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Substrate Properties of C5-Substituted Pyrimidine 2′-Deoxynucleoside 5′-Triphosphates for Thermostable DNA Polymerases During PCR☆

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Abstract—In order to enhance a collection of modified deoxynucleoside triphosphates useful for in vitro selection or SELEX (systematic evolution of ligands by exponential enrichment) techniques, we designed and synthesized modified analogues of 2′-deoxyuridine triphosphate and 2′-deoxycytidine triphosphate bearing a flexible and hydrophilic 7-amino-2,5-dioxaheptyl linker at a C5 position. Both analogues were found to be substrates for thermostable DNA polymerases which belong to an evolutional family B during PCR.

Much interest has been given to the development of functional nucleic acids such as ribozymes[1] and aptamers[2] by in vitro selection or SELEX techniques. DNA is an especially attractive material for functional molecules due to its chemical stability and availability for direct enzymatic amplification by polymerase chain reaction (PCR). However, the activities of reported functional DNAs are generally far inferior to those of protein enzymes or antibodies. Presumably, one cause may be the lack of functional groups found in proteins. In order to incorporate an additional functional group into DNA, a variety of chemically modified 2′-deoxynucleoside triphosphates were synthesized and demonstrated to be substrates for DNA polymerases used in polymerase extension reaction or PCR.[3,4] Moreover, some triphosphates were utilized for development of 'functional modified DNAs'.[5] Therefore, evaluation of the substrate properties of various modified nucleoside triphosphates for various DNA polymerases would help further development of functional nucleic acids.[4] In this paper, we report synthesis of the modified dUTP **1** and the modified dCTP **2** and their substrate properties for thermostable DNA polymerases during PCR.

The modified analogues **1** and **2** were synthesized according to Scheme 1. Radical bromination[6] of 3′,5′-diacetyl-2′-deoxythymidine **7** with NBS and AIBN, followed by alcoxy substitution with 2-[*N*-(*tert*-butyloxycarbonyl)-2-aminoethoxy]ethanol afforded **8**. Subsequently, 3′,5′-*O*-deacylation with methanolic ammonia afforded **9** in 30% overall yield for the three-step sequence starting from **7**. Removal of the *N*-Boc protecting group and reprotection of the free amino-linker terminus by reaction with ethyl trifluoroacetate provided **10** in 85% yield. The nucleoside **10** was phosphorylated directly with phosphorus oxychloride and pyrophosphate, followed by removal of the amino protecting group with aqueous ammonia to give **1** in 31% yield. Synthesis of the 2′-deoxycytidine derivative **11** proceeded through the corresponding 2′-deoxyuridine derivative **8**. Reaction of **8** with phosphorus oxychloride, followed by amination with aqueous ammonia and subsequent 3′,5′-*O*-deacylation afforded **11** in 17% overall yield starting from **7**. The nucleoside **11** was converted in four steps and 3.4% overall yield into the triphosphate **2**.

We investigated the incorporation of the analogues (**1**–**6**) in place of the corresponding natural nucleoside triphosphate (TTP or dCTP) during PCR, using pUC18 template DNA with the appropriate primers to provide 108 nt products (Figs. 1 and 2). The amplified region of

