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Synthesis and recognition of novel isonucleoside triphosphates by DNA polymerases

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Abstract—Isonucleosides have been attracting a lot of attention in recent years due to the chemical and enzymatic stability and potential anticancer and antiviral activities. We have reported some of the isonucleosides which exhibited significant anticancer activity and found that the oligonucleotide incorporated with isonucleoside could increase the enzymatic stability against the degradation by phosphodiesterase. In this paper, we investigated the recognition of the isonucleoside triphosphates **1**–**6** by *Taq*, Vent(exo$^-$), DeepVent(exo$^-$), 9° Nm, and Therminator DNA polymerases by a non-radioactivity method. We found that most of the isonucleoside triphosphates can be recognized by various DNA polymerase and act as terminators. Isonucleoside triphosphates **2** and **6** can be incorporated as substrates into the primer at 3′ terminus to lengthen the chain dependent on a DNA template by Vent(exo$^-$) and DeepVent(exo$^-$) DNA polymerases.

1. Introduction

Over the last decade, nucleoside and nucleotide drugs have attracted much attention due to a remarkable increase in the treatment of cancer and viral diseases. Currently more than 20 drugs fall into this category. Extensive research efforts have been directed toward the development of nucleoside therapeutics, structurally modified nucleoside inhibitors vary in either (or both) the ribose or base portion of the molecule.[1] Isonucleosides have been attracting a lot of attention in recent years due to the chemical and enzyme stability and potential anticancer and antiviral activities.[2] Nair reported the synthesis of isonucleoside, 1′,4′-anhydro-2′-deoxy-2′-nucleobase-D-arabinitol (Fig. 1), and found that oligodeoxynucleotides containing such isonucleoside could form a duplex with complementary DNA sequence and antagonize the degradation of enzyme.[3] Taktakishvili described the inhibition of HIV integrase by some dinucleotides containing isonucleoside.[4] We reported the synthesis of 1′,4′-anhydro-2′-deoxy-2′-nucleobase-L-arabinitol (**A**) (Fig. 1), the stereoisomer of 1′,4′-anhydro-2′-deoxy-2′-nucleobase-D-arabinitol (**B**), and found that 1′,4′-anhydro-2′-deoxy-2′-nucleobase-L-arabinitol showed significant cytotoxic activities in HL-60 cells, the ED_{50} being 1.60–8.06 μM.[2a,c] However, on the contrary, the oligonucleotide containing 1′,4′-anhydro-2′-deoxy-2′-nucleobase-L-arabinitol could not form regular base pair as described for its D-form partner.[5] We also investigated the synthesis of 4-deoxy-4-nucleobase-2,5-anhydro-L-mannitols (**C**) (Fig. 1) and the hybridization of the oligodeoxynucleotides incorporated with these isonucleosides. It indicated that the existence of hydroxymethyl group on the isonucleoside could stabilize duplex formed by the homo-oligodeoxynucleotide and its complementary sequence, and no obvious difference was observed when the 6′-hydroxyl group was free or protected.[5,6]

The deoxyribonucleoside kinases phosphorylate deoxyribonucleosides and thereby provide an alternative to de novo synthesis of DNA precursors. Their activities are essential for the activation of several nucleoside analogues.[7] Nucleoside anticancer and antiviral drugs are

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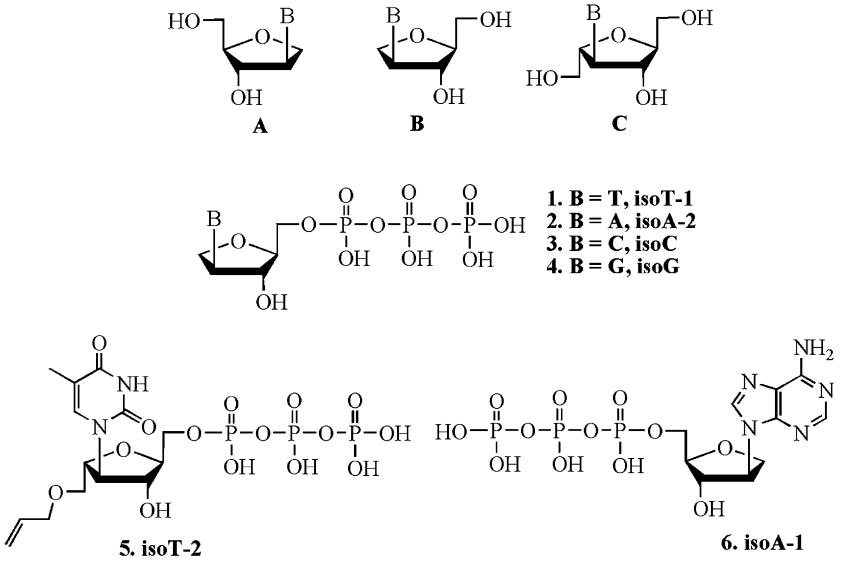


