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Solid-phase synthesis of 5′-O-β,γ-methylenetriphosphate derivatives of nucleosides and evaluation of their inhibitory activity against HIV-1 reverse transcriptase

Yousef Ahmadibeni [a,b], Chandravanu Dash [c], Stuart F. J. Le Grice [d], Keykavous Parang [a,*]

[a] Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, The University of Rhode Island, Kingston, RI 02881, USA
[b] Department of Chemistry, Columbus State University, Columbus, GA 31907, USA
[c] Centre for AIDS Health Disparities Research, Meharry Medical College, Nashville, TN 37208, USA
[d] Resistance Mechanism Laboratory, HIV Drug Resistance Program, National Cancer Institute at Frederick, National Institute of Health, Frederick, MD 21702, USA

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ABSTRACT

Bis(dichlorophosphino)methane was converted to a β,γ-methylenetriphosphitylating reagent. The reagent was immobilized on aminomethyl polystyrene resin-bound linker of 4-acetoxy-3-phenylbenzyl alcohol to afford a polymer-bound β,γ-methylenetriphosphitylating reagent, which was reacted with unprotected nucleosides followed by oxidation with *tert*-butyl hydroperoxide, deprotection of cyanoethoxy groups with DBU, and acidic cleavage to produce 5′-O-β,γ-methylene triphosphate nucleosides in 53–82% overall yields. Among all the compounds, cytidine 5′-O-β,γ-methylenetriphosphate inhibited completely RNase H activity of HIV-1 reverse transcriptase at 700 μM.

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Phosphate transfer is involved in several enzyme-catalyzed reactions[1–3] and therefore is a subject of considerable interest in biological systems. Triphosphate mimics, such as methylenetriphosphates, halogenated methylenetriphosphates, and imidotriphosphates, have been used to probe the mechanism of phosphoryl transfer in enzyme-catalyzed processes[2–4] and to target specific receptors or enzymes that bind or hydrolyze triphosphates.[5–8] Replacement of labile P–O–P bond in nucleoside triphosphates with a stable isosteric P–CH_2–P bond in nucleotide analogs results in enhanced metabolic stability. Synthesis of non-hydrolyzable triphosphate analogs of nucleosides is considered a challenge.

A number of solution-phase strategies have been previously reported for the synthesis of nucleoside 5′-O-β,γ-methylenetriphosphates including some of the compounds described here by the coupling reactions of nucleoside 5′-monophosphate salt forms or activated nucleoside monophosphates with diphosphonates (methylene diphosphonic acids). Some examples include the reactions of methylene diphosphonic acids with nucleoside 5′-monophosphates,[9,10] 5′-monophosphate-*N*-methylimidazolides,[11,12] or 5′-monophosphate-imidazolides.[13] Alternatively, sequential Michael–Arbuzov reactions on ethyl(bishalomethyl) phosphinates[8] were used for the synthesis of the bismethylene triphosphates. These synthetic strategies have been encountered by one or more of the following difficulties. (i) Activated nucleoside monophosphates or their specific salt forms are required for the synthesis of 5′-O-β,γ-methylenetriphosphate nucleoside analogs, respectively. (ii) The coupling reactions are mostly carried out in anhydrous organic solvents. Because of the poor solubility of most precursor phosphates in the reaction mixture, the yields are usually low. (iii) Stepwise lengthy purification of intermediates and final products from the reagents are required. (iv) These strategies require protection and deprotection reactions for most of the nucleosides. Thus, alternative convenient strategies for the preparation of 5′-O-β,γ-methylenenucleoside triphosphates are highly desired.

Herein, we report for the first time a solid-phase strategy for the synthesis of β,γ-methylenetriphosphate nucleoside analogs by using a novel solid-supported phosphitylating reagent. A β,γ-methylenetriphosphitylating reagent was prepared first. Aminomethyl polystyrene resin-bound linker of *p*-acetoxybenzyl alcohol[14] was used as solid support for immobilization of β,γ-methylenetriphosphitylating reagent. The unprotected nucleosides were reacted with the immobilized reagent. Subsequent oxidation, deprotection, and cleavage afforded the β,γ-methylenetriphosphate nucleoside analogs.

Scheme 1 illustrates the synthesis of β,γ-methylenetriphosphitylating reagent **6**. Phosphorus trichloride (**1**, 6 mmol) was reacted with diisopropylamine (2 equiv) to yield bis[diisopropylamino]phosphorochloridite (**2**). The subsequent reaction of **2** with water (1 equiv) afforded the intermediate bis[diisopropylamino]hydroxyphosphine (**3**).

