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Lost in Condensation: Poly-, Cyclo-, and Ultraphosphates

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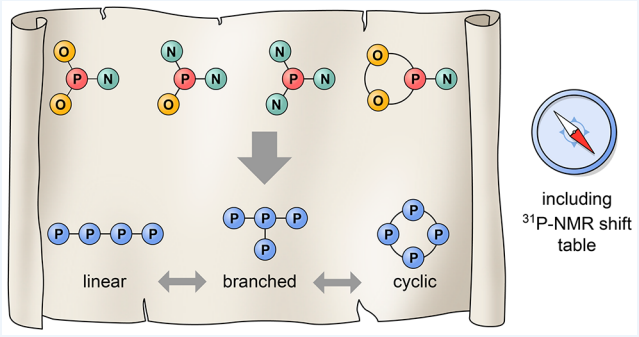
CONSPECTUS: Much like linear, branched, and cyclic alkanes, condensed phosphates exist as linear, branched, and cyclic structures. Inasmuch as alkanes are the cornerstone of organic chemistry, generating an inexplorably large chemical space, a comparable richness in structures can be expected for condensed phosphates, as also for them the concepts of isomerism apply. Little of their chemical space has been charted, and only a few different synthesis methods are available to construct isomers of condensed phosphates. Here, we will discuss the application of phosphoramidites with one, two, or three P−N bonds that can be substituted selectively to access different condensed phosphates in a highly controllable manner. Work directed toward the further exploration of this chemical space will contribute to our understanding of the fundamental chemistry of phosphates.



In biology, condensed phosphates play important roles in the form of inorganic representatives, such as pyrophosphate, polyphosphate, and cyclophosphate, and also in conjugation with organic molecules, such as esters and amidates. Phosphorus is one of the six biogenic elements; the omnipresence of phosphates in biology points toward their critical involvement in prebiotic chemistry and the emergence of life itself. Indeed, it is hard to imagine any life without phosphate. It is therefore desirable to achieve through synthesis a better understanding of the chemistry of the condensed phosphates to further explore their biology.

There is a rich but underexplored chemistry of the family of condensed phosphates *per se*, which is further diversified by their conjugation to important biomolecules and metabolites. For example, proteins may be polyphosphorylated on lysins, a very recent addition to posttranslational modifications. Adenosine triphosphate, as a representative of the small molecules, on the other hand, is well known as the universal cellular energy currency. In this Account, we will describe our motivations and our approaches to construct, modify, and synthetically apply different representatives of the condensed phosphates. We also describe the generation of hybrids composed of cyclic and linear structures of different oxidation states and develop them into reagents of great utility. A pertinent example is provided in the step-economic synthesis of the magic spot nucleotides (p)ppGpp. Finally, we provide an overview of ^{31}P NMR data collected over the years in our laboratories, helping as a waymarker for not getting lost in condensation.

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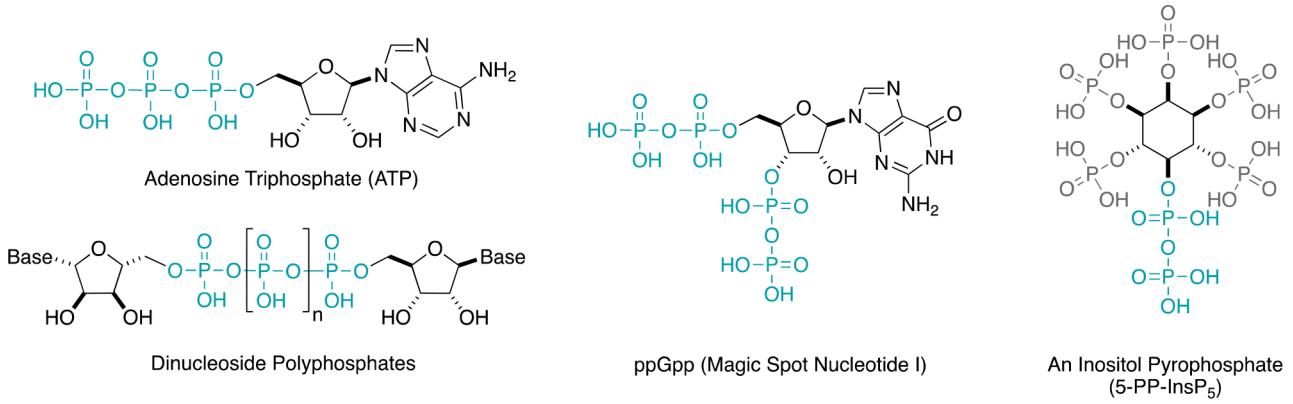

Figure 1. Examples of some modified condensed phosphates important in biology. Under physiological conditions, these will be partially deprotonated and complexed with different ions. To avoid confusion, we opted to show the fully protonated forms. Anhydrides are highlighted in turquoise, and esters, in gray.

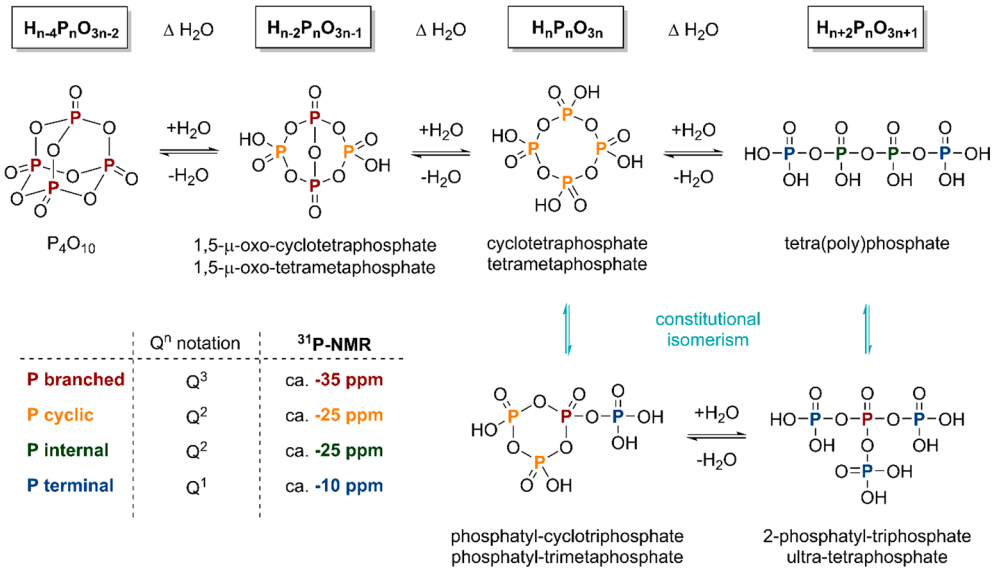

Figure 2. Possible structures of condensed phosphates with four phosphate units. Isomerism relationships among linear, cyclic, and branched condensed phosphates, signature ^{31}P NMR shifts, and Q^n notation, which is commonly used to describe terminal (Q^1), internal (Q^2), and branching (Q^3) phosphates.[18]

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1. INTRODUCTION

Phosphate is essential for survival; therefore, life has developed sophisticated mechanisms to acquire, distribute, utilize, store, and remove phosphate, maintaining a highly balanced phosphate homeostasis in organisms.[5,6] In biology, phosphates are ubiquitously attached to other molecular species to regulate, for example, their reactivity, function, shape, localization, and solubility. As a result, a rich array of chemical structures containing phosphates are now known. This includes phosphate diesters in the DNA backbone and phosphoric anhydrides found in adenosine triphosphate (ATP), the universal cellular energy currency.[7] Moreover, second messengers such as inositol pyrophosphates, dinucleoside polyphosphates, and alarmones such as the magic spot nucleotide ppGpp contain phosphoric anhydrides and esters (Figure 1).[8] Also, polydisperse inorganic polyphosphate (polyP), which has been identified across the kingdoms of life, is involved in diverse intra- and extracellular processes.[9] However, more can be found in biology than only linear condensed phosphates: the recently "rediscovered" metaphosphates (cyclic polyphosphates, for an example see Figure 2, and cyclotetraphosphate) of unknown function (e.g., in bacterial granules).[10,11] Because there is also the possibility of branched structures, we will now provide a comprehensive discussion of possible condensed phosphates.[12]

Condensed phosphates contain phosphoric anhydrides and occur in linear, cyclic, and branched structures or combinations thereof.[12−15] They can be additionally modified with (organic) residues, giving rise to a rich chemical space that is, for several reasons as we believe, underexplored. Among these reasons are a lack of efficient synthesis procedures in combination with purification issues aggravated by the inherent hydrolytic instability of the anhydrides.[16] For example, the simplest member of the noncyclic ultraphosphate class ultratetraphosphate uP_4 (a branched condensed phosphate without cyclic

