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Synthesis and polymerase incorporation of β,γ-modified α-L-threofuranosyl thymine triphosphate mimics

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ABSTRACT

Three β,γ-modified α-L-threofuranosyl nucleoside triphosphates were synthesized. The β,γ-modified tTTPs undergo a single incorporation event with HIV RT but undergo multiple incorporations to form full-length product with engineered thermophilic polymerases.

Alpha-L-threofuranosyl nucleic acid (TNA) is an artificial genetic polymer that has been proposed as a possible biological precursor to DNA and RNA.[1–3] In addition to its potential role in early forms of life, TNA has received great interest due to its unique chemical and biological properties. For example, the 2′–3′ backbone linkage found in TNA makes it highly resistant to cleavage by nucleases,[4] but still allows TNA to form stable Watson–Crick duplexes with DNA or RNA.[5] Furthermore, TNA can be transcribed from a DNA template using engineered versions of the thermophilic polymerases Deep Vent (exo-), 9°N, and KOD, which are capable of accepting α-L-threofuranosyl nucleoside triphosphates tNTPs as substrates.[6–9] These polymerases also retain their ability to utilize deoxyribose nucleoside triphosphates dNTPs, suggesting that the mutations made to the polymerases serve to relax their substrate specificity.

Considering the ability of tNTPs to be selectively accepted by polymerases having relaxed substrate specificity or lack of exonuclease editing ability, TNA provides a potentially useful scaffold for the design of anti-viral therapeutics. In 2003, Chaput and Szostak carried out DNA primer extension experiments using HIV reverse transcriptase (HIV RT). Interestingly, they found that the enzyme will incorporate two sequential tTTP monomers, but this appears to then cause chain termination.[6] In a subsequent study, Herdewijn and coworkers demonstrated that 2′-deoxythreosyl phosphonates show anti-HIV activity, presumably from incorporation by HIV RT, resulting in chain termination.[10]

Given the encouraging results from these studies, we were curious to explore the biological activity of tTTP analogues having modifications at the β,γ-bridging oxygen of the triphosphate (Fig. 1). β,γ-Modified dNTPs have been widely used as chemical probes to study the catalytic mechanisms of DNA polymerases, as the leaving group capability of the β,γ-diphosphate unit can be fine-tuned by changing the electron withdrawing capability of the unnatural bridging group.[11–13] Additionally, β,γ-modified dNTPs have been reported to serve as substrates for a variety of DNA polymerases. For example, Krayevsky and coworkers reported that dTTP having a CF_2 or CHF group replacing the β,γ-bridging oxygen are substrates for human DNA polymerases α and β, as well as Avian Myeloblastosis Virus (AMV) reverse transcriptase.[14] Taking a slightly different approach, Wang and coworkers synthesized and studied a series of modified AZT triphosphate mimics. Unsurprisingly, they found that modification of α,β-linkage led to poor inhibition of HIV RT. However, an AZT analogue having a β,γ-CF_2 modification and a non-bridging α-BH_3 modification showed a K_i value comparable to that of AZT, but with significantly enhanced stability in serum and cell extract.[15]

Despite these encouraging results, β,γ-modified triphosphates have yet to be widely explored as potential therapeutics, as nucleoside triphosphates are typically not capable of penetrating the cell membrane. However, Dinh and coworkers have reported a prodrug approach aimed at overcoming this limitation through acylation of the γ-phosphate with a fatty acid or cholesterol analogue.[16,17]

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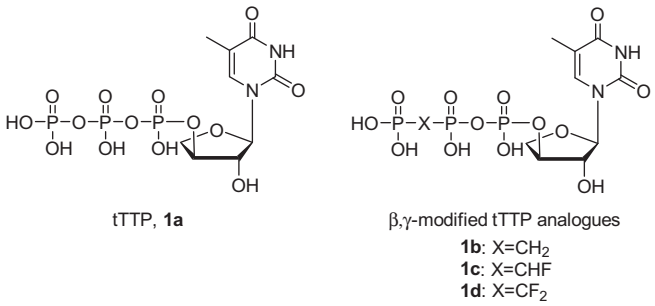


Figure 1. Chemical structure of tTTP **1a** and β,γ-modified tTTP analogues **1b–d**.

