Synthesis of nucleoside 5′-*O*-α,β-methylene-β-triphosphates and evaluation of their potency towards inhibition of HIV-1 reverse transcriptase†

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Received 2nd November 2009, Accepted 21st December 2009
First published as an Advance Article on the web 13th January 2010
DOI: 10.1039/b922846b

A polymer-bound α,β-methylene-β-triphosphitylating reagent was synthesized and subjected to reactions with unprotected nucleosides, followed by oxidation, deprotection of cyanoethoxy groups, and acidic cleavage to afford nucleoside 5′-*O*-α,β-methylene-β-triphosphates. Among all the compounds, cytidine 5′-*O*-α,β-methylene-β-triphosphate inhibited RNase H activity of HIV-1 reverse transcriptase with a K_i value of 225 μM.

Modified nucleoside triphosphates have received much attention as mimics of naturally occurring deoxyribo- and ribonucleoside triphosphates, as probes in several biochemical pathways involving DNA and RNA synthesis, and as potential diagnostic and therapeutic agents.[1,2] The structural similarity of modified nucleotides to natural nucleoside triphosphates make them useful reagents as substrates or inhibitors for DNA or RNA polymerases.[3,4] Although most natural polymerase enzymes incorporate natural nucleoside triphosphates into nucleic acids, there are certain polymerases that are capable of incorporating unnatural nucleoside triphosphates into nucleic acids.[5–7]

A number of approaches have been focused on modifications and/or substitution on the base,[8–9] carbohydrate,[10–13] and linear triphosphate moieties[14–17] to design modified nucleotides for diverse applications in nucleic acid and antiviral research.

Early in the life cycle of human immunodeficiency virus type 1 (HIV-1), viral RNA is reverse transcribed into double stranded DNA for integration into the genome of the infected cell.[18] This process is catalyzed by reverse transcriptase (RT), a virus encoded heterodimeric enzyme composed of 66 and 51 kD subunits (p66 and p51), possessing DNA polymerase and ribonuclease H (RNase H) activities.[19] DNA polymerase activity is required for the synthesis of RNA : DNA heteroduplex from the single stranded viral RNA. Whereas, RNase H activity is responsible for hydrolyzing the RNA strand of RNA : DNA heteroduplex molecules that are generated during reverse transcription and create the primer for plus strand DNA synthesis.[20] Both DNA polymerase and RNase H activities of HIV-1 RT have been considered as potential targets for antiretroviral therapy. 3′-Substituted modified nucleoside triphosphate analogues (*e.g.*, 3′-azidodeoxythymidine triphosphate) are incorporated into DNA, behave as chain terminators, and inhibit DNA synthesis by HIV-1 RT. On the other hand, the discovery of potent and selective inhibitors of HIV-1 RNase H has been challenging, and inhibitors of this enzyme function have yet to reach clinical development stage.[18] Although there are more than a dozen FDA approved drugs that inhibit the polymerase function of HIV-1 RT, there is not a single RNase H inhibitor in clinics. Therefore, a potential RNase H inhibitor can serve as a mechanistic probe and/or a lead compound against HIV-1 RT. Nucleotide analogues have been described as inhibitors of HIV-1 RNase H activity.[21,22]

Less attention has been given to the synthesis of modified nucleotides containing branched triphosphate groups that can have diverse applications in studying the enzymes (*e.g.*, RNase H) that use natural nucleoside triphosphates. Previously, we reported the synthesis of 5′-*O*-nucleoside β-triphosphates using a solid-phase β-triphosphitylating reagent.[23] Herein, we report the solid-phase synthesis of 5′-*O*-nucleoside β-triphosphates containing α,β-methylene triphosphate bridge by using a novel solid-phase phosphitylating reagent. The unusual branched triphosphate in nucleoside triphosphate analogues may be useful in elucidation of enzymatic functions and mechanism. Furthermore, the presence of an α,β-methylene group in the modified nucleotide may provide more stability to cleavage by cellular hydrolytic enzymes. We determined the potency of the modified analogues towards HIV-1 RT polymerase and RNase H activity.

α,β-Methylene-β-triphosphitylating reagent was first immobilized on polystyrene resin-bound linker of *p*-acetoxybenzyl alcohol. Coupling reactions of unprotected nucleosides with the immobilized reagent followed by oxidation, deprotection, and cleavage afforded nucleoside 5′-*O*-α,β-methylene-β-triphosphates. The advantages of this solid-phase strategy included major 5′-*O*-monophosphitylation, the facile isolation and purification of products, removal of unreacted reagents and starting materials in each step, and trapping of the linker on the resin in the final cleavage reaction.

