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One-Pot Synthesis of Nucleoside 5′-Triphosphates from Nucleoside 5′-*H*-Phosphonates

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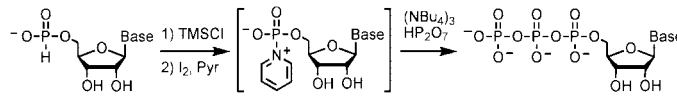
ABSTRACT



Nucleoside 5′-triphosphates (NTPs) play key roles in biology and medicine. However, these compounds are notoriously difficult to synthesize. We describe a one-pot method to prepare NTPs from nucleoside 5′-*H*-phosphonate monoesters via pyridinium phosphoramidates, and we used this approach to synthesize ATP, UTP, GTP, CTP, ribavirin-TP, and 6-methylpurine ribonucleoside-TP (6MePTP). Poliovirus RNA-dependent RNA polymerase efficiently employed 6MePTP as a substrate, suggesting that the cognate nucleoside, a poorly understood antiviral agent, may damage viral RNA.

Nucleoside 5′-triphosphates (NTPs) are critical mediators of myriad biological processes including DNA replication, transcription, and translation. Correspondingly, synthetic mimics of NTPs have been widely used as molecular probes and biological assay components and represent active metabolites of certain drugs such as the antiviral agent ribavirin. Despite their importance in biology and medicine, the diversity of commercially available NTPs is limited because these compounds are often difficult to prepare and isolate in pure form.[1]

Traditional approaches for the synthesis of NTPs include the "one-pot, three-step" method and the method of Ludwig and Eckstein.[2,3] Although these strategies work well for some substrates, others are plagued by low yields and difficulties in purification.[4] More recently, Ahmadibeni reported[5] a solid-phase route to NTPs, and Borch reported[6] a method for the preparation of activated phosphoramidates that can be converted to NTPs by reaction with tris(tetra-*n*-butylammonium) hydrogen pyrophosphate. The final coupling step employed by Borch was found to proceed in remarkably high yield, but the required four-step synthesis of the phosphoramidate precursor increased the complexity of the synthesis compared with other approaches.

We hypothesized that nucleoside 5′-*H*-phosphonates might provide novel and more readily synthesized precursors to NTPs. This hypothesis was based on reported syntheses of phosphates, phosphoramidates, and other phosphate derivatives from these precursors.[7−10] After conversion to silyl phosphites with TMSCl, *H*-phosphonate monoesters can be oxidized by elemental iodine and other reagents to generate

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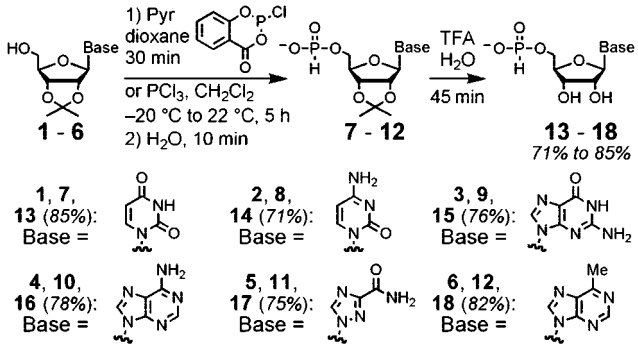
electrophilic intermediates.[11,12] These intermediates are known to react with a variety of nucleophiles to afford addition products, but surprisingly, this strategy has not been previously investigated for the synthesis of NTPs.

We report here a one-pot approach for the synthesis of NTPs from nucleoside 5′-*H*-phosphonate monoesters, relatively stable compounds that can be easily prepared from 2′,3′-*O*-isopropylidene-protected nucleosides.[13] We demonstrate that fully deprotected ribonucleoside 5′-*H*-phosphonate monoesters can be converted in situ to pyridinium phosphoramidate intermediates. Upon addition of nucleophilic tris(tetra-*n*-butylammonium) hydrogen pyrophosphate, NTPs can be isolated by use of a two-step purification protocol.