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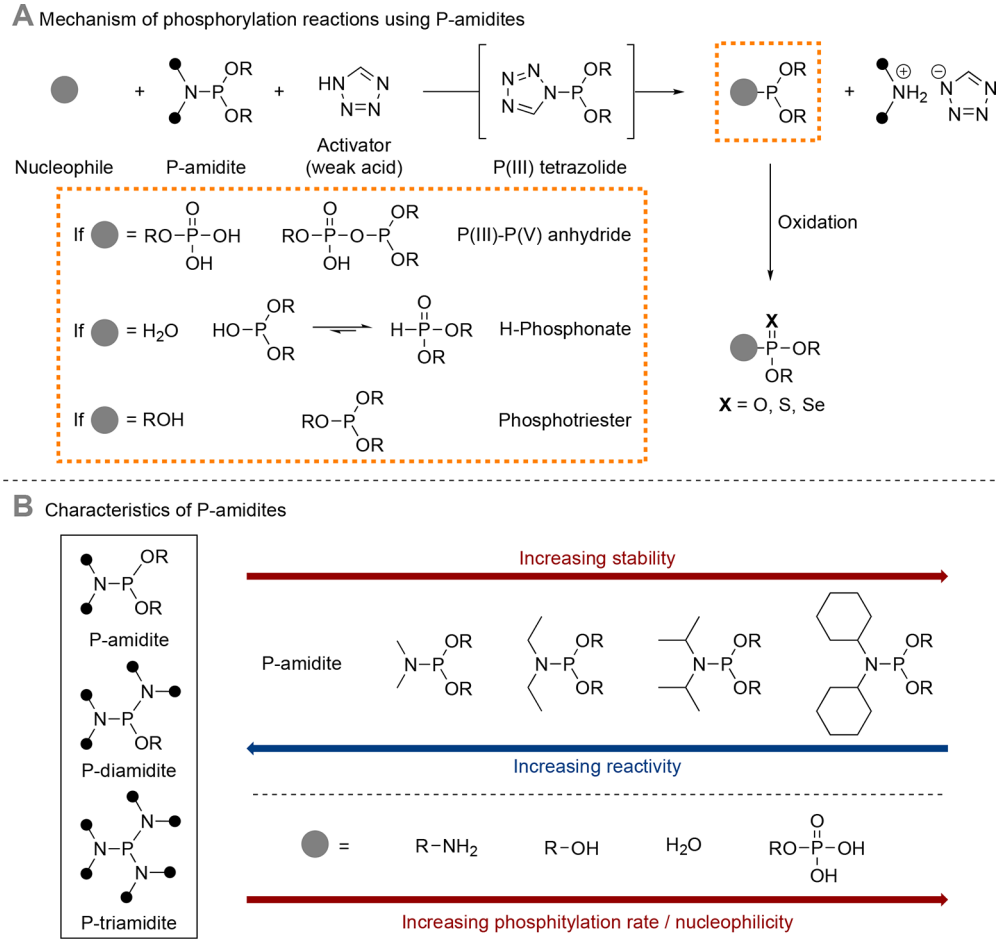

Figure 3. (A) Activation of P-amidites with tetrazole or other acidic activators and interception of the P(III)-tetrazolide intermediate with nucleophiles (orange box), followed by oxidation. (B) P-Amidites are versatile and tunable P(III) electrophiles that can be used for chemoselective mixed P-anhydride construction.

substructures composed of four phosphates, 2-phosphatyl-triphosphate) was only synthesized and characterized in 2021 (Figure 2),[1] and a DABCO adduct of that structure has also been reported recently.[17]

Condensed phosphates feature a unique chemistry, yet there are actually concealed similarities to the homologous series of alkanes and cycloalkanes. IUPAC defines the alkanes as "Acyclic branched or unbranched hydrocarbons having the general formula C_nH_{2n+2}, and therefore consisting entirely of hydrogen atoms and saturated carbon atoms." It has long been appreciated for alkanes that the number of possible linear or branched structures grows exponentially with the number of carbon atoms; for molecules with 10 carbon atoms, there are 75 possible structures, while for 12 carbon atoms there are 355 possibilities![19] A corresponding appreciation of the vastness of the phosphate space has been lacking. While the homologous alkanes are generated by consecutive formal removal of H_2 from minimal building block CH_4, the condensed phosphates are formed by the removal of water from minimal building block H_3PO_4. The formation of cycloalkanes is accompanied by another dehydrogenation (C_nH_{2n}), and every additional cyclization will require the formal removal of H_2. By analogy, the formation of cyclo-phosphates (the metaphosphates) requires the removal of one molecule of water for every cyclization.[20]

As a consequence, ultraphosphates (branched phosphates containing three connected, uncharged phosphate groups at the branch point) are constitutional isomers of linear polyphosphates ($H_{n+2}P_nO_{3n+1}$), whereas cyclic forms are not ($H_nP_nO_{3n}$; notice that we opted to describe the fully protonated acids in the Introduction). However, metaphosphates with side chains, which are branched phosphates with a cyclic subunit or (oligo)phosphatyl(oligo)metaphosphates, are constitutional isomers of metaphosphates ($H_nP_nO_{3n}$) with larger ring sizes. By extension, one would expect ultraphosphates of a certain length to give rise to stereoisomerism if a central P═O unit is connected to three different chains. The first potentially chiral ultraphosphate is 3-phosphatyl-hexaphosphate, but no such structures have ever been reported.

Despite the many hundreds of condensed phosphate structures for molecules containing from only 1 to 12 phosphorus atoms (cyclic, linear, branched in different ways, admixtures of rings and chains, fused rings, and stereoisomers), the number of crystallographically characterized polyphosphate structures is barely in double digits.[21−23] A ring containing 12 phosphorus atoms has been described and was structurally characterized as its guanidinium salt,[24] but beyond that lies uncharted territory.[25]

The analogy to (cyclo)alkanes helps us to grasp the structural richness that could potentially be generated within

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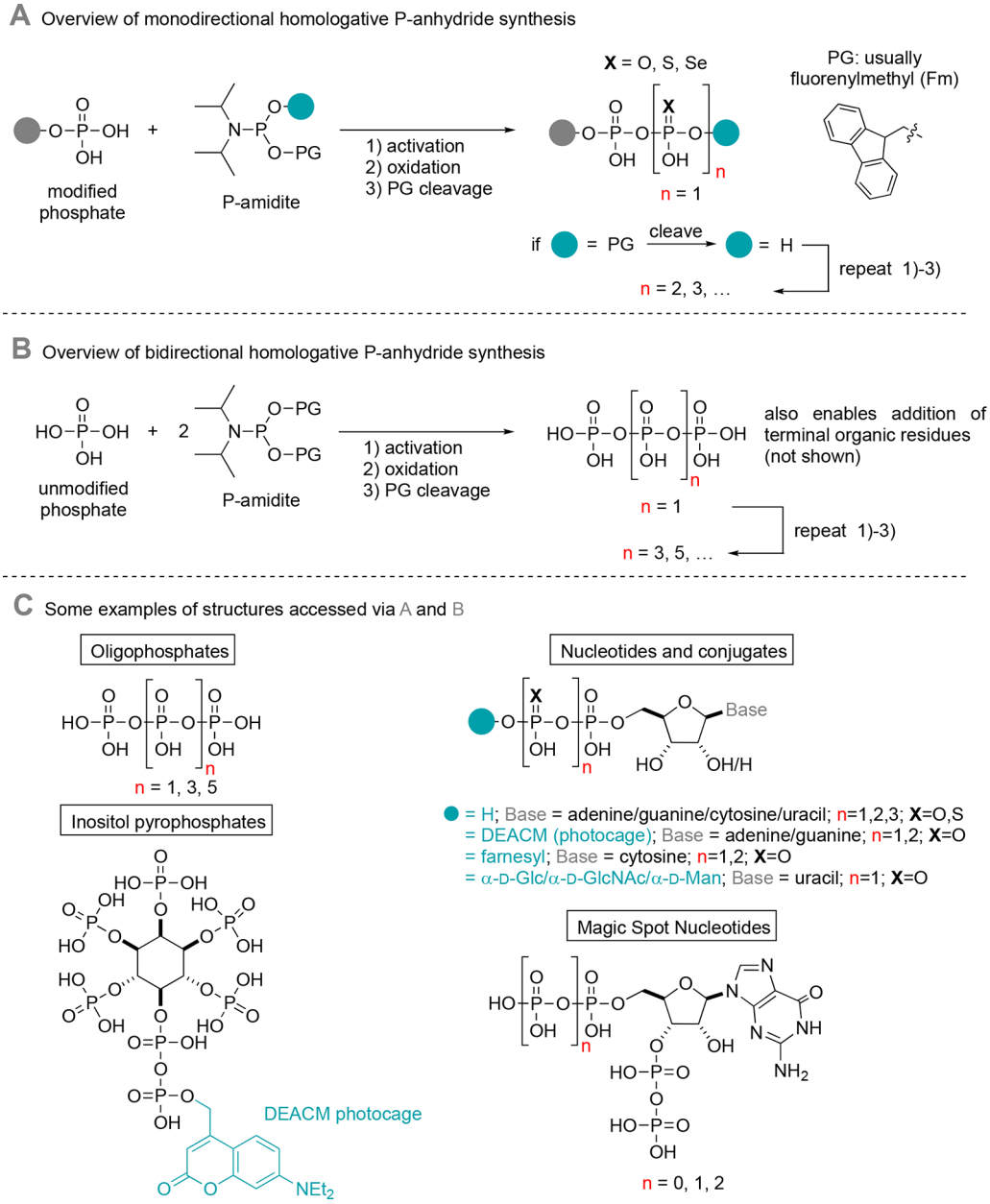