Scheme 1 illustrates the synthesis of α,β-methylene β-triphosphitylating reagent **6**. The reaction of bis(dichlorophosphino)methane (**1**) with diisopropylamine (1 equiv) in the presence of 2,6-lutidine (1 equiv) in anhydrous THF afforded **2**. Because of the bulkiness of the *N*,*N*-diisopropylamine group, the replacement of –Cl groups in the less hindered phosphorous group in **2** is significantly more desirable. Reaction of **2** with 3-hydroxypropionitrile (2 equiv) in the presence of 2,6-lutidine

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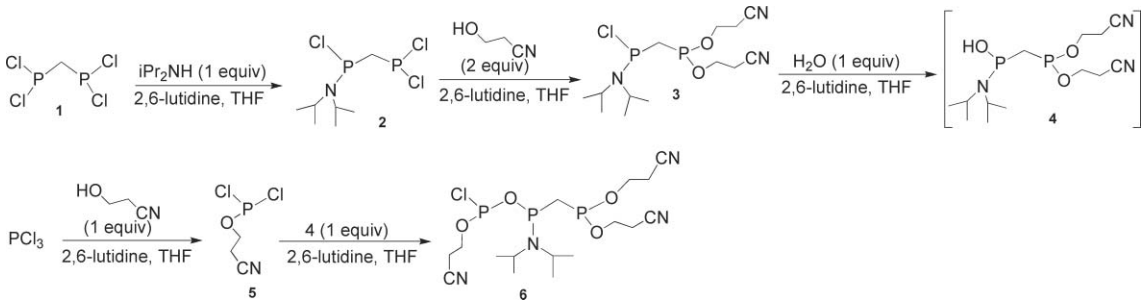
† Electronic supplementary information (ESI) available: Experimental procedures, characterization of resins with IR and final compounds with NMR, high-resolution mass spectrometry, and quantitative phosphorus analysis, DNA polymerase assay results. See DOI: 10.1039/b922846b



Scheme 1 Synthesis of α,β-methylene-β-triphosphitylating reagent **6**.

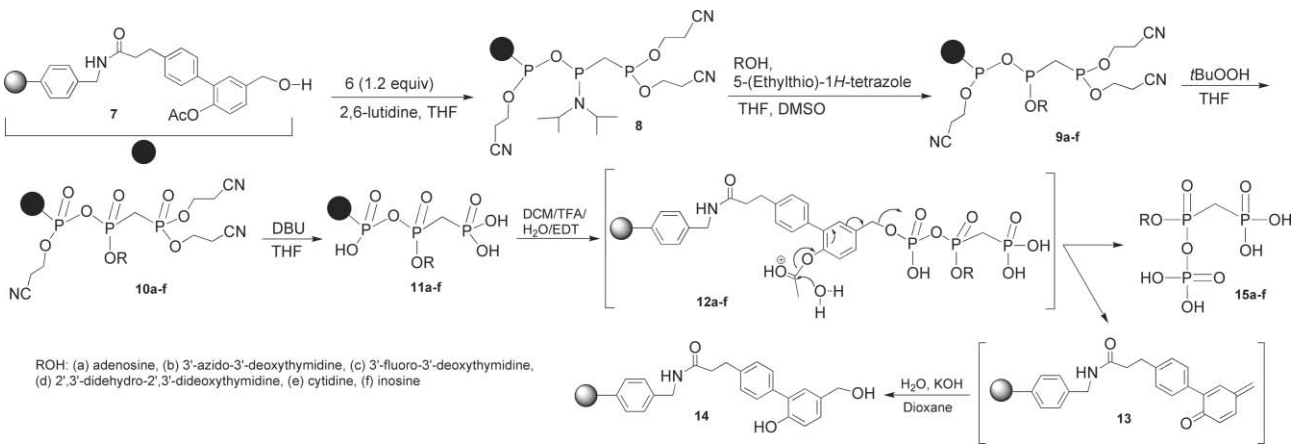
(2 equiv) afforded **3**, which was subjected to reaction with water (1 equiv) and 2,6-lutidine (1 equiv) to produce the intermediate **4.**

In a separate reaction, phosphorus trichloride was reacted with 3-hydroxypropionitrile (1 equiv) in the presence of 2,6-lutidine (1 equiv) in anhydrous THF to yield 2-cyanoethoxyphosphorodichloridite (**5**), which was reacted with **4** (1 equiv) to yield α,β-methylene-β-triphosphitylating reagent **6**, which was immediately used in the coupling reaction with polymer-bound *p*-acetoxybenzyl alcohol **7**. The chemical structure of **6** was confirmed by high-resolution time-of-flight electrospray mass spectrometry (ESI-TOF), but obtaining NMR for this compound was not feasible because of its rapid decomposition. Compound **6** was immediately used for the next coupling reaction with polymer-bound *p*-acetoxybenzyl alcohol **7**.