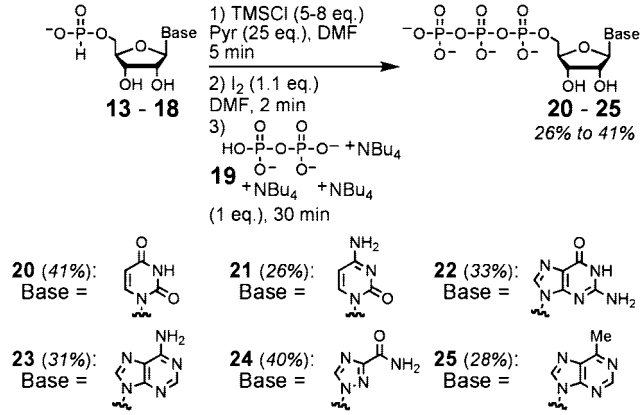
As shown in Scheme 1, starting with the known 2′,3′-*O*-isopropylidene-protected nucleosides **1**−**6**,[13−15] phosphitylation[16] with salicyl phosphorochlorodite or PCl_3 provided 2′,3′-*O*-isopropylidene-protected nucleoside *H*-phosphonate monoesters **7**−**12**. Treatment of these compounds with aqueous TFA yielded 5′-*H*-phosphonates derived from the four natural nucleosides uridine (**13**), cytidine (**14**), guanosine (**15**), and adenosine (**16**) as well as 5′-*H*-phosphonates derived from the antiviral agents ribavirin (**17**) and 6-methylpurine ribonucleoside (**18**).

Scheme 1. Synthesis of Nucleoside 5′-*H*-Phosphonates **13**−**18**



Based on literature precedent,[7−11,17] we hypothesized that addition of excess TMSCl and pyridine to the unprotected nucleoside 5′-*H*-phosphonate monoesters **13**−**18** would accomplish two objectives: (1) conversion of the *H*-phosphonate to a silyl-*H*-phosphonate or more reactive bis-silyl phosphite and (2) transient modification of spurious water and/or other reactive nucleophiles. Subsequent addition of elemental iodine would rapidly oxidize the phosphite and generate a reactive pyridinium phosphoramidate in situ. We further proposed that nucleophilic attack by tris(tetra-*n*-butylammonium) hydrogen pyrophosphate[18] would yield the NTP product. As shown in Scheme 2, applying this sequence to **13**−**18** indeed afforded triphosphates **20**−**25**. Athough the yields of the pure triphosphates were modest, ranging from 26% to 41%, the ability to easily control the production of the NTP in this one-pot process proved to be of significant benefit for the sythesis of certain substrates such as ribavirin triphosphate (**24**), which can be difficult to synthesize by nonenzymatic methods.[4]

Scheme 2. One-Pot Methodology for Conversion of Nucleoside 5′-*H*-Phosphonates **13**−**18** to Nucleoside 5′-Triphosphates **20**−**25**



To purify triphosphates prepared with this method, a simple prepurification step involving passage through a column of Sephadex LH-20 gel filtration media with aqueous triethylammonium bicarbonate (TEAB) buffer as the eluent was found to remove the majority of nonphosphate byproducts. Crude products were subsequently lyophilized and further purified by preparative reversed-phase HPLC with an C18 column to afford 10−30 mg quantities of triphosphates **20**−**25** as tetra(triethylammonium) salts in >90% purity.

To examine the mechanism proposed for formation of NTPs, a related reaction involving a more soluble, protected substrate was analyzed by ^{31}P NMR (Figure 1). In these experiments, 2′,3′-*O*-isopropylidene uridine 5′-*H*-phosphonate (**7**)[19] and tris(tetra-*n*-butylammonium) hydrogen pyrophosphate (**19**)[18] were prepared according to literature procedures. The 5′-*H*-phosphonate was treated with 3 equiv of TMSCl in anhydrous pyridine, followed by rapid oxidation with iodine to generate the pyridinium phosphoramidate in

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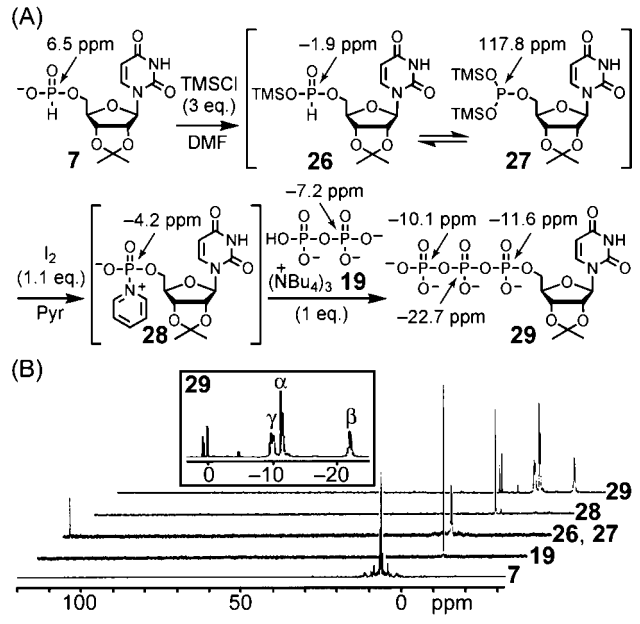

