The Chemistry of Branched Condensed Phosphates

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Condensed phosphates may exist as linear, cyclic or branched structures. Due to their important role in nature, linear polyphosphates have been well studied. In contrast, branched phosphates (ultraphosphates) remain largely uncharacterized, because they were already described in 1950 as exceedingly unstable in the presence of water, epitomized in the antibranching-rule. This rule lacks experimental backup, since no rational synthesis of defined ultraphosphates is known. Consequently, detailed studies of their chemical properties, reactivity and potential biological relevance remain elusive. Here, we introduce a general synthesis of monodisperse ultraphosphates. Hydrolysis half-lives up to days call the antibranching-rule into question. We provide evidence for the interaction of an enzyme with ultraphosphates and discover a rearrangement linearizing the branched structure. Moreover, ultraphosphate can phosphorylate nucleophiles such as amino acids and nucleosides with implications for prebiotic chemistry. Our results provide an entry point into the uncharted territory of branched condensed phosphates.

Polyphosphates (polyP) are polymers of orthophosphate linked by phosphoanhydride bonds. They are ubiquitous in living organisms with numerous biological functions.[1] PolyP may exist in three principally different structures: linear, cyclic (metaphosphates) or branched (ultraphosphates). Cellular polyphosphates are now defined as exclusively linear polymers[1–4]. This paradigm evolved, despite early reports on the presence of metaphosphates in cellular extracts[5–7] and was recently called into question by ^{31}P solid-state NMR data from whole *Xanthobacter autotrophicus*.[8] There have only been scattered comments on ultraphosphates in biology.[2,9] These, in turn, dismiss the occurrence of ultraphosphates by referring to the "antibranching-rule", which was coined in 1950 and has persisted since then.[10–14]

Some work has been done on vitreous and crystalline ultraphosphates[15–21] due to applications as laser materials.[10,22] Yet studies on monodisperse ultraphosphates are limited. In the 1970s, Glonek reported cyclic ultraphosphate structures from condensations of ortho- or metaphosphates.[23–27] The ultraphosphates could neither be obtained in pure form nor isolated and in part lack unambiguous analytical proof. Addition of water resulted in the instantaneous hydrolysis of the branches.[23]

In recent years, branched oligophosphates caught attention again: Cyclic ultraphosphates like **11** with modifications on the terminal phosphate can now be obtained and applied in the syntheses of linear nucleoside, dinucleoside and inorganic polyphosphates.[28–32] Cummins et al. synthesized **7** as its [PPN] (bis(triphenylphosphine)iminium) salt, which allowed the isolation of the product.[33] Application of **7** was demonstrated in the tetraphosphorylation of nucleotides and enabled the synthesis of another ultraphosphate species containing four phosphates in the cyclic subunit.[34] While cyclic ultraphosphates have thus recently become accessible, there is no synthesis of ultraphosphates available that are devoid of cyclic substructures and therefore are true constitutional isomers of linear polyphosphates.[35] This fundamental type of condensed phosphates remained unstudied.

Here, we report the synthesis of non-cyclic ultraphosphates using phosphoramidite chemistry. This approach provides access to both symmetrically modified ultraphosphates containing three equal

modifications on each terminal phosphate, including the smallest possible unmodified ultraphosphate uP_4 (**2**) as well as unsymmetrical analogues containing two different residues. The synthetic approach enables the generation of thio- and seleno-ultraphosphates. To interrogate the antibranching-rule, hydrolysis studies were conducted including enzymatic degradation. Furthermore, the phosphorylation of nucleosides with inorganic ultraphosphate was studied with implications for prebiotic chemistry. We also study the reactivity of modified ultraphosphates in organic solvent by using a combination of ^{31}P-NMR and capillary electrophoresis mass spectrometry (CE-MS) and discover an ultraphosphate rearrangement, which we name the "phosphate walk".

