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Protection-Free One-Pot Synthesis of 2′-Deoxynucleoside 5′-Triphosphates and DNA Polymerization

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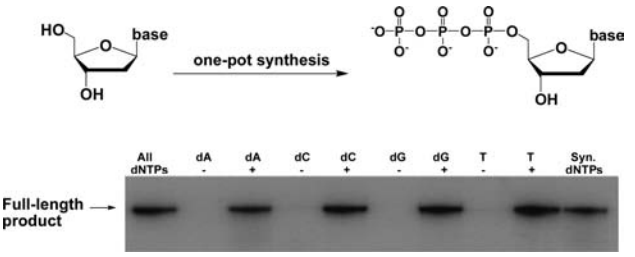
ABSTRACT



By differentiating the functional groups on nucleosides, we have designed and developed a one-pot synthesis of deoxyribonucleoside 5′-triphosphates without any protection on the nucleosides. A facile synthesis is achieved by generating an *in situ* phosphitylating reagent that reacts selectively with the 5′-hydroxyl groups of the unprotected nucleosides. The synthesized triphosphates are of high quality and can be effectively incorporated into DNAs by DNA polymerase. This novel approach is straightforward and cost-effective for triphosphate synthesis.

Nucleoside 5′-triphosphates (dNTPs and NTPs) are the building blocks for the synthesis of nucleic acids (DNA and RNA) and are also utilized in many important biological systems, including DNA replication, RNA transcription, purinergic signaling, neurotransmission, and signal transduction.[1] To better understand the roles of triphosphates and meet the needs in nucleic acid research, the first chemical synthesis of nucleoside 5′-triphosphates was achieved over six decades ago.[2] Although numerous strategies have been continuously developed in recent decades,[3] a convenient synthesis of the nucleoside 5′-triphosphates remains as a long-standing challenge. This is primarily due to the multiple functionalities (hydroxyl and amino groups) of the nucleosides, which generally requires many synthetic steps because of the protection and deprotection of these functionalities. Moreover, the synthesis of nucleoside 5′-triphosphates generates many byproducts that are very difficult to remove. Thus, there is an urgent need to develop straightforward strategies for

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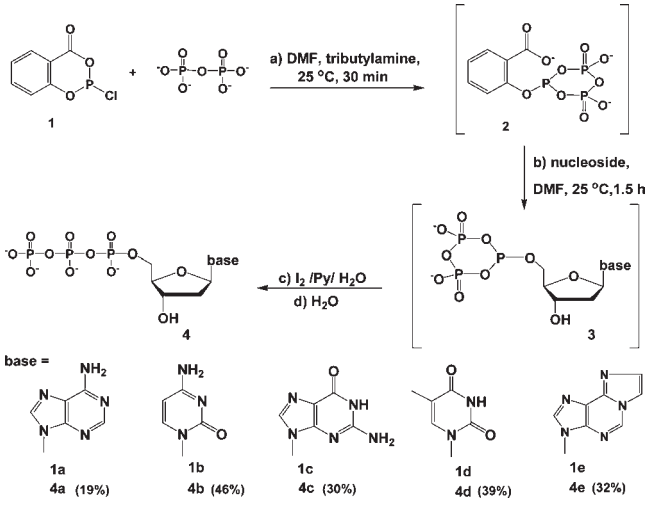
synthesizing nucleoside 5′-triphosphates to reduce the cost of triphosphates (especially modified ones) significantly and meet the growing needs in signal regulation research and studies of nucleic acid structure, function, and detection.[4]

Nucleoside triphosphates are currently synthesized via three major approaches: phosphitylation (or phosphoramiditylation),[3a−c,5] monophosphate isolation/activation,[3c−e,6] and phosphorylation.[3b,c,7] The first strategy employs a highly reactive phosphitylating agent, thereby requiring the protection of both the sugar and nucleobase moieties[3c,5a−5c,5f,5g] to reduce unwanted byproducts. Since the 5′-hydroxy groups of the nucleosides are more reactive than the 3′- and 2′-hydroxyl groups, selective protection of the 3′- and 2′-hydroxyl groups, as well as the amino groups, on the nucleobases are problematic. Nucleosides undergo lengthy selective protection and deprotection to obtain the partially protected intermediates containing the free 5′-hydroxyl groups, before the phosphitylation reaction. The protecting groups are normally removed after the phosphite oxidation and cyclic triphosphate hydrolysis, affording the nucleoside 5′-triphosphates.