In a separate reaction, bis(dichlorophosphino)methane **4** (6 mmol) was treated with 3-hydroxypropionitrile (3 equiv) to yield **5**, which was reacted with **3** (1 equiv) to yield β,γ-methylenetriphosphitylating reagent (**6**). The chemical structure of **6**

* Corresponding author. Tel.: +1 401 874 4471; fax: +1 401 874 5787.
E-mail address: kparang@gmail.com (K. Parang).

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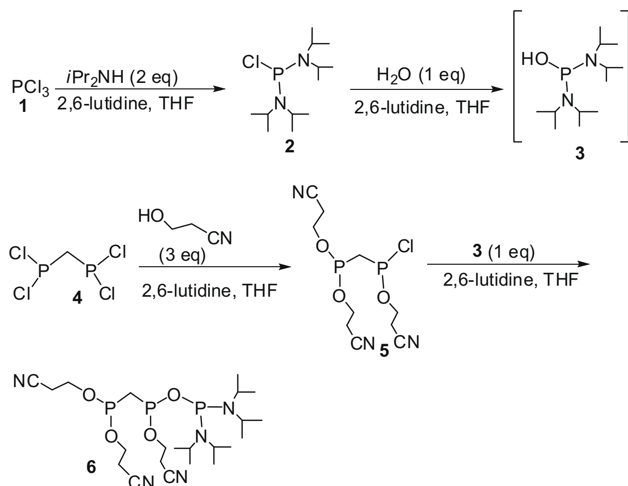
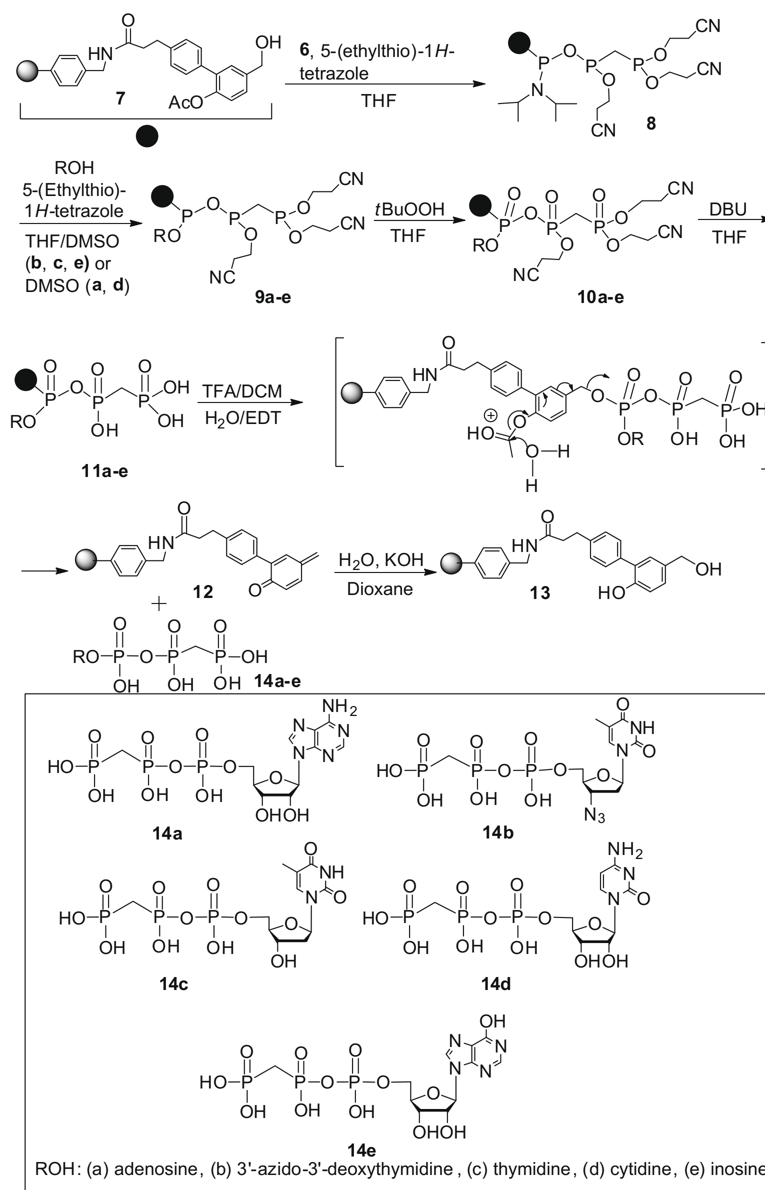


Scheme 1. Synthesis of β,γ-methylenetriphosphitylating reagent **6**.

was confirmed by high-resolution time-of-flight electrospray mass spectrometry (ESI-TOF) and elemental phosphorus analysis. Compound **6** was immediately used for the next coupling reaction with polymer-bound *p*-acetoxybenzyl alcohol **7** to avoid any decomposition.

