 254 nm in the presence of aqueous hydrogen sulfide as a reductant (Fig. 5a). Flux measurements from the same experimental setup have been carried out within our group previously, from which we determined a flux rate of 2.5×10^{16} cm^{-2} s^{-1} (19 mW cm^{-2}) for the 254-nm-centred emission[24]. Inspection of ^{1}H NMR spectra showed that, under these conditions, thioanhydrocytidine **21a** gave 2-thiocytosine **23** in 29% yield after 3 h of irradiation. However, thioanhydrouridine **15a** afforded two new nucleoside derivatives, as well as thiouracil **19** as major products. Promisingly, one nucleoside showed two 2′-H signals shifted upfield (2.64 and 2.32 ppm) in the ^{1}H NMR spectrum (Supplementary Fig. 38), strongly suggesting that a deoxyribosyl moiety was present. Comparison of NMR spectra with literature values for deoxyribosyl pyrimidines suggested the formation of 2′-deoxy-2-thiouridine **11**. We subsequently verified this assignment by synthesizing **11** via a literature route[25] and using this material to spike our samples (Supplementary Fig. 40). We speculate that the second nucleoside product is 2′-α-thio-2-thiouridine **30**, but attempts to isolate and characterize this compound have so far been thwarted by its facile decomposition to 2-thiouracil **19** as the only identifiable product. The instability of the glycosidic bonds of 2′-thioribonucleosides has previously been documented[26]. Addition of the NMR integration standard, pentaerythritol, to the crude mixture indicated yields of 33, 25 and 28% for **11**, **19** and presumed **30**, respectively. The 5′- and 3′-phosphates of 2′,2-anhydro-2-thiouridine (**15b** and **15c**, respectively) undergo the same photoreduction, in similar yields to **15a** (Supplementary Figs. 41 and 42).

A possible mechanism for this remarkable photoreduction is proposed in Fig. 5b. Separate control reactions in the dark with H_2S and irradiation without H_2S gave no reduction products nor **30**, suggesting that initiation is by an electron released from hydrosulfide under ultraviolet irradiation[4,27,28], rather than homolysis of the C–S bond of **15a**. Transfer of this electron to thioanhydrouridine **15a** to form the radical anion **31**, and subsequent homolysis of the C–S bond would give intermediate **32**. The newly formed C2′ radical can abstract a hydrogen atom from H_2S (pictured) or react with a hydrogen atom (not pictured), to give **11**. Alternatively, reaction with an HS$^\bullet$ radical equivalent (HS$^-$ with loss of an electron, or H_2S_2 or a

