A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition

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Accepted Article

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To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201814366
Angew. Chem. 10.1002/ange.201814366

Link to VoR: http://dx.doi.org/10.1002/anie.201814366
http://dx.doi.org/10.1002/ange.201814366

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10.1002/anie.201814366

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A phosphoramidite analog of *cyclo*triphosphate enables iterative polyphosphorylations

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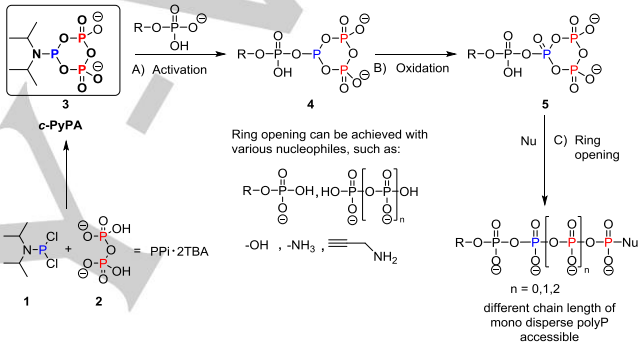
Abstract: An iterative polyphosphorylation approach is described, which is based on a phosphoramidite (P-amidite) derived reagent (*c*-PyPA) obtained from the cyclization of pyrophosphate with a reactive di*iso*propylamino dichlorophosphine. This type of reagent is unprecedented as it represents a reactive P-amidite without protecting groups. The reagent proved to be stable in solution over several weeks. Its utility is described in the context of iterative monodirectional and bidirectional polyphosphorylations. The ensuing functionalized *cyclo*triphosphate can be opened with a variety of nucleophiles providing ready access to diverse functionalized polyphosphate chains of defined length with several tags, including both P-N and P-O labels. Their interaction with exo- and endopolyphosphatases is described.

Condensed phosphates, such as found in nucleoside triphosphates (NTPs), are of central importance in biology.[1] In addition, higher homologs, such as adenosine tetra- and pentaphosphates[2] have been identified and synthetically modified nucleoside hexaphosphates are used in next generation sequencing.[3,4] The pervasiveness of this type of modification in biology, however, extends far beyond nucleotides.[5] For example, inorganic polyphosphate (polyP) is a polydisperse biopolymer that can be composed of tens to thousands of phosphate unit.[6] PolyP has been well characterized in bacteria where it was shown to serve crucial functions, e.g. during the bacterial stress response, in biofilm formation, and as a phosphate storage molecule. In yeast, polyP synthesis is regulated by inositol pyrophosphates[7] via activation of the SPX domain of the vacuolar transporter chaperone complex.[8] In mammals, polyP is involved in the regulation of the blood-clotting cascade, but the enzymes generating it remain elusive.[9] Reports suggest polyphosphorylation as a new posttranslational modification of proteins,[10,11] and cellular delivery of a polydisperse, labeled polyP has recently been achieved.[12]

Therefore, access to modified polyphosphates is highly desirable, yet the synthesis of such labile structures in monodisperse form is significantly understudied.[13] Approaches to analogs of polyP exist, but they rely on the biochemical synthesis of polydisperse polyP followed by modification resulting in P-amidate end-labeling.[14] An efficient chemical approach would require being rapid, iterative, high-yielding and additionally enable the introduction of orthogonal modifications. A recent report has made use of the activation of *cyclo*triphosphate (also often referred to as trimetaphosphate, tmp) as an *N*-methylimidazolium salt according to Taylor[15] followed by nucleophilic displacement and ring opening with an amine nucleophile to obtain a modified P_8 in a one-flask operation. A drawback of this procedure is the low yield obtained (ca. 10%).[11] Very recently, a new approach for the activation of *cyclo*triphosphate has been developed by Cummins.[16] In a wider context, the development of a P-(III) based triphosphorylation procedure including mixed oxidation states has been pioneered by Ludwig and Eckstein[17] and a phosphoramidite (P-amidite) for the introduction of methylene bisphosphonates has recently been reported by Filippov.[18] However, no P-amidite reagents[19] without protecting groups are available for triphosphorylations.

Here, an approach is presented based on the development of a P-amidite derived reagent, which we dubbed *c*-PyPA (*cyclic* pyrophosphoryl P-amidite, Scheme 1) that proceeds through P(III)-P(V) intermediates[20] in a rapid, modular, and high-yielding fashion. It does not require the use of protecting groups.



