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COMMUNICATION

Incorporation of 4′-*C*-aminomethyl-2′-*O*-methylthymidine into DNA by thermophilic DNA polymerases†

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The dual modified nucleotide 4′-*C*-aminomethyl-2′-*O*-methylthymidine 5′-triphosphate was synthesized and enzymatically incorporated into DNA by the thermophilic DNA polymerases Pfu and Therminator III. The dual ribose modification imparted increased exonuclease resistance to DNA compared to the well-known 2′-*O*-methyl modification.

Functional nucleic acids such as deoxyribozymes (DNA enzymes) and aptamers are attracting interest as potential therapeutic tools.[1] In order to impart drug-like properties to these molecules, judicious incorporation of chemical modifications is warranted.[2] Site-specifically installed nucleoside modifications are often deleterious at key residues in the catalytic core of DNA enzymes or in binding motifs of aptamers. Improved performance can be achieved by incorporating nucleotide analogs during *in vitro* selection.[3] This strategy requires DNA polymerases that can efficiently incorporate modified nucleotides, as well as copy and extend modified DNAs with high fidelity. Though many commercially available polymerases are known to tolerate nucleobase modifications,[4] their ability to accommodate ribose modifications met with limited success.[5] In many cases, efficient utilization has been achieved by directed evolution of polymerases.[6] A particularly challenging modification is presented by 2′-*O*-methyl nucleotides, which could only be used with engineered polymerases[7] for *in vitro* selection of 2′-*O*-methyl-modified aptamers.[8]

Nucleotide analogs that harbour dual ribose modifications offer special benefits in terms of nuclease resistance and target affinity. Here we investigate dual modified 4′-*C*-aminomethyl-2′-*O*-methylthymidine (ammT) in comparison to 2′-*O*-methylthymidine (mT) (Fig. 1). We report the synthesis of ammT triphosphate **1** and its enzymatic incorporation into DNA for potential *in vitro* selection applications. The primary amino group at the 4′-substituent is expected to improve nuclease resistance and enhance the catalytic ability of DNA enzymes, as has been reported for a number of nucleoside analogs that carry amino acid-like functional groups in RNA-cleaving deoxyribozymes.[9]

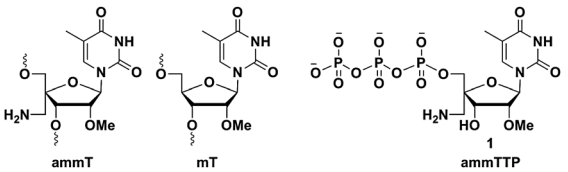

Fig. 1 Structures of thymidine analogs 4′-*C*-aminomethyl-2′-*O*-methyl thymidine (ammT) and 2′-*O*-methylthymidine (mT), and structure of the target nucleotide triphosphate **1** (ammTTP).

We have recently demonstrated advantageous properties of the corresponding 4′-*C*-aminomethyl-2′-*O*-methyl-modified uridine and cytidine nucleosides to enhance the therapeutic potential of small interfering RNAs (siRNA).[10] Inspired by these results, we seek to select DNA enzymes that contain amm-modified nucleotides in the catalytic core. The analogously modified thymidine has been previously investigated as modification of antisense oligonucleotides,[11] but the corresponding triphosphate and its enzymatic recognition have not been reported. Gaining access to the triphosphate **1** and exploring its competence as a substrate for DNA polymerases are the first steps towards evolving amm-modified DNA enzymes.