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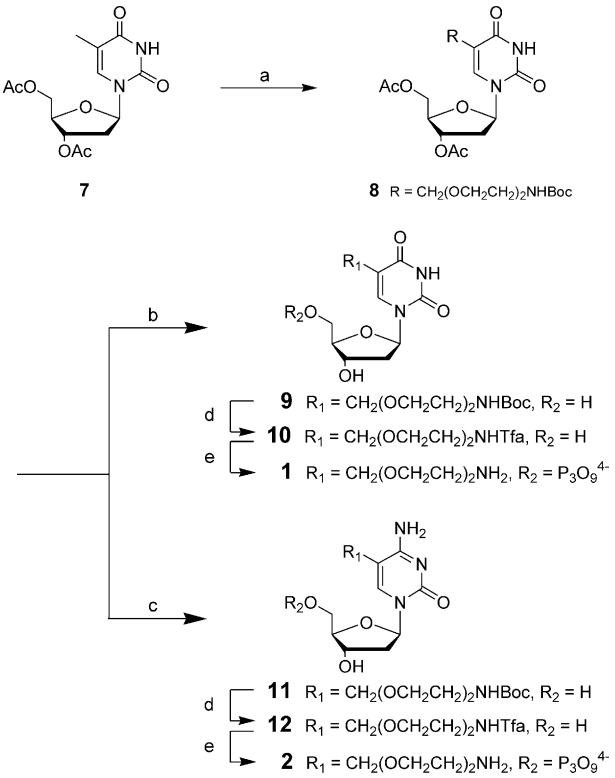
the double stranded template contains 25 places of non-serial T-sequence 'T', four places of two serial T-sequence 'TT', a place of three serial T-sequence 'TTT', a place of four serial T-sequence 'TTTT', 27 places of non-serial C-sequence 'C', seven places of two serial C-sequence 'CC', a place of three serial C-sequence 'CCC' and a place of four serial C-sequence 'CCCC' excluding the primer sequences. The following commercially available analogues, propynyl dUTP **3**, dUTP **5**, propynyl dCTP **4**, and methyl dCTP **6** were used for comparative studies on substrate properties. The direct PCR-mediated synthesis of modified DNA was performed using seven polymerases that belong to families A (*Taq*, *Tth* and *Thermo Sequenase*) and B [*Pfu*, *Pwo*, *Vent(exo-)* and *Deep Vent(exo-)*], respectively. The PCR products were resolved by 2% agarose gel electrophoresis, visualized by ethidium bromide staining; gel images were recorded with Molecular Imager® FX (Bio-Rad). The intensity of each band corresponding to full-length DNA product was quantified using the Quantity One® software. The amount of full-length PCR product formed by positive control reaction was set at 100% (Table 1). During PCR, both of the analogues dUTP **1** and dCTP **2** were found to be suitable substrates for all family B polymerases but not for all family A polymerases. Methyl dCTP **6** was found to be a good substrate for all polymerases, as was natural TTP of which a methyl group is at the C5 position. In contrast, propynyl dUTP **3** was accepted as a good substrate by all polymerases, whereas propynyl dCTP **4** was not. It is interesting that modification of dCTP with the propynyl group caused a loss of favorable substrate properties for the polymerases whereas modification of dUTP with the identical C5-substituent barely affected them. These results suggest that dCTP may be adopted into the substrate-binding site with a manner different from TTP; therefore, it is likely that the polymerases are



Scheme 1. Synthesis of C5-substituted pyrimidine nucleoside triphosphates from 3′, 5′-di-*O*-acetyl-2′-deoxythymidine: (a) (i) NBS, AIBN, dry benzene, reflux, 2 h; (ii) 2-(*N*-boc-2-aminoethoxy)ethanol, $KHCO_3$, dry DMF, 90 °C, 3 h; (b) methanolic ammonia, rt, 4 h; (c) (i) $POCl_3$, dry pyridine, rt, 4 h, then concd aqueous ammonia, 50 °C, 2 h; (ii) methanolic ammonia, rt, 4 h; (d) (i) CF_3COOH, rt, 30 min; (ii) CF_3COOEt, TEA, MeOH, rt, 1 h; (e) (i) $POCl_3$, protonsponge, $(MeO)_3PO$, 0 °C, 45 min, then *n*-Bu_3N PPi, DMF, rt, 1 h; (ii) 2 N aqueous ammonia, rt, 2 h.