Scheme 1. ***c*yclic-*py*rophosphoryl-*P*-*a*midite (*c*-PyPA) **3** enables the synthesis of polyP with defined chain lengths.

The synthesis of *c*-PyPA **3** comprises the reaction of di*iso*propylamino dichlorophosphine **1** and pyrophosphate **2** as the 2 × tetrabutylammonium (TBA) salt in equimolar amounts in the presence of a base under strictly dry conditions. *c*-PyPA **3** can be prepared both in DMF or MeCN and is stable over weeks in solution if stored under argon at -20 °C. The ^{31}P NMR of reagent *c*-PyPA **3** is shown in figure 1 B (i) with a diagnostic shift of 130 ppm and a ^{3}J P-P coupling (triplet) for the P(III) nucleus and -20 ppm (doublet) for the P(V) nuclei with an integration of 1:2. The decomposition product is the corresponding cyclic H-phosphonate that forms by hydrolysis of the P-N bond (see scheme S6 in the supporting information).

Next, in analogy to established P-amidite chemistry,[21] the activation of **3** with acidic activators was studied. 4,5-Dicyanoimidazole (DCI) and 5-(ethylthio)-1*H*-tetrazole (ETT)[22] proved to be efficient in this process. Initial studies were conducted with phenylphosphate as readily available nucleophile, which reacted immediately with activated *c*-PyPA **3** giving a 1-deoxy*cyclo*triphosphate **4** (for Hantzsch Widman Nomenclature see the supporting information, table S1). This intermediate was then oxidized using *meta*-chloroperbenzoic acid (*m*CPBA) to the *cyclo*triphosphate **5**, which can be traced due to its characteristic ^{31}P chemical shift at the trifurcation of -35 ppm and the coupling pattern (doublet of triplet with identical *J* values) as shown in figure 1 B (iii). These structures can then be ring-opened with different nucleophiles, and we investigated several conditions that would be well suited for obtaining linearized oligophosphate chains with different modifications. The hydrolysis of *cyclo*triphosphate **5**

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simply by addition of water is slow and even after three hours significant amounts of *cyclo*triphosphate **5** were still detectable (figure 1 C (i)). Hydrolysis with NaOH was much faster and required only 15 minutes almost exclusively resulting in the linear phenyl tetraphosphate **6** (figure 1 C (ii). Hydrolysis with aqueous HCl was equally rapid compared to the basic conditions (see the supporting information, NMR page 14), but did not occur as selectively. Treatment with aqueous ammonia led to linearization and formation of a terminal phosphoramidate **7** with high selectivity. Of note, such structures can be hydrolysed to the terminal phosphates by simple adjustment of pH.[23] Apparently, (modified) amines react much faster than alcohols as nucleophiles with the *cyclo*triphosphate **5**, which was demonstrated by the addition of ethanolamine, exclusively leading to P-amidate **8**. Instead of amines as nucleophiles, also phosphates can be used (scheme 2). The scope of this strategy is shown in table 1. For example, prop-2-yn-1-yl phosphate worked well as nucleophile in the reaction sequence when a divalent metal ion (e.g. Mg^{2+}, Zn^{2+}) was used as additive, giving compound **11** after purification by strong anion exchange chromatography (SAX) in 41% yield. Not only phenylphosphate but also alkyne tagged phosphates such as pent-4-yn-1-yl phosphate were successfully used in this sequence. The introduction of three additional phosphate units by linearization of **5** with *cyclo*triphosphate gave **15** in 35% isolated yield. This demonstrates that it is also possible to linearize **5** by addition of modified phosphate, pyrophosphate and triphosphate nucleophiles in the presence of Mg^{2+} or Zn^{2+}. In all cases, the addition of Mg^{2+} gave better results. These reactions thus provide access to modified polyPs, both with terminal P-N and P-O modifications, making this approach especially versatile. Additionally, the introduction of a non-hydrolysable terminal methylene bisphosphonate was possible and afforded **14** in 47% isolated yield enabling access to non-hydrolysable analogs. Why these systems prefer linearization over branching is currently unknown. One reason could be steric crowding at the trifurcation, thereby repelling incoming nucleophiles.