The synthesis of triphosphate **1** (Scheme 1) started from the known 4′-*C*-azidomethyl-modified thymidine nucleoside **2**, which was prepared from D-glucose using reported procedures.[11] Nucleoside **2** was converted to the dual modified precursor **7**[11] using a five-step procedure. To prevent alkylation of the nucleobase, the 4-methoxybenzyl (PMB) protecting group was installed in compound **3**. Cleavage of the 2′-acetyl group generated the crude hydroxyl precursor, which was subsequently methylated to obtain compound **4**. After cleavage of the PMB group by ceric ammonium nitrate (CAN), the azide functionality of compound **5** was reduced to an amino group by triphenylphosphine (PPh_3). The amine was protected by a trifluoroacetyl group to produce compound **6**, which was subsequently debenzylated with 10% Pd/C in EtOH. For synthesis of the 5′-triphosphate,[12] the thymidine nucleoside **7** was first treated with phosphoryl chloride ($POCl_3$) in trimethyl phosphate, and the dichlorophosphate intermediate was reacted *in situ* with tetrabutylammonium pyrophosphate. The *N*-trifluoroacetyl-protected nucleotide triphosphate **8** was isolated by ion exchange chromatography and purified by RP-HPLC. Cleavage of the trifluoroacetyl group by aqueous

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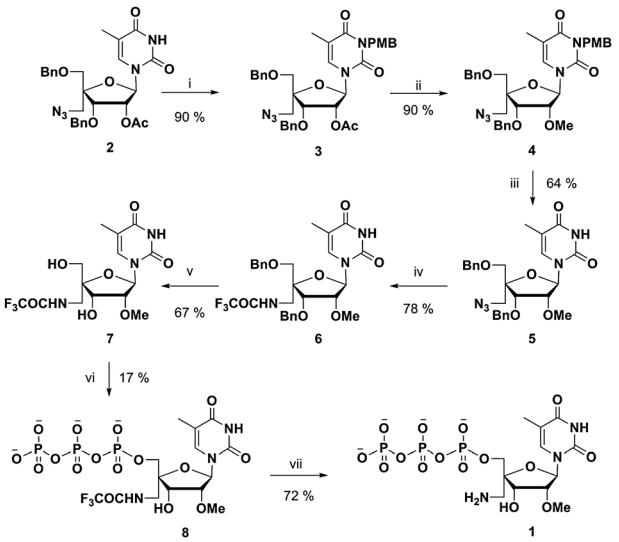
† Electronic supplementary information (ESI) available: Experimental details, figures, and NMR spectra. See DOI: 10.1039/c2cc35222b



Scheme 1 Synthesis of ammTTP **1**. *Reaction conditions*: (i) 4-methoxy benzyl chloride, DBU, CH_3CN, rt, 7 h; (ii) (a) NaOMe, MeOH, rt, 3 h; (b) NaH, MeI, THF, rt, 3 h; (iii) ceric ammonium nitrate, CH_3CN–H_2O (9 : 1) rt, 50 h; (iv) (a) PPh_3, THF, H_2O, 45 °C, 4 h, (b) $EtOCOCF_3$, Et_3N, rt, 16 h; (v) 10% Pd/C, EtOH, 70 °C, 48 h; (vi) (a) proton sponge, $PO(OMe)_3$, $POCl_3$, −20 to −13 °C, 3 h, (b) $(nBu_4N)_2H_2P_2O_7$, nBu_3N, DMF, 0 °C, 1.5 h; then $Et_3NH^+ \cdot HCO_3^-$; (vii) Et_3N–MeOH–H_2O 1 : 3 : 7, rt, 14 h.

NH_3 under various reaction conditions was challenging due to partial degradation of the triphosphate. Successful deprotection was finally accomplished by treatment with NEt_3–MeOH–H_2O (1 : 3 : 7).[13] The target nucleotide **1** was purified by RP-HPLC and characterized by NMR and MS.

The nucleoside triphosphate **1** was then studied as a substrate for various DNA polymerases, including Klenow fragment, Taq, Vent(exo$^-$), Pfu, KOD, Phusion, Therminator, and Therminator III. The primer extension experiments used the 23 nucleotide (nt) DNA primer P1 and the 35 nt template T1 containing a single adenosine in the coding region (Fig. 2).[14] The results are here shown for Pfu polymerase. As expected, the positive control reaction (lane 2) using the four natural dNTPs resulted in a full-length extension product.

