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Incorporation of an inducible nucleotide analog into DNA by DNA polymerases

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ABSTRACT

Non-natural nucleotides with diverse functionalities are highly useful in many areas of basic research and practical applications. We have previously developed an efficient method for post-synthetic modifications of 2-amino-6-vinylpurine (AVP)-containing oligonucleotides, which permits conjugations of a variety of useful functional appendages to the AVP moiety in DNA. Here we report an investigation on the ability of various DNA polymerases to use 5′-triphosphate of 2′-deoxyribosyl-2-amino-6-(2-methylthioethyl)purine (a stable precursor of AVP) as the substrate for templated DNA synthesis.

1. Introduction

Chemically modified (or functionalized) DNA probes, such as those having fluorophores, magnetic moieties and other functions, have been increasingly used in biotechnology[1,2] medicine[3,4] diagnostics and nanotechnology.[5,6] For example, some chemically modified DNA probes have been used as unique tools to bring proteins into proximity of a target nucleic acid.[7] Modified DNA probes of 20–30 nucleotides in length are normally synthesized chemically by an automated DNA synthesizer using phosphoramidite derivatives of non-natural nucleosides. However, as the chemical method is not ideally suited for the synthesis of large DNA or RNA molecules, a better way to introduce a functionalized nucleotide into long nucleic acid molecules is to use DNA polymerases that can recognize modified nucleoside 5′-triphosphates as substrates.[8,9] This enzymatic approach is particularly important for the development of functional nucleic acids (such as DNAzymes,[10] ribozymes,[11] and aptamers[12]) that contain modified nucleotides because they are typically generated by an in vitro selection process that involves multiple rounds of functional selection and DNA amplification.[13,14] Therefore, there is a high demand for the development of 5′-triphosphate derivatives of artificially designed and functionally diverse nucleoside analogs that can be taken as excellent substrates of commercially available DNA polymerases.

We have previously reported that 2-amino-6-methylthioethylpurine (AVP (SMe)), a stable sulfide derivative of 2-amino-6-vinylpurine (AVP),[15,16] within DNA duplexes can be activated to selectively form a cross-link with cytidine.[17,18] This inducible alkylating system has been demonstrated to be effective in inhibiting gene expression in cells.[19] Furthermore, we have shown that an AVP moiety in ODNs can be exploited for post-synthetic conjugation with a variety of nucleophiles under mild conditions (Fig. 1).[20]

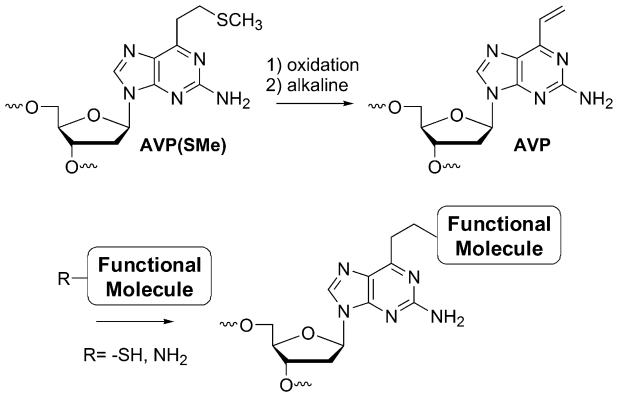

Figure 1. Post-synthetic conjugation of suitable nucleophiles to oligodeoxy-nucleotides (ODNs) containing 2-amino-6-methylthioethylpurine, a stable precursor of 2-amino-6-vinylpurine.

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Advantages offered with AVP as a conjugation element include its stability and high efficiency for conjugation under neutral solution conditions. A good example to illustrate this point is the conjugation of a DNA probe with luciferase without a significant loss of enzyme activity.[21] Thus, we believe that the incorporation of AVP into large DNA molecules can offer many useful downstream applications where such modified DNA molecules can be conjugated to a protein or nucleic acid of interest. In this study, we have sought suitable DNA polymerases for incorporating 2-amino-6-methylthioethylpurine nucleoside 5′-triphosphate (abbreviated as dAVP(SMe)-TP).

2. Results and discussion

2.1. Synthesis of 5′-triphosphate of 2′-deoxyribosyl-2-amino-6-(2-methylthioethyl)purine

2′-Deoxyribosyl-2-amino-6-(2-methylthioethyl)purine (**1**, Scheme 1) was prepared by the protocol described in our previous reports.[16,17] This nucleoside was converted to the corresponding 5′-triphosphate (Scheme 1) by a conventional procedure using $POCl_3$ and tributyl ammonium pyrophosphate. After deprotection of the phenoxyacetyl (Pac) group of the 2-amino group, **2** was purified by HPLC and thoroughly characterized by mass spectrometry as well as ^1H and ^{31}P NMR spectroscopies (see Section 4).