Furthermore, the application of P(III) chemistry provides a platform to access sulfur-containing analogs as well: 1-deoxy*cyclo*triphosphate **4** was oxidized using Beaucage's reagent which resulted in the formation of **16** as judged by the chemical shift and the coupling pattern (26 ppm, dt, see the supporting information, NMR page 13). 1-Deoxy-1-thio*cyclo*triphosphate **16** was treated with propargyl amine resulting in the linear terminal phosphoramidate **17** isolated in 40% yield**.** This strategy therefore enables the extension of a phosphate-containing precursor by three phosphate units using **3** followed by an additional extension in the linearization step with the potential of further diversification (e.g. oligophosphate introduction, thiophosphorylation, amidation). This part of the study revealed the usefulness of **3** as a reagent for monodirectional phosphoric anhydride syntheses in which one can introduce six phosphate units in a one-flask operation using monophosphates as starting materials.

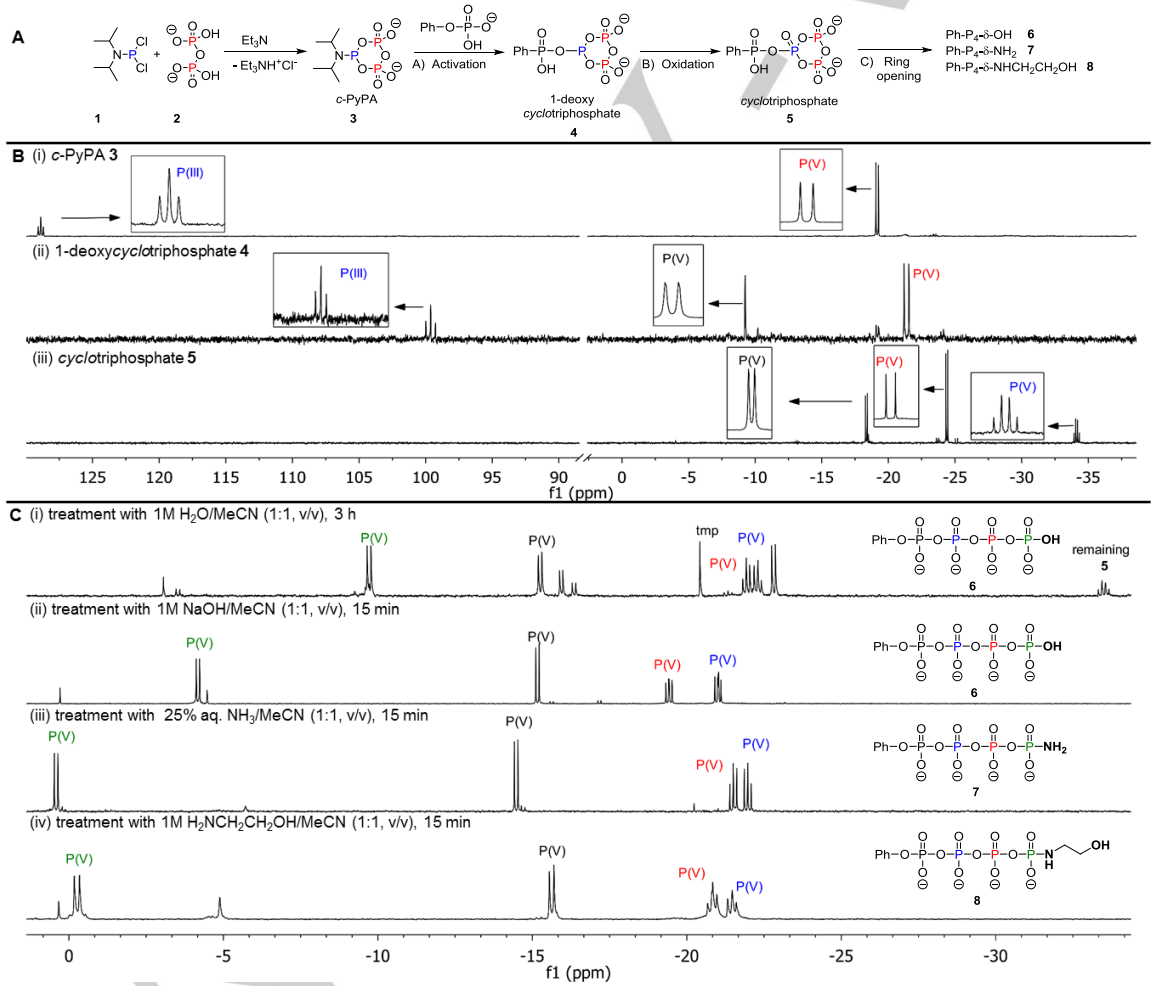

Figure 1: A) General scheme for NMR reactions. **B)** $^{31}P\{^{1}H\}$ NMR study monitoring the formation of a *cyclo*triphosphate intermediate **5** under ambient conditions (for more details see supporting information) (i) *c*-PyPA **3** in reaction mixture. (ii) The 1-deoxy*cyclo*triphosphate **4** (signal at 101 ppm) formed after adding an equimolar amount of phenylphosphate TBA salt to *c*-PyPA **3.** ETT was used as an activator, r.t., 10 min. (iii) Addition of *m*CPBA at 0 °C gave *cyclo*triphosphate **5**, showing a characteristic peak doublet of triplet at -35 ppm. **C)** Ring opening reactions under different conditions. (i) Treatment with water resulted in a slow and unselective reaction. (ii) Treatment with NaOH afforded the phenyl