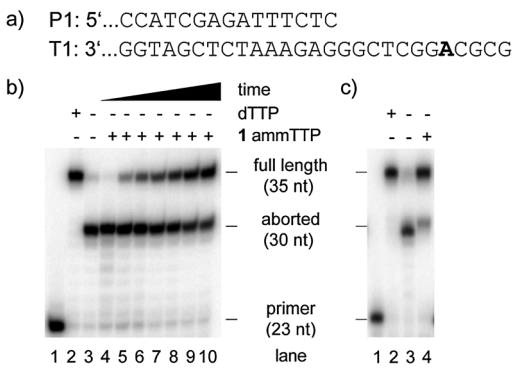


Fig. 2 Primer extension reactions using Pfu polymerase. (a) Partial sequences of primer and template. (b) Lanes (1): primer only, (2): pos. ctrl. (all dNTPs), (3): neg. ctrl. (no dTTP), 4–10: timecourse of primer extension at 72 °C, for 3 min to 12 hours, using 0.2 mM **1**, 0.03 u μL^{-1} Pfu. (c) Lanes 1–3: same as in (b), (4): 0.8 mM **1**, 0.1 u μL^{-1} Pfu, 50 mM LiCl, 72 °C, 12 h.

The negative control reaction (lane 3) in the absence of dTTP yielded the expected 30 nt product due to termination at the templating adenosine. With this primer–template complex, several of the tested DNA polymerases in the absence of dTTP extended the primer significantly beyond the pause site.[15] This indicates mismatch incorporation at the templating adenosine and calls for caution in interpreting primer extension experiments with modified nucleotide triphosphates. For this reason, we continued our experiments with Pfu, which performed accurately in the initial screening.[16] Gratifyingly, Pfu was able to accept **1** as a substrate and incorporated ammT into full-length modified DNA (Fig. 2b). The incorporation and the extension of the modified oligonucleotide are rather slow reactions (lanes 4–10); practically useful amounts of full-length product were produced with 200 μM **1** after incubation for up to 12 h at 72 °C. We observed batch-to-batch variability in performance of Pfu polymerases and found that the reaction is enhanced and well reproducible in the presence of 50 mM LiCl and 800 μM **1** (Fig. 2c).

For further investigation of efficiency and selectivity of ammT incorporation, single-nt incorporation experiments were performed using primer P1 and template T2 (Fig. 3a, N = A). Extension of the primer with ammT occurred efficiently with Therminator III DNA polymerase. This enzyme is a commercially available variant of 9° N DNA polymerase described to incorporate 3′-modified nucleotide analogs. We found that Therminator III completely extended the primer P1 with ammT within 10 min in the presence of 100 μM **1**, which was much more efficient than Pfu. The modified DNA was isolated and incorporation of ammT was confirmed by ESI-MS (Fig. S1, ESI†).

Since 2′-*O*-methyl-modified nucleotides are generally difficult substrates for DNA and RNA polymerases, we examined the effect of the 4′ modification by comparing single-nt extension experiments with dTTP, mTTP and ammTTP. Interestingly, mT was also efficiently incorporated by Therminator III DNA polymerase,

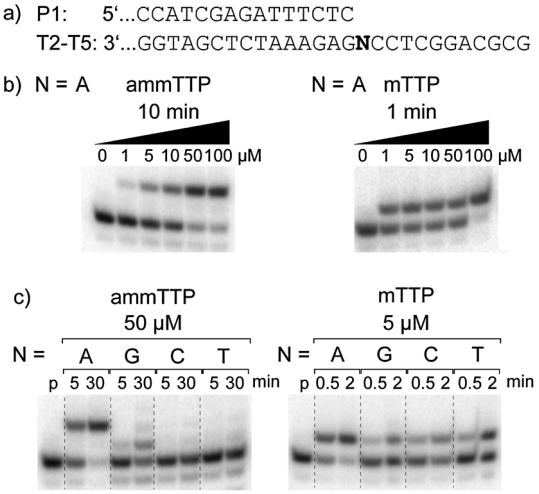


Fig. 3 Single-nucleotide primer extension reactions using Therminator III DNA polymerase. (a) Partial sequences of primer and templates; N = A, G, C, T. (b) Concentration dependence for ammTTP (**1**) and mTTP using matched template T2 (N = A). (c) Selectivity of incorporation using matched (N = A) and mismatched (N = G, C, T) templates. All reactions run at 72 °C with 0.02 u μL^{-1} Therminator III. p = primer.