Aminomethyl polystyrene resin-bound linker of *p*-acetoxybenzyl alcohol (**7**) was synthesized from aminomethyl polystyrene resin in multiple-step reactions[14] and was selected as a solid support for immobilization of the β,γ-methylenetriphosphitylating reagent. We have previously reported the application of the *p*-acetoxybenzyl alcohol linkers for the solid-phase synthesis of diverse number of compounds, such as carbohydrate and nucleoside monophosphates,[15–17] diphosphates, diphosphodithioates, triphosphates, or triphosphotrithioates,[18] nucleoside β-triphosphates,[19] oligodeoxynucleotides containing internucleotide diphosphodiester linkages,[20] sulfonamides,[14] symmetrical 5′,5′-dinucleoside mono-, di-, tri-, and tetraphosphodiesters,[21] and nucleoside 5′-*O*-α,β-methylene-β-triphosphates.[22]

Scheme 2 shows the synthesis of nucleoside α,β- and β,γ-methylenetriphosphates. Polymer-bound linker **7** was reacted with



Scheme 2. Synthesis of nucleoside 5′-*O*-β,γ-methylenetriphosphates **14a–e** using polymer-bound reagent **8**.

Table 1
Overall isolated yields and purity of crude products for **14a–e**

No.	Overall yield (%) calcd from **8**	Purity of crude products
14a	75	87
14b	81	92
14c	82	90
14d	57	78
14e	53	67

β,γ-methylenetriphosphitylating reagent (**6**, 1.2 equiv) in the presence of 5-(ethylthio)-1*H*-tetrazole to produce the corresponding polymer-bound β,γ-methylenetriphosphitylating reagent **8**. The treatment of unprotected nucleosides (4 mmol, e.g., adenosine (**a**), 3′-azido-3′-deoxythymidine (**b**), thymidine (**c**), cytidine (**d**), and inosine (**e**)) with **8** (1 mmol) in the presence of 5-(ethylthio)-1*H*-tetrazole gave **9a–e**, respectively. Oxidation with *tert*-butyl hydroperoxide followed by removal of the cyanoethoxy group with DBU afforded the corresponding polymer-bound nucleoside β,γ-methylenetriphosphates **11a–e**. Subsequent steps were supported by FT-IR peaks for all the intermediate resins. The cleavage of polymer-bound compounds was carried out under mild acidic conditions, DCM/TFA/water/EDT (72.5:23:2.5:2 v/v/v/v). The crude products had a purity of 67–92% and were purified on the C_{18} Sep-Pak cartridges to afford nucleoside β,γ-methylenetriphosphates **14a–e** (Scheme 2) in 53–82% overall yield (calculated from **8**) (Table 1) (269–411 mg). The purity of compounds was calculated based on the weight of pure and crude compounds.

Among all the synthesized methylenetriphosphate derivatives, **14d** is a novel compound. Adenosine, cytidine, and inosine contain a secondary (2′ and/or 3′) alcohol, a primary (5′) alcohol, or a free amino group. Only 5′-O-substituted compounds were purified with high selectivity as a result of this sequence. The presence of the phosphitylating reagents on the solid support having a hindered structure allowed for the regioselective reaction of the unprotected nucleosides with the polymer-bound phosphitylating reagents. There was no need to protect the free aromatic amino group in adenosine and cytidine. The most reactive hydroxyl group of unprotected nucleosides reacted selectively with hindered and bulky polymer-bound reagents when an excess of nucleoside was used. The compounds were characterized by ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, ESI-TOF, and phosphorus elemental analysis.

Nucleoside methylenetriphosphates remain as attractive probes and non-hydrolyzable triphosphate mimics in studying and inhibiting the phosphate transfer in biological systems. The synthesized compounds were evaluated to determine whether they can inhibit human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT), a critical enzyme in HIV life cycle.

HIV-1 RT converts the single-stranded RNA genome into double-stranded DNA that is later integrated into the host genome.[23] Retroviral RTs are multifunctional enzymes possessing DNA polymerase activities on both DNA and RNA templates, as well as a ribonuclease H (RNase H) activity that hydrolyzes the RNA of RNA/DNA replication intermediates. HIV-1 RT is a heterodimer consisting of p66 and p51 subunits that share the same sequence at the N-terminus. The RNase H domain resides in the C-terminus of p66 subunit, which is not present in p51.[24] Both DNA polymerase and RNase H activities of HIV-1 RT have been considered as potential targets for antiretroviral therapy. However, all currently used HIV-1 RT inhibitors are directed against the polymerase function of the enzyme.[25] Designing compounds that block the RNase H activity of the HIV-1 RT has been challenging. Although recent screening efforts led to the discovery of few RNase H inhibitors, none of them advanced into clinical trials. RNase H has been a challenging target for a number of reasons. Inhibitors that block the RT-associated RNase H function have higher cytotoxicity, since they interfere with the functions of cellular counterparts. RNase H proteins, including human RNase H, are homologous and share a similar three-dimensional fold. To address these challenges and to discover potent RNase H inhibitors we examined the potency of synthesized modified nucleoside analogs against RNase H and polymerase function of HIV-1 RT.