tetraphosphate- δ-OH **6** almost exclusively. (iii) + (iv) Ring-opening under basic conditions delivered phenyl tetraphosphate-δ-amidates **7**, **8** within 15 min. Furthermore the phenyl tetraphosphate-δ-amidates **7** were treated with 1 M HCl, which resulted in cleavage of P-N amidate bond and afforded phenyl tetraphosphate **6** (see the supporting information, NMR page 16). Abbreviations: Ph: phenyl; tmp: tmp (*cyclo*triphosphate); ETT: 5-(ethylthio)-1*H*-tetrazole; *m*CPBA: *meta*-chloroperbenzoic acid; TBA: tetrabutyl ammonium.

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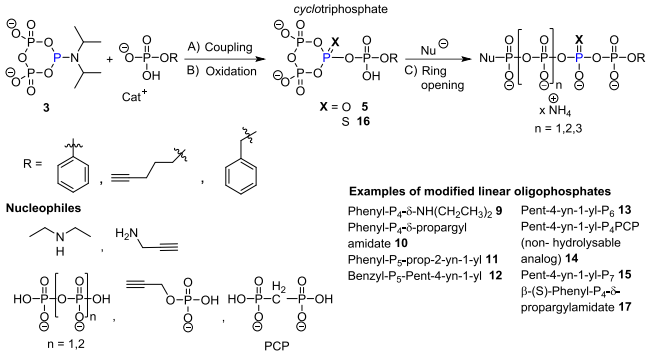
Monodirectional approach I



Scheme 2. Overview of the monodirectional triphosphorylation using *c*-PyPA **3**. Intermediate **5** can be diversified into several polyphosphate conjugates. All reactions were conducted in MeCN or DMF.

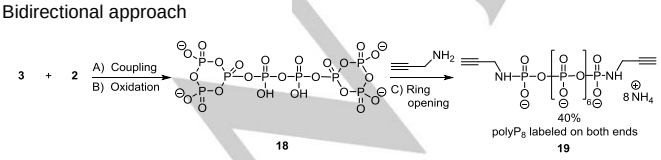
Table 1: Conditions and yields obtained using monodirectional approach I

Entry	Monophosphate[a]	Conditions[b][c]	Nucleophile (equiv.)	Yield	Product
1	Phenyl-1.0 TBA	MeCN/2/1.5	NaOH	99%[e]	**6**
2	Phenyl-1.0 TBA	MeCN/2/1.5	NH_4OH	98%[e]	**7**
3	Phenyl-1.0 TBA	MeCN/2/1.5	$NH_2CH_2CH_2OH$	95%[e]	**8**
4	Phenyl-1.0 TBA	MeCN/2/1.5	$NH(CH_2CH_3)_2$	91%[e]	**9**
5	Phenyl-1.2 TBA	MeCN/2/1.5	$HC{\equiv}CCH_2NH_2$ (3.5)	80%[f]	**10**
6	Phenyl-1.0 TBA	MeCN/2/1.5	H_2O (1)[d]	65%[f]	**6**
7	Phenyl-1.2 TBA	MeCN/2/1.2	$HC{\equiv}CCH_2\text{-}PO_4^{2-}$ (1.5)[d]	41%[f]	**11**
8	Benzyl-1.5 TBA	MeCN/2/1.5	$HC{\equiv}C(CH_2)_3PO_4^{2-}$ (1.5)[d]	29%[f]	**12**
9	Pentynyl-2CyNH+	MeCN/5/1.5	$P_2O_7^{2-}$ (1.5)[d]	44%[f]	**13**
10	Pentynyl-2CyNH+	MeCN/5/1.5	PCP (1.5)[d]	47%[f]	**14**
11	Pentynyl-2CyNH+	MeCN/5/1.5	$P_3O_{10}^{4-}$ (1.5)[d]	35%[f]	**15**
12	Pentynyl-1.0 TBA	MeCN/2/1.2	$HC{\equiv}CCH_2NH_2$ (3.5)	40%[f]	**17**