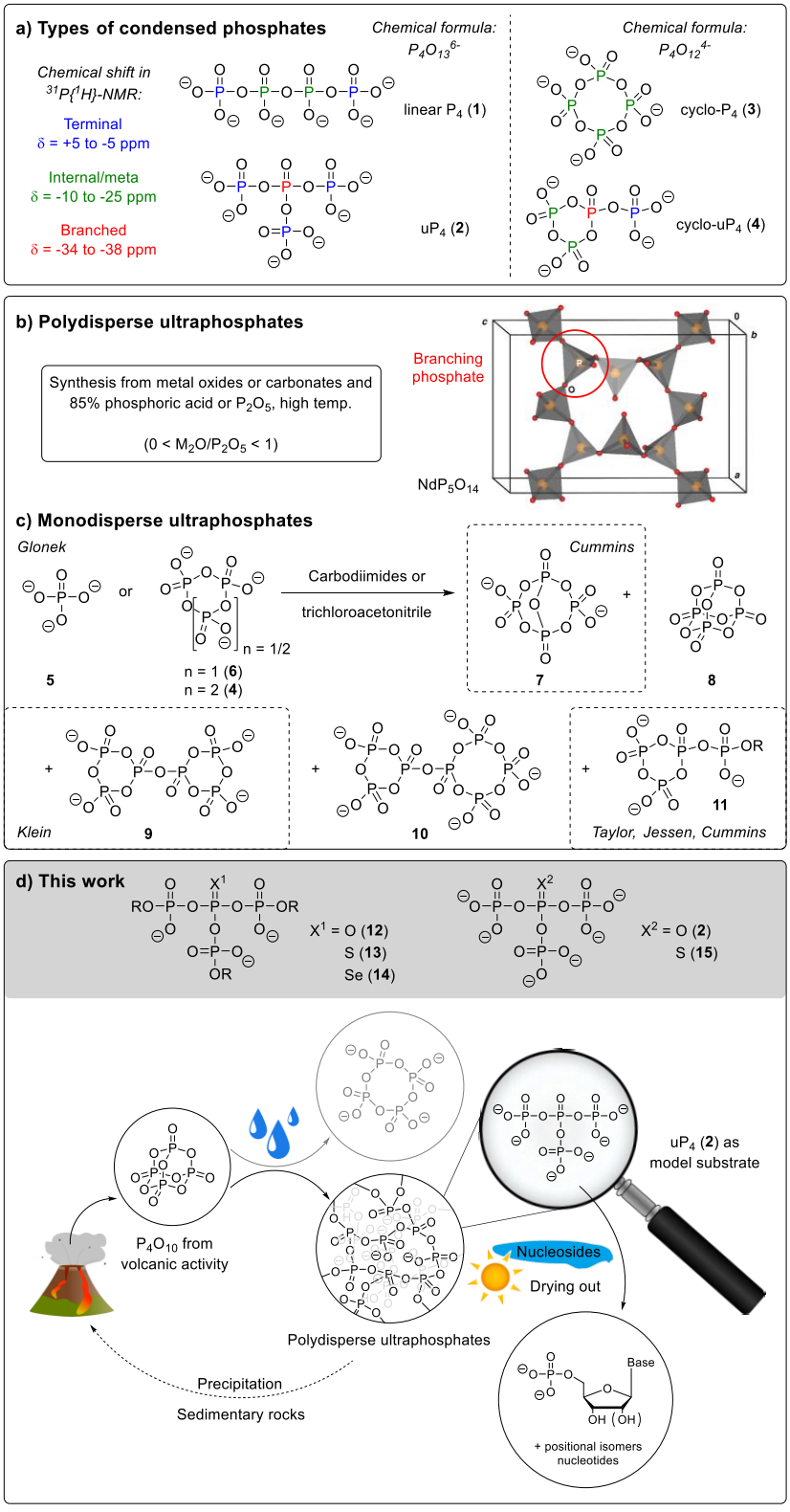


Fig. 1 | Previous work on cyclic ultraphosphates and non-cyclic ultraphosphates in this work with prebiotic implications. **a**, Types of condensed phosphates and their chemical shift in $^{31}P\{^1H\}$-NMR. **b**, General synthesis protocol for polydisperse ultraphosphates and crystal structure of NdP_5O_{14}[36]. **c**, Monodisperse ultraphosphates detected by Glonek in the condensation reactions of orthophosphoric acid or metaphosphates using carbodiimides or trichloroacetonitrile.[23–27] The

structures in dashed boxes were further described in publications by Klein[37], Taylor[28], Jessen[30,31] and Cummins[32]. **d**, General structures of ultraphosphates in this work and suggestion for prebiotically plausible phosphorylation reactions using ultraphosphates: uP_4 (**2**) was used as a model substrate for polydisperse ultraphosphates arising from reactions of P_4O_{10} in the presence of water.[38] The prebiotic phosphate cycle including phosphorus pentoxide from volcanic activity has already been proposed and meets the challenge of making phosphate available from low-solubility minerals.[39–42]

Symmetrical ultraphosphates

Phosphordiamidites are used in linear polyphosphate syntheses by twofold activation and reaction with (modified) phosphates.[43] A phosphortriamidite should therefore enable threefold activation and reaction with three phosphates to produce a mixed P(III)-P(V)-anhydride intermediate **18** (hereafter called ultraphosphite, Fig. 2a), which can further be oxidized resulting in an ultraphosphate (**12**).

Initially, the reaction of three equivalents of tetrabutylammonium (TBA) phenyl phosphate with tris(diethylamino)phosphine (**16**) and ethylthiotetrazole (ETT) was studied: $^{31}P\{^1H\}$-NMR of the mixture showed consumption of **16** within a few minutes, giving rise to the ultraphosphite intermediate. The absence of peak splitting due to homonuclear P-P coupling for this mixed P(III)-P(V)-anhydride is in accordance with earlier observations for P-amidite couplings.[43] Oxidation with *m*CPBA gave phenyl-modified ultraphosphate **20**, which was isolated by precipitation with Et_2O (71% yield, 78% purity, measured by $^{31}P\{^1H\}$-NMR). As decomposition products, diphenyl triphosphate (10%) and phenyl phosphate (10%) were detected. The central ultraphosphate signal, now showing the expected multiplicity (quartet) with a chemical shift of ca. $\delta = -35$ ppm, was detected in water. This finding is in sharp contrast to the antibranching-rule, claiming the instantaneous hydrolysis of ultraphosphates.[2,9,23] Purification was possible in aqueous buffer (NH_4HCO_3) using strong anion exchange chromatography (SAX) and $^{31}P\{^1H\}$-NMR analysis of fractions showed pure **20** in solution. Lyophilization resulted in significant decomposition. We also studied $NaClO_4$ or LiCl solutions as eluents in SAX and attempted to isolate the product by precipitation from acetone.[31] While we were unable to isolate trisphenyl uP_4 **20** using this procedure, trisadenosine ultraphosphate (**21**)

precipitated and afforded the product in 55% yield. $^{31}P\{^{1}H\}$-NMR of the precipitate showed pure ultraphosphate, but after drying, decomposition (> 15%) was detected.

Next, we evaluated the scope of ultraphosphate synthesis (see Fig. 2b) by changing modifications on the terminating phosphates. Since not all products precipitated readily, reaction yields as derived for **21** cannot be given and Fig. 2b lists the purities of crude products according to $^{31}P\{^{1}H\}$-NMR. Alkyne modified ultraphosphates (**24** and **25**) were readily accessible. Efforts towards the synthesis of amino acid- and carbohydrate-modified ultraphosphates using *O*-phospho-L-tyrosine or α-D-glucose-1-phosphate resulted in side-reactions of the phosphoramidite with the amine or the primary alcohol, respectively. Application of Fmoc-*O*-phospho-L-tyrosine followed by deprotection of the amine gave access to amino-acid modified ultraphosphate **27**. The use of D-glucose-6-phosphate enabled access to a carbohydrate containing ultraphosphate **28**. Thiamine-derived ultraphosphate **29** could be obtained from the tetrakis[3,5-bis(trifluoromethyl)phenyl]borate (BArF) salt of thiamine phosphate. Modification of the oxidation (S_8 or KSeCN) facilitated entry into thio- (in green) and seleno- (**23**) ultraphosphates.