We have previously reported the synthesis of aminomethylpolystyrene resin-bound *p*-acetoxybenzyl alcohol **7** (Scheme 2).[24] Our earlier studies revealed the application of polymer-bound *p*-acetoxybenzyl alcohol linkers in the synthesis of a diverse number of compounds.[25] Polymer-bound linker **7** was selected for immobilization of reagent **6**.

Scheme 2 shows the synthesis of nucleoside 5′-*O*-α,β-methylene-β-triphosphates. Aminomethyl polystyrene resin-bound linker of *p*-acetoxybenzyl alcohol **7** was reacted with reagent **6** in the presence of 2,6-lutidine to produce the corresponding polymer-bound α,β-methylene-β-triphosphitylating reagent (**8**). The treatment of excess of unprotected nucleosides (adenosine (**a**), 3′-azido-3′-deoxythymidine (**b**), 3′-fluoro-3′-deoxythymidine (**c**), 2′,3′-didehydrothymidine (**d**), cytidine (**e**), and inosine (**f**)) in the presence of 5-(ethylthio)-1*H*-tetrazole gave **9a–f**. Oxidation with *t*-butyl hydroperoxide followed by removal of the cyanoethoxy group with DBU, afforded the corresponding polymer-bound nucleosides 5′-*O*-α,β-methylene-β-triphosphosphate diester derivatives, **11a–f**. The cleavage of polymer-bound compounds was carried out under acidic conditions, DCM/TFA/water/EDT (72.5 : 23 : 2.5 : 2 v/v/v/v). The intramolecular cleavage mechanism of final products from **12a–f** is shown in Scheme 2. The linker-trapped resin (**13**) was separated from the final products by filtration and was converted to **14** by reaction with potassium hydroxide. The crude products had a purity of 68–83% and were purified on the C_{18} Sep-Pak cartridges to afford 5′-*O*-nucleoside α,β-methylene-β-triphosphates (**15a–f**, Scheme 2) in 47–73% overall yield (calculated from **8**, 212–344 mg, Table S1, see the Supporting Information†).

Nucleoside 5′-*O*-α,β-methylene-β-triphosphates were synthesized with high selectivity as a result of this sequence because of the presence of the phosphitylating reagent on the solid support having a hindered structure, thereby allowing for the regioselective reaction for unprotected nucleosides (*e.g.*, adenosine, inosine, and cytidine). The most reactive hydroxyl group of unprotected nucleosides reacted selectively with hindered polymer-bound reagents when an excess of nucleoside was used. There was no need to protect the free amino group in adenosine and cytidine. The compounds were characterized by ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, ESI-TOF, and phosphorus elemental analysis. ^{31}P NMR indicated the presence of only one diastereoisomer, suggesting that one of the diastereoisomers of each compound was purified from the



Scheme 2 Synthesis of nucleoside 5′-*O*-α,β-methylene-β-triphosphates **15a–f** using polymer-bound linker **7**.

crude as the major product. On the other hand, there is a slight possibility that diastereoisomers may not be distinguishable by ^{31}P NMR because of overlapping peaks.

Purification of HIV-1 RT and enzyme assays was performed according to the previously reported procedures.[26–28] Initially, these compounds were screened towards the polymerase and RNase H activity of HIV-1 RT at a fixed concentration of the compound (1 mM). In comparison to the wild type enzyme, the polymerase activity was not affected in the presence of these compounds **15a–f**, however a few compounds exhibited inhibitory activity against RNase H function. The results of the polymerase assay are presented in Figure S1 (see Supporting Information†).

