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Synthesis of Nucleoside Tetraphosphates and Dinucleoside Pentaphosphates via Activation of Cyclic Trimetaphosphate

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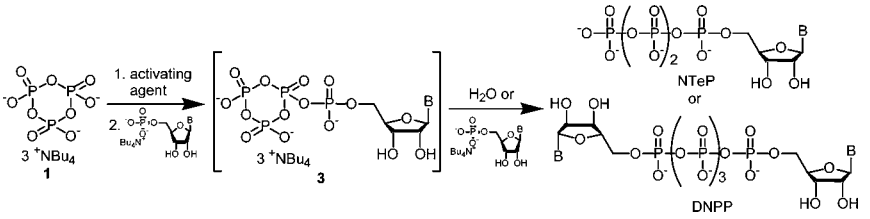
ABSTRACT



A procedure for the synthesis of dinucleoside 5′-pentaphosphates (Np_5N) and nucleoside 5′-tetraphosphates (Np_4) is described. The procedure relies on the activation of cyclic trimetaphosphate followed by a reaction with a nucleoside 5′-monophosphate (NMP) to give intermediates of type 3. Reaction of 3 with water or an NMP gives the desired products in yields ranging from 77 to 86%.

Dinucleoside and nucleoside polyphosphates (Np_XN and Np_X) have been the focus of studies for many years due to their important biological properties, their potential as therapeutics, and their biotechnological applications. Among such compounds are dinucleoside 5′-pentaphosphates (Np_5N's) and nucleoside 5′-tetraphosphates (Np_4). Np_5N's such as Ap_5A and Ap_5T are potent inhibitors of enzymes such as adenylate kinase, thymidine kinase, thymidylate kinase, and ribonucleotide reductase.[1-a−f] The crystal structure of *Aquifex aeolicus* adenylate kinase complexed with Ap_5A was used to determine how the enzyme achieves a catalytically competent state.[2] Np_5N's can also be enzyme substrates. For example, Ap_5A is a good substrate for *Humulus lupulus* adenylate isopentyltransferase exhibiting a higher affinity than AMP, ADP, and ATP.[3] Recently, considerable attention has also been directed toward Np_4's, as these species have been shown to be selective agonists of the P2Y4 receptor.[4] They are also used as synthons to prepare 2′-deoxynucleoside-5′-tetraphosphates containing terminal fluorescent labels which, as a result of being excellent substrates of DNA polymerase, have found use in various applications in DNA sequencing and labeling.[5−7]

Various approaches have been reported for the preparation of Np_5N's and Np_4's; however, for the most part, these procedures are either lacking in scope and/or do not produce the desired products in good yield. For example, the highest reported yield for the synthesis of Ap_5A is only 52%.[1e] This was achieved by treating ATP with DCC to give adenosine 5′-trimetaphosphate and then reacting the tributyl ammonium salt of this compound with the tributyl ammonium salt of ADP. Much lower yields of Ap_5A were

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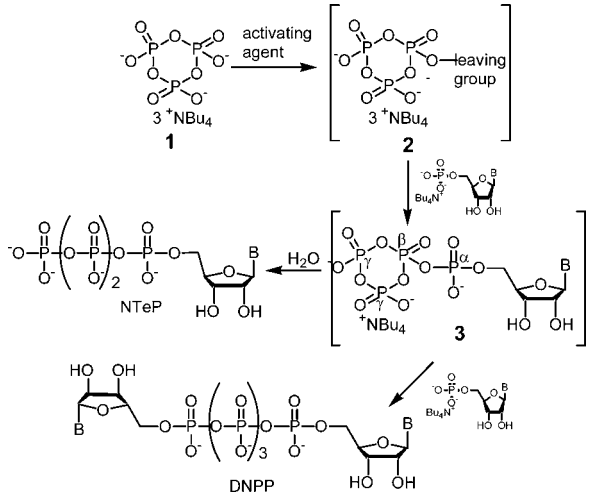
reported using other procedures.[1c,8−10] The reported yields of other Np_5N's such as Up_5U and Ap_5T have also been much lower (10−22%).[1f,11a,11b]

A number of methodologies have also been reported for preparing Np_4's.[12−17] The yields are also generally low to modest though; recently, good yields were reported for Ap_4 (82%) by reacting ATP with the imidazolide of cyanoethylphosphate under microwave irradiation followed by phosphate deprotection with DBU.[15] Np_4's have also been prepared using enzymatic methodologies; however, this approach is limited by the scale and substrate specificity of the enzymes.[18]

A high yielding synthesis of Np_5N's and Np_4's of broad scope would facilitate studies of their biochemical, medical, and biotechnological applications. Here we report such a synthesis that utilizes an activated form of cyclic trimetaphosphate as the phosphate donor.