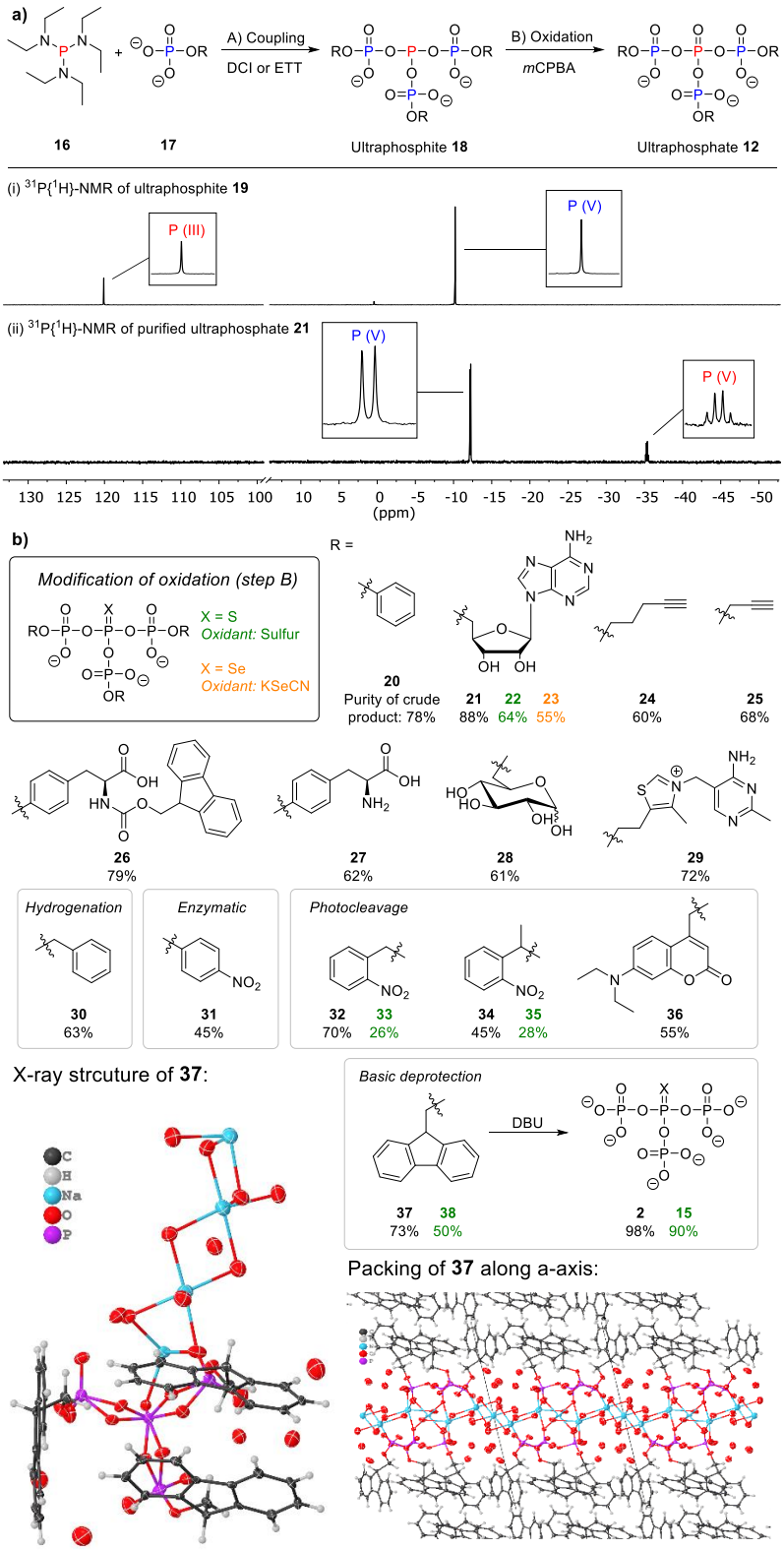


Fig. 2| **Symmetrical ultraphosphates. a**, Synthesis of ultraphosphates by threefold coupling of phosphates with tris(diethylamino)phosphine (**16**) and subsequent oxidation. $^{31}P\{^{1}H\}$-NMR spectra of the intermediary ultraphosphite **19** and the ultraphosphate **21** after oxidation and purification for R = adenosine. **b**, Synthetic scope of the synthesis of symmetrically

modified ultraphosphates and modification of the oxidation step to yield thio- and seleno analogues. The purity of the crude products are given in percent according to the $^{31}P\{^{1}H\}$-NMR spectra. Yields were not determined due to uncertain quantities of counterions but the respective crude masses are reported in the supporting information along with the spectra.

Unmodified and unsymmetrical ultraphosphates

We envisioned the synthesis of inorganic ultraphosphate uP_4 (**2**) as the defining minimal unit of this substance class. Reactions of phosphoric acid with **16** were unsuccessful thus requiring protected precursors for **2**. Different cleavage strategies for the protected phosphates were considered, including hydrogenolytic (**30**), enzymatic (**31**, discussed later), phototriggered (**32**-**36**) and basic (**37**) deprotection (see Fig. 2b). We found that (9*H*-fluorenyl-9-yl)methyl (Fm) dihydrogen phosphate was readily accessible[44] and that the corresponding ultraphosphate **37** could be synthesized under ambient conditions. **37** could also be stored indefinitely in solution at −20°C after purification, enabling screening of several bases for deprotection. Only DBU enabled deprotection to **37**, but precipitation of uP_4 **2** resulted in decomposition.

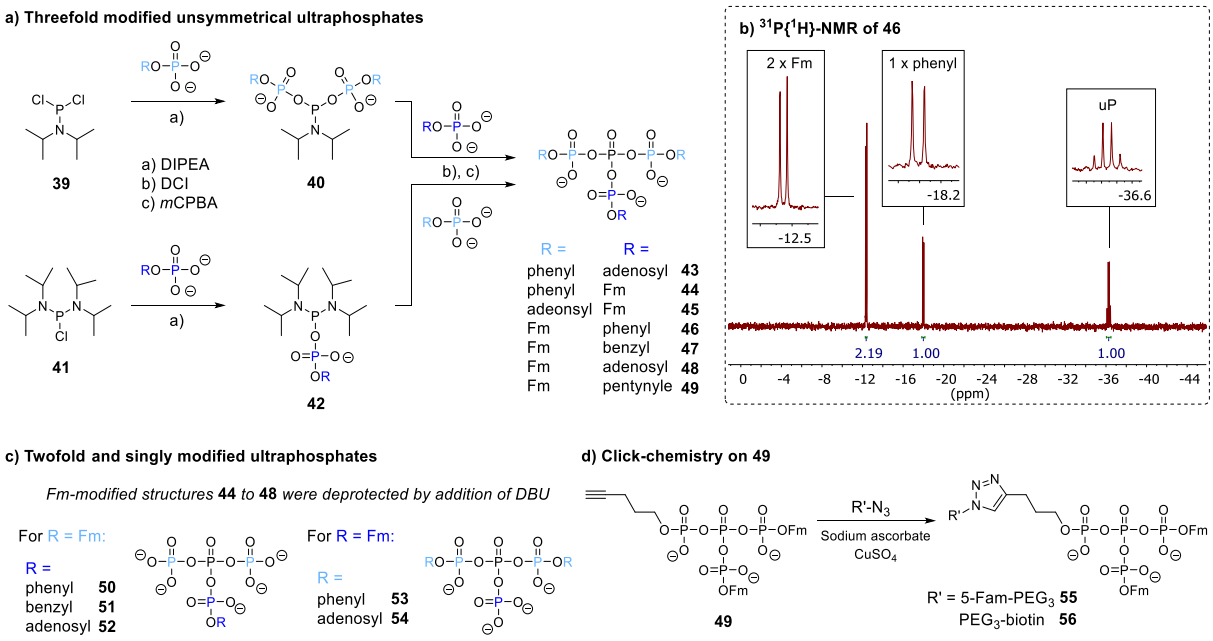


Fig. 3 | Unsymmetrical ultraphosphates. For the discussion of yields see Supplementary Chap. 12.2. **a**, Syntheses of threefold modified unsymmetrical ultraphosphates using either a chloro phosphormono- or diamidite. **b**, $^{31}P\{^{1}H\}$-NMR spectrum of unsymmetrically modified ultraphosphate **46**. **c**, Twofold and singly modified ultraphosphates

by deprotection of Fm-modified, unsymmetrical ultraphosphates using DBU. **d**, Application of click-chemistry on ultraphosphate **49**.