Figure 4. (A) Monodirectional homologation of functionalized (gray circle) phosphates with P-amidites. The P-amidite may contain one or two protecting groups (PG; e.g., Fm) and zero or one residue (turquoise circle; e.g., a nucleoside). The intermediacy of a P(III)−P(V) anhydride enables the variation in the oxidation (e.g., mCPBA, S_8, KSeCN) to introduce **X** in different positions. (B) Bidirectional homologation is possible with unmodified (oligo)phosphates. (C) Some examples of modified condensed phosphates accessible with the strategies.

the family of condensed phosphates, let alone the fact that also metal coordination to deprotonated OH groups will create further diversity.[22] Given the many opportunities, our groups have embarked on a synthesis program that is aiming toward further exploring the condensed phosphates and their potential application to (modified) metabolite synthesis and inorganic and analytical biochemistry. This account will give an overview of synthesis approaches from the Jessen laboratory to the diverse members of this family and discuss applications in the synthesis and analysis of phosphorylated metabolites.

2. P-AMIDITE CHEMISTRY

Phosphormonoamidites (P-amidites), introduced by Marvin Caruthers in 1981,[26] are best known for enabling automated DNA/RNA synthesis by providing a storable yet easily activatable form of a reactive P(III) electrophile. This breakthrough technology marks the advent of modern molecular biology, and it has been so successful that only very recently have alternative, potentially competitive, approaches based on P(V) chemistry been introduced.[27]

The mechanism by which P-amidites are activated with weak acids has been comprehensively reviewed by Nurminen and Lönnberg.[28] Briefly, activation with 1*H*-tetrazole leads to the rapid formation of a P(III) tetrazolide, which is intercepted with nucleophiles. In DNA synthesis, the 5′-OH group of the solid-supported (oligo)nucleotide is coupled to the P-amidite, giving rise to a P(III) triester on a minute time scale, which is then oxidized to the P(V) triester. Short reaction times of activated P-amidites with alcohols is a basic necessity of

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oligonucleotide synthesis: longer reaction times would render the assembly of long oligonucleotides impractical.

The introduction of additional P−N bonds as compared to a P-monoamidite generates phosphordiamidites (P-diamidites) and phosphortriamidites (P-triamidites) as shown in Figure 3B. They can be activated to react with nucleophiles two and three times, respectively. Such building blocks are uniquely suited to construct condensed phosphates (Q^1, Q^2, and Q^3), as will be discussed in sections 2.1−2.4.

A challenge we struggled with back in 2012 was the synthesis of stereochemically defined inositol pyrophosphates, such as 1- and 3-PP-InsP_5 and meso-compound 5-PP-InsP_5 (Figure 1).[29−31] These syntheses have been reviewed,[32] and here we will only briefly discuss a key step: the construction of the P-anhydride (shown in blue in Figure 1). There are two basic strategies for approaching this challenge: P(III) chemistry, including oxidation, for example, using a P-amidite approach, and P(V) chemistry.[33]

There are advantages and disadvantages for both approaches, but in this case, the P(III) approach was more successful, particularly when P-amidites were used as an electrophile and a phosphate was used as a nucleophile. This strategy gives rise to a mixed P(III)−P(V) anhydride, which is then oxidized to a P(V)−P(V) anhydride (Figure 3A).[8] It benefits from the rapid reaction of activated P-amidites so that rates on a second to minute timescale can be achieved. The oxidation step enables the introduction of several heteroatoms (Figure 3A, **X**). P-amidite chemistry had been used before to construct P-anhydrides, but it had not found widespread application.[34,35]

During the reaction, we noticed that the phosphate nucleophile did not have to be dry, simplifying the synthetic protocols, as most phosphates contain several equivalents of water. We challenged the reaction and ran the P-anhydride-forming step in methanol. It still worked. This was surprising because P-amidites are known to react with alcohols, the very reason for their invention in DNA synthesis.[4] Excess water (5% in DMF) was not tolerated and resulted only in the hydrolysis of the P-amidite to the corresponding H-phosphonate (Figure 3A, orange box). Even so, we were able to run the reactions in organic solvents without the removal of water. By NMR studies, we also delineated that, after consumption of the phosphate nucleophile, water (present as crystal water and traces in the solvent) reacted faster than other alcohols (primary, secondary, and tertiary), thiols, or amines (Figure 3B, gray circle). The sum of these findings allowed us to develop an enabling technology for the general construction of modified condensed phosphates based on P-amidite chemistry.[4,36]

2.1. Construction of Linear Oligophosphates by Mono- And Bidirectional Homologation

Because P-amidites react more rapidly with phosphates than with alcohols and water, protecting groups are not required on phosphate-containing substrates. Moreover, crystal water bound to phosphorylated substrates does not have to be removed. In fact, it helps to further simplify the synthesis procedure, as now a slight excess of P-amidite can be used to completely consume the starting material, after which the remaining P-amidite is hydrolyzed before reacting with other nucleophilic groups (Figure 3B).[4,36] Only phosphomonoesters or terminal OH groups of condensed phosphates react with P-amidites so that branching is avoided, which would be the result of a nucleophilic attack of an internal phosphate. This observation generally holds for the many reactions we studied, but we have not yet understood why this is the case. Thiophosphates react as oxygen nucleophiles; the sulfur atom remains unmodified.[36] Overall, it is possible to follow two related strategies in phosphate homologations: mono- and bidirectional (Figure 4A,B).

We have used these strategies to assemble different important phosphorylated metabolites and also unnatural and modified analogs for studies into their biological functions.[4,36,37] For example, it is possible to generate monodisperse oligophosphates, such as P_3, P_5, and P_7 using the bidirectional approach (Figure 4C, oligophosphates; unpublished data; a more efficient approach is discussed in section 2.4 and Figure 7B,C). Such defined compounds will be invaluable for looking into the potentially different biological functions of monodisperse linear inorganic polyphosphates.

We have accessed adenosine-modified oligophosphates beyond ATP, such as adenosine tetraphosphate Ap_4.[4,38,39] Interestingly, adenosine tetraphosphates and higher homologues up to nucleoside nonaphosphates have been described independently by us, Andexer,[38] and Jendrossek[40] as products of *in vitro* reactions using bacterial polyphosphate kinases (PPKs). The potential biological functions of such compounds are unknown.

The monodirectional homologation has also been used to generate and apply novel photocaged nucleotides, such as [7-(diethylamino)coumarin-4-yl]methyl-ATP (DEACM-ATP), thio-DEACM-ATP, farnesylated CDP, and CTP as lipophilic analogs, and the important group of nucleoside diphosphate sugars, notably without protecting groups on the sugar−phosphate substrate (Figure 4C).[36] DEACM-caged and cell-permeant inositol pyrophosphates,[41] such as symmetric DEACM 5-(PP)-InsP_5,[42] 2-(PP)-InsP_5,[43] unsymmetric DEACM 1-(PP)-InsP_5,[44] and even caged 1,5-$(PP)_2$-InsP_4,[45] the most densely phosphorylated second messenger known to date, have been accessed using this strategy (Figure 4C). Their biological functions, particularly their impact on Ca^{2+} oscillations, have been studied using *in cellulo* DEACM uncaging.[44−46] Furthermore, we have demonstrated the application of monodirectional iterative homologations in the chemoenzymatic synthesis of magic spot nucleotides, which will be discussed in more detail in section 3.

