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Efficient Solid-Phase Chemical Synthesis of 5′-Triphosphates of DNA, RNA, and their Analogues

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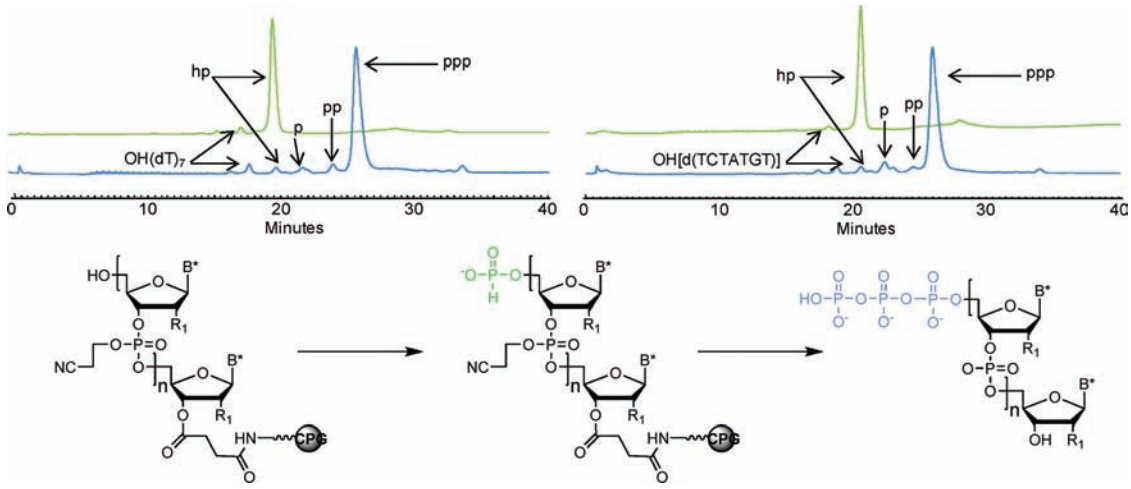
ABSTRACT



A robust, reproducible, and scalable method for the solid-phase synthesis of 5′-triphosphates of DNA, RNA, and their chemically modified analogues using 5′-*H*-phosphonate intermediates is described. 5′-Triphosphates of oligonucleotides with varying lengths and sequences containing different 5′-terminal nucleotides, with and without internal sugar-backbone modifications, were efficiently prepared as crude products or further purified by HPLC.

DNA and RNA 5′-triphosphates (TPs) serve as important substrates for many biochemical applications. For example, DNA TPs are used in the biotechnology industry[1] as substrates for the preparation of synthetic genes.[2] In addition, RNA TPs are used for the ligation of RNA molecules,[3−5] the detection of viral responses via activation of the RIG-I protein,[6] the induction of antiviral immunity,[7] and in the

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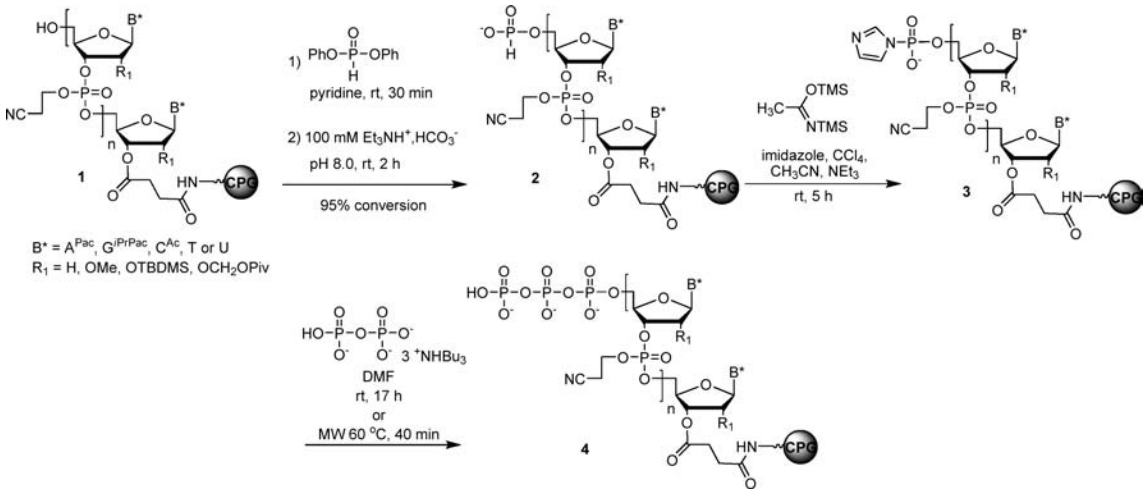
Scheme 1. General Synthesis of ON 5′-TPs on Solid Support