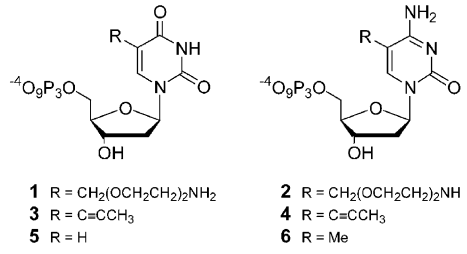


Figure 1. The C5-substituted pyrimidine nucleoside triphosphates employed in this study.

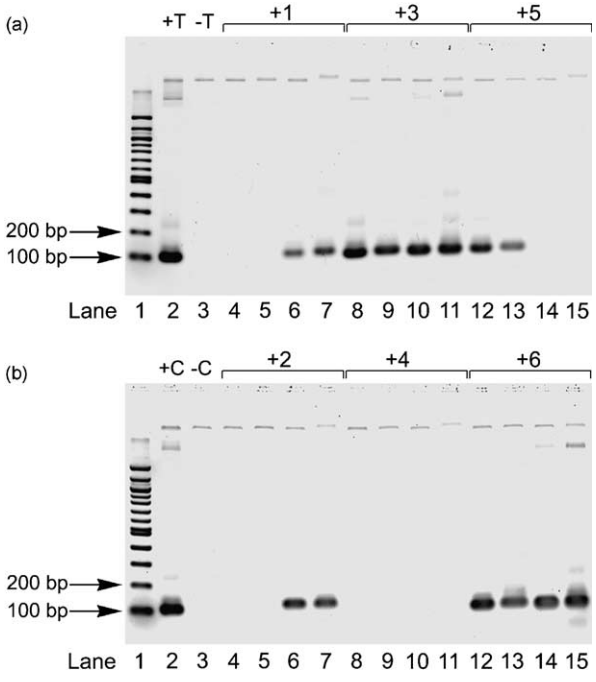


Figure 2. Ethidium bromide-stained 2% agarose gel of 108 nt PCR products. All amplifications were performed as follows; a hot start (1 min at 94 °C), followed by 20 cycles of amplification (0.5 min at 94 °C, 0.5 min at 52 °C, 1 min at 74 °C) and a final incubation for 5 min at 74 °C: (a) lane 1, molecular weight markers; lane 2, a PCR containing all four natural triphosphates, dATP, dGTP, dCTP and TTP (positive control); lane 3, a PCR containing dATP, dGTP and dCTP (negative control); lanes 4–7, a PCR containing dATP, dGTP, dCTP and 1; lanes 8–11, a PCR containing dATP, dGTP, dCTP and 3; lanes 12–15, a PCR containing dATP, dGTP, dCTP and 5. Thermostable DNA polymerases used; Taq (lanes 2–4, 8 and 12), Thermo Sequenase (lanes 5, 9 and 13), Pwo (lanes 6, 10 and 14), Vent(*exo*-) (lanes 7, 11 and 15); (b) lane 1, molecular weight markers; lane 2, a PCR containing all four natural triphosphates, dATP, dGTP, TTP and dCTP (positive control); lane 3, a PCR containing dATP, dGTP and TTP (negative control); lanes 4–7, a PCR containing dATP, dGTP, TTP and 2; lanes 8–11, a PCR containing dATP, dGTP, TTP and 4; lanes 12–15, a PCR containing dATP, dGTP, TTP and 6. Thermostable DNA polymerases used; Taq (lanes 2–4, 8 and 12), Thermo Sequenase (lane 5, 9 and 13), Pwo (lanes 6, 10 and 14), Vent(*exo*-) (lanes 7, 11 and 15).