Our general approach is outlined in Scheme 1. The tetrabutylammonium salt of trimetaphosphate (TriMP, **1**) is activated with a reagent to give intermediate **2**. Reaction of **2** with a nucleoside 5′-monophosphate (NMP) would yield intermediate **3** which would be either hydrolyzed to the Np_4's or reacted with an additional nucleoside monophosphate to give the Np_5N's.

Scheme 1. General Procedure for Preparing Np_5N and Np_4



The tetrabutylammonium salt of TriMP (**1**) has been prepared in 72% yield by passing the trisodium salt of TriMP through a cation exchange resin in the tetrabutyl ammonium form followed by concentration of the effluent and then a series of washing and filtering steps or in an unspecified yield by passing the trisodium salt of TriMP through a cation exchange resin in the H^+ form followed by titration to pH 7 with TBAH.[19,20] We prepared **1** in almost quantitative yield by first converting the sodium salt of TriMP to its pyridinium form using an ion exchange column followed by titrating the eluent solution to pH 7 with a dilute solution of TBAH followed by freeze-drying to a white powder. The ^{31}P NMR of TriMP in D_2O prepared in this manner exhibited a singlet at −19.2 ppm.

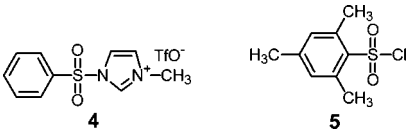


Figure 1. Activating agents used for Np_5N and Np_4 syntheses.

We examined sulfonyl imidazolium salt **4** as an activating agent, as we have recently reported that it is, in the presence of *N*-methylimidazole (NMI), a very effective reagent for the synthesis of other nucleoside polyphosphates and their conjugates (Figure 1).[21] We also investigated the combination of mesitylene chloride (**5**) and NMI as an activating agent, as compound **5**, unlike reagent **4**, is commercially available, is relatively inexpensive, and has been used as a coupling agent for phosphate ester bond formation.[22]

Reagent **4** or **5** (0.86 equiv) was added to a solution of 1.0 equiv of **1** and 3.2 equiv of *N*-methylimidazole (NMI) in DMF, and the reaction was followed by ^{31}P NMR. With reagent **5** after 10 min much of the TriMP, which appears at approximately δ −19, was consumed and the NMR, in addition to a few very minor peaks, consisted of a relatively small triplet at δ −31.4, a doublet at δ −20.7, and a triplet at δ −21.5 (for example, see Figure 2A). After 30 min the triplet at δ −31.4 had disappeared leaving the doublet at δ −20.7 and the triplet at δ −21.5 though the relative peak heights within the triplet at δ −21.5 had changed (Figure 2B). No further change occurred after 30 min. We propose that these signals can be accounted for by a reaction between **1** and **5** to give mixed anhydride **6** which reacts relatively rapidly with NMI to give imidazolium

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intermediate **7** (Scheme 2). The triplet at δ −31.4 corresponds to the α-phosphorus atom of **6**. The doublet that should be associated with the two β-phosphorus atoms of **6** coincides with the central and right side peaks of the triplet that is due to the α-phosphorus atom in compound **7** which appears at δ −21.5. The doublet at δ −20.7 is due to the two β-phosphorus atoms of **7**. The α- and β-phosphorus atoms in **7** have very similar chemical shifts and are strongly coupled to one another which results in the doublet at δ −20.7 and triplet at δ −21.5 to be highly skewed toward one another. The relative simplicity of the spectra and the fact that some unreacted TriMP remained suggested that the formation of dimers or higher order oligomers is not readily occurring.