Overall, the serendipitous finding in our initial inositol phosphate syntheses that P-amidites react chemoselectively with phosphates in the presence of other nucleophiles has simplified the usually cumbersome process of modified P-anhydride synthesis. On the basis of this strategy, we have been able to access very difficult highly negatively charged synthetic targets. It is important, though, to notice that recently many exciting developments in P-anhydride construction, relying mostly on P(V) chemistry, have been disclosed and are generating new impetus and opportunities in the field.[23,34,47,48]

2.2. Construction of Modified Linear Oligophosphates by Homologative Dimerization

Whereas the P-monoamidite originates from a P-monocation synthon, a P-diamidite reacts as a synthetic equivalent of a P-dication. It can therefore be used in homologative dimerizations of oligophosphates with n and m phosphate units, giving products with a combined chain length of $n + m + 1$ after oxidation and deprotection.[37] In the laboratory, it is easy to conduct reactions where $n = m$ (homodimerization; also

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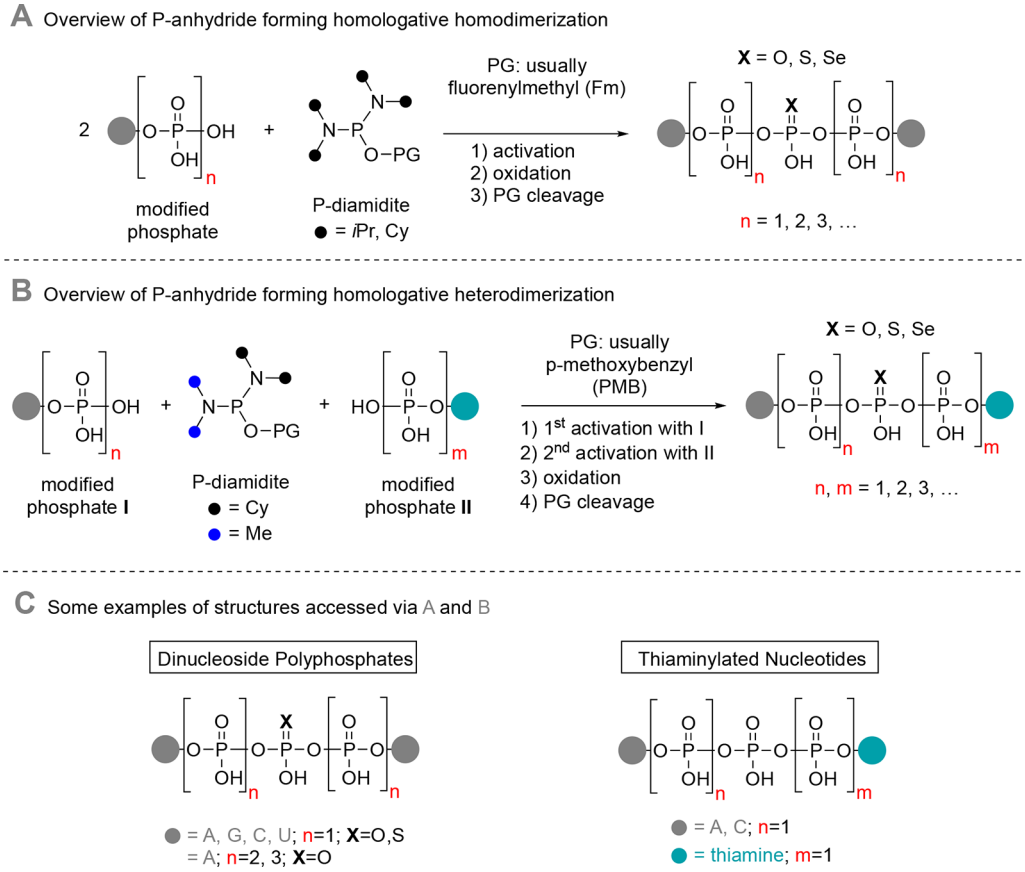

Figure 5. (A) Homologation by the homodimerization of functionalized (gray circle) phosphates with P-diamidites. (B) Homologation by the heterodimerization of functionalized (gray and turquoise circles) phosphates with P-diamidites by the consecutive exchange of NMe_2 (blue circles) and NCy_2 (black circles). (C) Examples of synthesized structures. Abbreviations: Cy, cyclohexyl; PG, protecting group.

residues on the phosphate chains are identical) and much more difficult, though possible, to achieve heterodimerizations ($n \neq m$ and/or different residues on the phosphate chains). This is summarized in Figure 5. We have used this strategy to rapidly construct several members of the dinucleoside polyphosphate family and also thiaminylated nucleotides, such as ApppTh (Figure 5C).[37]

Dinucleoside polyphosphates (Np_xN) are connected through a short oligophosphate chain (p_x), ranging from 2 to 8 units, and are capped by different nucleosides (often A and/or G but not limited to these). Given the structural diversity, comparably little is known about their biological functions, yet they have been observed across the kingdoms of life. In mammals, some seem to target the purinergic system, most notably as agonists of the P2Y receptors. Moreover, Ap_xGs have recently been shown to serve as noncanonical initiating nucleotides in RNA synthesis and were identified in distinct RNA caps in *E. coli*.[49] The chemical syntheses of Np_xN have been summarized recently by Roy.[47]

The growing recognition of the importance and utility of Np_xN in several areas of research and our limited knowledge of their biological function have motivated us to develop a method based on a homologative homodimerization that provides ready access to this family of molecules (Figure 5A).[37] While we have demonstrated the principal applicability of homologative heterodimerizations, for example, in the synthesis of ApppTh (thiaminylated nucleotides), more optimization is required to avoid significant byproduct formation via concurrent homodimerization (Figure 6B,C).

2.3. Construction of Branched Condensed Phosphates without Cyclic Subunits

P-Triamidites represent synthetic equivalents of a P-trication synthon and hence should enable the reaction with three phosphate nucleophiles to give branched phosphate oligomers, called ultraphosphates (Q^3 phosphates).[1] The synthesis of defined ultraphosphates without cyclic subunits has not received attention, mainly due to the antibranching rule coined in 1950. It postulates an extremely rapid hydrolysis of branched phosphates in aqueous solution.[13] This rule has been reiterated in the recent literature.[50] In contrast to this paradigm, we were recently able to purify several modified and unmodified ultraphosphates, obtained by P-triamidite chemistry, in aqueous buffers using strong anion exchange (SAX) chromatography. However, ultraphosphates indeed hydrolyze over time, and if four phosphates are involved, they do so into a mono- and a triphosphate (Figure 6A).[1]

We tracked this decomposition by $^{31}P(^{1}H)$ NMR for several modified ultraphosphates and found varying pH-dependent half-lives. For example, a 3-fold adenosyl-modified ultratetraphosphate had a half-life of 16 h at pH 10.0. We found even higher stability up to several days for the unmodified ultratetraphosphate (uP_4), which is synthetically accessible by basic deprotection using a fluorenylmethyl (Fm)-protected phosphate (Figure 6A).

Since the [PPN] (bis(triphenylphosphine)iminium) cation is known to stabilize anions, we developed a method to isolate ultraphosphate [PPN] salts directly from fractions after SAX purification using NaCl as an eluent.[1] This enabled the storage