Figure 1. Analysis of sequential conversion of *H*-phosphonate monoester **7** to triphosphate **29** by ^{31}P NMR. Panel A: Synthetic route illustrating measured ^{31}P chemical shifts of reagents, proposed intermediates, and product. Panel B: ^{31}P NMR spectra of compounds prior to and after sequential addition of reagents shown in panel A. From the bottom of the spectral stack: the first two spectra are of purified starting materials (**7**, **19**), the third spectrum was obtained after addition of TMSCl, the fourth spectrum was obtained after addition of I_2, and the fifth spectrum was obtained after addition of **19**. Inset: expansion of resonances of **29** showing assignments of phosphorus atoms.

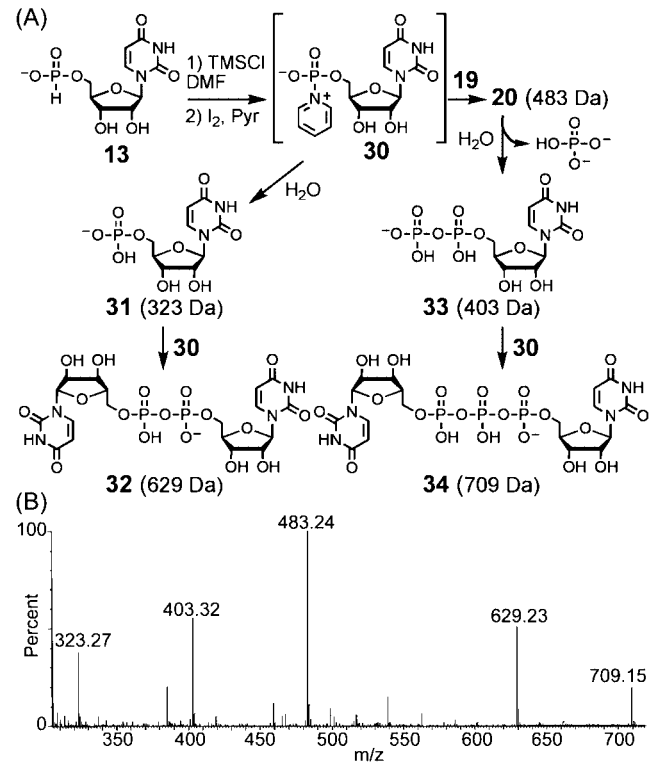

Figure 2. Mass spectral analysis of the reaction mixture obtained after conversion of *H*-phosphonate **13** to triphosphate **20**, using 3 equiv of TMSCl, followed by partial purification by gel filtration (Sephadex LH-20). Panel A: Structures of compounds detected by mass spectrometry. Panel B: Mass spectrum obtained with a Waters ZQ-4000 single quadrupole instrument using electrospray ionization (negative-ion mode).

situ. As shown in Figure 1, chemical shifts observed by ^{31}P NMR were consistent with the clean generation of two silylated intermediates (**26**, **27**) that upon oxidation are efficiently converted to the activated pyridinium phosphoramidate (**28**). Addition of tris(tetra-*n*-butylammonium) hydrogen pyrophosphate afforded the triphosphate **29** as the major product with 60% conversion (as calculated by integration). In contrast, if the less nucleophilic tris(tri-*n*-butylammonium) pyrophosphate was employed as the nucleophile, only a trace of triphosphate was produced (data not shown), demonstrating the importance of the more nucleophilic[6,18] tris(tetra-*n*-butylammonium) hydrogen pyrophosphate in the reaction.