The second strategy relies on nucleoside 5′-monophosphate synthesis, isolation, and activation prior to the pyrophosphate treatment.[3b,d,e,6] Clearly, this strategy is not ideal for a convenient synthesis. In the third strategy, highly reactive phosphorus oxychloride ($POCl_3$) is employed as the phosphorylating agent, and the trimethylphosphate solvent used in the synthesis aids at reducing the $POCl_3$ reactivity, primarily generating the dichlorophosphate at the 5′-position (equivalent to an activated monophosphate) prior to the pyrophosphate treatment. In theory, the third strategy requires no protection of the nucleosides.[7a−c] In practice, however, this strategy usually generates numerous byproducts (such as the regio-isomers and oligophosphates) and causes purification difficulties, which have even been reported recently.[8]

To minimize the unwanted byproducts and achieve high-quality products, we designed a mild and selective phosphitylating reagent to differentiate these functionalities. To create a differentiating reagent, we decided to take advantage of the high reactivity of salicyl phosphorochloridite[5]

Scheme 1. One-Pot Synthesis of dNTPs



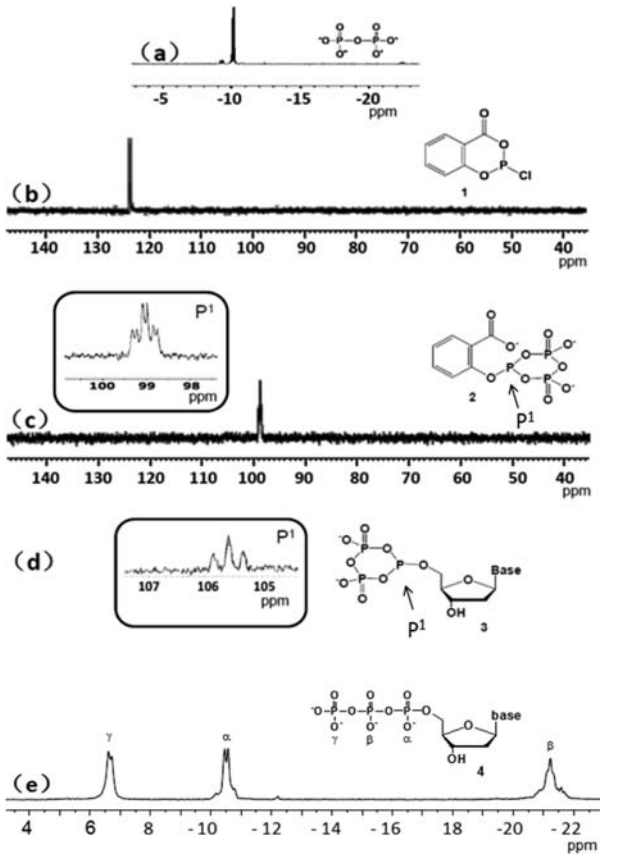


Figure 1. ^{31}P NMR monitoring of 5′-triphosphate **4d** formation at room temperature (in DMF-d_7; reference: H_3PO_4 as an external standard). (a) Tributylammonium pyrophosphate (−10.2 ppm); (b) salicyl phosphorochloridite (124.7 ppm); (c) proposed formed phosphitylating reagent **2** *in situ*, with chemical shift observed at 98.9 ppm; (d) the trivalent phosphorus of cyclic triphosphite **3d** (105.7 ppm); (e) thymidine 5′-triphosphate **4d**.