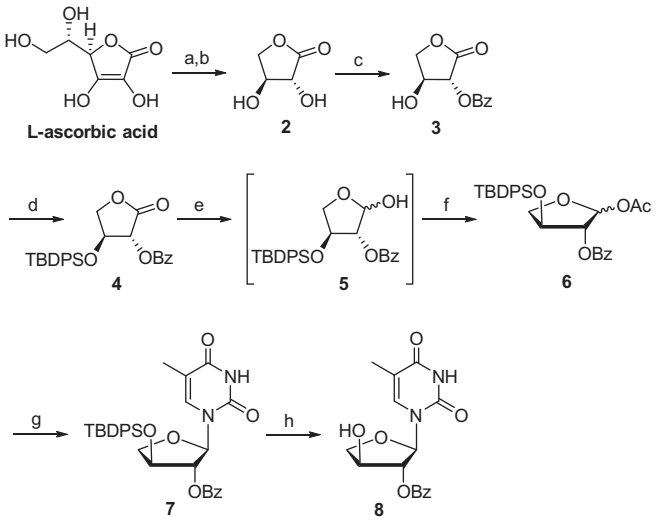
Taking a similar approach, Meier and coworkers have recently reported the successful cellular delivery of nucleoside triphosphates having a lipophilic acyloxybenzyl group at the γ-phosphate, which can be cleaved by intracellular esterases to reveal the nucleoside triphosphate therapeutic.[18,19] These recent developments suggest that viable prodrug approaches could be implemented for β,γ-modified nucleoside triphosphates, which encouraged us to further explore their ability to be incorporated by DNA polymerases.

Here we report the synthesis and study of three β,γ-modified tNTP analogues, which represents the first example of β,γ-modifications in the context of a non-native carbohydrate scaffold. We synthesized 'wild-type' tTTP **1a** and tTTP analogues having a CH_2, CHF, or CF_2 group replacing the β,γ-bridging oxygen (**1b–d**), and compared their incorporation efficiencies in primer extension reactions using a variety of polymerases. Excitingly, we find that the β,γ-modified tTTPs are substrates for HIV RT, and unlike **1a**, which requires two incorporations before chain termination, **1b–d** show no further elongation after only a single incorporation event. Additionally, we demonstrate that a mutant KOD polymerase is capable of generating full length product in a primer extension experiment using β,γ-CF_2 tTTP **1d**. Together, these experiments explore new chemical space by combining carbohydrate modifications with triphosphate modifications, and demonstrate that this approach can lead to enhanced inhibition of a viral reverse transcriptase.

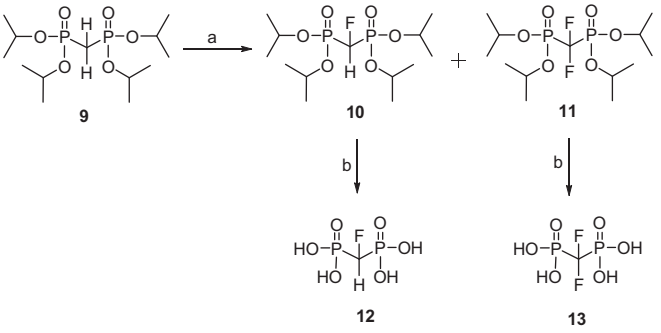
To synthesize tTTPs **1a–d**, we began by following a recently published procedure for generating 2′-*O*-Bz protected α-L-threose nucleoside **8** (Scheme 1).[20] Starting from L-ascorbic acid, we were able to synthesize **8** in high yield over 8 steps, providing the mono-protected nucleoside precursor necessary for elaboration into tTTP **1a** and analogues **1b–d**.