[a] Monophosphates were used as TBA or *cyclo*hexylammonium salt. [b] 1 - 1.2 equiv. of c-PyPA **3** were added to the reaction mixtures. [c] The entries are given as: solvent/ equiv. of ETT/ equiv. of oxidant (*m*CPBA) only in entry 12 use of Beaucage's reagent for the oxidation. [d] All reactions were treated with $MgCl_2$ (1.5 equiv) except for the first five entries where the ring opening was performed under basic conditions. [e] Yield after precipitation; compounds were essentially pure and did not require further workup. [f] Yield after ion exchange chromatography on Q-Sepharose using an aqueous solution of ammonium bicarbonate for gradient eluent. Abbreviations: MeCN: acetonitrile, *m*CPBA: *meta*-chloroperbenzoic acid, TBA: tetrabutylammonium, $CyNH^+$: *cyclo*hexylammonium salt.

Conceptually, such an extension as described above should also work in a bidirectional fashion, if the starting phosphate/pyrophosphate units were unmodified. In this scenario, the addition of at least two equivalents of **3** would enable the rapid generation of even longer modified polyP chains in a one-flask procedure. To study this potential reactivity, unmodified pyrophosphate **2** was reacted with an excess of **3**. The use of 3.5 equivalents of *c*-PyPA **3** indeed facilitated complete consumption of pyrophosphate **2** and, after oxidation, led to the bidirectionally extended intermediate **18** that was then linearized using amine nucleophiles (scheme 3). In a recent report, this structure had been obtained using P(V) chemistry, albeit in a low yield (ca. 10%).[11] *c*-PyPA **3** enabled the synthesis of compound **19** in 40% isolated yield.

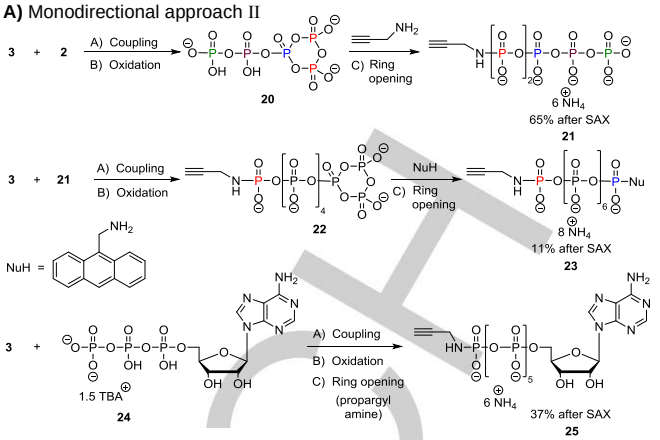
Bidirectional approach



Scheme 3. Reagents and intermediates involved in the synthesis of $polyP_8$ **19** modified on both termini by applying 3.5 eq of *c*-PyPA **3**.

Using the bidirectional approach, one will always obtain polyP with identical modifications at both termini. In this context, it is of

A) Monodirectional approach II



B) $^{31}P\{^1H\}$ NMR of intermediate **20**

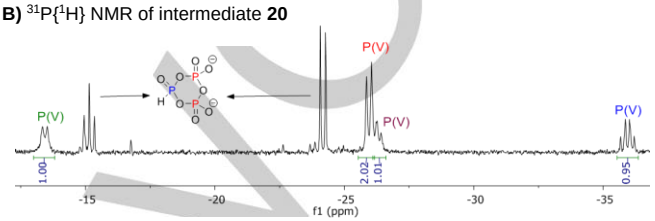
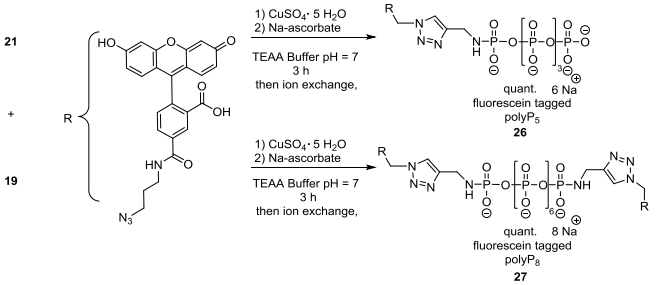