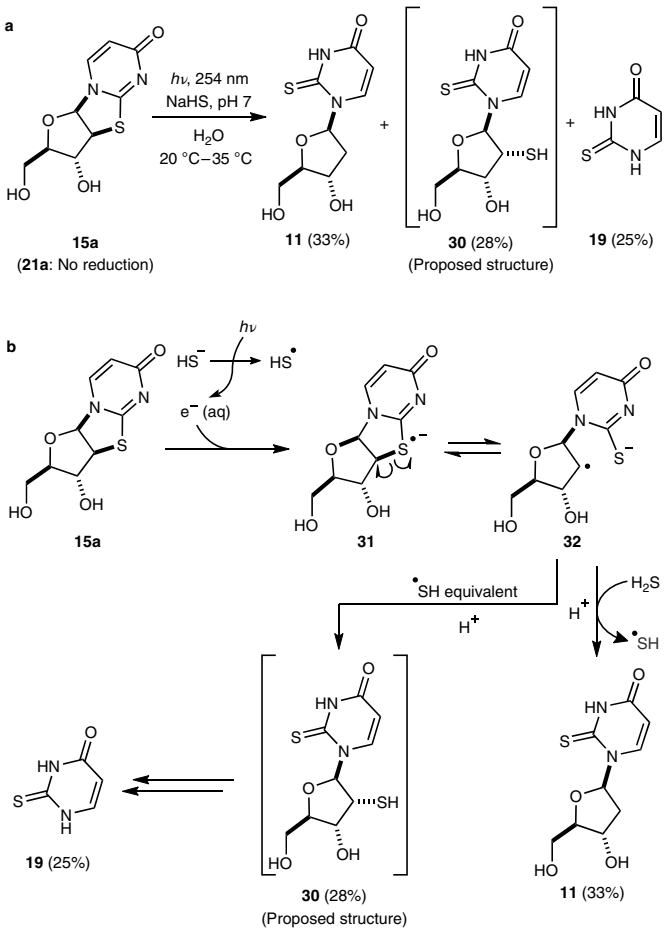

Fig. 5 | Photoinduced reduction of thioanhydrouridine with hydrosulfide (HS^-) and its proposed mechanism. a, Photoreduction of **15a** gives the major product 2′-deoxy-2-thiouridine **11**, as well as 2-thiouracil **19**, which is possibly formed via facile nucleobase loss from proposed intermediate **30** (see ref. [26]). Nucleotides **15b** and **15c**—minor products of the phosphorylation of **8** in Fig. 3—undergo photoreduction under the same conditions, in similar yields (Supplementary Figs. 41 and 42). **b**, Our proposed mechanism is initiated by photodetachment of an electron from aqueous hydrosulfide to generate a solvated electron. Reduction of **15a** by this electron generates radical anion **31**, which undergoes C–S bond homolysis to generate stabilized radical anion **32**. **32** can react with a hydrogen atom donor or combine with a HS• radical to give major isolated product **11** or proposed intermediate **30**, respectively, which we observed in crude spectra but were unable to isolate.

HS• radical, generated in previous steps) forms 2′-thionucleoside **30**. We conjecture that **21** probably does not undergo this reduction because the first step, were it to occur, would lead to a radical anion intermediate with less resonance stabilization than **31**.

Syntheses of deoxyribose and β-2′-deoxyadenosine. Since our synthesis of 2′-deoxyribonucleoside **11** takes place under the same conditions under which amino acid[4] and sugar[29] precursors and RNA pyrimidines[5,22] are formed, we immediately questioned whether DNA building blocks such as **11**, and related (canonical) derivatives, may also have been present at the advent of life. In an attempt to form the canonical 2′-deoxyribonucleoside, deoxyuridine, we investigated the hydrolysis of **11** under the same conditions under which β-2-thioribocytidine **5** is converted to cytidine (Fig. 1)[5]. However, in the presence of phosphate buffer, **11** is cleanly hydrolysed to 2-deoxyribose **12** and thiouracil **19**. A detailed study of the hydrolysis of 2′-deoxy-2-thiouridine **11** at 60 °C revealed half-lives of 32 and 31 h for 2′-deoxy-2-thiouridine **11** in 0.27 M phosphate (pH 7.0) and acetate (pH 4.1) buffers, respectively, and 28 h in unbuffered solution (Fig. 6a and Supplementary Figs. 43–50). The complete and clean conversion of 2′-deoxy-2-thiouridine **11** to 2-thiouracil **19** and deoxyribose **12** suggests that **11** may have been a prebiotic source of deoxyribose, both in abiotic reactions and/or for early enzymes, akin to the use of activated ribose in modern phosphoribosyltransferases[30–32]. This reactivity of **11** presented an opportunity for a plausible synthesis of purine deoxynucleosides via transglycosylation[33–35]. Indeed, heating of **11** with excess adenine **33** in the dry state[36] at 100 °C for 31 h led to the formation of both alpha and beta isomers of 2′-deoxyadenosine **13**, in 6 and 4% yield respectively, accompanied by nucleobase loss to give 2-thiouracil **19** in 72% yield (Fig. 6b and Supplementary Figs. 54 and 55) Notably, replacement of **11** with 2-deoxyribose **12** led only to a complex mixture that did not contain β-2′-deoxyadenosine (Supplementary Fig. 56). Such a demonstration of a plausible abiotic pathway stemming from the ribo-pyrimidine synthesis to DNA is significant, and further efforts are underway in our laboratory to discover more favourable transglycosylation conditions. Additionally, if promiscuous proto-enzymes with ribosylase activity existed, **11** may have been an efficient source of deoxyribose in an early biosynthesis of deoxyribonucleotides via transglycosylation. Thus, there are various plausible pathways by which the C2′-reduced nucleoside **11** could have facilitated the chemical progression of RNA into DNA.