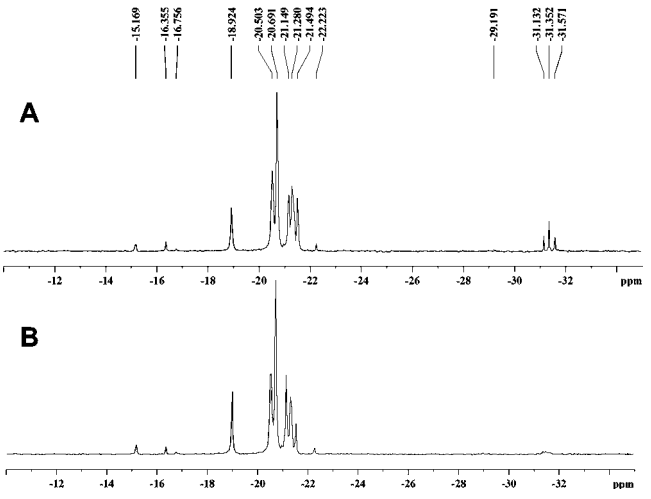
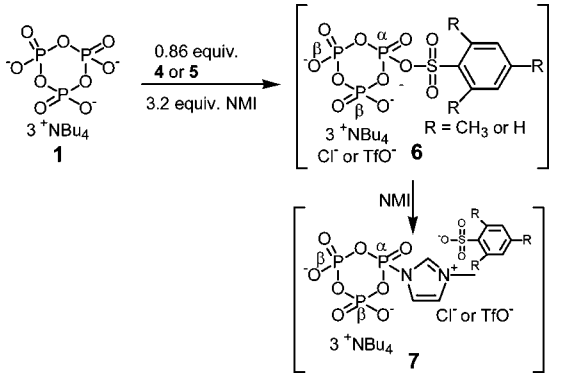


Figure 2. ^{31}P NMR of the reaction of 0.86 equiv of reagent **5** with 1.0 equiv of compound **1** in the presence of 3.2 equiv of NMI in DMF. (A) Spectrum recorded after 10 min. (B) Spectrum recorded after 30 min. See the text for details.

Scheme 2. Activated Forms of TriMP



The ^{31}P NMR of the reaction between reagent **4** and compound **1** after 10 min was almost identical to the ^{31}P NMR of the reaction between reagent **5** and **1** after 30 min. The NMR spectra recorded after 20 and 30 min showed no change. No triplet at approximately δ −31 was evident in any of the spectra suggesting that the reaction between reagent **4** and compound **1** and the subsequent reaction of the mixed anhydride to give the intermediate imidazolium salt of type **7** is very fast possibly because the mixed anhydride formed with reagent **4** is less sterically hindered than the one formed with reagent **5**.

The above solutions were added to a solution of 0.64 equiv of the tetrabutylammonium salts of nucleoside monophosphates (NMPs) in DMF at 0 °C. The reactions were allowed to warm to rt and monitored by ^{31}P NMR. Unreacted TriMP was always evident at approximately δ −18.7. The peaks corresponding to the NMPs (at approximately δ 0.8−2), and the doublet at δ −20.7 and triplet at δ −21.5 corresponding to intermediate **7**, decreased with time, and in addition to a few minor peaks, several new peaks appeared, most notably two doublets at approximately δ −9.8 and δ −21.4 and a quartet at approximately δ −31.3 (for example, see Figure 3). We propose that these new peaks can be attributed to intermediate **3** (Scheme 1). The doublet at δ −9.8 can be assigned to the α-phosphorus atom of **3**. The quartet can be assigned to the β-phosphorus of **3** and is actually an overlapping doublet of triplets. The large doublet at δ −21.4 is assigned to the two γ-phosphorus atoms of **3**. After 2.5−3 h, when almost all of the NMP had been consumed, 100 mM triethylammonium acetate (TEAA) buffer was added, the solutions were washed with chloroform and the aq. layer was left for 2 h at room temperature. ^{31}P NMR analysis of the mixtures revealed major peaks at approximately δ −8, δ −9, and δ −21 suggesting the formation of Np_4's. HPLC purification of this material using TEAA as eluent and conversion of their triethylammonium salts to their ammonium salts using an ion-exchange resin resulted in the isolation of the Np_4 in excellent yields (**8**–**11**, Table 1). It is interesting to note that Ng and Orgel reported that AMP was the major product when Ap_4 was treated with l-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in HEPES buffer at pH 6.5. They postulated an intermediate of type **3** forming first followed by hydrolysis of **3** to give AMP.[23] The ^{31}P NMR of the reaction mixture between **3** and AMP after addition of TEAA followed by a chloroform wash and stirring for 2 h indicated that only ~5% of **3** was being hydrolyzed to AMP.

