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# Convenient synthesis of nucleoside 5′-triphosphates for RNA transcription†

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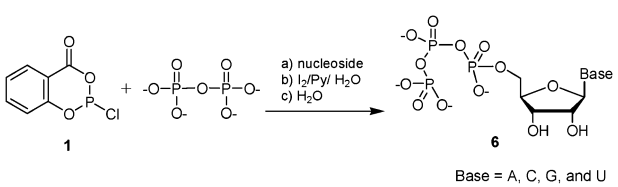
**By generating a selective phosphitylating reagent *in situ*, nucleoside 5′-triphosphates can be conveniently synthesized in one pot. This novel strategy without nucleoside protection has been developed to largely simplify synthesis of the nucleoside triphosphates. This demonstrated principle can be applied to the 5′-triphosphate synthesis of both native and modified nucleosides.**

In addition to participation in RNA transcription as the substrates, nucleoside 5′-triphosphates (NTPs) are involved in many biological regulations and pathways.[1] To transcribe RNAs and investigate the biological systems, many native and modified NTPs are synthesized either chemically or enzymatically. Since the first chemical synthesis of NTPs was accomplished six decades ago,[2] a large number of chemical strategies have been developed to effectively synthesize nucleoside triphosphates in the past decades.[3–13] However, many challenges remain in chemically selective triphosphate synthesis due to the multiple functionalities of nucleosides, *i.e.*, 5′-, 2′-, and 3′-sugar hydroxyl groups and nucleobase amino groups. These functional groups require the protection and deprotection steps during the triphosphate synthesis in order to minimize the formation of by-products and regioisomers, which are usually difficult to remove.[3] For instance, in the recent phosphate triester strategy,[7] these functional groups of the starting materials need to be fully protected in order to ensure good yields and minimal by-products. To increase the synthetic selectivity, nucleoside and nucleotide kinases have also been explored.[14–18] Because of the drawbacks of enzymatic synthesis, such as the substrate specificity, yield and cost, chemical synthesis is still a better choice for preparing a large quantity of nucleoside triphosphates, especially those with modifications.[5,19,20]

The 3′- and 2′-triphosphates are the major regioisomers and by-products in the 5′-triphosphate synthesis, and the mono-, di- and even oligo-phosphates can also form as undesired products during the triphosphate synthesis.[3] In spite of many synthetic strategies developed, including one-pot synthesis,[4,8,9,21–23] a convenient synthesis of the 5′-triphosphates directly from unprotected nucleosides with high regioselectivity still remains as a challenge. To address the selectivity issue, avoid the protection and deprotection, and simplify the triphosphate synthesis, we decided to generate a mild and selective reagent for the 5′-hydroxyl phosphitylation. We found that we can tailor the reactivity of many phosphitylating reagents by reacting them with phosphate or pyrophosphate first. The generated phosphitylating reagent *in situ* is mild and can selectively react with the 5′-hydroxyl groups of nucleosides without any protection. Thus, we report here a convenient synthesis of NTPs in one pot and without any protection and deprotection of the nucleosides. Our transcription experiments indicate that the synthesized NTPs are of high quality and well recognized by RNA polymerase to synthesize full-length RNAs.

Though the primary 5′-OH is a better nucleophile than the secondary 2′- and 3′-OH groups, the difference between them is small. We hypothesized that a bulky phosphitylating reagent can offer a better selectivity to distinguish them. In addition, we found that these nucleobase amino groups are not very reactive in a mild phosphitylation reaction. Thus, our novel synthetic strategy designed for the 5′-triphosphates (**6**, Scheme 1) starts from reacting **1**[21,24] with pyrophosphate, generating a bulkier phosphitylating reagent than **1**, which selectively phosphitylates nucleosides. After the phosphite oxidation and hydrolysis, the 5′-triphosphates (**6**) are then straightforwardly synthesized.

This is a very convenient synthesis. A synthetic mechanism is proposed in Scheme 2. The reaction of 2-chloro-4-H-1, 3,2-benzodioxaphosphorin-4-one (salicyl phosphorochoridite, **1**) and tributylammonium pyrophosphate generates a mild phosphitylating reagent **2** *in situ*, which selectively phosphitylates the non-protected nucleosides (**3**, including A, C, G and U) to offer 5′-phosphite **4**. This cyclic phosphite (**4**) is oxidized by iodine to generate the 5′-cyclic triphosphate (**5**), followed by hydrolysis to afford the 5′-triphosphates (**6**).