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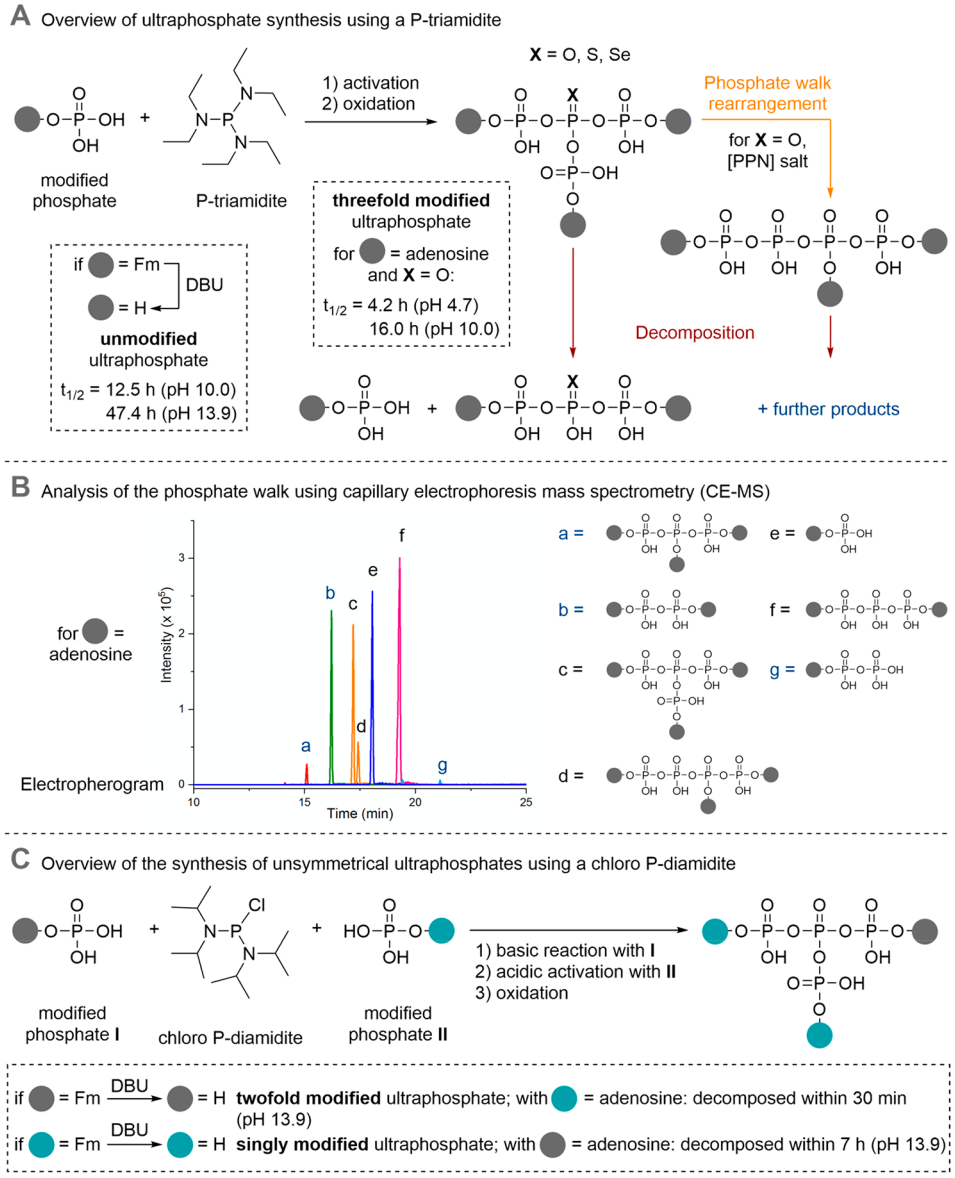

Figure 6. (A) Synthesis of 3-fold-modified ultraphosphates using a P-triamidite and the rearrangement of their [PPN] (bis(triphenylphosphine)-iminium) salts by the phosphate walk. Deprotection of Fm-modified ultraphosphate enables the synthesis of unmodified ultraphosphate. Exemplary hydrolysis half-lives ($t_{1/2}$) are given and were calculated by assuming pseudo-first-order reaction kinetics. (B) Analysis of the phosphate walk using capillary electrophoresis mass spectrometry (CE-MS). (C) Synthesis of unsymmetrical ultraphosphates using a chloro-P-diamidite and the deprotection of Fm-modified structures to yield 2-fold and singly modified ultraphosphates.

of ultraphosphates in solid form without decomposition and also solubilized the salts in several polar organic solvents. Interestingly, when ultraphosphates were allowed to stand for several days to weeks in solution in organic solvents, we detected unexpected decomposition products (Figure 6B, products a, b, and g) that appeared in addition to the expected hydrolysis products (modified mono- and triphosphates, see Figure 6B, products e and f).

A combination of ^{31}P-HMBC and capillary electrophoresis mass spectrometry (CE-MS) analysis allowed the identification of a linearized tetraphosphate (Figure 7B, compound d) as the product of a novel rearrangement, which we christened the "phosphate walk". In this rearrangement, one phosphate branch "walks into the line", potentially via a phosphatyl-cyclotriphosphate intermediate (which was supported by DFT calculations) or, alternatively, a one-step mechanism. The phosphate walk appears to be a common rearrangement of ultraphosphates in organic solvents, observed for a range of different ultraphosphate starting materials, leading to linearization.[1]

The successful analysis of phosphate walk products provides a striking example of the unique separation efficiency of CE-MS for highly charged small organic and inorganic molecules. We are confident that CE-MS will increase its already significant impact in this area of research by enabling the rapid analysis of complex mixtures containing condensed phosphates without degradation. Its ability to separate highly charged and labile small molecules based not only on charge but also on shape was recently demonstrated by our group in the analysis of inositol phosphate and pyrophosphate regioisomers.[51] For the construction of unsymmetrically modified ultraphosphates, we used a chloro P-diamidite (Figure 6C). This reagent enabled us to first introduce one modified phosphate in a basic environment under which the

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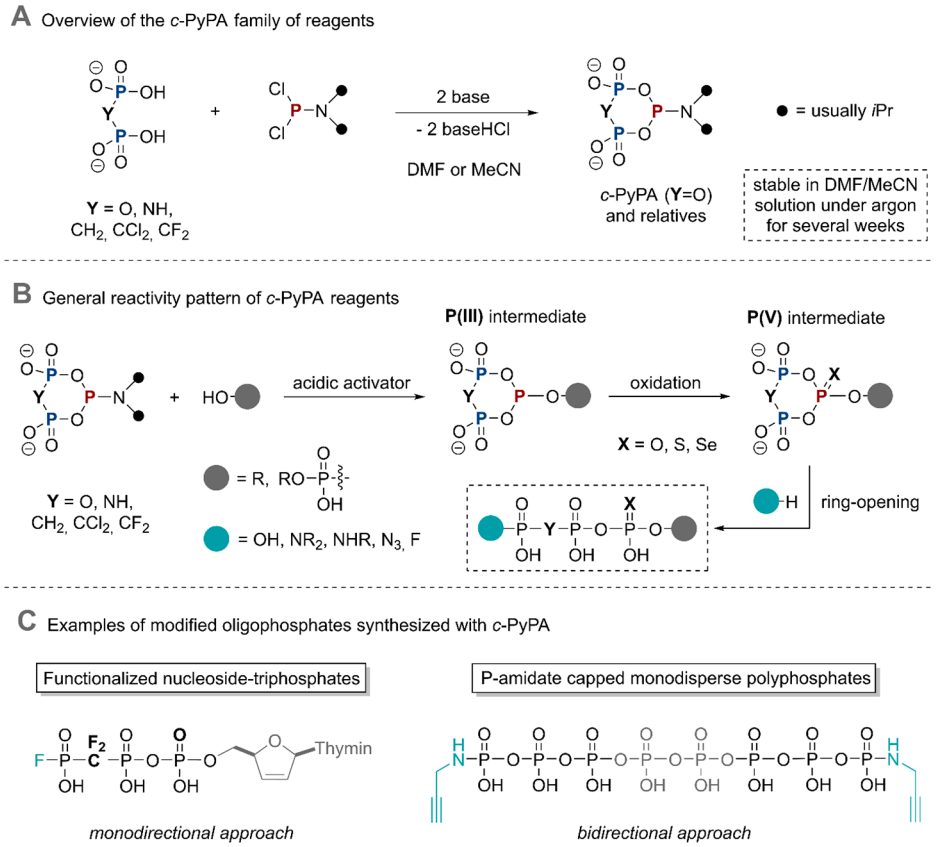

Figure 7. (A) Family of c-PyPA reagents. (B) c-PyPA can be used to modify O-nucleophiles (gray circle) followed by oxidation and ring opening with another nucleophile (turquoise circle). (C) Two examples of different molecules accessed using c-PyPA and its derivatives in either monodirectional triphosphorylation (nucleoside analog d4T) or bidirectional triphosphorylation (polyphosphate analog P_8).

two P−N bonds remain stable, followed by the reaction of the intermediate P(V)−P(III)-diamidite with differently modified phosphates under acidic conditions (Figure 6C). Although over-reaction in step 1 and unselective phosphate exchange in step 2 caused byproduct formation, the products could be isolated after SAX purification. Deprotection of Fm-modified structures gave access to 2-fold and singly modified ultraphosphates, which decomposed within 30 min (two adenosines connected to the ultraphosphate) to 7 h (one adenosine connected to the ultraphosphate) at pH 13.9. While this demonstrates that ultraphosphates indeed undergo hydrolysis, it is not instantaneous, and we are confident that a rich chemistry awaits further exploration. In conclusion, now that we discovered methods to construct and purify ultraphosphates without cyclic subunits, the time has come for an in-depth experimental elucidation of their chemical properties and even their potential biology.