Np_5N's were synthesized by first preparing intermediate **3** as described above. Instead of quenching intermediate **3** with buffer, 1.3 equiv of an NMP in DMF and 0.7 equiv of anhydrous $MgCl_2$ were added. ^{31}P NMR analysis of the mixture indicated the reactions were complete after 3 days. Quenching with 100 mM TEAA buffer followed by washing with chloroform, purification by RP-HPLC, and then ion exchange chromatography gave Np_5N's as their ammonium salts in very good yield using either reagent (**12**–**16**, Table 2). The reaction was extremely slow in the absence of magnesium ions. We also found that unsymmetrical Np_5N can be produced using this procedure (entry 5, Table 2)

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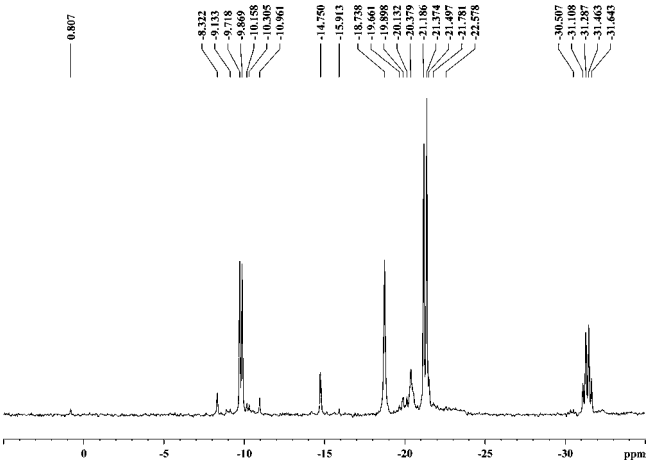

Figure 3. ^{31}P NMR of the reaction of intermediate **7** with UMP in DMF after 2.5 h. See the text for details.

In summary, we report a novel approach to the synthesis of Np_5N's and Np_4's via activation of cyclic trimetaphosphate. This procedure has several advantages over current methods. Unlike some procedures[11,15] it does not require protection of the nucleoside or phosphate groups or special conditions (i.e., microwaves). Readily available and inexpensive mesitylene chloride/NMI can be used as an activating agent. It utilizes very inexpensive sodium TriMP as a substrate which is easily converted into its tetrabutylammonium salt in almost quantitative yields. It employs 5′-monophosphates which are considerably less expensive or easier to prepare than nucleoside 5′-di- or triphosphates. The products are relatively easy to purify by RP-HPLC. Most significantly, the products are produced in excellent yield. This procedure may prove to be a convenient method for preparing 2′-deoxynucleoside-5′-tetraphosphates containing terminal labels. Such studies as well as additional mechanistic studies are in progress and will be reported in due course.

Table 1. Synthesis of Nucleoside Tetraphosphates

entry	product	yield (%)
1	Ap_4 (**8**)	83,[a] 84[b]
2	Cp_4 (**9**)	84,[a] 86[b]
3	Gp_4 (**10**)	84,[a] 84[b]
4	Up_4 (**11**)	84,[a] 86[b]

[a] Isolated yields using reagent **4**. [b] Isolated yields using reagent **5**.

Table 2. Synthesis of Dinucleoside Pentaphosphates

entry	product	yield (%)
1	Ap_5A (**12**)	85,[a] 81[b]
2	Cp_5C (**13**)	82,[a] 84[b]
3	Gp_5G (**14**)	85,[a] 84[b]
4	Up_5U (**15**)	80,[a] 84[b]
5	Gp_5U (**16**)	77[b]

[a] Isolated yields using reagent **4**. [b] Isolated yields using reagent **5**.

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Supporting Information Available. Preparation procedures and characterization data for all Np_4 and Np_5N. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.