**Scheme 1** Synthesis of nucleoside 5′-triphosphates.

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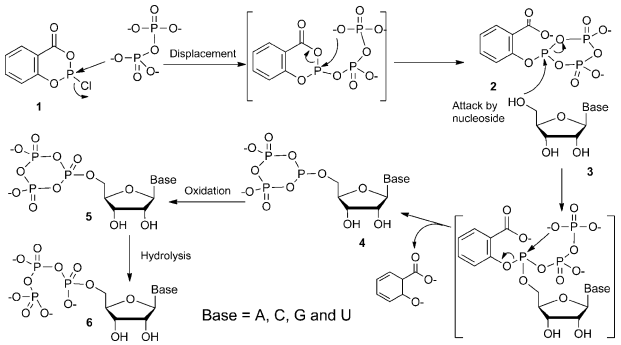


**Scheme 2** Proposed mechanism for nucleoside 5′-triphosphates.

Before establishing the synthetic system, initially, the solubility problem of the nucleosides was encountered when pyridine and dioxane were used as solvents. Since DMF can facilitate regioselectivity,[24] DMF was also used as a solvent. Unfortunately, the purine nucleosides (A and G) were not readily soluble in DMF under the reaction conditions, and many attempts of using a single solvent failed to generate desired products. Thus, we decided to examine mixtures of solvents. After many trials, we found that a mixture of DMF and DMSO (1 : 1) dissolved the purines well and generated the desired 5′-triphosphates. In contrast, no solubility problem was encountered for the pyrimidine nucleosides (C and U), and their reactions were performed in DMF. Moreover, the reaction of **1** and pyrophosphate was carried out in DMF in order to generate the selective phosphitylating reagent (**2**, Scheme 2).

Due to the multiple functionalities of the nucleosides, we did not expect to conveniently achieve a clean triphosphate synthesis in one pot with satisfied selectivity. To our pleasant surprise, this 5′-phosphitylation is selective and the synthesized crude triphosphate products are relatively clean with a fine ratio (85 : 15) of 5′-triphosphate *vs.* 3′- (and 2′-) triphosphate (Fig. 1 and S1-3 in Supporting Information†). Moreover, excess pyrophosphate was used to ensure complete consumption of the highly reactive phosphitylating agent (**1**), since unreacted **1** can compromise the phosphitylation selectivity. We found that the phosphitylation reaction can be carried out at room temperature for 1 h and still offers satisfactory selectivity on the 5′-hydroxyl group of each nucleoside. We also found that

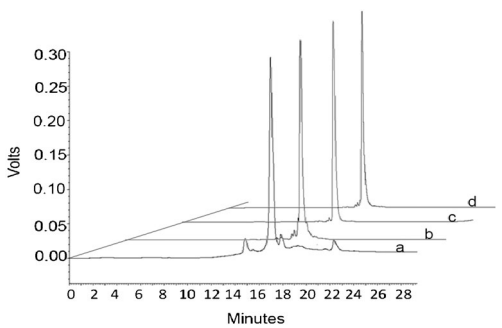


**Fig. 1** HPLC profiles of the synthesized nucleoside 5′-triphosphate. (a) crude ATP synthesized; (b) the synthesized (test) ATP after purification (injected 20 μL, 1.0 mM, retention time 16.4 min); (c) standard ATP (injected 20 μL, 1.0 mM, retention time 16.6 min); (d) co-injection of the synthesized and standard dATPs (injected 10 μL each, 1.0 mM, retention time 16.3 min).

the reduced reaction temperature (0 or −10 °C) further increased the 5′-selectivity, indicated by HPLC analysis (Fig. S2 in Supporting Information†), which is consistent with the literature.[3] In order to consume the nucleosides at a lower temperature, the phosphitylation time was extended (at least 3 h). After the $I_2$ oxidation of the phosphite (**4**), the cyclic triphosphate (**5**) was finally hydrolyzed to afford the triphosphates (**6**) in this one-pot synthesis.

The synthesized crude NTPs were first precipitated with ethanol to remove most of the reaction reagents and by-products, followed by reversed-phase HPLC purification. The purified NTPs were analyzed by RP-HPLC, and a typical HPLC profile is presented in Fig. 1. The HPLC profiles of the crude 5′-NTP products were relatively clean (Fig. S1-3 in Supporting Information†). To confirm the high quality of these NTPs, we used RP-HPLC to analyze the synthesized NTPs, and each one of them was also compared with its corresponding commercial NTP standard. The synthesized NTPs (ATP, CTP, GTP and UTP) were also confirmed by Electron-spray Mass spectrometry (Table 1).