Unsymmetrically modified ultraphosphates were accessible by orthogonal activation strategies using chlorophosphoramidites (**39** or **41**, see Fig. 3a). Although over-reaction in the first and unselective phosphate exchange in the second step were observed, a series of unsymmetrical ultraphosphates were obtained after purification. Fm-modified structures further allowed deprotection by addition of DBU to yield twofold or singly modified ultraphosphates (**50** to **54**, see Fig. 3c). The number of modifications influenced the stability of the ultraphosphate towards hydrolysis, showing faster decomposition for twofold modified structures. For example, **54** was completely hydrolysed in the deprotection mixture (5% DBU in water, pH 13.9) within 30 min while **52** had a half-life of 180 min. Copper-catalyzed click reactions on **49** were performed with biotin and fluorescein-conjugates and allowed preservation of the ultraphosphate (Fig. 3d).

Salt metatheses and crystallization

To obtain a single crystal of an ultraphosphate, we examined salt metatheses reactions to yield [PPN] salts, since [PPN] is well-known for its high crystallinity and its capability to stabilize anions[45]. We developed a method to isolate the ultraphosphate [PPN] salts from fractions after SAX purification (Supplementary Fig. 2) and found that they can be dried and stored indefinetly, in contrast to non-[PPN] salts. Only uP_4 [PPN] decomposed as soon as residual water was removed.

While no crystals of sufficient quality for crystal structure analysis could be obtained from the [PPN] salts, it was found that Fm-protected **37** can be salted out from solutions after reversed-phase purification (55% water, 35% MeCN, 10% TEAA (100 mM, pH 7.0)) by addition of NaCl and that these conditions supported formation of single-crystals. X-ray diffraction studies established the identity of **37** (Fig. 2). The packing along the a-axis (Supplementary Fig. 3) shows that the ultraphosphate is arranged along a chain of water-bridged sodium cations to form distinct layers where hydro- and lipophilic interactions are maximized. We attribute the increased stability of **37** compared to other

symmetrically modified ultraphosphates to a steric shielding of the ultraphosphate moiety. Accordingly, the comparably lower stability of singly or twofold modified ultraphosphates can be explained. However, as a counter-effect, the higher negative charge enables longer half-lives of singly compared to twofold modified structures. Although [PPN] salts are stable storage forms for ultraphosphates, their insolubility in water can be disadvantageous. The metathesis to water-soluble sodium salts was possible using NaOTf. Only for uP_4, the salt metathesis was unsuccessful and led to the formation of inorganic mono- and triphosphate. Unexpectedly, also pyro- (14%) and linear tetraphosphate (12%) were detected.

Stability of ultraphosphates in water

The antibranching-rule describes ultraphosphates as exceedingly unstable in the presence of water.[11,12] Our observations, however, revealed a significant hydrolytic stability for short-chain ultraphosphates expressed in half-lives ranging from several hours to days. The decomposition of uP_4 and trisadenosine uP_4 **21** was tracked by $^{31}P\{^1H\}$-NMR at different pH values and in the presence of different cations (see Fig. 4a and Supplementary Fig. 4). There is a high pH-dependency on the decomposition rates, with longer half-lives obtained with increasing pH-values. In the presence of Mg^{2+}, the decay was about twice as fast, and Ca^{2+} accelerated the decay even further. In contrast, monovalent cations had only little effects.

The stability of ultraphosphates suffices to subject them to polyacrylamide gel electrophoresis on dense gels (PAGE; Supplementary Fig. 5 and Supplementary Fig. 6) without significant decomposition followed by staining with toluidine blue.[46] Such separations take several hours in aqueous buffer. Threefold modified ultraphosphates gave clear bands without any observed decay and even uP_4 could be analysed by PAGE, pointing towards a possible analytical approach to detect ultraphosphates in biological samples. Singly or twofold modified structures **52** and **54** decomposed under these conditions.