Initially we evaluated the potency of the compounds with modified phosphates toward the RNase H activity of HIV-1 RT at a fixed concentration (1 mM) of the compounds. RNase H assay was performed using recombinant RT and RNA/DNA hybrids as previously described.[26–28] The results of the RNase H cleavage are presented in Figure 1A. Lane W represents the RNase H cleavage products in the absence of any inhibitor. Lanes **a–e** represent the RNase H cleavage products in the presence of the compounds **14a–e**, respectively. These results indicate that the RNase H activity of HIV-1 RT is inhibited by the cytidine derivative **14d** (lane 4). Therefore, we tested the potency of compound **14d** by performing concentration-dependent inhibition assays. RNase H inhibition was not observed up to 100 μM with the compound. The results from 100 μM to 1 mM are presented in Figure 1B. Compound **14d** achieved complete inhibition of the RNase H activity at 700 μM (Fig. 1B, lane 6). These results are consistent with those of our

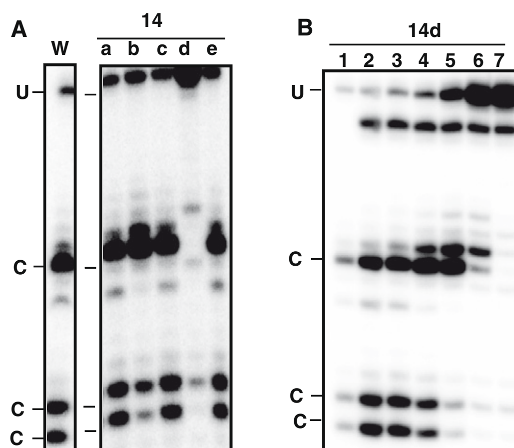


Figure 1. (A) RNase H analysis of HIV-1 RT in the presence of compounds **14a–e**. Lane w represents no inhibitor. The RNase H cleavage of HIV-1 RT was inhibited by **14d**; (B) RNase H activity with increased concentration of compounds **14d**. Lane 1 (100 μM), lane 2 (200 μM), lane 3 (300 μM), lane 4 (400 μM), lane 5 (500 μM), lane 6 (700 μM), and lane 7 (1 mM). The unhydrolyzed substrate is marked as U, whereas the cleavage products are marked as C.

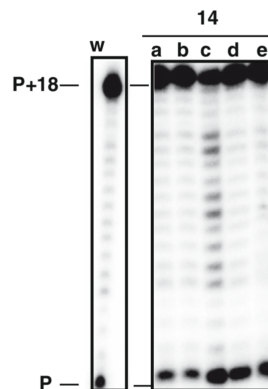


Figure 2. DNA polymerase activity of HIV-1 RT in the presence of compounds **14a–e** (1 mM). Lane w represents no inhibitor. The radiolabeled primer is marked as P, whereas the fully extended product is marked as P + 18.

earlier studies that showed another modified nucleoside triphosphate derivative, cytidine 5′-*O*-α,β-methylene-β-triphosphate inhibited RNase H activity of HIV-1 reverse transcriptase.[22]

We have also tested the potency of these compounds against the polymerase function of HIV-1 RT (Fig. 2). In comparison with the wild-type enzyme (lane w), the polymerase activity was not affected in the presence of the compounds **14a**–**e**, (lanes a–e). However, compound **14c** caused the polymerase complex to pause several positions during DNA synthesis (lane c). Since compound **14d** did not inhibit the polymerase activity of HIV-1 RT (lane 4), it can provide insights for designing of additional compounds, which may have better inhibitory activity against the RNase H activity.

To the best of our knowledge, this is the first report of the synthesis of nucleoside β,γ-methylenetriphosphates by using solid-phase reagents without the need for precursors, such as nucleoside monophosphates and 5′-methylenediphosphonate analogs. Unreacted reagents were removed by extensive washing of the resins in each step. This solid-phase strategy offered the advantages of expeditious synthesis, 5′-O-substitution, high selectivity, facile isolation, and purification of final products.

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Supplementary data

Supplementary data (experimental procedures and characterization of resins with IR and final compounds with NMR, high-resolution mass spectrometry, and quantitative phosphorus analysis, enzyme assay) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.04.005.

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