enzymatic synthesis of m^{7}G-5′-capped RNAs.[1,8,9] It was recently demonstrated that the immune response triggered by RNA TPs binding to RIG-I synergizes with gene silencing mediated by small interfering RNAs (siRNAs).[10] This data emphasizes the therapeutic potential of immunostimulatory nucleic acids,[11] including siRNA TPs. Recent insights into the nature of the RIG-I substrate were made possible using synthetic RNA TPs rather than 5′-TP products generated by in vitro RNA transcription.[12−14] There are several advantages to synthetic RNA TPs over those from in vitro transcriptions, such as purity, reproducible yield (independent of RNA sequence), the potential for large-scale synthesis, and the ability to introduce chemical modifications into the RNA.[15]

Despite the existence of an arsenal of methods for the synthesis of nucleoside triphosphates (NTPs),[16−19] there is no efficient and universal method for the enzyme-free chemical synthesis of DNA and RNA TPs. The few approaches describing DNA and RNA TP synthesis on solid support[1,8,16,20] are dependent on oligonucleotide (ON) length and/or sequence and require difficult separation procedures resulting from low conversions and poor yields. These approaches are also limited to the small-scale production of ON TPs and have not been demonstrated with chemically modified ONs. All these reports are based on the use of the highly reactive classical phosphitylation reagent discovered in 1989 by Ludwig and Eckstein.[21] A versatile and scalable method for synthesis of ON TPs is needed.

Here, we report a highly efficient and simple procedure for the solid-phase synthesis of DNA and RNA TPs of various lengths, sequences, and chemical modifications that provides reasonably good yields and satisfactory purity of the crude products so that difficult chromatography purifications can be avoided. This method uses stable, inexpensive, commercially available reagents and is based on robust and facile chemical reactions that are compatible with the synthesis of natural or chemically modified DNA or RNA. Based on several reported approaches for the solution-phase syntheses of NTPs,[19,22] we opted for the use of the 5′-*H*-phosphonate ON (Hp-ON) as a stable and easily accessible intermediate for triphosphate synthesis.

The general synthetic route is depicted in Scheme 1. The solid-supported 5′-OH ON **1** was prepared by standard automated solid-phase ON synthesis using controlled pore glass support (CPG) and phosphoramidites.[23] Treatment of **1** with a solution of diphenyl phosphite in pyridine, followed by hydrolysis, afforded the solid-supported Hp-ON **2**. The

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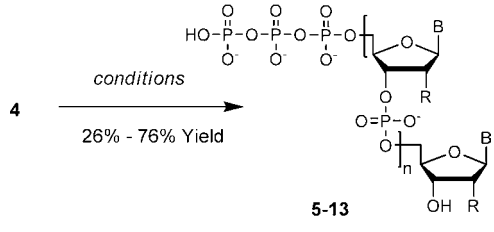
5′-TP ON **4** was then efficiently obtained using a two-step activation/phosphorylation procedure. First, **2** was oxidized to an activated 5′-phosphoroimidazolidate **3** using CCl_4 in the presence of imidazole and *N*,*O*-bis-trimethylsilylacetamide (BSA), which was subsequently reacted with excess of (tri-*n*-butylammonium) pyrophosphate affording **4**. Substitution of the imidazole by pyrophosphate was successfully accomplished either at rt for 17 h or upon microwave activation at 60 °C for 40 min (see the Supporting Information). Final release from the CPG support and deprotection yielded the crude ON 5′-TPs **5**−**13** (Tables 1 and 2).

Table 1. Release and Deprotection of ON 5′-TPs



product	substrate **4**, R_1	conditions
5, **6**, **11**	H, OMe	NH_4OH, rt, 2 h
7−**10**	OTBDMS	(1) NH_4OH−EtOH (3:1), rt, 2 h; (2) 1 M TBAF, rt, 24 h
12, **13**	OCH_2OPiv	(1) 1 M DBU in CH_3CN, rt, 1 min; (2) NH_4OH, rt, 3 h; (3) isopropylamine

We initiated our study with the synthesis of hepta-2′-deoxythymidinyl-5′-TP **5**, which was readily prepared on solid support as depicted in Scheme 1. Treatment with ammonia for 2 h at room temperature removed the ON from the CPG support. **5** was analyzed by ion-exchange HPLC, MALDI-TOF MS, and ^{31}P NMR (Figure 1). The phosphitylation of the starting 5′-OH$(dT)_7$ to the corresponding 5′-Hp$(dT)_7$ was virtually quantitative (Figure 1A.a). The crude purity of the ppp$(dT)_7$ was approximately 80%, with $(dT)_7$, Hp$(dT)_7$, p$(dT)_7$ and pp$(dT)_7$ all below 5%, (Figure 1A.b). These impurities were removed by HPLC (Figure 1A.c).