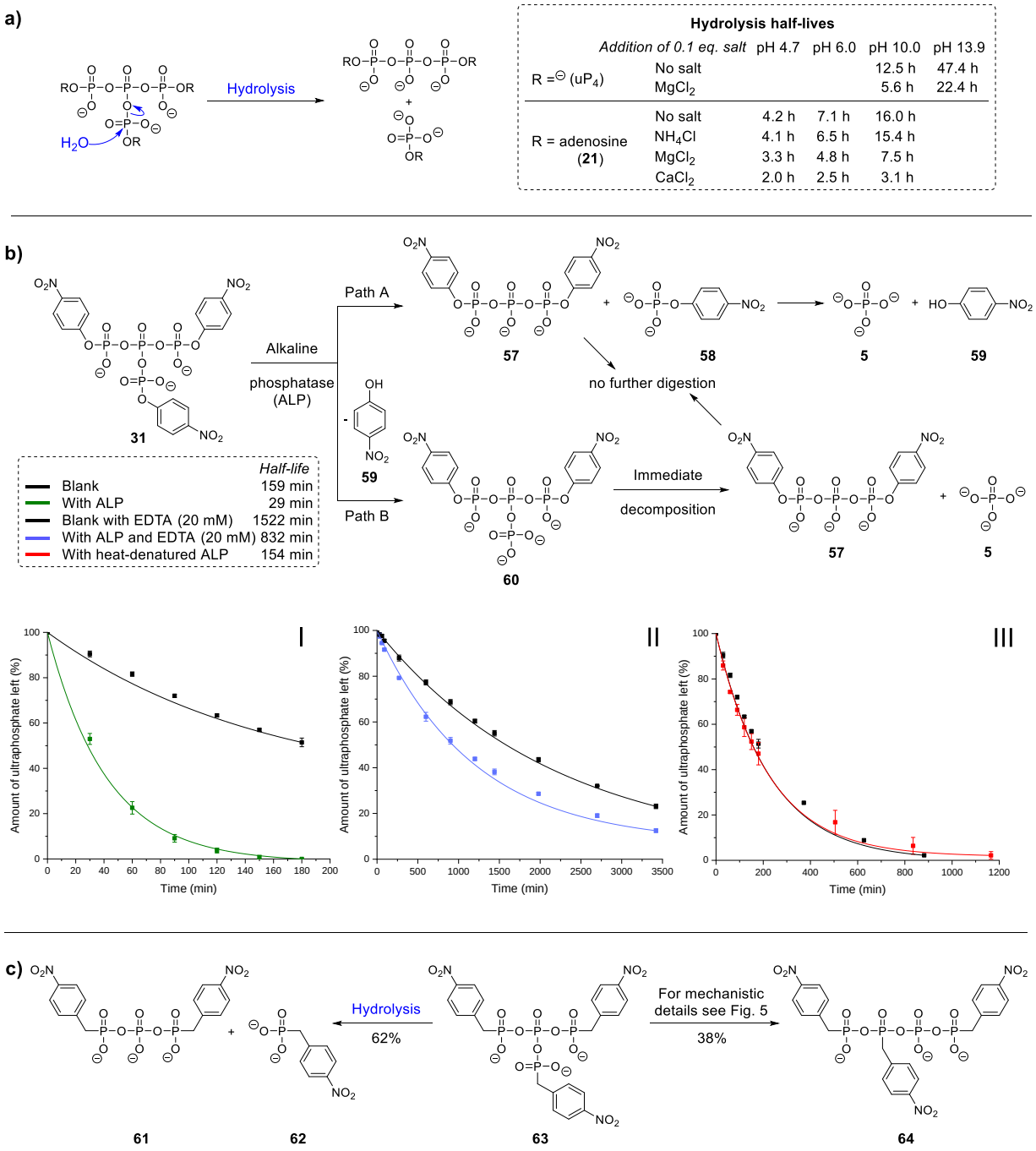


Fig. 4 | Stability of ultraphosphates in aqueous media and enzymatic digestion. **a**, Decomposition of trisadenosine ultraphosphate (**21**) and uP_4 under different pH values and in presence of 0.1 eq. of different cations. Half-lives were calculated assuming pseudo-first order reaction kinetics. **b**, Enzymatic digestion of tris(*para*-nitrophenyl) ultraphosphate (**31**) by alkaline phosphatase (ALP) from bovine intestinal mucosa. Kinetics were recorded using $^{31}P\{^{1}H\}$-NMR and the [PPN] signal was used as internal standard. The results are means ± standard deviation from experiments performed in triplicates. Half-lives were calculated assuming pseudo-first order reaction kinetics. **I**, **31** in the presence and absence of ALP. **II**, **31** with 20 mM EDTA in the presence and absence of ALP. **III**, **31** in the presence and absence of heat-inactivated ALP. **c**, Decomposition of tris(*para*-nitrobenzyl phosphonyl) ultraphosphate (**63**).

Enzymatic digestion of ultraphosphates

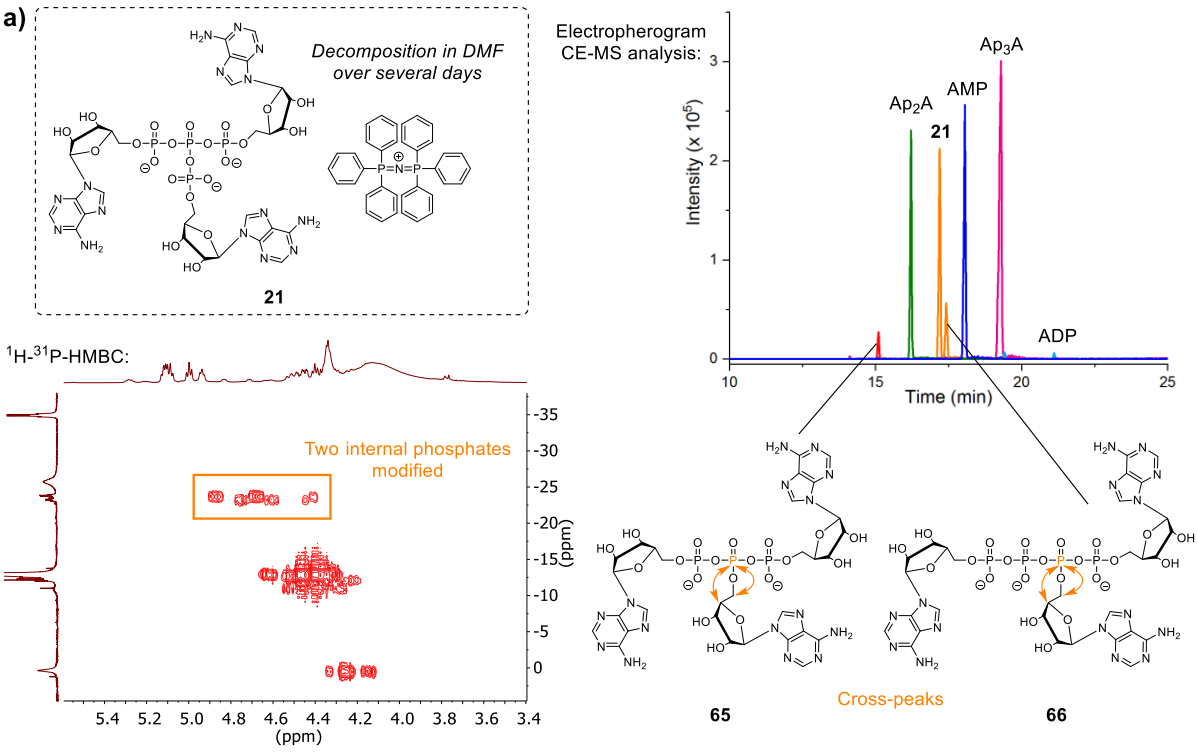
Ultraphosphates have not been reported to occur in biological systems. Since polyphosphate extraction protocols usually include acidic conditions, nucleophilic reagents, divalent cations and drying – all of which accelerate ultraphosphate decomposition – one would not expect to detect ultraphosphates. The publication by Hong provides evidence for this case already for the more stable metaphosphates.[8] In accordance, we could not find any ultraphosphate signal at ca. δ = –35 ppm in $^{31}P\{^{1}H\}$-NMR analyses of polyphosphate extracts from different yeast strains modified in their polyphosphate metabolizing enzymes (the polyP overexpressor GPD-vtc5, the polyP devoid Δ vtc4 and polyP accumulating Δ ppn1, ppn2, ppx1, that lack phosphatases degrading polyP; Supplementary Fig. 7 and Supplementary Fig. 8). We hypothesized that in addition to chemical conditions, enzymes might also degrade ultraphosphates, which would further complicate strategies to extract them from biological sources. We studied the enzymatic digest of ultraphosphates using alkaline phosphatase (ALP). We initially examined the hydrolysis of *para*-nitrophenylphosphate (pNPP) and its ultraphosphate **31**. We synthesized ultraphosphate **31** as its [PPN] salt and tracked the decomposition in the presence and absence of alkaline phosphatase by $^{31}P\{^{1}H\}$-NMR (Fig. 4b).[47] **31** showed a half-life of $t_{1/2}$ = 159 min in the absence of the enzyme, which was significantly reduced upon its addition ($t_{1/2}$ = 29 min). We were interested as to whether the ultraphosphate **31** binds to the active site or whether surface effects are responsible for the accelerated hydrolysis. We measured additional kinetics using either heat-denatured enzyme or enzyme treated with EDTA to remove Mg^{2+} and Zn^{2+} ions. The decay of **31** in presence of heat-inactivated enzyme copies the kinetics of the blank. Only a low residual hydrolytic activity in the presence of EDTA was found. We conclude that ultraphosphate **31** binds to the active site and is enzymatically digested, providing evidence for an ultraphosphate-enzyme interaction. Similar results were obtained for the adenosine-modified ultraphosphate **21** (Supplementary Fig. 9). However, it was still unclear whether ALP first hydrolyses the branching point of ultraphosphates, or one of the three phosphorester bonds (path A or B in Fig. 4b). The latter would result in an unstable twofold modified ultraphosphate **60** that would rapidly decay further. uP_4 **2** was