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and converted it into a weak phosphitylating reagent. After many trials (data not shown), we found that phosphates, such as monophosphate and pyrophosphate, were capable of effectively reducing the reactivity of salicyl phosphorochloridite. Finally, we chose pyrophosphate to both tailor the reactivity and generate the triphosphates after the oxidation and hydrolysis in a one-pot synthesis (Scheme 1). Our selective phosphitylating reagent (**2**) can be generated *in situ*, and without any purification, it can selectively react with the 5′-hydroxyl groups of nucleosides that contain no protection on the sugar and nucleobases. To demonstrate the proof of principle, we report here a convenient synthesis of the native and modified deoxyribonucleoside triphosphates and demonstrate DNA polymerase extension and PCR reactions using the synthesized native 5′-triphosphates.

Nucleoside 5′-triphosphates (**4**) were synthesized conveniently (Scheme 1). After reacting salicyl phosphorochloridite with pyrophosphate to generate the selective phosphitylating reagent (**2**), an unprotected nucleoside was added, followed by iodine oxidation and hydrolysis. The developed synthetic conditions are mild, and this strategy was extended to the one-pot synthesis of ethanodeoxyadenosine 5′-triphosphate (EdATP, **4e**) successfully. A ^{31}P NMR study was also performed to monitor the entire synthesis (Figure 1), where thymidine (**d**) was used as the model compound. The NMR study of the reactions was conducted by using deuterated DMF (DMF-d_7) as the solvent. After the reaction proceeded for 30 min, intermediate **2** was observed as the major product, revealed by an upfield chemical shift from singlet 124.7 ppm (Figure 1b) to 98.9 ppm (td, Figure 1c). Intermediate **2** was formed by two nucleophilic reactions between **1** and pyrophosphate, which displaced the chloride and carboxylate. This mild phosphitylating reagent (**2**) is also bulky and exhibits selectivity toward the more reactive 5′-hydroxyl group. After thymidine addition, the other key intermediate (5′-cyclic triphosphite **3d**) was observed as the major product 1 h later, revealed by 105.7 ppm (triplet, Figure 1d). Since nucleosides are much cheaper compared to the corresponding synthesized triphosphates, we have purposely allowed only a majority of nucleosides to be consumed (approximately 70%) to maintain the high regioselectivity of 5′-cyclic triphosphite intermediates. After the oxidation, hydrolysis, and NaCl-ethanol precipitation, the overall reaction yields of dNTPs are 19−46% (determined by HPLC), and the precipitated dNTPs are capable of DNA polymerase and PCR reactions. Furthermore, to confirm their integrity, all synthesized dNTPs were analyzed by NMR and MS, shown in Table 1 and the Supporting Information.

Because of the high selectivity of the 5′-triphosphate synthesis, synthesized dNTPs (crude products) can be easily purified by RP-HPLC to offer high purity (typical HPLC shown in Figure 2 and the Supporting Information), while an ion exchange strategy is not very effective in removing the minor 3′-triphosphates from the 5′-triphosphates. The nucleoside 3′-triphosphate (approximately 5−10%) is the major byproduct. The minor peak shown in Figure 2 was isolated via HPLC and characterized by comparison with HPLC and MS of the previously synthesized thymidine 3′-triphosphate. The presence of an excess of **1** in the reaction could result in a nucleophilic attack by the unprotected 3′-hydroxyl group of a deoxynucleoside, producing the undesired 3′-product. We later found that adding excess pyrophosphate (such as 2 equiv) to **1** could completely convert **1** to **2** and minimize the byproduct formation. In addition, a lower temperature (0 or −10 °C), which further reduces the reaction rate, can generally minimize formation of byproducts, as observed by RP-HPLC analysis (data not shown). RP-HPLC analysis and coinjection with the dNTP standards revealed that the byproducts, commonly observed with the conventional strategies, are significantly reduced when our new approach is used.