Table 1. Substrate properties of the C-5 modified pyrimidine nucleoside triphosphates for thermostable DNA polymerases during PCR[a]

Evolutionary family	A	A	A	B	B	B	B
Thermostable DNA polymerase	*Taq*	*Tth*	*Thermo Sequenase*	*Pwo*	*Pfu*	*Vent(exo-)*	*Deep vent(exo-)*
1 (7-amino-2,5-dioxaheptyl dUTP)	–	–	–	+	+	++	+
2 (7-amino-2,5-dioxaheptyl dCTP)	–	–	–	++	+	++	+
3 (propynyl dUTP)	+++	++	++	+++	+++	+++	++
4 (propynyl dCTP)	–	–	–	–	–	–	–
5 (dUTP)	+++	+++	++	–	–	–	–
6 (methyl dCTP)	+++	++	++	+++	+++	+++	+++

[a]The symbols indicate the ratio of the amount of full-length product generated by a polymerase with all four natural triphosphates, dATP, dGTP, dCTP and TTP to the amount of full-length product generated utilizing a C-5 modified pyrimidine nucleoside triphosphate instead of the corresponding natural triphosphate (TTP or dCTP). +++, >70%; ++, 35–70%; +, <35%; –, no PCR product was detected.

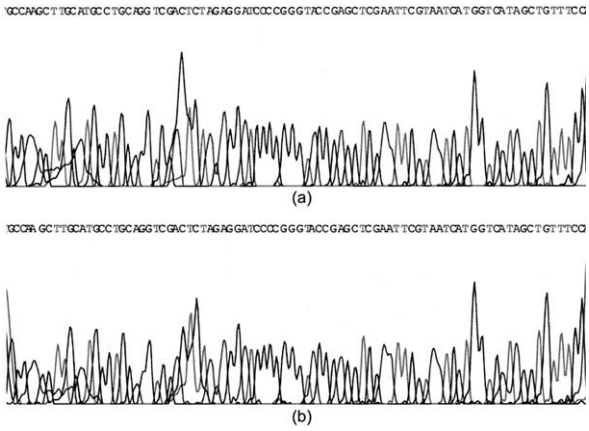


Figure 3. Sequencing of DNA fragments (a) transferred from the PCR product using the modified dUTP **1** instead of TTP, (b) transferred from the PCR product using the modified dCTP **2** instead of dCTP. Both figures show DNA sequences (25–108) excluding the primer region.

not capable of adequate binding to propynyl dCTP 3 because of steric hindrance resulting from the rigid projecting group. It can also be inferred that the generation of PCR product depends on template activity of DNA containing C5-modified bases. In addition, PCR using family B polymerases and dUTP 5 bearing the smallest substituent at the C5 position did not afford any observable products presumably because of the inhibitory effect[7] of uracil-containing DNA product generated in the first few cycles. It seems that the nature of modified base-containing DNA product as well as steric hindrance due to a C5 substituent may greatly affect the generation of PCR product when family B polymerases are involved. In regards to biological evolution, it is interesting to compare the different substrate properties of the modified nucleotides using both family A and B polymerases; bacteria use family A polymerases for DNA synthesis, whereas archaea use family B polymerases.[8]

In order to provide evidence of accurate incorporation of modified triphosphates, the modified DNAs as PCR products were enzymatically converted into natural DNA, and then sequenced with a Genetic Analyzer (ABI). The results show that the modified triphosphates were accurately incorporated to yield the corresponding modified DNAs in which the original sequence was conserved (Fig. 3).

In conclusion, we successfully designed and synthesized the novel modified pyrimidine nucleoside triphosphates (**1** and **2**) that are substrates for family B polymerases such as *Pfu*, *Pwo*, *Vent(exo-)* and *Deep Vent(exo-)*. As for modified dCTP, there are some examples of PCR-mediated synthesis of DNA using a modified dCTP under coexistence of natural dCTP.[9,10] However, such modified dCTPs are not suitable for SELEX but for probe attachment of DNA. PCR using the modified dCTP **2** yielded modified DNA in which all cytosine residues on a DNA strand, excluding the primer region, were completely replaced by modified cytosine residues. Thus, to the best of our knowledge, this is the first report of the direct PCR-mediated synthesis of DNA using a modified dCTP in the absence of natural dCTP.

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10. Substrate properties of 5-propargylamino dCTP for the polymerases during PCR are same as those of propynyl dCTP **4**.