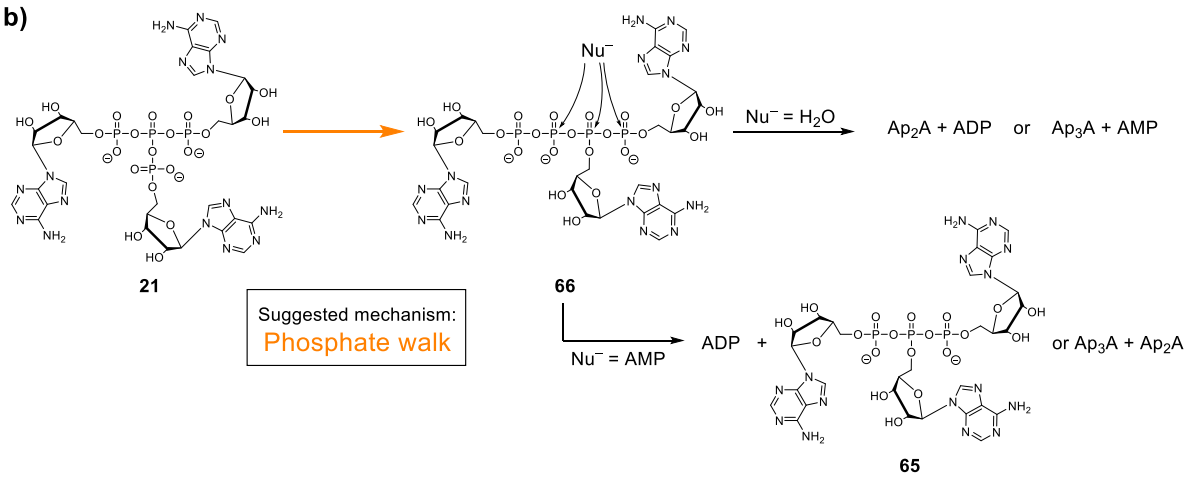
enzymatically digested as well (Supplementary Fig. 10), pointing towards the cleavage of the anhydride bond. To further study the enzymatic digestion of modified ultraphosphates, we envisioned the synthesis of the "nonhydrolyzable" analogue **63** with a CH_2-group as an oxygen replacement, which the enzyme cannot cleave, while still being capable of hydrolysing the central anhydrides (Fig. 4c). *para*-Nitrobenzyl phosphonic acid was transformed to ultraphosphonate **63**. After oxidation, $^{31}P\{^1H\}$-NMR suggested a clean reaction to the product, but after precipitation, ultraphosphonate **63** was no longer present (Supplementary Fig. 11). We identified the usual hydrolysis products **61** and **62** but also another product, which was purified by AIEX chromatography and found to be a linearized mixed tetraphosphonate/phosphate analogue **64**. This is reminiscent of the linear tetraphosphate we found for uP_4 **2** decomposition described earlier and pointed towards a rearrangement typical for ultraphosphates.

Ultraphosphates rearrange by phosphate walk

Apart from linearized products such as P_4 from uP_4 [PPN] **2** and the synthesis of an ultraphosphonate (**63** and **64**, Fig. 4c and Supplementary Fig. 11), trisadenosine ultraphosphate [PPN] (**21**) also showed another decay mechanism accompanying simple hydrolysis of the branching point after several days in DMF. Next to AMP and Ap_3A as expected products of hydrolysis, further signals were detected and ^1H-^{31}P-HMBC cross-peak analysis revealed that internal phosphates must carry an adenosine (Fig. 5a). The mixture was analysed by capillary electrophoresis mass spectrometry (CE-MS), which enabled the separation of different components and determination of their mass. We confirmed masses matching Ap_2A, a threefold modified triphosphate **65** as well as a threefold modified tetraphosphate **66**. Since **21** was analytically pure, a rearrangement must lead to linearization of the ultraphosphate that "walks into the line"[48], which we dub the "phosphate walk" (Fig. 5b). We propose, that the ultraphosphate linearizes by an attack of one terminal phosphate at another accompanied with the cleavage of either a phosphoanhydride or phosphorester bond. The latter would form a cyclic ultraphosphate intermediate and requires a second nucleophilic attack of the released alcoholate to form **66** as the central intermediate of the mechanism. Depending on the nucleophile (AMP or H_2O) and the attacked

phosphate of **66** (Fig. 5b), both the internally modified phosphate **65** and Ap_2A as well as standard decomposition products can be explained.