Figure 1. The structures of isonucleosides and isonucleoside triphosphates.

pro-drugs in that they are actively transported into cells and then activated by cellular kinases to form the nucleotide triphosphate (NTP). For example, a variety of human T- and B-lymphocyte cell lines efficiently converted the prodrug to the AZT metabolites. Phosphorylation of AZT to AZTMP is carried out through the cytosolic salvage enzyme thymidine kinase.[8] Stavudine (d4T) is metabolized in cells to the mono-, di-, and triphosphate nucleotides. Thymidine kinase 1 (TK1) is responsible for the phosphorylation of stavudine but it is not the only enzyme involved in its activation.[9] Human TMPK can phosphorylate 1-(2′-Deoxy-2′-fluoro-β-L-arabinofuranosyl)-5-methyluracil (L-FMAU) and play a critical role in L-FMAU metabolism in cells.[10] Amdoxovir [(−)-β-D-2,6-diaminopurine dioxolane, DAPD] is the prodrug of dioxolane guanosine (DXG). Joy et al. examined the phosphorylation pathway of DXG using 15 purified enzymes, most of which can phosphorylate DAPD.[11] Abacavir is phosphorylated by adenosine phosphotransferase to a monophosphate and further metabolized in several steps to the triphosphate dGTP analogue carbovir triphosphate (CBVTP). CBVTP is thought to be the agent responsible for antiviral activity.[12] Those NTPs are now able to competitively inhibit the enzyme or, more commonly, act as a substrate and be incorporated into the growing polynucleotide chain which subsequently prevents (chain-terminates) the further replication of the viral genome. Therefore, it would be interesting to investigate the recognition of isonucleoside triphosphate by DNA polymerases in the DNA synthesis; the results will imply the relationship of molecular factors of isonucleoside with the DNA polymerases in the DNA chain elongation.

We have reported the synthesis of isonucleoside triphosphates **1**–**4**.[13] In this paper, we continued to synthesize another two isonucleoside triphosphates **5** and **6** (Fig. 1), and investigated the recognition of **1**–**6** by *Taq*, Vent(exo$^-$), DeepVent(exo$^-$), 9° Nm and Therminator DNA polymerases with a non-radioactivity method. Compounds **2** and **6** are a pair of stereoisomers which could be used as different substrates to understand the stereochemical requirements for the recognition of enzymes and the protected isonucleoside triphosphate **5** is used as a substrate to study the influence of allyl group on the recognition of enzymes.