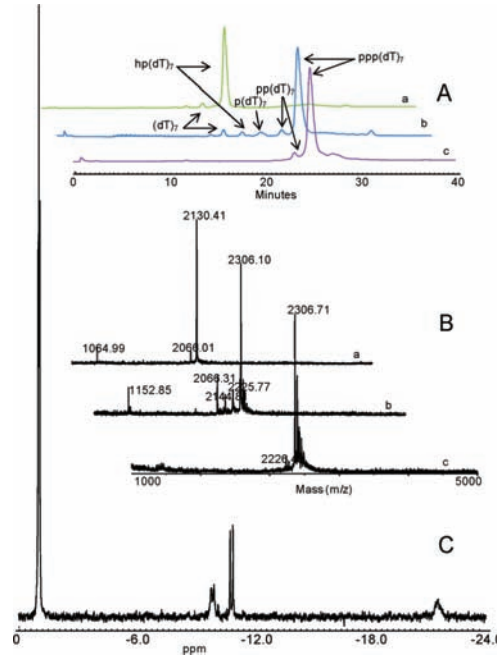


Figure 1. (A) Ion-exchange HPLC profiles of (a) crude hp$(dT)_7$, (b) crude **5**, and (c) pure **5**. (B) Negative-mode MALDI-TOF MS profiles of (a) crude hp$(dT)_7$, calcd *m*/*z* 2130.40, found 2130.41; (b) crude **5**, calcd *m*/*z* 2306.35, found 2306.10; (c) pure **5**, found 2306.70. (C) ^{31}P NMR spectrum of **5**. hp = 5′-*H*-phosphonate; *p* = 5′-phosphate; pp = 5′-diphosphate; ppp = 5′-triphosphate. Note: the large peak around 0 pppm is due to a signal from the phosphodiester linkages.

The approach was successfully applied to the synthesis of a 5′-TP DNA with mixed base composition **6**. Due to the low thermal stability of triphosphates,[16] labile nucleobase protecting groups were used. This allowed efficient deprotection and release from the solid support of the crude **6** at room temperature in 72% yield (Table 2).

Table 2. Data for Synthesized DNA and RNA 5′-TPs

no.	5′-sequence-3′	scale (μmol)	yield[c] (%)	isolated yield (%)[d]
5	ppp$(dT)_7$	0.25	81.9	76
6	ppp[d(TCTATGT)]	0.5	83.8	72
7	pppU_7[a]	0.25	74.6	33
8	pppUUGUCUCUGGUCCUUACUUAA[a,e]	2.0	93.2	26
9	pppUUGUCUCUGGUCCUUACUUAA[a,e]	10.0	81.2	30
10	pppAccGAAGuGuuGuuuGuccdTsdT[a,e]	1.0	89.2	32
11	pppaaguaaggaccagagacaadTsdT	4.0	77.0	40
12	pppU_7[b]	0.25	77.3	66
13	pppAGUUGUUCCC[b]	0.5	77.2	50

[a] Prepared using 2′-*O*-TBDMS-protected phosphoramidites. [b] Prepared using 2′-*O*-PivOM-protected phosphoramidites. [c] Percentage yield of triphosphate in the crude as calculated from the integration of the ion-exchange chromatogram. [d] Isolated yield of crude material. [e] Purified full-length material; d = 2′-deoxy; upper case nucleotides = 2′-OH; lower case nucleotides = 2′-*O*-methyl; s = phosphorothioate; ppp = 5′-triphosphate.

The chemical synthesis of RNA is more complex than that of DNA due to the 2′-hydroxyl group on each sugar, which requires protection during the synthesis and an additional deblocking procedure, typically performed as a final step. We evaluated our synthetic scheme with standard RNA synthesis, employing 2′-*O*-*tert*-butyldimethylsilyl[24] (TBDMS) 3′-phosphoramidite building blocks (Scheme 1). After synthesis of the 2′-TBDMS 5′-TP of U_7, demasking of the 2′-TBDMS groups was performed using 1 M TBAF at room temperature for 24 h (Table 1), yielding the target U_7 TP **7** in moderate 33% yield (Table 2). Alternative desilylation methods resulted in significant to total hydrolysis of the TP moiety (see the Supporting Information). Despite the long TBAF treatment (24 h), **7** was successfully recovered with acceptable purity (74.6%). However, tedious desalting procedures reduced its isolated yield (Table 2).