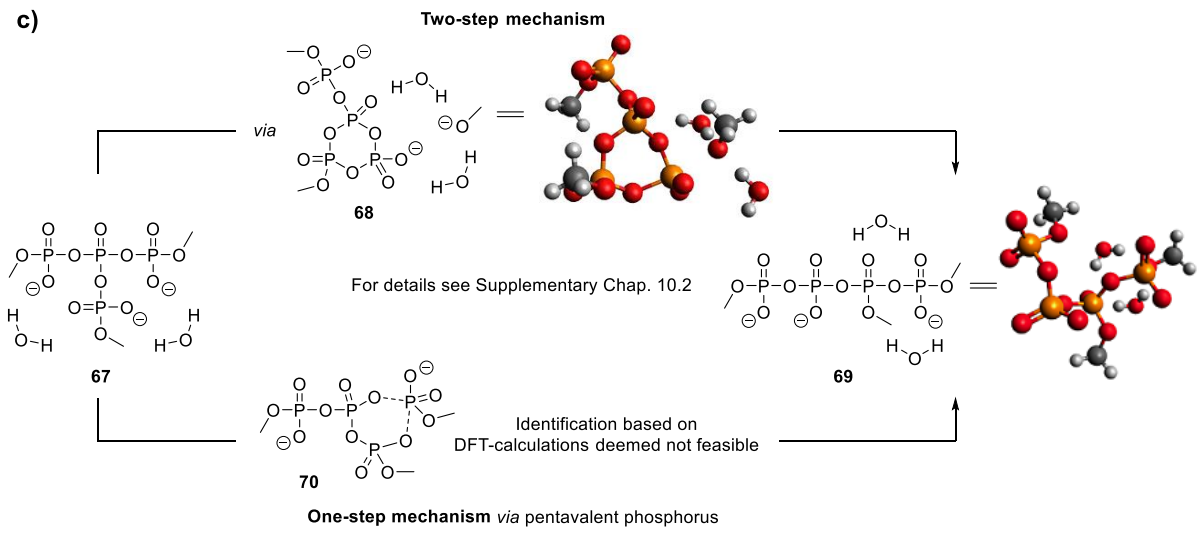


Fig. 5 | Rearrangement of ultraphosphates. a, Analytical results for trisadenosine ultraphosphate (**21**) [PPN] salt after 14 days in DMF: ^{1}H-^{31}P-HMBC with two cross-peaks for internally modified oligophosphates. CE-MS analysis of the product mixture and proposed structures **65** and **66** for the internally modified compounds. **b**, Phosphate walk rearrangement for trisadenosine ultraphosphate (**21**) and nucleophilic attack of the linearized product **66**. **c**, Possible mechanistic pathways of the phosphate walk rearrangement for trimethyl ultraphosphate dihydrate (**67**). Mechanistic studies were carried out at the B97D/Def2-TZVPD(water) level of theory; for details see Supplementary Chap. 10.2.

Bisphenyladenosine (**43**) and trisbenzyl ultraphosphate (**30**) [PPN] (Supplementary Fig. 12 and Supplementary Fig. 13) also showed phosphate walk products. For **30**, the rearrangement could only be induced by heating to 80°C.

Computation was invoked to corroborate the feasibility of the internal phosphate walk mechanism. The history of mechanistic disputes surrounding substitution reactions at phosphorous include a myriad of experimental results and *ab initio* computations.[49–51] The energy of highly charged species, with variable counter ions in high vs. moderate dielectrics involving hydrogen bond donors, are sensitive to intricate changes in speciation, constitutional and conformation isomerism, and explicit viz continuum environmental effects.[51–53] Mechanistic computational studies involving trimethyl ultraphosphate (**67**) at the B97D/Def2-TZVPD(water) level of theory support the ultraphosphate **67** and linearized product **69** having energies compatible with both species being accessible under normal conditions. A cyclic intermediate **68** (Fig 5c) was found that results formally from the loss of methoxide and the formation of a six-membered ring by association of a formally negative oxygen of one

phosphate to the phosphorous where methoxide is removed. These three states (**67**-**69**) were the basic minima considered.

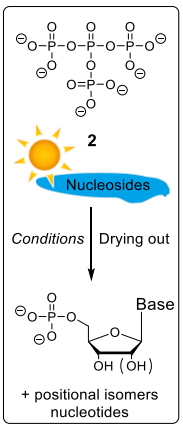
The "walk" could occur through this higher energy cyclic state that serves as a possible bridge between branched and linear forms and has transition states leading to the ring-formation and -opening. Alternatively, the association could form a ring with a pentavalent phosphorous atom (**70**); however, numerous attempts to identify such an intermediate by DFT-calculations were unsuccessful. Mono- and dihydrates were considered, where the explicit waters were placed in positions consistent with prevailing models[51] bringing down the relative activation energies. The data support a trend where reaction in water coupled to specific acid catalysis would show fast substitution rates. Considering effective molarities, relative nucleophilicities and the high concentration of water in water (55 M), hydrolysis as the dominant reaction path is not surprising. Taken out of the context of water, the effective molarity of internal nucleophile remains constant; the nucleophilic strength of solvent is reduced and the concentration of solvent molecules as nucleophiles in bulk solvent drops to on the order of 10 M.[54,55] All these factors presage parallel rates of acceleration in the presence of acid but a product distribution in which internal attack becomes competitive if not fully dominant. This shift in product distribution opens the way for the phosphate walk. For the ultraphosphonate analogue **63**, however, the one-step mechanism must be energetically favoured.

Reactivity of uP_4 as phosphorylating agent

Ultraphosphates could potentially serve as phosphorylating reagents if suitable nucleophiles are present, as has been previously studied for poly- and metaphosphates.[56–59] Earlier studies suggested the formation of branched phosphates on primitive earth as partial hydrolysis products of P_4O_{10}, which in turn can be volatilised from magma.[6,39–41] uP_4 **2** was used as a model substrate for polydisperse ultraphosphates arising from P_4O_{10} in the presence of water[38] to study the potential contribution of branched phosphates to prebiotic phosphorylation reactions (Fig. 6a and Supplementary Fig. 15). Aliphatic nucleophiles were studied for general reactivity patterns of ultraphosphates. Ethanolamine

was used to screen the required stoichiometry to favour the phosphorylation reaction over simple hydrolysis by water. We observed monophosphorylation of the amine moiety with a phosphorylation ratio of up to 85:15 (phosphorylated ethanolamine vs. orthophosphate as hydrolysis product) with 3000 eq. ethanolamine. Decreasing ethanolamine gave ratios of 70:30 for 500 eq. and 27:73 phosphorylated ethanolamine for 100 eq., respectively. For secondary amines and alcohols, we additionally detected a phosphoramidate arising from the reaction of DBU – which was present due to the deprotection conditions – with uP_4. This side-reaction was avoided using polymer-bound DBU for the deprotection, affording DBU free uP_4 in 81% purity (see Supplementary Fig. 15). Interestingly, uP_4 **2** was found to be less stable in the absence of DBU. Thus, further experiments were performed with DBU in solution due to the better repeatability. For different amino acids, the primary or secondary amine and – if present – the alcohol or thiol moiety were phosphorylated (Supplementary Fig. 15). For glycine, only 8% phosphorylation of the amine was observed at pH 8.8 but adjustment to pH 13.0 increased the phosphorylation to 66%, in accordance with the longer half-life of uP_4 at higher pH (see Fig. 4a). Although it is unclear whether this pH condition is relevant to prebiotic chemistry[60], the result still shows a general reactivity trend. Freeze-drying experiments increased the phosphorylation of glycine to its phosphoramidate to 52% at a pH of ca. 9.