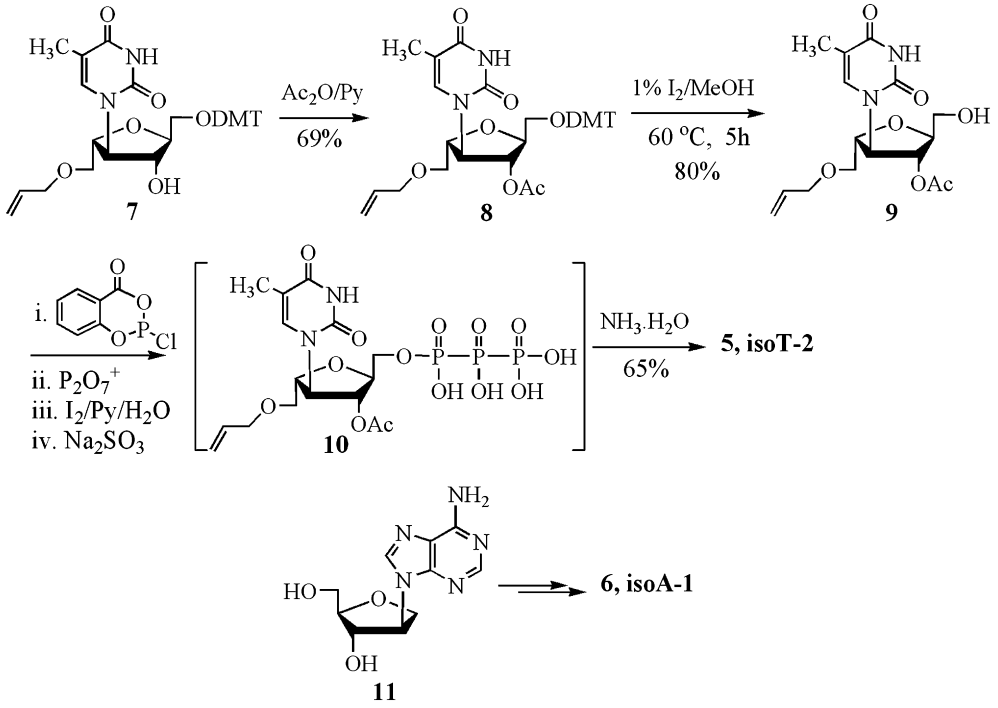
2. Results and discussion

2.1. Chemistry

Isonucleoside triphosphates **5** and **6** were synthesized according to a published procedure in our laboratory for the synthesis of isonucleoside triphosphates **1**–**4**[13] (Scheme 1). Compound **7**[6b] was reacted by Ac_2O/pyridine to give compound **8**, which was treated by 1% I_2/MeOH to give compound **9** in 80% yield.[14] Compound **9** was phosphorylated via a one-pot reaction and followed deprotection to give compound **5** (Scheme 1) in 65% yield. By a similar procedure,[13] compound **6** was prepared from compound **11**.[5]

2.2. Incorporation of isonucleoside triphosphates 1–6 into DNA by various DNA polymerases

To investigate the recognition of DNA polymerase, corresponding isonucleoside triphosphates **1**–**6** were used as substrates instead of the natural dNTP, respectively, in a general polymerization reaction on a 25 oligomer DNA template and a 17 oligomer primer (Scheme 2). The primer was labeled by 5′-FITC. *Taq*, Vent(exo$^-$), DeepVent(exo$^-$), 9° Nm, and Therminator DNA polymerases were used in the polymerization. Denaturing polyacrylamide gel electrophoresis was used to resolve single base in an arbitrary DNA sequence and permitted the analysis of a variety of incorporation phenomena of isonucleoside triphosphates into the elongated DNA chain catalyzed by DNA polymerase.



Scheme 1. Synthesis of isonucleoside triphosphates **5** and **6**.

3'-G ATA TCA CTC AGC ATA A-5' (FITC) (17 mer)
5'-GAC ACG CXC TAT AGT GAG TCG TAT T-3' (25 mer)

DNA Polymerase, Compound **1-6**,
Natural nucleoside triphosphate(dNTP)

3'-CTG TCC GYG ATA TCA CTC AGC ATA A-5' (FITC)
5'-GAC ACG CXC TAT AGT GAG TCG TAT T-3' (25 mer)

Scheme 2. DNA polymerase assay: 25-mer oligodeoxynucleotide is used as DNA template and a 5′-FITC labeled 17-mer oligodeoxynucleotide is used as primer. Template 1, X = A; template 2, X = T; template 3, X = G; template 4, X = C; Y = T, A, C, G corresponding to each template.

The experimental design allows one to compare the insertion and prolongation extent of isonucleoside triphosphate into the elongated DNA chain at the first target site with four diverse bases along four diverse DNA template strands. The results of isonucleoside triphosphates **1**–**6** incorporating into DNA by various DNA polymerase were, respectively, analyzed by denaturing polyacrylamide gel electrophoresis in Figures 2–6. In Figures 2–6, each line was labeled as follows: N represents negative control, enzyme was free; P1 represents positive control, four natural dNTP were used as substrates; P2 represents another positive control, only one natural dNTP was used as substrate; the numeral **1**–**12** represents that isonucleoside triphosphates were added instead of natural nucleoside triphosphates, respectively, in which **1**, **3**, **5**, **7**, **9**, **11**: *iso*T-1, *iso*T-2, *iso*A-1, *iso*A-2, *iso*C, *iso*G, only; **2**, **4**, **6**, **8**, **10**, **12**: *iso*T-1, *iso*T-2, *iso*A-1, *iso*A-2, *iso*C, *iso*G, and other three natural dNTP, respectively. The reaction conditions were presented in the experimental section.