2.4. Construction of Cyclic Oligophosphates with Cyclic Pyrophospho-P-amidites (*c*-PyPA) and Their Application to Modified Nucleotide Synthesis

When analyzing the reactivity of mixed chloro P-amidites as discussed (section 2.3), it occurred to us that dichloro-P-monoamidites (better known as phosphoramidous dichlorides) could potentially be used to construct cyclic oligophospho-P-amidites under basic conditions as activatable P(III) analogs of cyclophosphates. We studied the smallest possible member of this family of compounds, which we named cyclic-pyrophosphoryl P-amidite (*c*-PyPA) as well as its phosphonate analogs (Figure 7A).[3,52]

We were particularly interested in studying the potential reactivity of *c*-PyPA toward diverse nucleophiles under weakly acidic activation, followed by oxidation and ring opening with another nucleophile. If successful, this approach would provide access to a range of modified nucleoside oligophosphates or other modified polyphosphates.

In terms of reactivity, *c*-PyPAs principally resembles a P-monoamidite as discussed in section 2.1. Therefore, we studied both mono- and bidirectional extensions. Such approaches are particularly efficient for the introduction of several phosphate units in a single reaction sequence: each *c*-PyPA delivers three phosphate units onto a nucleophile, without protecting groups. This general reactivity is summarized in Figure 7B, and two examples that were accessed using *c*-PyPA and analogs are shown in Figure 7C.[3,52]

In a single reaction sequence, several modifications can be introduced. For example, the α-position of an NTP can be modified (in this case, d4T), the bridging oxygen between $\beta-\gamma$ phosphates can be replaced (in this case, CF_2 as a nonhydrolyzable analog), and diverse nucleophiles can be introduced into the γ-phosphate (in this case, F). The bidirectional extension, applied to construct a P_8 - analogue (Figure 7C, P-amidate-capped monodisperse polyphosphates), has made available defined and clickable oligophosphates that we used to identify novel targets of protein polyphosphorylation on a protein microarray.[53] The accessibility of defined, modified polyphosphates will help to further study polyphosphate biology by providing access to probes that were previously difficult to obtain.

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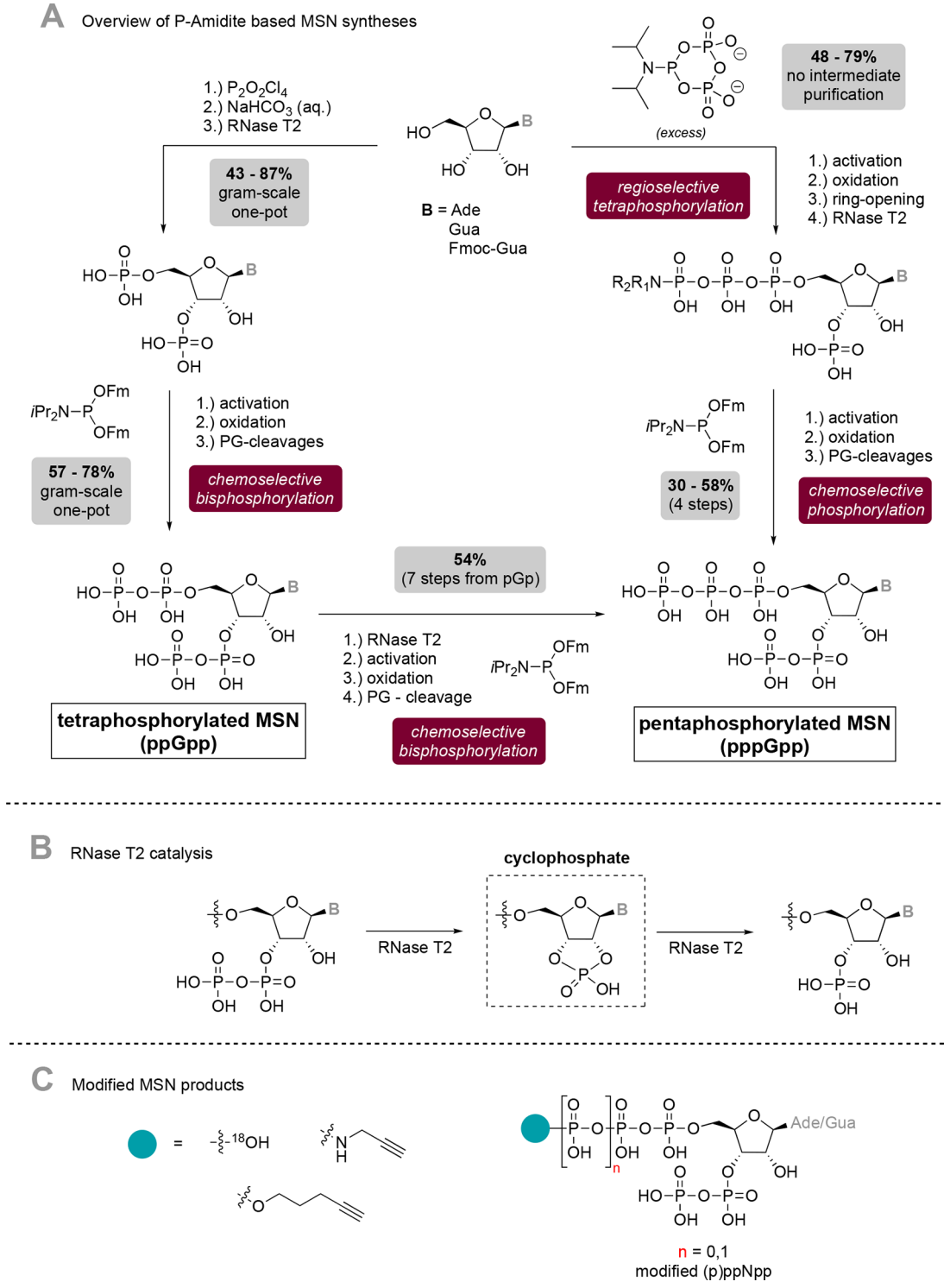

Figure 8. (A) Left: MSN synthesis by a sequential bisphosphorylation approach based on monodirectional P-amidite extensions. Right: MSN synthesis by regioselective tetraphosphorylation with c-PyPA followed by a monodirectional P-amidite extension. (B) Illustration of two-step RNase T2 catalysis. (C) Structural illustration of modified MSN products.

3. APPLICATIONS TO COMPLEX SYNTHETIC TARGETS: MAGIC SPOT NUCLEOTIDES

There are many intriguing densely phosphorylated second messengers to which synthetic access is severely limited. The application of the above-described approaches can lead to elegant solutions to long-standing synthetic problems and can additionally make useful molecular tools accessible to better understand biological functions of certain densely phosphorylated metabolites. Among these molecules are the magic spot nucleotides (MSN). They constitute a class of hyperphosphorylated guanosine and adenosine nucleotides present in bacteria and plants. These nucleotides are key regulators of the bacterially stringent response that is initiated to adapt to adverse conditions, such as starvation and sudden changes in pH and temperature. MSN are characterized by their 3′,5′-bis-

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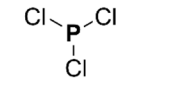
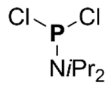
Reagents

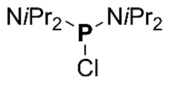
shifts are given in ppm (solvent = $CDCl_3$)



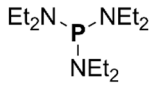
δ (^{31}P) = 220



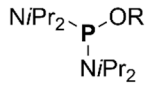
δ (^{31}P) = 169



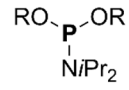
δ (^{31}P) =135



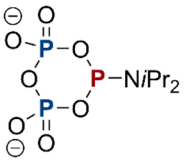
δ (^{31}P) = 118



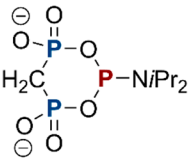
δ (^{31}P) = 123



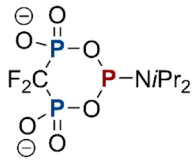
δ (^{31}P) = 148



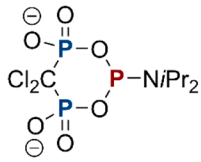
δ (^{31}P) = 130 (t)
δ (^{31}P) = -17 (d)



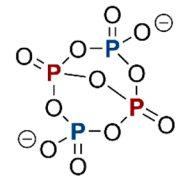
δ (^{31}P) = 126 (t)
δ (^{31}P) = 6 (d)



δ (^{31}P) = 127 (m)
δ (^{31}P) = -5 (ddd)



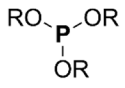
δ (^{31}P) = 129 (t)
δ (^{31}P) = -1 (d)



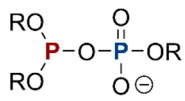
δ (^{31}P) = -33 (t)
δ (^{31}P) = -24 (t)