The results of the RNase H analysis are presented in Fig. 1A. Lane W represents the RNase H cleavage products representative of the primary and secondary cleavages in the absence of any inhibitor. Lanes a–f represent the RNase H cleavage products in the presence of the compounds **15a–f**, respectively. Although compounds **15a–d** and **15f** did not show any inhibitory activity, the RNase H cleavage of HIV-1 RT was inhibited by 5′-*O*-cytidine α,β-methylene-β-triphosphate (**15e**) (Fig. 1A, Lane e). To further evaluate the inhibitory potency of **15e**, RNase H assay was performed at increasing concentrations of **15e**. Since RNase H inhibition was not observed up to 100 μM concentration, the results from 100 μM–1mM are presented in Fig. 1B.

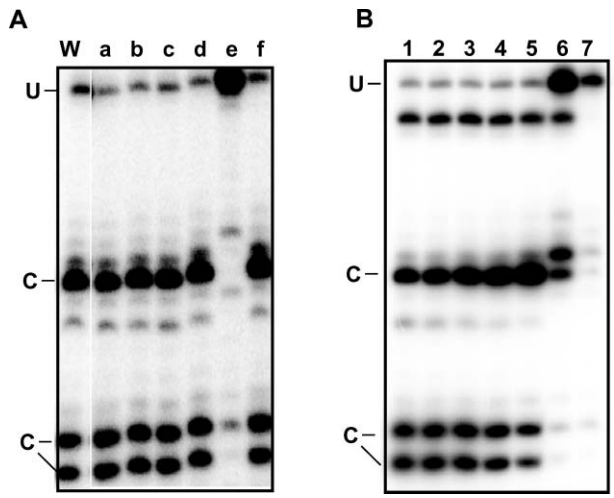


Fig. 1 **A.** RNase H analysis of HIV-1 RT in the presence of compounds **15a–e**. Lane w represents no inhibitor, lane a (**15a**), lane b (**15b**), lane c (**15c**), lane d (**15d**), lane e (**15e**), and lane f (**15f**), at 1 mM concentrations. **B.** RNase H activity as a function of compound **15e** concentration. 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. The RNase H cleavage of HIV-1 RT was inhibited by 5′-*O*-cytidine α,β-methylene-β-triphosphate (**15e**).

To further characterize the nature of inhibition, we performed Dixon analysis for the compound **15e**.[29a] The results of this analysis indicated a competitive mode of inhibition with a K_i value of 225 μM (Fig. 2). Subsequently, the IC_{50} value was calculated to be 585 μM for compound **15e** by the Cheng–Prusoff equation[29b] for competitive inhibition [*i.e.*, $K_i = IC_{50}/(1+ [S]/K_m)$] towards the RNase H activity of HIV-1 RT.

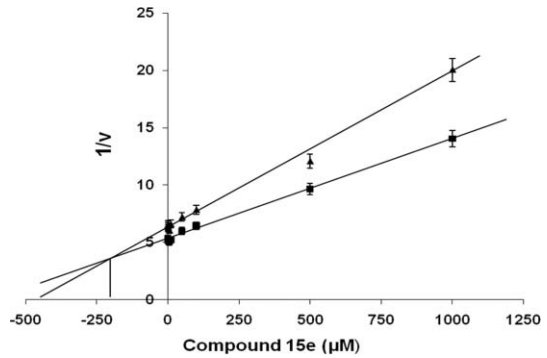


Fig. 2 Inhibition kinetics analyses of compound **15e** with RNase H of HIV-1 RT. The enzyme activity was estimated using the substrate at 200 nM (▲) and 400 nM (■) at increasing concentrations of **15e**. Rate of reaction represents rate of RNase H hydrolysis. Reciprocals of the rate of hydrolysis were plotted *versus* the inhibitor concentrations. The straight lines indicated the best fit of the data obtained. The inhibition constant K_i was calculated from the point of the intersection of the plots.

To the best of our knowledge, this is the first report of the synthesis of nucleoside 5′-*O*-α,β-methylene-β-triphosphates. This solid-phase methodology using novel polymer-bound phosphitylating reagent (**8**) allowed for the expeditious development of these analogues in a short synthetic route without the need for the nucleoside phosphate precursors or protected nucleosides. The solid-phase strategy offered the advantages of monosubstitution, high selectivity, and facile isolation and purification of products. Further exploring and optimization of 5′-*O*-cytidine α,β-methylene-β-triphosphate is required to design cell-permeable compounds that can potentially inhibit the RNase H activity of HIV-1 RT at lower concentrations.

Acknowledgements

We acknowledge the financial support from National Science Foundation, Grant Number CHE 0748555. The work at NCI was supported in part by the Intramural Research Program of the NIH, NCI and Center for Cancer Research.

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