The procedure was then applied to the synthesis of four 21-mer RNA TPs: two unmodified RNAs (**8** and **9**), a chimeric 2′-OH/2′-*O*-methyl ON (**10**), and a fully 2′-*O*-methyl-modified ON (**11**). Each was obtained in good isolated yield (26−40%) and good purity (77−93%), demonstrating that chemically modified ON 5′-TP constructs of the type used as siRNA could be synthesized. The overall efficiency in terms of chemical conversion, recovery, and final purity was similar at synthesis scales ranging from 1 to 10 μmol (Table 2 and Figure 2). RNA TPs **8**−**10** were purified by ion-exchange HPLC (Figure 2A.b,A.c). To the best of our knowledge, this is the first report on an efficient and reproducible synthesis of natural and chemically modified RNA 5′-TPs employing standard TBDMS chemistry.

However, there are some drawbacks related to the 2′-*O*-TBDMS RNA TP synthesis, such as long desilylation reaction times, low stability of the triphosphate moiety to fluoride treatments, and tedious desalting or purification procedures. Therefore, we sought an alternative for 2′-OH protection of RNA during the triphosphate synthesis. Recently, Debart et al. developed the base-labile 2′-*O*-pivaloyloxymethyl (PivOM) group for 2′-OH protection of RNA.[25] Compared to the TBDMS approach, PivOM protection provides better coupling yields and can be removed by ammonia treatment rather than fluoride treatment.[25] We first prepared **12** and compared our results to **7**, demontrating that the PivOM chemistry provided U_7 TP with higher purity (77.3% vs 74.6%) and higher isolated yield (66% vs 33%) (Table 2). We then synthesized the 10-mer mixed base RNA TP **13**, a sequence difficult to access using in vitro transcription methods. Yields and purity of **13** were acceptable (Table 2). Synthesis of longer RNA 5′-TP using the PivOM approach is currently in progress.

In conclusion, we report a robust, reproducible, and scalable method for the synthesis of 5′-triphosphates of DNA, RNA, and their chemically modified analogues. The solid-supported synthesis is efficient and provided ON 5′-TPs of various lengths and sequences with different 5′-terminal nucleotides and sugar and backbone chemical modifications. ON 5′-TPs were typically obtained in high yields (26−76%) and acceptable crude purity (ca. 80% of triphosphate). Further automation of the method, scale-up, and application to ONs with other chemical modifications and/or other protecting groups will make this approach broadly useful for various biological evaluations. Work along these lines is in progress.

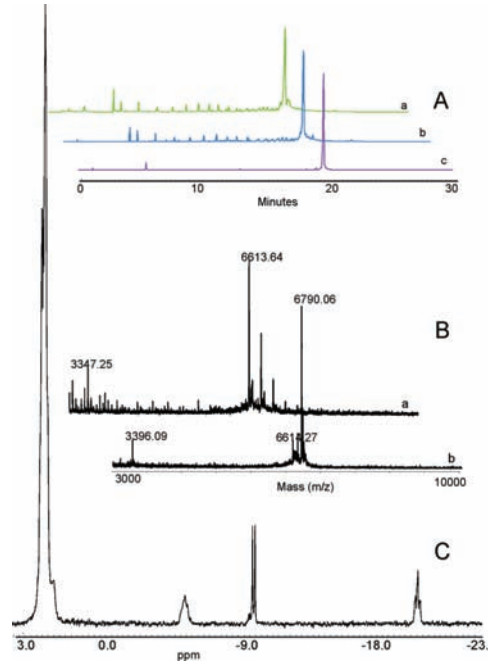


Figure 2. (A) Ion-exchange HPLC profiles of (a) crude hpUUGUCUCUGGUCCUUACUUAA; (b) crude **8**; (c) pure **8**. (B) MALDI-TOF MS profiles of (a) crude hpUUGUCUCUGGUCCUUACUUAA, calcd *m*/*z* 6611.87, found 6613.64; (b) pure **8**, calcd *m*/*z* 6787.87, found 6790.06. (C) ^{31}P NMR spectrum of **9**.

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Supporting Information Available: Experimental procedures, HPLC, MS, and ^{31}P NMR spectra, additional figures, and a table. This material is available free of charge via the Internet at http://pubs.acs.org.

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