P(III) - Intermediates

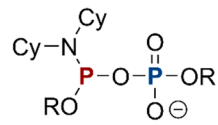
shifts are given in ppm (solvent = $CDCl_3$, DMF-d7, MeCN-d3)



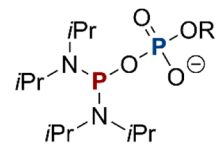
δ (^{31}P) = 138



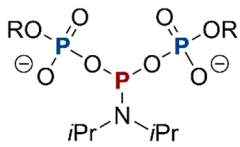
δ (^{31}P) = 128 (s)
δ (^{31}P) = -9 (s)



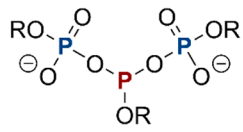
δ (^{31}P) = 137 (d)
δ (^{31}P) = -7 (d)



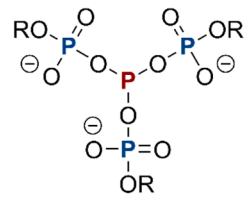
δ (^{31}P) = 120 (d)
δ (^{31}P) = -9 (d)



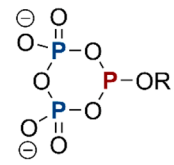
δ (^{31}P) = 126 (t)
δ (^{31}P) = -9 (d)



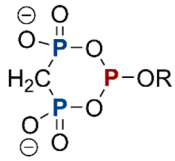
δ (^{31}P) = 118 (s)
δ (^{31}P) = -9 (s)



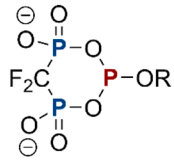
δ (^{31}P) = 120 (s)
δ (^{31}P) = -10 (s)



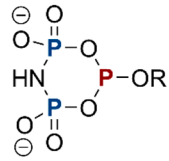
δ (^{31}P) = 106 (t)
δ (^{31}P) = -23 (d)



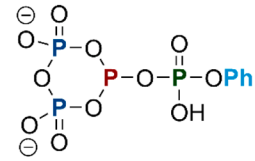
δ (^{31}P) = 106 (t)
δ (^{31}P) = 5 (d)



δ (^{31}P) = 108 (td)
δ (^{31}P) = -7 (ddd)



δ (^{31}P) = 107 (t)
δ (^{31}P) = -12 (d)



δ (^{31}P) = 98 (td)
δ (^{31}P) = -22 (d)
δ (^{31}P) = -16 (d)

General remarks: if R = aryl: downfield shift for ^{31}P of about 5 ppm compared to R = alkyl

Figure 9. continued

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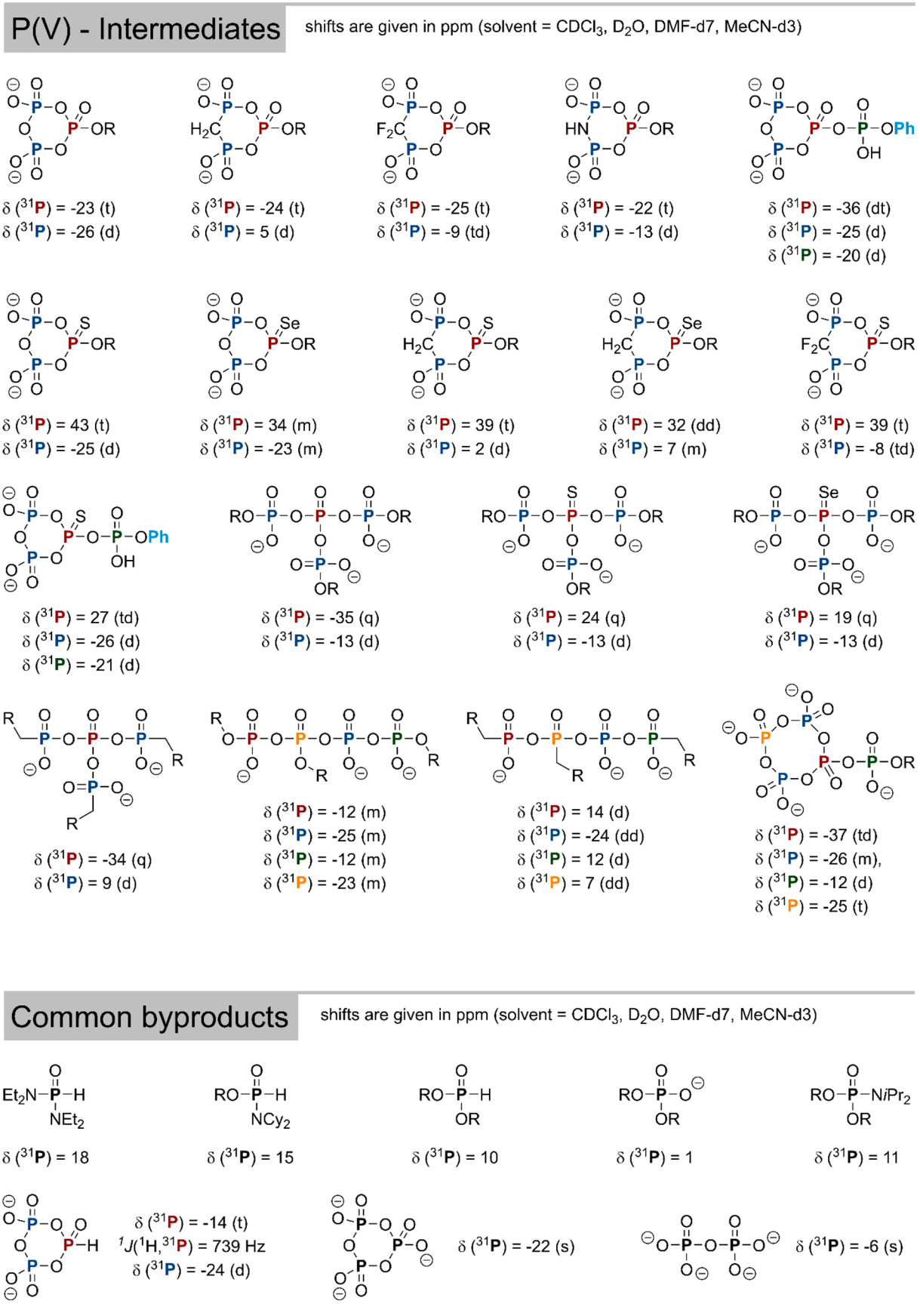

Figure 9. continued

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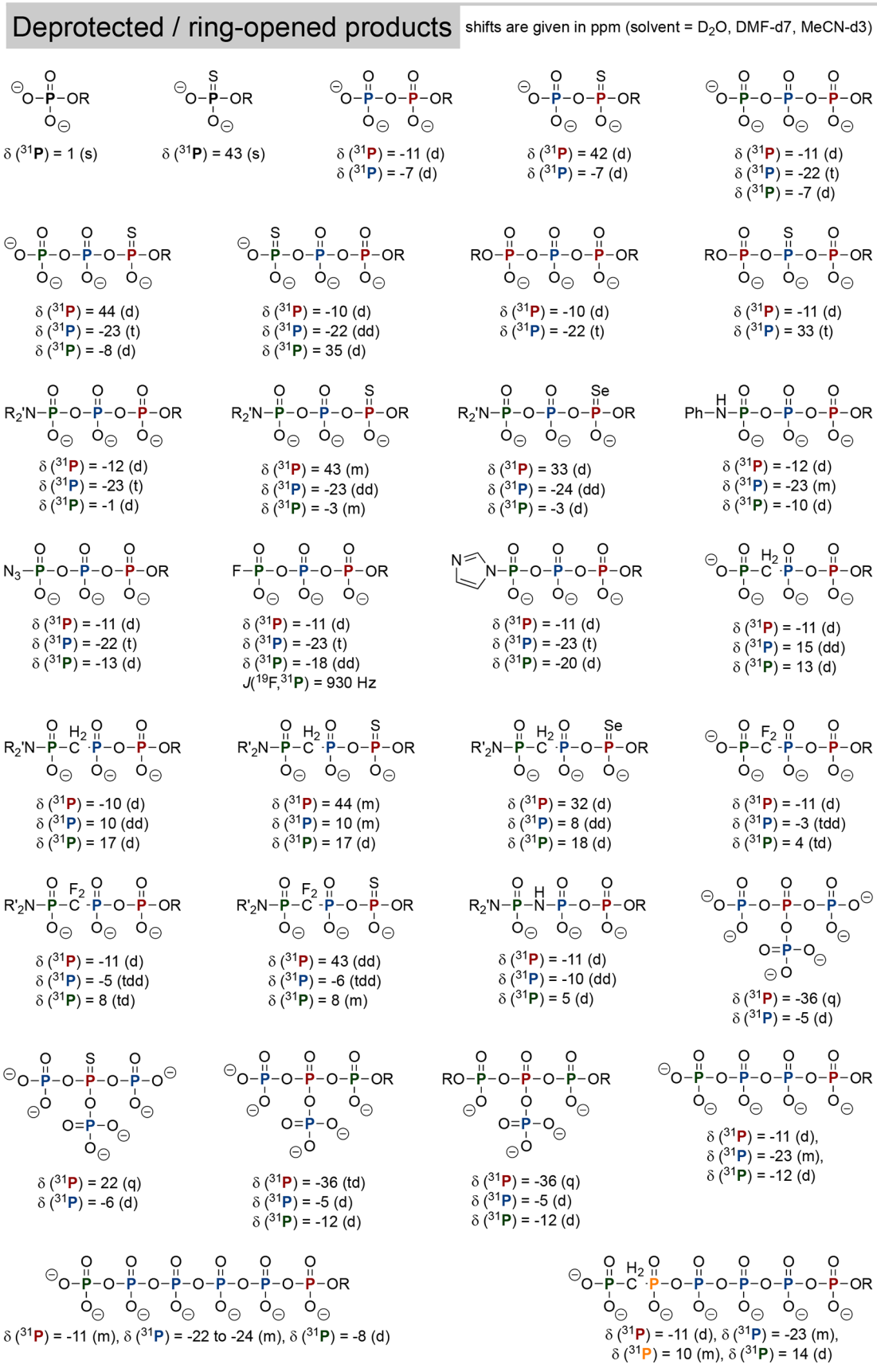