In Figures 2 and 3, the primer+1 bands are clearly shown and the DNA synthesis was arrested at this site (line 1–12), which indicated that isonucleoside triphosphates **1–6** could be incorporated, respectively, into the first elongated position of the primer at 3′-terminus, same as the case of dTTP (line P2), on the corresponding template **1**–**4** catalyzed by *Taq* and 9° Nm DNA polymerases. However, in Figure 2, no full-length and full-length+1 products were observed, which meant that isonucleoside triphosphates **1**–**6** acted only as chain terminators for *Taq* DNA polymerase. In comparison with lane 5 and lane 6 (*iso*A-1, **6**) of Figure 3, it showed that *iso*A-1 could stop the elongation of primer at the primer+1 site (lane 5), and when *iso*A-1 and another three dNTP were used for the DNA synthesis, the primer+1 and full-length bands appeared (lane 6). It may give a clue that *iso*A-1 could be recognized as a poor substrate by 9° Nm DNA polymerase, after the incorporation at the primer+1 site, the oligomer chain could elongate continuously at the primer 3′ terminus to the full-length product. Compounds **1**–**5** were also chain terminators for 9° Nm DNA polymerase, the main bands observed at primer+1 site. When the elongation of primer chain was arrested, some shorter fragments (Fig. 2, line 5 and Fig. 3, line 5, 9, 11) were exhibited due to the degradation of primer by DNA polymerase in the reaction period.

An extensive DNA polymerase assay screening is shown in Figures 4–6. In all cases, compounds **1**–**6** exhibited as terminators to incorporate to primer at 3′ terminus and arrest the elongation of the primer at different stage to give the limited stretches on a DNA template. It was observed full-length and sometime full-length+1 bands are observed in Figure 4, lane 6 (*iso*A-1, **6**) and in Figures 5 and 6, lane 6 (*iso*A-1, **6**) and lane 8 (*iso*A-2, **2**).[15] It was indicated that *iso*A-1 and *iso*A-2 were able to be substrates for the recognition by Therminator

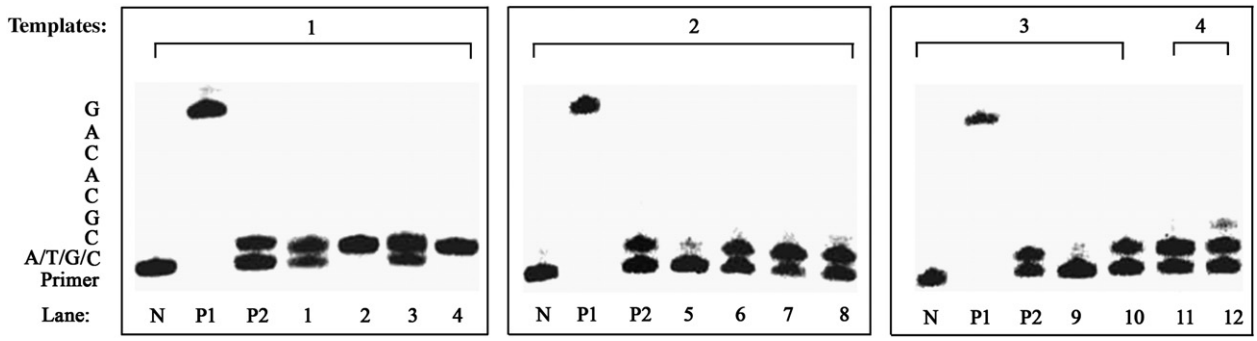


Figure 2. DNA incorporation by *Taq* DNA polymerase.

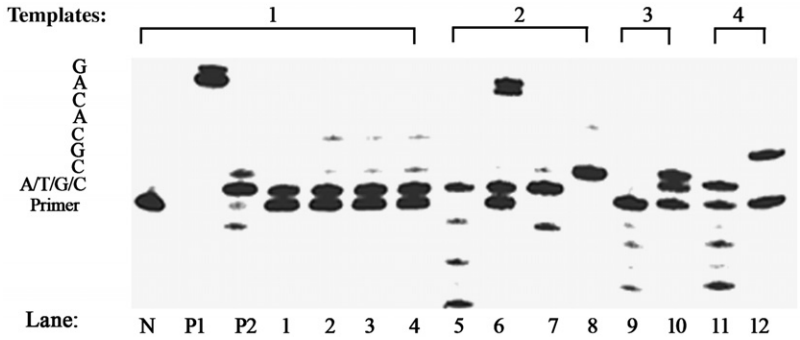


Figure 3. DNA incorporation by 9° Nm DNA polymerase.