Table 1. ESI-TOF $[M\text{-}H^+]^-$ Mass Spectrometry Analysis of 2′-Deoxynucleoside 5′-Triphosphates

entry	dNTP	chemical formula	measured (calcd) $[M\text{-}H^+]^-$ *m/z*
4a	dATP	$C_{10}H_{16}N_5O_{12}P_3$	489.9938 (489.9936)
4b	dCTP	$C_9H_{16}N_3O_{13}P_3$	465.9814 (465.9817)
4c	dGTP	$C_{10}H_{16}N_5O_{13}P_3$	505.9893 (505.9885)
4d	TTP	$C_{10}H_{17}N_2O_{14}P_3$	480.9831 (480.9820)
4e	EdATP	$C_{12}H_{16}N_5O_{12}P_3$	513.9940 (513.9936)

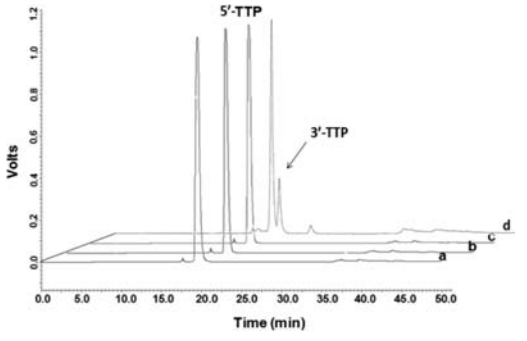

Figure 2. RP-HPLC profiles of chemically synthesized and commercial 2′-deoxynucleoside 5′-triphosphates. (a) Synthesized 5′-TTP after NaCl-ethanol precipitation and RP-HPLC purification (retention time: 19.8 min); (b) standard 5′-TTP (retention time: 19.4 min); (c) coinjection of a and b (retention time: 19.4 min); and (d) crude 5′-TTP and 3′-TTP after NaCl-ethanol precipitation (retention time: 19.4 and 20.7 min), respectively.

To further confirm the quality of the synthetic dNTPs, we have demonstrated their ability as substrates for DNA polymerization. By using a ^{32}P-labeled primer, we performed the DNA polymerization on a DNA template

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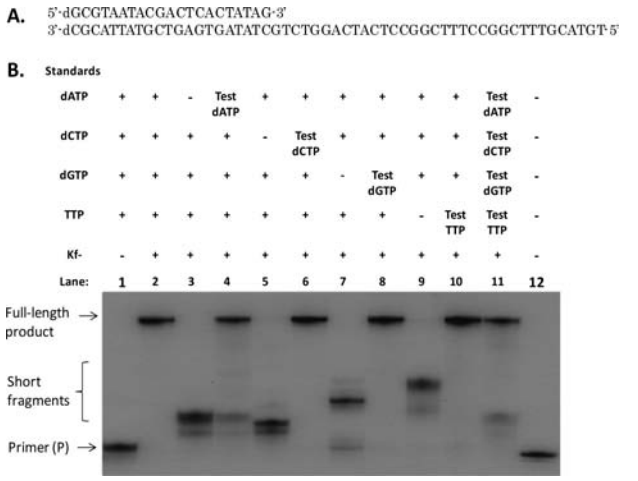

Figure 3. (A) Primer and template sequences used in the polymerization experiment. (B) Primer extension reaction of chemically synthesized dNTPs and commercially available dNTPs into DNA by Klenow fragment exo(-) (Kf-). The primer was 5′-end labeled using polynucleotide kinase and [γ-^{32}P] ATP. Polymerization reactions were performed with primer (3.5 μM), template (5 μM), all dNTPs (1.0 mM each), and Kf- (0.05 U/μL) at 37 °C for 1 h. Reactions were analyzed by 19% polyacrylamide gel electrophoresis. Lane 1: primer (P) and all dNTPs, but no Kf-; Lane 2 (positive control): P, template (T), all commercial dNTPs, and Kf-; Lanes 3, 5, 7, and 9 (negative controls) omitted dATP, dCTP, dGTP, and TTP from Lane 2, respectively; Lanes 4, 6, 8, and 10 were compensated with the synthesized dATP, dCTP, dGTP, and TTP (respectively) to the corresponding Lanes 3, 5, 7, and 9. Lane 11: P, T, all synthesized dNTPs, and Kf-; Lane 12: 5′-^{32}P-labled primer.