2.2. Primer extension using exo⁻ Klenow Fragment

The ability of DNA polymerases to incorporate **2** into DNA was first investigated using the exonuclease-free (exo⁻) Klenow Fragment (KF). Eight reactions were carried out, each of which used the ^{32}P-labelled 20-nt (nt: nucleotide) primer hybridized with one of the four 16-nt templates with a base variation at the 5′ end (Fig. 2, indicated by 'N'; N = A, T, G, C), along with KF and 2′-deoxyadenosine-5′-triphosphate (dATP) or **2**. The reaction conditions were optimized for the incorporation of dAVP(SMe)-TP (**2**). The products of the reactions were analyzed by denaturing polyacrylamide gel electrophoresis (PAGE), and the results are shown in Figure 2. In these reaction conditions, dAVP(SMe)-TP (**2**) was incorporated into the DNA chain across T on the template strand with high selectivity and dATP was incorporated into DNA chain opposite T. By further analysis, we found that the K_m for the incorporation of dAVP(SMe)-TP against T with Klenow Fragment is about tenfold lower than that of dATP against T (data not shown). When the incorporation reaction of dAVP(SMe)-TP was carried out with higher concentration of KF, a small amount of the product was observed against C (data not shown). These characteristics of dAVP(SMe)-TP were similar to that of *O*-6-methyldeoxyguanosine triphosphate,[22] and dAVP(SMe) would act as an analog dATP with Klenow Fragments. The methylsulfide group of dAVP(SMe) is likely to be remained in the extended DNA, because any bands derived from cross-linking products were not observed in gels after the polymerase reactions. The above results led us to further investigate whether a longer DNA can be synthesized by the primer extension. The new extension reaction was

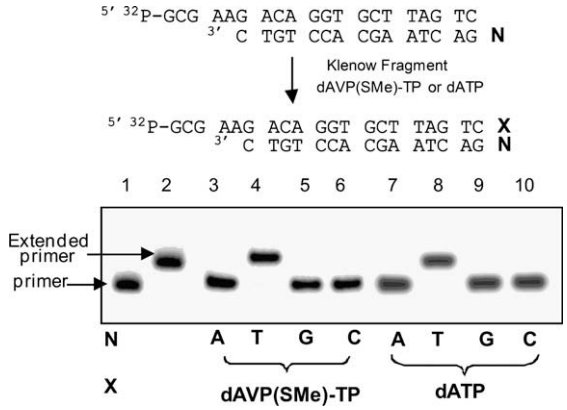


Figure 2. Gel analysis of primer extension reactions using exo⁻ Klenow Fragment (KF, 0.1 U/μL). Lanes 3–10: the reaction products (carried out at 37 °C for 15 min) using one of the four 16-nt templates differing by the 'N' (N = C, G, A, T) at the 5′ end, the 20-nt primer (^{32}P-labelled), **2** (lanes 3–6) or dATP (lanes 7–10) and KF. Lane 1: ^{32}P-labelled primer alone; lane 2: ^{32}P-labelled 21-nt oligonucleotides with the sequence of $^{5'}$GCG AAG ACA GGT GCT TAG TCG$^{3'}$ as a size marker.

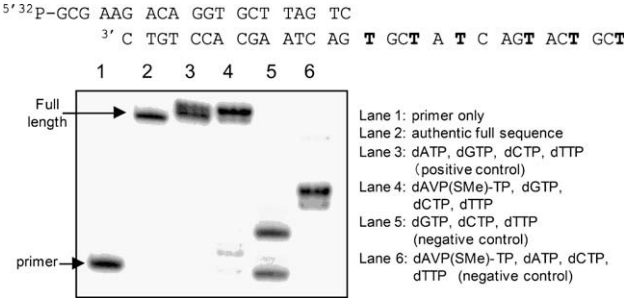
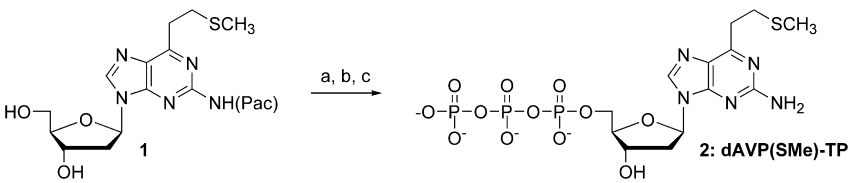