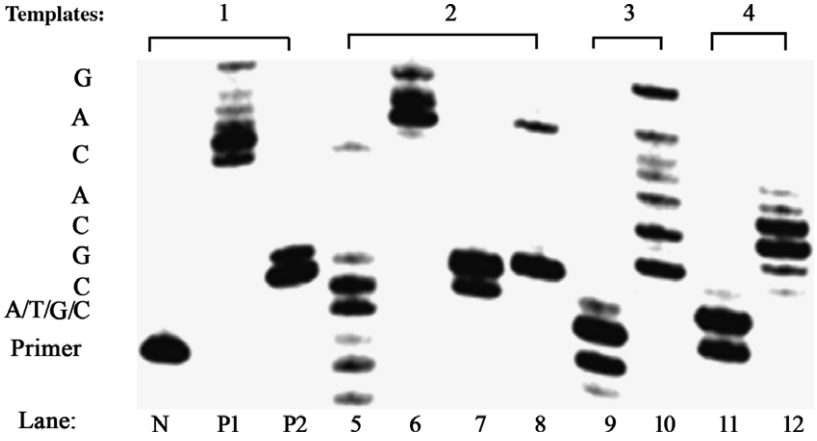


Figure 4. DNA incorporation by Therminator DNA polymerase.

DNA polymerase, Vent(exo$^-$) DNA polymerase, and Deep Vent(exo$^-$) DNA polymerase. *iso*A-1 was more accepted as substrate by Therminator DNA polymerase (Fig. 4, line 6), Vent(exo$^-$) DNA polymerase (Fig. 5, line 6) and Deep Vent(exo$^-$) DNA polymerase (Fig. 6, line 6) and *iso*A-2 was more accepted as substrate by Vent(exo$^-$) DNA polymerase (Fig. 5, line 8) and Deep-Vent(exo$^-$) DNA polymerase (Fig. 6, line 8). *iso*-C, **3** is a poor substrate for Therminator DNA polymerase.

To determine the kinetic parameters of the incorporation of *iso*A-2 or dATP, a steady-state kinetic assay was performed.[16] The kinetic parameters for incorporation of one *iso*A-2 by 9° Nm DNA polymerase were studied, the K_m being a little higher than that of its natural one (Table 1).

It was reported that modified nucleosides were the poor substrates for the recognition by natural DNA polymerase. From this study, we found that isonucleoside triphosphates **1**–**6** were able to be recognized by diverse DNA polymerases and incorporated into the DNA chain and elongated in various degrees, the *iso*A-1 could be used as a substrate as the natural dNTP to incorporate and elongate the primer at 3′ terminus to full-length and full-length+1 products by Vent(exo$^-$), Deep-Vent(exo$^-$), 9° Nm, Therminator DNA polymerases (Fig. 3–6, lane 6), and the *iso*A-2 was a substrate for

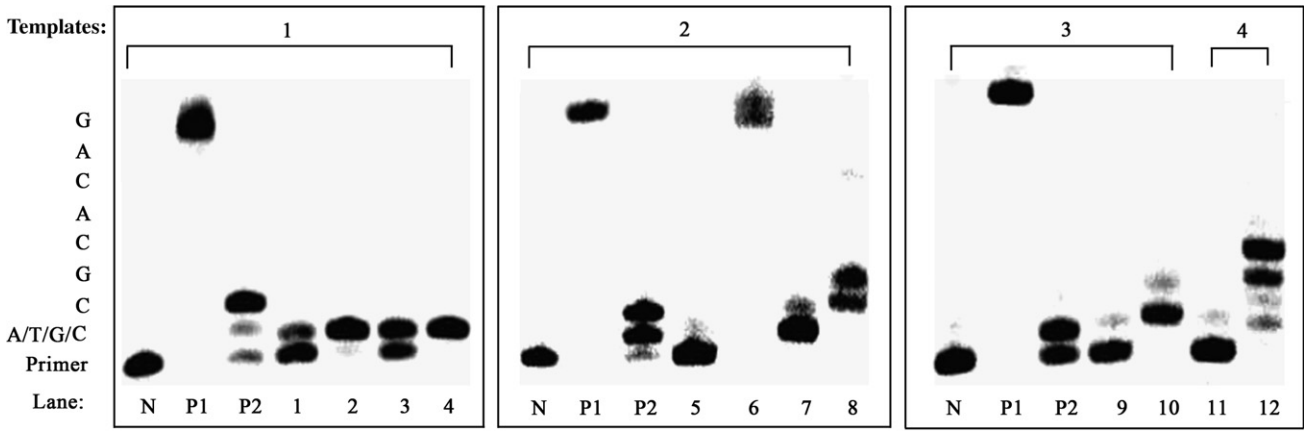


Figure 5. DNA incorporation by Vent(exo$^-$) DNA polymerase.