To optimize the reaction and probe the mechanism of conversion of nucleoside 5′-*H*-phosphonates to triphosphates by mass spectrometry, reaction of deprotected uridine 5′-*H*-phosphonate monoester (**13**) was investigated. Initial attempts to synthesize UTP (**20**) explored the addition of only 3 equiv of TMSCl in the first step. As shown in Figure 2, analysis of the crude products by mass spectrometry revealed the formation of the monophosphate (**31**), the diphosphate (**33**), and dimeric derivatives (**32** and **34**). Since tris(tetra-*n*-butylammonium) hydrogen pyrophosphate typically contains more than 3 equiv of water,[18] we reasoned that the phosphoramidate intermediate might be partially hydrolyzed and form these byproducts through the routes shown in Figure 2. To suppress these side reactions, the addition of 5−8 equiv of TMSCl, depending on the nucleoside, was found to minimize the formation of undesired side products without adversely affecting the nucleophilicity of the pyrophosphate. For example, during the synthesis of UTP (**20**) from **13**, a 31% yield was obtained using 3 equiv of TMSCl, whereas the yield was increased to 41% with 5 equiv of TMSCl. We also explored the utility of other nucleophilic bases known to yield stable phosphoramidate intermediates[20–22] including *N*-methylmorpholine, DMAP, *N*-methylimidazole, and *N*-methylpyrrolidine, but pyridine proved to be superior, likely because these alternatives are presumably too basic or nucleophilic for compatability with TMSCl. Correspondingly, anhydrous DMF containing 25 equiv of anhydrous pyridine proved to be the optimal solvent/base combination for both solubilization and preparation of NTPs from deprotected nucleoside 5′-*H*-phosphonates such as **13**−**18**.

To examine the biological activity of a triphosphate prepared by this method, we investigated the incorporation

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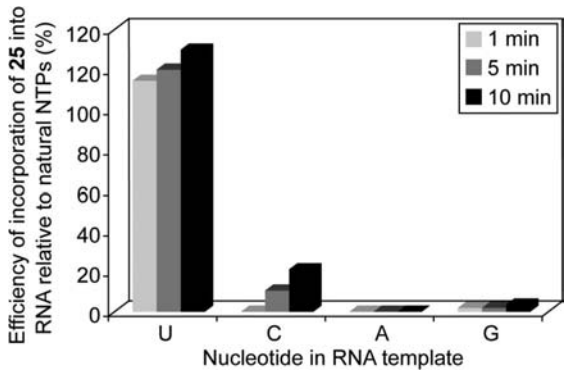

Figure 3. Evaluation of 6-methylpurine ribonucleoside triphosphate (**25**) as a substrate of $3D^{pol}$ from poliovirus. Bars illustrate the amount of incorporation of **25** into RNA relative to correct natural NTPs templated by U, C, A, or G (efficiency of correct incorporation of natural NTPs = 100%). Data was acquired by first assembling $3D^{pol}$ on a symmetrical RNA primer−template duplex (end-labeled with ^{32}P ATP) for 2 min at 30 °C. Incorporation of nucleotides into RNA was initiated, and the reaction was quenched after 1, 5, or 10 min. Extension products were separated from unmodified RNA by denaturing PAGE, bands were visualized with a phosphorimager, and radioactivity was quantified using ImageQuant software.

of 6-methylpurine ribonucleoside triphosphate (**25**) into RNA by RNA-dependent RNA polymerase from poliovirus ($3D^{pol}$). The cognate ribonucleoside, an antiviral and anticancer agent with activity against RNA viruses, lacks a clearly defined mechanism of action.[23] As shown in Figure 3, using an assay that we previously employed to investigate other NTPs,[24,25] **25** was found to be efficiently incorporated by $3D^{pol}$ into a symmetrical RNA substrate, with activity comparable to natural ATP. These results establish that a high level of biological activity of NTPs can be obtained following synthesis from a 5′-*H*-phosphonate precursor. Additionally, the ability of **25** to efficiently mimic ATP during synthesis of RNA by $3D^{pol}$ suggests that if 6-methylpurine ribonucleoside were converted into **25** in mammalian cells, it could become incorporated into viral RNA, potentially altering RNA structure, function, or coding. Although further study is needed to determine the mechanism of action of **25**, related purine analogues such as ribavirin are known to function by a mechanism that is dependent on incorporation of the analogue into the viral genome.[26–28]

In summary, we developed a one-pot method for the synthesis of biologically active nucleoside 5′-triphosphates from nucleoside 5′-*H*-phosphonates. These precursors can be readily synthesized in high yield from 2′,3′-*O*-isopropylidene-protected nucleosides. Nucleoside 5′-*H*-phosphonates silylated with excess TMSCl (5−8 eq.) are efficiently activated under mild conditions with I_2/pyridine, enabling conversion to triphosphates upon addition of nucleophilic tris(tetra-*n*-butylammonium) hydrogen pyrophosphate. This oxidative coupling method does not require protection of ribose or nucleobases and has the potential to be applied to a variety of nucleosides and analogues.

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Supporting Information Available: Experimental procedures and characterization data are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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