For all canonical (deoxy)nucleosides, even 10 eq. were sufficient to detect phosphorylated nucleosides by CE-MS after freeze-drying. For full consumption of uP_4 **2** in one single freeze-drying experiment 500 eq. were necessary. These conditions allowed phosphorylation of up to 54% while evaporation of solvent at room temperature resulted in 33% nucleoside phosphorylation. The product distribution was analysed by CE-MS, and spiking experiments revealed product identities (Fig. 6b). We found phosphorylation in the 5´- and also 2´- and 3´-positions. To rule out phosphorylation arising from inorganic mono- and triphosphate, these compounds were lyophilized with 3000 eq. uridine, leading to no phosphorylation. Ultraphosphate **2** as a model substrate - and by extension other branched polyphosphates - may thus have served as phosphate donors in prebiotic chemistry.



a)

	Conditions	Phosphorylation/ orthophosphate
Uridine	3000 eq., pH 13 (DBU)	4:96
	3000 eq., pH 13 (DBU), 10 eq. $MgCl_2$	11:89
	3000 eq., pH 13 (NaOH)	7:93
	3000 eq., pH 13 (NaOH), freeze-drying	32:68
	3000 eq., freeze-drying	29:71
	3000 eq., 80°C	7:93
	10 eq. $MgCl_2$, freeze-drying	11:89
	100 eq., freeze-drying	45:55 (incomplete)
	500 eq., freeze-drying	44:56
	500 eq., evaporation	25:75
	Control experiment with each 3000 eq. of inorganic mono- and triphoshpate and freeze-drying gave no UMP	
Adenosine	pH 13 (NaOH), freeze-drying	32:68
	100 eq., freeze-drying	56:44 (incomplete)
	500 eq., freeze-drying	54:46
	500 eq., evaporation	16:84

	Conditions	Phosphorylation/ orthophosphate
Thymidine	500 eq., freeze-drying	41:59
	500 eq., evaporation	19:81
Deoxyadenosine	500 eq., freeze-drying	23:77
	500 eq., evaporation	14:86
Guanosine	500 eq., freeze-drying	40:60
	500 eq., evaporation	23:77
Deoxyguanosine	500 eq., freeze-drying	41:59
	500 eq., evaporation	23:77
Cytidine	500 eq., freeze-drying	34:66
	500 eq., evaporation	29:71
Deoxycytidine	500 eq., freeze-drying	40:60
	500 eq., evaporation	33:67

b) Electropherograms CE-MS analysis:

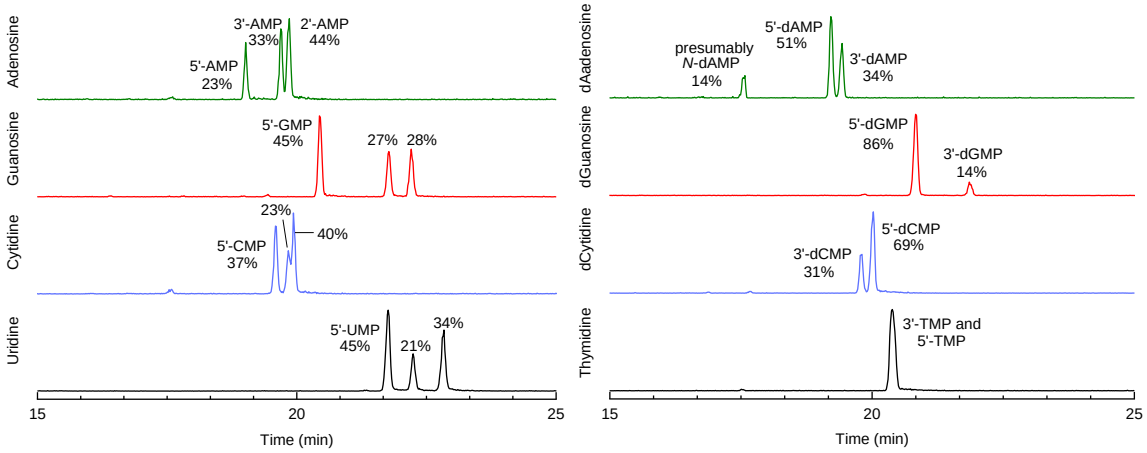


Fig. 6 | Phosphorylation of nucleosides by uP_4 (2) and electropherograms of the CE-MS analysis. a, Conditions and phosphorylation rates for the reaction of nucleosides with uP_4. If the pH of the nucleophile solution was adjusted, the applied base is indicated in brackets. Phosphorylation rates were calculated without consideration of the phosphoramidate by-product arising from the reaction of DBU with uP_4. **b**, Electropherograms of the CE-MS analysis of the product distribution. The positional isomers were determined by spiking-experiments.

Conclusion

Life relies on condensed phosphates, but ultraphosphates as a fundamental type and true constitutional isomers of the linear polyphosphates remained unstudied. In this study, we disclose the synthesis of defined monodisperse ultraphosphates containing zero to three modifications. For those short-chain ultraphosphates, we found significant hydrolytic stability expressed in half-lives up to days, which calls the antibranching-rule into question. We provide evidence for the interaction of an enzyme with ultraphosphates and describe the phosphate walk, which linearizes branched phosphates. Ultraphosphate was applied as a phosphorylating reagent for nucleophiles such as amino

acids and nucleosides with implications for prebiotic chemistry. With synthetic access to this class of molecules, the chemistry – and potential biology – of the branched phosphates can finally be studied.

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Author Contributions

T.D. and H.J.J. conceived the research project and wrote the manuscript. T.D. prepared the samples and applied them in follow-up studies. N.S. and A.H. gave input on the synthetic procedure. D.Q. developed the CE-MS method. D.Q. and M.H. analysed samples using CE-MS and supported data analysis. V.B.E. analysed samples using PAGE. A.M. provided polyphosphate extracts from yeast. J.S.S. and K.K.B. performed DFT calculations.

Competing Interests

The authors declare no competing interests.

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