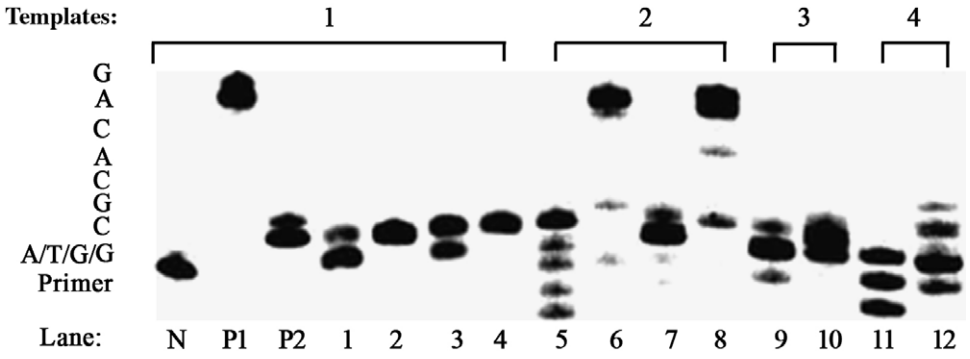


Figure 6. DNA incorporation by DeepVent(exo$^-$) DNA polymerase.

Table 1. Steady-state kinetic parameters for 9° Nm DNA polymerase extension

Substrate	K_m (μM)	V_{max} (%/min)	V_{max}/K_m (M^{-1})
dATP	0.78 ± 0.25	6.73 ± 1.37	8.63×10^6
*iso*A-2	3.70 ± 1.40	2.95 ± 0.32	7.97×10^5

Vent(exo$^-$) and DeepVent(exo$^-$) DNA polymerases. It was also indicated that compounds **1**–**5** were the terminators to arrest the elongation of the primer chain which was consistent with the general mechanism of antitumor nucleosides and cytotoxicity of compounds **1**–**4** we reported previously.[13] Though we reported that oligodeoxynucleotides incorporated with 4-deoxy-nucleobase-2,5-anhydro-L-mannitols (Fig. 1) could hybridize with the complementary sequence and the existence of hydroxymethyl group on the isonucleoside could stabilize duplex forming, it was obvious that compound **5** could not be used as a substrate for DNA polymerization. By comparison with the substrate properties of compound **2** (*iso*A-2) and **6** (*iso*A-1) for the various DNA polymerases described above, it seemed that compound **6** (*iso*A-1) was the more likely substrate as the natural dATP in the polymerization. The computer simulation of homo-oligonucleotide containing *iso*A-1 or *iso*A-2 was investigated. The results indicated that the homo-oligonucleotide containing *iso*A-1 could form the stable duplex with its complementary DNA sequence. The molecular factors such as backbone distortion were important parameters that determine the capacity of the duplex formation, in the case of *iso*A-1 the change of backbone distortion was accepted by the recognition of complementary sequence.[17] Furthermore, it was reported that antisense oligonucleotide constructed from isonucleotide could antagonize the hydrolysis of nuclease and give acceptable binding ability.[18] A practical way is to build a mixed backbone, in which the isonucleotide is placed at the critical position of the sequence.[19] In this study, we provided a possibility to construct antisense oligodeoxynucleotide with mixed backbone by DNA polymerization.

3. Conclusion

All of the six isonucleoside triphosphates **1**–**6** were recognized by diverse DNA polymerases and incorporated into the DNA chain and elongated in various degrees, the *iso*A-1 was the more likely substrate as the natural dATP in the polymerization. It was proved that isonucleoside triphosphates **1**–**5** were the terminators to arrest the elongation of DNA chain by all of DNA polymerases in this study. DNA polymerases belonging to the B-family were more efficient in this capacity. We found that certain DNA polymerases were able, despite the significant differences of the substrates in the sugar-phosphate backbone, to copy limited stretches dependent on a DNA template. Vent(exo$^-$) and DeepVent(exo$^-$)

DNA polymerases were considered to be the most promising DNA polymerases for the polymerization reactions with *iso*A-1 and *iso*A-2 (comparable with the natural one) as substrates. In a word, based on the above results, we may continue to modify *iso*A-1 or *iso*A-2, etc., especially their monophosphates, to search new antiviral or anticancer agents in this field.

4. Experimental

^{1}H NMR spectra were recorded on a Varian Mercury 300 or 500 NMR spectrometer. Tetramethylsilane was used as internal reference for ^{1}H NMR, 85% phosphoric acid as external reference for ^{31}P NMR. High-resolution ESI mass spectra were obtained at Bruker DALTONICS APEX IV 70e instruments, and the data are reported in *m/e* (intensity to 100%). Vent(exo$^-$), DeepVent(exo$^-$), 9° Nm, Therminator DNA polymerases were purchased from New England Biolabs. *Taq* DNA polymerase was purchased from Promega. Primer and template were purchased from TaKaRa.