To further confirm the high quality of these NTPs, they were subjected to RNA transcription. Each NTP was used as a substrate for T7 RNA polymerase. The experiment was designed to individually incorporate each of the test NTPs (synthesized NTPs). From the autoradiograph (Fig. 2), we observed that all the test NTPs (Lanes: 4, 6, 8, 10, 11) were incorporated. It shows that the RNA transcripts were identical to the RNA product from the transcription using all standard NTPs, which served as the positive control in this experiment. When one NTP was omitted from the positive control, no full-length RNA product was observed. However, when one synthesized NTP was added to compensate for the corresponding missing NTP, the full-length RNA was efficiently synthesized similar to the positive control, indicating that the synthesized 5′-triphosphates can be successfully incorporated into RNA. Furthermore, when the entire standard NTPs were replaced with the synthesized ones, the full-length RNA was still synthesized similarly to the positive control, confirming the high quality of the synthesized NTPs. Excitingly, we found that the crude NTPs purified only by ethanol precipitation, without HPLC purification, can also be directly used for RNA transcription. This largely simplifies the preparation of the nucleoside triphosphates, further indicating the selectivity and convenience of this novel synthetic strategy.

In summary, we have generated a new phosphitylating reagent *in situ* by reacting salicyl phosphorochoridite with pyrophosphate, which enables establishment of a convenient strategy for the nucleoside triphosphate synthesis in one pot. We have demonstrated the proof of principle using the native nucleosides (A, C, G and U) without any protection. It is worth pointing out that similar to the native triphosphate synthesis with the iodine oxidation, our strategy can likely be used to conveniently synthesize the nucleoside triphosphate containing modifications at the α-phosphate, such as 5′-(α-*P*-thio-triphosphates),[21,22,25,26] the 5′-(α-*P*-borano-triphosphates)[4,22,27,28] or the 5′-(α-*P*-seleno-triphosphates),[29–31] when the phosphite intermediate (**4**) is oxidized with the corresponding sulfur, borane or selenium oxidants. This mild synthetic strategy allows convenient synthesis of many modified and non-modified

**Table 1** HRMS (ESI-TOF) analysis of the synthesize NTPs

| Nucleoside triphosphate | Chemical formula | Measured (calcd.) $[M - H]^-$ $m/z$ |
|---|---|---|
| ATP | $C_{10}H_{16}N_5O_{13}P_3$ | 505.9891 (505.9885) |
| CTP | $C_9H_{16}N_3O_{14}P_3$ | 481.9781 (481.9772) |
| GTP | $C_{10}H_{16}N_5O_{14}P_3$ | 521.9828 (521.9834) |
| UTP | $C_9H_{15}N_2O_{15}P_3$ | 482.9615 (482.9612) |

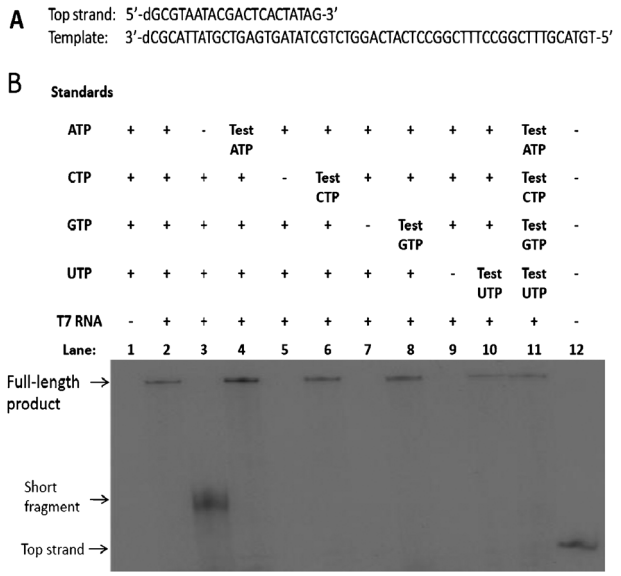


**Fig. 2** (A) DNA template and promoter sequences used in the transcription experiment. B. RNA transcription reactions using commercial and synthesized (test) NTPs by T7 RNA polymerase. RNA transcripts were bodily labelled using [$\alpha$-$^{32}$P]-ATP. These transcription reactions (5.0 μL each) containing the DNA promoter (1.0 μM), the DNA template (1.0 μM), NTPs (0.50 mM each) and RNA polymerase (0.1 μL per microlitre reaction) were incubated at 37 °C for 2 h. Each reaction was analyzed by 19% polyacrylamide gel electrophoresis. Lane 1: containing DNA template/promoter and all NTPs, but no T7 RNA polymerase; Lane 2: containing DNA template/promoter, all NTPs, and T7 RNA polymerase; Lane 3, 5, 7 and 9: containing DNA template/promoter, T7 RNA polymerase, but missing ATP, CTP GTP and UTP, respectively; Lane 4, 6, 8 and 10: ATP, CTP GTP and UTP were added to the missed reactions, respectively. Lane 11: using all synthesized NTPs (test); Lane 12: DNA promoter as the marker.

triphosphates and opens new opportunities for investigating transcription and NTP-participated signal transduction pathways.

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