Figure 9. Overview of ^{31}P NMR chemical shifts and multiplicities of reagents, intermediates, and products as encountered in the family of the condensed phosphates.

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oligophosphate frameworks as found in ppGpp, pppGpp, ppApp, and pppApp.[54]

Synthetic chemistry approaches were developed in the 1970s and 1980s but suffered from relatively long sequences and single-digit yields.[55] While MSN are accessible using enzymatic approaches, there are still issues regarding efficiency and the accessibility of analogs. Figure 8 summarizes our complementary solutions to this problem: the chemoenzymatic synthesis of several MSNs based on 2′,3′-cyclophosphate ring opening with RNase T2 (Figure 8B), chemoselective monodirectional extensions, and c-PyPA.[2,56] Using these approaches in telescoping reactions and one-flask transformations, we have been able to access ppGpp on a gram scale in an overall yield of 68% and with only one intermediate product requiring purification.

In our sequential bisphosphorylation protocol from 2019 (Figure 8A, left part), commercially available nucleosides were transformed with pyrophosphoryl chloride followed by basic hydrolysis and RNase T2 catalysis toward the key intermediate pNp in single-flask operations. Subsequently, the chemoselective nature of P-amidite phosphitylations enables an efficient bisphosphorylation of pNp to ppNpp on a gram scale, again in a one-flask reaction. The deprotection of Fm groups must be carried out with DBU to prevent the formation of the corresponding 2′,3′-cyclophosphate byproducts. The treatment of ppNpp with RNase T2 generates ppNp structures, that again function as bisphosphorylation substrates toward pentaphosphorylated MSN (pppNpp).

In 2020, we presented a synthetic shortcut toward pppNpp by applying c-PyPA in a regioselective simultaneous tri- and monophosphorylation process (Figure 8A, right part).[2] Treating nucleosides with an excess of c-PyPA, followed by the oxidation and addition of amine nucleophiles, led to the exclusive formation of nucleoside-5′-amidotriphosphate-2′,3′-cyclophosphate structures. This can be explained by an amine-induced ring-opening cyclization event. Subsequent RNase T2 catalysis selectively delivered amido-pppNp structures. The chemoselective phosphorylation of amido-pppNp with Fm-P-amidite at the 3′-phosphate provided amido-pppNpp, relying on the blocked reactivity of terminally capped 5′-phosphates (section 2). The amidate products can then be hydrolyzed at pH 3, leading to pppNpp natural products or to isotopomers with $H_2{}^{18}O$ as the solvent. RNase T2 catalysis is pivotal in all of these approaches, allowing facile and selective regiodiscrimination between 2′- and 3′-OH based on the corresponding cyclophosphates (Figure 8B).

In addition to various MSNs (e.g., ppGpp, ppApp, pppGpp, pppApp, ppAp, ppGp, and pGpp), a variety of modified MSN structures (Figure 8C) were accessible by using either mixed P-amidites or ring opening with propargylamine. These "clickable" analogues can then be transformed into biotinylated or fluorescent MSN-versions that are applicable in the context of chemical biology.

4. ANALYSIS OF CONDENSED PHOSPHATES BY ^{31}P NMR

A powerful analytical tool for following reactions using all the above-described methods is ^{31}P NMR. Samples can simply be drawn from reaction mixtures and analyzed after the addition of deuterated solvent (usually DMF-d_7, $CDCl_3$, or MeCN-d_3). We hope that the list provided below (Figure 9) will be useful for those navigating in the realm of condensed phosphates. It summarizes data mostly collected in our laboratory and also includes some very useful data on related species obtained in other laboratories.[1,4,22,23,31,36,37,48,52,57] The chemical shift values will vary depending on the concentrations, pH, solvent, and particularly the attached residues but will by and large revolve around the values indicated. However, this list is not meant to be comprehensive, and it will not be applicable to all possible modifications. We are confident, though, that it will be very useful for initial putative assignments.

5. CONCLUDING REMARKS

The countless possible combinations of linear, branched, and cyclic polymeric hydrocarbon structures are the cornerstone of organic chemistry. We argue that condensed phosphates share reminiscent features; therefore, a vast and largely uncharted territory lies before us. The importance of condensed phosphates in biology, their contribution to the emergence of life,[58] and their societal impact are beyond doubt.

New methodology to deliberately connect and disconnect different condensed phosphate structures, enabling the synthetic chemist to add and subtract water molecules on demand, will greatly spur the realization of the great potential of condensed phosphate chemistry. It should be possible to create a polyphosphate toolbox for chemical biology comprising a wealth of available structures, together with methods for interconverting them and for making connections between them and a variety of organic molecules. By ushering into existence many new, well-defined condensed phosphate structures and reactions, synthetic chemists will set the stage for new classes of hybrid organic−inorganic materials that might prove to be valuable as battery electrolytes or as biodegradable, environmentally friendly polymers, in addition to the obvious biological and medicinal chemistry applications. In this context, we hope that this Account will serve others as a compass to navigate within the large realm of the condensed phosphates.

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Notes

The authors declare no competing financial interest.

Biographies

Henning J. Jessen has been a professor of bioorganic chemistry at the University of Freiburg, Germany since 2015. He received his Ph.D. in 2008 from the University of Hamburg, working with Professor Chris Meier. He was postdoctoral fellow in the group of Professor Karl Gademann at EPFL and the University of Basel and an oberassistent with Professor Jay Siegel at the University of Zürich. His research interests include the bioorganic chemistry of condensed phosphates.

Tobias Dürr received his B.Sc. and M.Sc. in chemistry from the University of Freiburg with Prof. Reinhard Brückner and Prof. Henning J. Jessen. He is currently a Ph.D. candidate at Albert-Ludwigs-Universität Freiburg (2018−present) with Prof. Henning J. Jessen, focusing on the chemistry of branched condensed phosphates, called ultraphosphates.

Thomas M. Haas received his B.Sc. and M.Sc. in chemistry from the University of Freiburg working with Prof. Jan Streuff and Prof. Henning Jessen. After a research stay at the University of Bologna with Professor Cavani, he has been a Ph.D. candidate with Professor Jessen since 2017. His research is focused on the development of synthetic methods for magic spot nucleotides and phosphate isotopomers.

Alexander Ripp received his B.Sc. in chemistry in 2016 from the University of Freiburg. He obtained his M.Sc. degree in 2019 under the guidance of Professor Henning J. Jessen and continued in the group as a Ph.D. student within the DFG Cluster of Excellence *liv*MatS. His research is focused on selective polyphosphorylations and light manipulation of phosphates within the context of adaptive materials.

Christopher C. Cummins is the Henry Dreyfus Professor of Chemistry at the Massachusetts Institute of Technology (MIT) in Cambridge, Massachusetts. He received his Ph.D. in inorganic chemistry in 1993 from MIT, working with Professor Richard R. Schrock. His research interests are in the area of exploratory synthetic chemistry and reactivity studies.

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