4.1. Synthesis

4.1.1. 6-*O*-Allyl-1-*O*-(4,4′-dimethoxy)trityl-4-deoxy-4-(thymin-1-yl)-2,5-anhydro-L-mannitol (7). Compound **7** (white solid foam) was synthesized by the reported method.[6b] ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.26 (s, 1H, 3-NH), 7.55 (s, 1H, H-6), 7.41 (d, J = 7.8, 2H, Ph–H), 7.21 (m, 7H, Ph–H), 6.86 (d, J = 8.6, 4H, Ph–H), 5.82 (m, 1H =CH–), 5.53 (d, J = 5.6, 1H, 4′-H), 5.12 (m, 2H, CH_2=), 4.66 (t, 1H, H-3′), 4.16 (m, 2H, H-5′, H-2′), 3.99 (m, 2H, 2′-C–O–CH_2–), 3.73 (s, 6H, –OCH_3), 3.49 (d, J = 2.4, 2H, 2′-CH_2–), 3.10 (m, 2H, 5′-CH_2), 1.71 (s, 3H, 5-CH_3). MS (ESI) for $C_{35}H_{38}N_2O_8$ ([M]$^+$) Calcd: 614. Found: 614. Elemental analysis for $C_{35}H_{38}N_2O_8$ Calcd: C, 68.39; H, 6.23; N, 4.56. Found: C, 68.30; H, 6.30; N, 4.34.

4.1.2. 3-*O*-Acetyl-6-*O*-allyl-1-*O*-(4,4′-dimethoxy)trityl-4-deoxy-4-(thymin-1-yl)-2,5-anhydro-L-manitol (8). To the solution of compound **7** (127 mg, 0.21 mmol) in anhydrous pyridine (2 ml), Ac_2O (1.0 ml) was added. The mixture was stirred at rt for 24 h. After usual procedure, white solid foam **8** was obtained (89 mg, 69%).

^{1}H NMR (300 MHz, DMSO-d_6) δ = 11.35 (s, 1H, N–H), 7.12–7.50 (m, 10 H, 3-NH, 9 Ph–H), 6.85 (m, 4H, Ph–H), 5.88 (m, 1H, =CH–), 5.37 (t, J = 6.3, 1H, 3′-H), 5.18 (m, 2H, CH_2=), 4.69 (m, 1H, 4′-H), 4.35 (m, 1H, H-2′), 4.10 (m, 1H, H-5′), 3.99 (m, 2H, 2′-C–O–CH_2–), 3.73 (m, 6H, –OCH_3), 3.53 (m, 2H, 2′-CH_2–), 3.37 (d, 2H, 5-CH_2–), 1.95 (s, 3H, –$COCH_3$), 1.65 (s, 3H, –$COCH_3$). MS (ESI) for $C_{37}H_{40}N_2O_9$ ([M]$^+$) Calcd: 656. Found: 656. Elemental analysis for $C_{37}H_{40}N_2O_9$ Calcd: C, 67.67; H, 6.14; N, 4.27. Found: C, 67.75; H, 6.24; N, 4.13.

4.1.3. 3-*O*-Acetyl-6-*O*-allyl-4-deoxy-4-(thymin-1-yl)-2,5-anhydro-L-mannitol (9). Compound **8** (160 mg, 0.125 mmol) was dissolved into a solution of I_2 (200 mg, 0.79 mmol) in methanol (20 ml). The solution was heated at 60° for 6 h. A small quantity of sodium thiosulfate was added to quench the reaction. The solvent was removed and the residue was purified by column chromatography on silica gel (dichloromethane/methanol) to give compound **9** (white solid syrup, 64 mg, 80%).

^{1}H NMR (300 MHz, DMSO-d_6) δ = 11.31 (s, 1H, 4-NH), 7.63(s, 1H, 6-H), 5.85 (m, 1H, =CH), 5.35 (t, 1H, 3′-H), 5.09–5.26 (m, 3H, CH_2=, 5′-C–OH), 4.854 (t, 1H 4′-H), 4.30 (m, 1H, 5′-H), 4.00 (m, 1H, 2′-H), 3.95 (m, 2H, -5′-CH_2–), 3.65 (t, 2H, 2′-C–O–CH_2), 3.48 (m, 2H, 2′-CH_2–), 2.01 (s, 3H, $COCH_3$), 1.77 (s, 3H, 5-CH_3). MS (ESI) for $C_{16}H_{22}N_2O_7$ ([M]$^+$) Calcd: 354. Found: 354. Elemental analysis for $C_{16}H_{22}N_2O_7$ Calcd: C, 54.23; H, 6.26; N, 7.91. Found: C, 54.33; H, 6.29; N, 7.70.