Figure 3. Gel analysis of the primer extension reaction using 31-nt template by KF (0.5 U/μL). The reactions were carried out at 37 °C/30 min.

thus carried out using a 31-nt DNA template containing all four nucleotides with multiple T and C residues, and the results are shown in Figure 3. The 36-nt, full-length DNA product was obtained when dATP was replaced by **2** (comparing lanes 3 and 4 in Fig. 3). However, prematurely terminated products were observed when **2** was omitted (lane 5), suggesting that 2′-deoxyguanosine-5′-triphosphate (dGTP) was not incorporated against multiple T in the longer DNA template. These results clearly indicate that KF can make multiple incorporations of **2** against T residues in a DNA template. We also performed a reaction where dGTP was replaced by **2** and retained the other three bases (dATP, 2′-deoxycytidine-5′-triphosphate (dCTP) and thymidine-5′-triphosphate (dTTP)) (lane 6). The facts that only partially extended products but no fully extended products were seen clearly suggest that **2** cannot be efficiently incorporated by KF against multiple C residues in a long DNA template.



Scheme 1. Synthesis of the required 5′-triphosphate. Reagents and conditions: (a) proton sponge, $(MeO)_3PO$, $POCl_3$, 0 °C for 1 h; (b) tributylammonium pyrophosphate, nBu_3N, DMF, room temperature (rt) for 2 h; 1 M triethylammonium bicarbonate buffer, 14 h; (c) 28% NH_3, rt for 3 h.

2.3. Primer extension using thermophilic DNA polymerases

Next we examined the incorporation of **2** against 4 bases using 4 commercially available thermophilic DNA polymerases: Deep vent (exo$^-$), Bst, Taq and Tth polymerases. We performed the primer extension reactions using a protocol similar to that for KF except that a higher reaction temperature was chosen (as described in Section 4). When we used the standard conditions recommended by manufacturers for the extension reaction with 4 polymease, dAVP(SMe)-TP (**2**) was not incorporated into the DNA chain. Therefore, the reaction conditions were optimized for the incorporation of dAVP(SMe)-TP (**2**). The results, shown in Figure 4A, illustrate that each polymerase exhibits a unique signature in incorporating **2** against T and C. Deep vent DNA polymerase can incorporate **2** against T and C; however, the incorporation efficiency was much higher against T than against C (lanes 4 vs 6). Bst, Tth and Taq polymerase can selectively incorporate **2** against T (lanes 8, 12 and 16). Under the same conditions, we performed the primer extension reactions as control experiments using dATP instead of dAVP(SMe)-TP, and the results are shown in Figure 4B. We found that Deep vent DNA polymerase retained its original fidelity but other polymerases lost their original fidelity under these conditions. These results have suggested that all polymerases used in our experiments incorporate dAVP(SMe)-TP against T efficiently under our experimental conditions. However, as dATP was less selectively incorporated except by Deep vent DNA polymerases (Fig. 4B), the experimental conditions should be optimized for applying dAVP(SMe)-TP to the synthesis of long DNA with thermophilic DNA polymerase.

2.4. dAVP(SMe) modified ODN as template

We also investigated the incorporation of the natural 2′-deoxynucleoside-5′-triphosphates (dNTPs; dATP, dCTP, dGTP and dTTP) on a template containing dAVP(SMe) as the terminal nucleobase. The template having the dAVP(SMe) moiety at its 5′ end was prepared by the automated solid-phase synthesis.[17] After annealing, the primer-template duplex was subjected to primer extension in the presence of one single dNTP along with KF, Deep vent, Bst and Taq polymerase in individual reactions, and the results are summarized in Figure 5. It was found that KF, Bst and Taq polymerase selectively incorporated dTTP opposite to dAVP(SMe) (Fig. 5, lanes 4, 12 and 16). On the other hand, Deep vent DNA polymerase incorporated dTTP and dCTP (Fig. 5, lanes 8 and 10). These reaction conditions were suitable for retaining their original fidelity of KF, Deep vent and Bst polymerase, except that Taq polymerase incorporated dTTP against all four bases (data not shown). These results indicate that KF, Bst and Taq polymerase can selectively recognize dAVP(SMe) as an analog of dA and can be used for accurately translating dAVP(SMe) into a T residue during in vitro DNA synthesis. It may be speculated that, as dAVP(SMe) has a hydrogen bonding pattern to dT, KF, Bst and Taq polymerases can recognize dAVP(SMe) as an analog of dA.