Figure 2: A) Synthesis of $polyP_5$ **21** by using monodirectional approach II: *c*-PyPA **3** was selectively coupled to pyrophosphate **2** which results in intermediate **20**. Intermediate **20** was reacted with propargylamine and thus transformed into $polyP_5$ **21**. Synthesis of the tagged $polyP_8$ **23** involved $polyP_5$ **21** used as a starting substrate for the second extension and ring opening with 9-(aminomethyl)anthracene.This sequence gave modified $polyP_8$ **23.** ATP **24** was selectively coupled to **3** and the ring opening was performed with propargylamine resulting in **25**. **B)** $^{31}P\{^1H\}$ analysis of reactive intermediate **20** is shown.

note that it is also possible to initiate a selective monodirectional extension even on an unmodified pyrophosphate by using reduced amounts of *c*-PyPA **3** (figure 2). In this case, ^{31}P NMR analysis of the intermediate **20** after oxidation indicated the presence of a trifurcation (-36 ppm), a terminal phosphate (-13 ppm) and internal phosphoric anhydrides (-25 ppm, two signals) in a 1:1:1:2 ratio. Therefore, it is also possible to introduce selectively only one equivalent of c-PyPA **3** to an unprotected pyrophosphate **2**. Intermediate **20** was then linearized by the addition of propargylamine. The use of propargylamine afforded product **21** in 65% isolated yield. This strategy enables the selective synthesis of polyP modified on one end only that can then be extended again on the remaining unmodified other end. For example, alkyne tagged **21** was extended again using *c*-PyPA **3** followed by linearization with the amine nucleophile 9-(aminomethyl)anthracene leading to $polyP_8$ **23** in 11% isolated yield with different tags at both termini (figure 2). Due to solubility issues of **21** in organic solvents, the second extension generally suffered from lower yields. In addition, **3** was applied to the extension of ATP by three phosphate units and introduce an alkyne tag at the terminal phosphate in a one-flask procedure. This directly resulted in a modified nucleoside hexaphosphate in 37% yield after SAX. Such nucleotides are used in single molecule real time sequencing.[3]



Scheme 4. Alkyne tagged polyPs **19**, **21** can be transformed into fluorescein labeled polyPs **26** and **27** using click chemistry.

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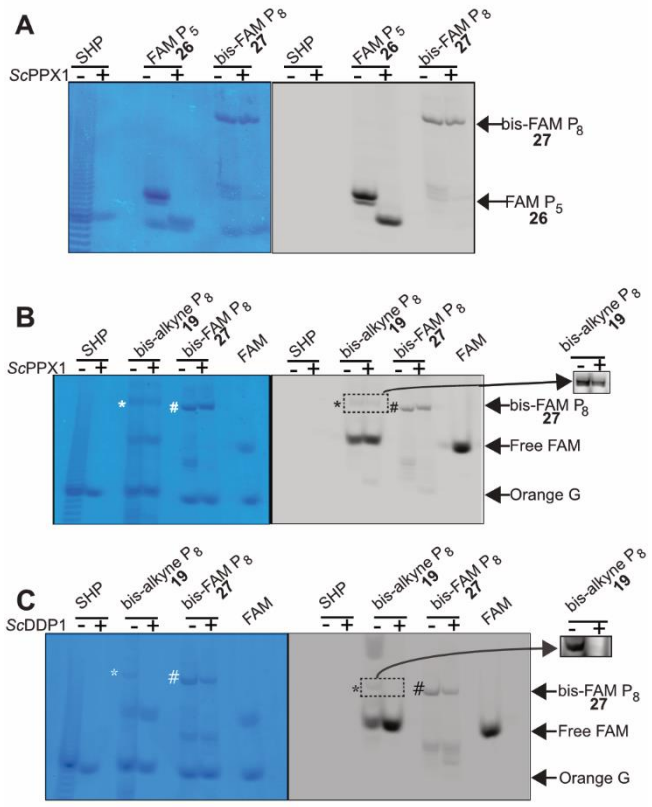