In parallel, we synthesized pyrophosphate analogues (fluoromethylene)bisphosphonic acid **12** and (difluoromethylene)-bisphosphonic acid **13** starting from the commercially available tetraisopropyl methylenediphosphonate (**9**) according to a previously reported procedure (Scheme 2).[11] While synthesis of deoxyribose nucleoside 5′ triphosphates is relatively straightforward, synthesis of threose nucleoside 3′ triphosphates (tNTPs) is much more difficult, owing to the increased steric hindrance of the 3-hydroxyl group.[21] Thus, once all of our key intermediates were in hand, we chose to explore both one-pot and stepwise synthetic routes to access the target compounds tTTP **1a** and β,γ-bridging oxygen modified tTTP analogues **1b–d**.

To investigate the possibility of a one-pot synthesis of tTTP **1a** and analogues **1b–d** from nucleoside intermediate **8**, we first focused on the synthesis of dichlorophosphoridate **14**, which we envisioned as a key intermediate (Scheme 3). The widely used method for synthesizing nucleoside 5′ triphosphates involves formation of a dichlorophosphoridate intermediate via reaction of the 5′ hydroxyl with $POCl_3$ in $PO(OMe)_3$ using Yoshikawa's procedure.[22] However, this approach failed to yield the desired



Scheme 1. Synthesis of 2′-O-Bz protected thymine nucleoside **8**. Reagents and conditions: (a) (i) 30% H_2O_2, $CaCO_3$, H_2O, 18 h, 0 °C-rt; (ii) active charcoal, 70 °C, 2 h, 80%; (b) 10% H_2SO_4 in H_2O, pTsOH, CH_3CN, 2 h, reflux, 90%; (c) BzCl, pyridine, CH_2Cl_2; 0 °C, 0.5 h, 75%; (d) TBDPS-Cl, imidazole, DMAP (cat), CH_3CN, 18 h, 0 °C-rt 86%.; (e) DIBAL-H, THF, −78 °C, 0.5 h; (f) Ac_2O, DMAP, pyridine, CH_2Cl_2, −78 °C-rt, 1.5 h, 90% from **4**; (g) (i) thymine, BSA, CH_3CN, 60 °C, 15 min; (ii) TMSOTf, 60 °C, 2 h, 82%; (h) TBAF, THF, 0 °C, 1 h, 86%.



Scheme 2. Synthesis of modified diphosphate building blocks **12** and **13**. Reagents and conditions: (a) (i) NaH, THF, DMF, 0 °C-rt 1 h; (ii) Selectfluor, rt, 3 h; (iii) NH_4Cl, H_2O, 18% (**10**) and 30% (**11**); (b) CH_2Cl_2, TMSBr, rt, 48 h, 70–85%.

intermediate **14** when applied to the sterically hindered 3′ hydroxyl of threose nucleoside **8**. To compensate for the increased steric hindrance of **8**, we investigated the use of a variety of organic bases in conjunction with the $POCl_3$ and $PO(OMe)_3$, and observed that this significantly improved the efficiency of the phosphorylation reaction. Specifically, we screened a series of nitrogen-containing organic bases including pyridine, Et_3N, Bu_3N, DMAP, and proton sponge (1,8-bis(dimethylamino)natpthalene), and found that proton sponge provided the highest yield of **14**, with 80% conversion observed by HPLC. Moreover, the hydrophobicity of the proton sponge base enabled it to be easily removed at the conclusion of the reaction sequence via extraction with Et_2O under weakly basic aqueous conditions. Having optimized conditions for generating intermediate **14**, we next turned to investigating the remainder of the one-pot synthetic method. To couple the β,γ-diphosphate unit, we added to the reaction mixture tributylamine and either tributylammonium pyrophosphate, methylenediphosphonic acid, **12**, or **13**, which yielded the benzoyl protected precursors to **1a–d**, respectively. The benzoyl group was then removed using concentrated ammonium hydroxide, and **1a–d** were each purified via aqueous extraction followed by reverse phase HPLC using a