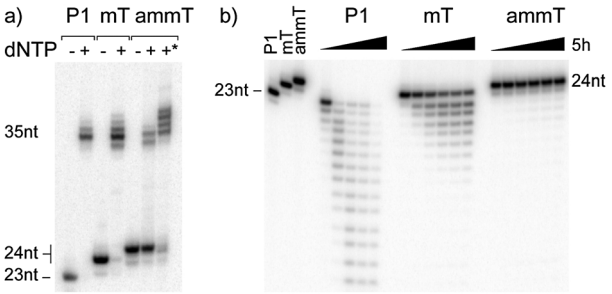


Fig. 4 (a) Extension of modified primers with 200 μM dNTPs and 0.02 u μL^{-1} Therminator III DNA polymerase for 1 h (*6 h) at 72 °C. (b) SVPD digestion of unmodified DNA in comparison to mT- and ammT-terminated DNA. SVPD 10^{-4} u μL^{-1}, 1 mM Mg^{2+}, 22 °C, time points up to 5 h (experiments up to 18 h are shown in Fig. S4, ESI†).

even at lower concentrations than ammT (Fig. 3b). The primer was fully extended with only 1 μM mTTP in 5 min (Fig. S2, ESI†). While the primer extension with dTTP did not stop efficiently after the first nt extension,[17] mT was only incorporated once.[18]

We also investigated mismatch extension of primer P1 with thymidine and its analogs ammT and mT, using templates T3–T5, which each contain a different nucleotide directly after the primer (Fig. 3c). Using two different concentrations of mTTP and ammTTP, we found that ammT was incorporated only with the matched template T2, while mT was also incorporated with all other templates, demonstrating low specificity of Therminator III with 2′-*O*-methylated analogs (Fig. S2, ESI†). When tested with all mNTPs, Therminator III was able to extend the primer by 6–7 methylated nucleotides (Fig. S3, ESI†).[19] DNA with 3′-terminal ammT and mT could be further extended with unmodified nucleotides to give a full-length product (Fig. 4a). The extension reaction was slower than the incorporation of the modification. While the mT-modified primer was fully extended within 1 h at 72 °C, up to 6 h were needed for full extension of ammT-terminated DNA.

The ammT- and mT-modified DNAs were characterized with respect to their resistance against 3′-exonuclease degradation, using snake venom phosphodiesterase (SVPD). Interestingly, the ammT-modified DNA showed improved stability compared to mT-modified DNA, which was already more stable than unmodified DNA (Fig. 4b). Similar results were found for the internally ammT-modified DNA generated by Pfu (Fig. S4, ESI†).

In summary, we have synthesized ammTTP **1** from the nucleoside precursor **7**, which was prepared in overall 19 steps from D-glucose. We found that ammT is incorporated into DNA by recombinant thermophilic DNA polymerases Pfu and Therminator III. This is the first report that shows a nucleoside analog with two functionally distinct ribose modifications being a competent substrate for DNA polymerases. For the incorporation of multiple dual-modified nucleotides, further engineering of DNA polymerases will be required. Mutation of recently identified gate keeper amino acids in thermophilic DNA polymerases enabled efficient RNA synthesis.[19,20] It is conceivable that analogous mutations in Therminator III could promote synthesis of multiply ammT- or mT-modified DNA. The observed increased resistance against nuclease degradation makes ammT-modified DNA a promising candidate for further investigation towards *in vitro* selection of functional nucleic acids.

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