Conclusions

Our investigation into the phosphorylation of prebiotic RNA pyrimidine intermediate **8** has resulted in several significant discoveries. We have shown that 5′-phosphoryl-2-thiouridine **14** can be obtained from kinetically selective phosphorylation of **8**, demonstrating its viability as a potentially important primordial non-canonical component of nucleic acids. Other phosphorylation conditions lead to the until-now elusive prebiotic functionalization of the C2′ position of a nucleoside with sulfur. Moreover, we successfully reduced such a sulfur-containing derivative to a non-canonical 2′-deoxyribonucle-

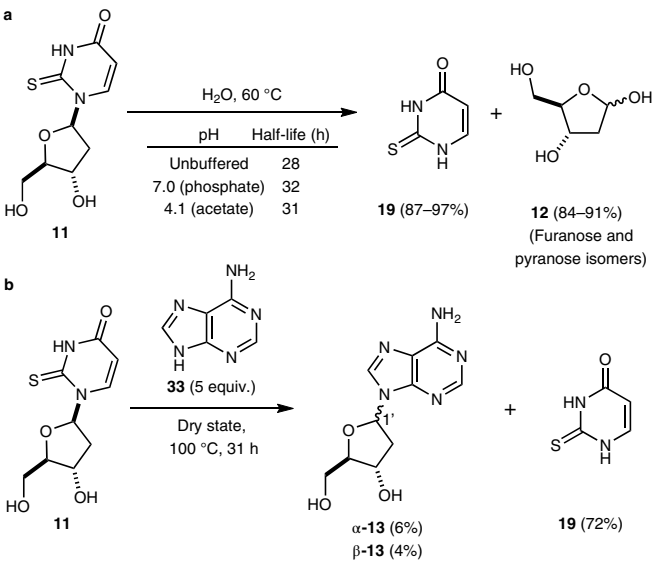

Fig. 6 | Hydrolysis and transglycosylation reactions of 2′-deoxy-2-thiouridine 11. a, Hydrolysis of **11** at different pH values gives deoxyribose **12** and the corresponding nucleobase, 2-thiouracil **19**. **b**, Transglycosylation in the dry state with adenine **33** gives a mixture of the alpha- and beta- stereoisomers of the canonical deoxynucleoside, deoxyadenoisine **13**, as well as nucleobase loss to yield **19**.

oside, **11**, using a prebiotically plausible, hydrogen sulfide-mediated photoreduction. This photochemical reaction emulates the critical biochemical conversion of ribonucleotides to 2′-deoxyribonucleotides, but without the involvement of any complex enzymes. Finally, we have demonstrated that this deoxyribonucleoside reacts with adenine to form β-deoxyadenosine **13b**—a canonical DNA purine deoxynucleoside. Thus, 2-thioribonucleosides are key intermediates in a synthesis of both RNA and DNA building blocks, providing a long sought-after prebiotic link between DNA and its biological and chemical progenitor, RNA.

Data availability

Full experimental details and data are provided in the Supplementary Information. The raw data for Supplementary Figs. 49–53 are available from the authors upon reasonable request. Correspondence and requests for materials should be addressed to J.D.S. and R.K.

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Author contributions

J.D.S. and R.K. supervised the experimental research. J.X., N.J.G. and C.G. performed the experiments. All of the authors co-wrote the paper and assembled the Supplementary Information.

Competing interests

The authors declare no competing interests.

Additional information

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