4.1.4. 6-*O*-Allyl-4-deoxy-4-(thymin-1-yl)-2,5-anhydro-L-mannitol-1-triphosphate (5, *iso*T-2).[13] By the same method as the preparation of compounds **1**–**4**, compound **5** (*iso*T-2) was synthesized from compound **9**. ^{1}H NMR (300 MHz, DMSO-d_6) δ = 7.50 (s, 6-H, 1H), 4.86 (m, =CH, 1H), 4.65 (m, 3′-H, 1H), 4.55 (m, CH_2=, 2H), 4.15 (m, 4′-H, 1H), 4.055 (m, 5′-H, 1H), 3.973 (m, 2′-H, 1H), 1.75 (s, 5-CH_3, 3H); ^{31}P NMR (121.41 MHz, D_2O) δ = −4.60 (d, $J_{\beta,\gamma}$ = 50.0, γP), −8.92 (m, $J_{\alpha,\beta}$ = 52.0, αP), −20.91 (βP). HRMS (ESI) for $C_{14}H_{23}N_2O_{15}P_3$ ([M]$^+$) Calcd: 552.0311. Found: 552.0291.

4.1.5. 6-O-Allyl-4-deoxy-4-(adenin-9-yl)-2,5-anhydro-L-mannitol- 1- triphosphate (6, *iso*A-1). $[\alpha]_D^{25}$ −10.10; ^{1}H NMR (300 MHz, D_2O) δ = 8.18 (s 1H H-2); 8.04 (s, 1H, H-8); 4.90 (m, 1H, H-3′), 4.60 (m, 1H, H-4′), 4.24 (m, 2H, H-2′), 4.08 (m, 2H, H-5′-CH_2–), 3.95 (m, 1H, H-5′). ^{31}P NMR (121.41 MHz, D_2O) δ −4.50 (d, $J_{\gamma,\beta}$ = 48, 1P, γP), −8.90 (d, $J_{\alpha,\beta}$ = 52.8, 1P, αP), −19.8 (m, 1P, βP). HRMS (ESI) for $C_{10}H_{16}N_5O_{12}P_3$ ([M]$^+$) Calcd: 449.0008. Found: 449.0021.

4.2. DNA polymerase catalyzed incorporation of isonucleoside triphosphate into DNA

Primer extension experiment with DNA polymerase. The template (20 pmol) was annealed with 5′-FITC-primer(20 pmol) in buffer containing a final concentration of 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris–HCl (pH 8.8, 25 °C), 2 mM $MgSO_4$, and 0.1% Triton X-100 by cooling from 90 to 0 °C temperature in ice bath quickly, for *Taq* DNA polymerase buffer containing a final concentration of 50 mM KCl, 10 mM Tris–HCl (pH 9.0, 25 °C), 1.5 mM $MgCl_2$, 0.1% Triton X-100, and then incubated with dNTP(1 nmol each), isonucleoside triphosphate (1 nmol), and DNA polymerase (2 U) at 50 °C for 1.5 h (total volume 10 μL). The template sequence was 5′-GAC ACG C<u>X</u>C TAT AGT GAG TCG TAT T-3′ (25 mer) (template 1, X = A; template 2, X = T; template 3, X = G; template 4, X = C), the primer sequence was 3′-G ATA TCA CTC AGC ATA A-(**FITC**)-5′ (17 mer).

Polyacrylamide gel electrophoresis has been performed under denaturing conditions 7 M urea with 20%

polyacrylamide (v/v), 1× TBE buffer and run at 400 V until the bromophenol blue had migrated 15 cm. The samples have been denatured by heating to 100 °C with formamide (30%, v/v) for 5 min before loading on the gel. The results were analyzed by Storm 840.

4.3. Kinetic experiments

The reaction mixture was prepared by adding 9° Nm DNA polymerase to primer annealed to template 2 (Fig. 2), buffer, and dATP or *iso*A-2. The final mixture (10 μL) contained 0.01 U/μL 9° Nm DNA polymerase, the commercially supplied buffer, 2 μM primer–template complex, and various concentrations of dATP or *iso*A-2. A concentration range of 0.5–10 μM was used. Reaction mixtures were incubated at 50 °C. Reaction times were between 3 and 15 min.

Acknowledgments

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