(Figure 3) with all commercial dNTPs as a positive control, and the HPLC purified or precipitated dNTPs were synthesized as test substrates. We conducted four polymerization reactions by omitting one dNTP each time to serve as negative controls. As expected, the positive control gave the full-length DNA product (Lane 2 in Figure 3), while the negative controls with one dNTP missing did not generate any full-length DNA (Lanes 3, 5, 7, and 9) and the DNA synthesis stopped after synthesizing short fragments. We then used the HPLC purified individual dNTPs to compensate for the corresponding missing commercial dNTPs in each polymerization reaction and compared them with the positive control. We observed that DNA polymerase recognized the synthesized dNTPs as well as the standard dNTPs. Polymerase efficiently extended the primer for each reaction containing an individual synthesized dNTP (Lanes 4, 6, 8, and 10) to the expected full-length DNA products identical to the DNA synthesized by using the commercial 5′-triphosphates as standard (Lane 2). The incorporation efficiency of the synthesized dNTPs was virtually identical to that of the standard ones. More convincingly, in the reaction containing all synthetic triphosphates (Lane 11), the full-length DNA was generated similarly to the reaction containing all of the standard dNTPs (Lane 2). Since a DNA polymerase does not recognize 3′-triphosphates as substrates, we conducted polymerase reactions with the crude dNTPs. Our results show that the precipitated synthetic dNTPs can also allow both DNA primer extension and PCR reactions (Figures S16 and S17). Our typical synthesis scale is approximately 0.1 mmol, which can generate sufficient dNTP for 200 PCR reactions (50 μL, 1 mM dNTP each). Furthermore, the synthesis can be easily scaled up; we have done the synthesis at the 100-mg scale without any difficulties.

In summary, by generating a selective phosphitylating reagent *in situ*, we have developed a straightforward approach to conveniently synthesize deoxynucleoside 5′-triphosphates without any protection of the nucleosides. The proof of principle was demonstrated by using modified and nonmodified nucleosides. This *in situ* phosphitylating reagent, generated by reacting salicyl phosphorochloridite with pyrophosphate, is mild and permits high regioselectivity at the 5′-hydroxyl group for the triphosphate synthesis. The quality of the synthesized dNTPs was also confirmed by DNA polymerase primer extension and PCR reactions. Our one-pot synthesis is convenient and cost-effective and eliminates the lengthy protection and deprotection steps associated with most conventional approaches. Moreover, it reduces byproduct formation and purification difficulty. This mild chemistry makes synthesis of sensitive modified triphosphates possible. Such a strategy is also applicable to the synthesis of nucleoside α-modified 5′-triphosphates. For instance, intermediate **3** may be oxidized with either sulfur or selenium (data not shown) and may likely be oxidized by borane reagents to afford the corresponding nucleoside 5′-(α-P-thiotriphosphates),[5a,d,e] nucleoside 5′-(α-P-selenotriphosphates),[9] or nucleoside 5′-(α-P-boranotriphosphates).[5b,c,f,g] This general strategy enables scale-up synthesis of a large number of modified triphosphates to meet the emerging needs in nucleic acid structure and function studies and detection research.

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Supporting Information Available. Experimental procedures; ^{1}H, and ^{31}P NMR, HRMS, and MALDI-TOF MS analytical data; HPLC profiles. These materials are available free of charge via the Internet at http://pubs.acs.org.

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