Figure 3: Terminal alkyne groups protect polyP from degradation by exopolyphosphatase. (A) Sodium hexametaphosphate (SHP; a commercially available polydisperse polyP), FAM P_5 **26** and bis-FAM P_8 **27** were incubated with or without *S. cerevisiae* exopolyphosphatase (*Sc*PPX1), and the products were resolved on a 35.8% polyacrylamide gel. Fluorescent bands were visualized, and the gel was subsequently stained with Toluidine blue. The fluorescence image (right panel) and Toluidine blue-staining (left panel) show that SHP and FAM P_5 **26** are digested by *Sc*PPX1, but bis-FAM P_8 **27** is protected. (B, C) SHP, bis-alkyne P_8 **19**, and bis-FAM P_8 **27** (#) were incubated with or without *Sc*PPX1 (B), or the *S. cerevisiae* endopolyphosphatase (*Sc*DDP1; C). 5-FAM azide was then conjugated with bis-alkyne P_8 **19** (*) using click chemistry, and all the products were resolved on a 35.8% polyacrylamide gel. The fluorescence image (right panel) and Toluidine blue-staining (left panel) show that bis-alkyne P_8 **19** is protected from digestion by exopolyphosphatase but is degraded by endopolyphosphatase, whereas bis-FAM P_8 **27** is resistant to both enzymes. Inset in (B) and (C) shows bis-alkyne P_8 **19** with a higher contrast for ease of comparison. Orange G dye added in the sample loading buffer indicated the extent of the gel run. Unconjugated 5-FAM azide was used as a marker for comparison with FAM released after degradation of the conjugated polyP chain. To improve visualization, Toluidine blue stained gel images were subjected to level adjustment using Affinity Photo software. All images are representative of three independent experiments.

We then applied click chemistry to attach fluorescein (FAM) to one or both termini of alkyne modified P_5 **21** and bis alkyne modified P_8 **19** (scheme 4).

In order to serve as useful tools, we wanted to understand the interaction of some exemplary probes with endo- (DDP)[24] and exopolyphosphatases (PPX)[25] that processively cleave polyPs either internally or externally (Figure 3). FAM labelled P_5 **26** blocked at one end is digested by *Saccharomyces cerevisiae* *Sc*PPX but bis-FAM P_8 **27**, blocked at both ends, is resistant to its activity (figure 3A). Presence of an alkyne group at both ends of P_8 **19** is sufficient to prevent *Sc*PPX digestion (figure 3B). However, endophosphatases like *Sc*DDP1 can digest bis-alkyne modified P_8 **19** while bis-FAM-P_8 **27** resists *Sc*DDP1 activity (figure 3C). The bulky FAM groups at both ends of **27** may block access of DDP1 to the polyphosphate chain, whereas alkyne groups in **19** do not pose any hindrance.

In summary, a novel triphosphorylation reagent **3** has been developed. Its usefulness in the context of one-flask polyphosphorylation reactions has been described, culminating in the synthesis of several modified polyphosphates with or without P-N and P-O end labels. This novel reagent opens new possibilities to target densely charged and unstable modified polyphosphates, as exemplified by mono- and bidirectional extensions followed by selective nucleophilic ring-opening. The obtained polyP analogs were studied in the context of their interaction with phosphatases.

Acknowledgements

We gratefully acknowledge financial support from the HFSP grant (Human Frontier Science Program RGP0025/2016). We thank MagRes of the University of Freiburg for significant amount of time for NMR spectroscopy. We thank Prof. G. M. Blackburn for helpful discussions concerning nomenclature.

Keywords: *Cyclic* phosphoramidite, Triphosphorylation, Monodisperse polyP, Phosphoric anhydrides.

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10.1002/anie.201814366

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Entry for the Table of Contents

Layout 2:

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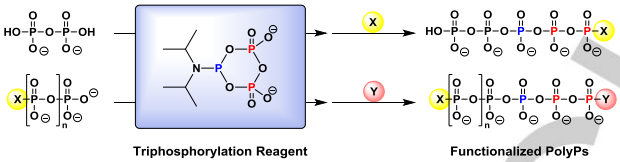
Going long

A triphosphorylation reagent was developed, which enables access to defined polyphosphates in a one-flask operation. These polyphosphate chains are modified on either one end or both ends with different tags.



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A phosphoramidite analog of *cyclo*triphosphate enables iterative polyphosphorylations