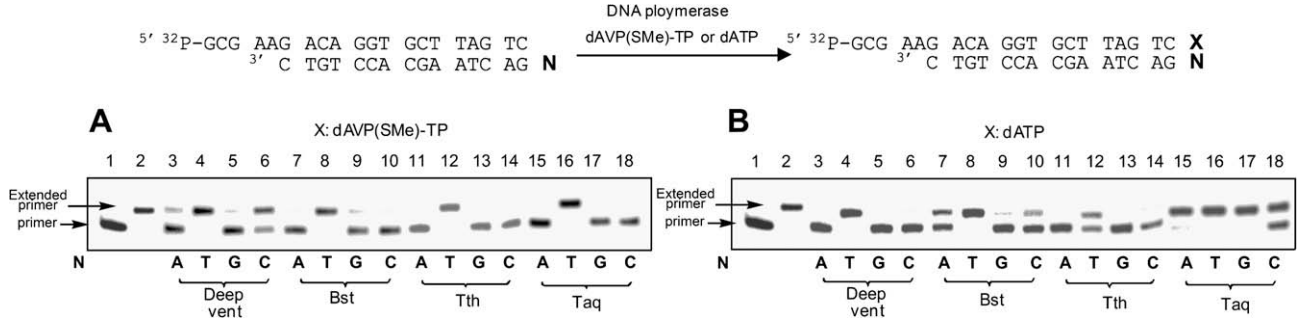


Figure 4. Gel analysis of the primer extension reactions using thermophilic DNA polymerases. The reaction temperatures were: 65 °C for Bst polymerase (0.16 U/μL) and 70 °C for Taq (0.1 U/μL), Tth (0.25 U/μL), and Deep vent DNA polymerases (0.04 U/μL). The reaction time was 15 min.

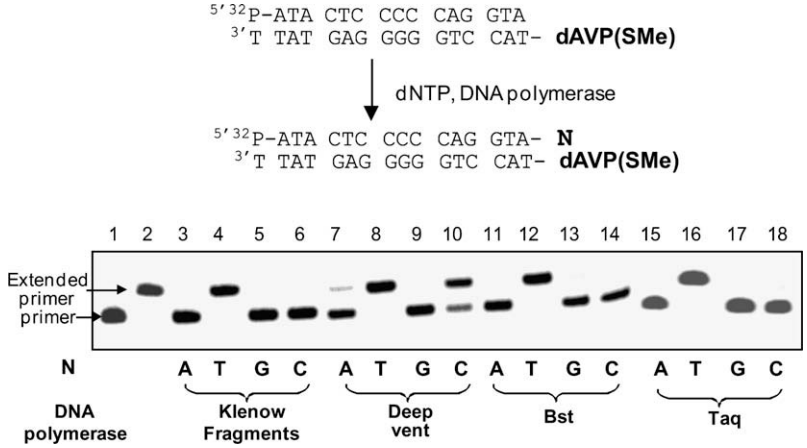


Figure 5. Gel analysis of primer extension reactions on a DNA template containing dAVP(SMe) at 5′-terminal position with 1 mM of each separate dNTP and a ^{32}P-labelled 15-nt primer. The reaction temperatures were: 37 °C for KF (0.1 U/μL), 70 °C for Deep vent DNA polymerases (0.04 U/μL) and Taq (0.1 U/μL), 65 °C for Bst polymerase (0.16 U/μL). The reaction time was 15 min.

3. Conclusion

In this work, we have examined the ability of various DNA polymerases to perform template-directed incorporation of 2′-deoxyribosyl-2-amino-6-(2-methylthioethyl)purine (dAVP(SMe)) into a DNA chain using 2′-Deoxyribosyl-2-amino-6-(2-methylthioethyl)purine-5′-triphosphate (dAVP(SMe)-TP). The polymerases that we have surveyed include exonuclease-free Klenow Fragment (KF) and thermophilic Deep vent, Bst, Taq and Tth DNA polymerases. We have found that all these DNA polymerases have the ability to incorporate dAVP(SMe)-TP. In particular, KF exhibited both high incorporation efficiency and exclusive selectivity for thymine nucleotide on the template under the condition where the polymerases keep their original fidelity. We have also examined the ability of KF and thermophilic Deep vent, Bst and Taq DNA polymerases to incorporate a natural nucleotide against a dAVP(SMe) unit in a DNA template. All these DNA polymerases have incorporated a natural nucleotide against a dAVP(SMe) unit in a DNA template. We have found that KF, Bst and Taq DNA polymerases selectively incorporate a thymine nucleotide across dAVP(SMe) in the template showing that dAVP(SMe) can be recognized as an analog of dA by these polymerases. Although the enzyme reaction conditions should be optimized for application of dAVP(SMe)-TP to polymerase chain reaction (PCR), these results show the possibility for the synthesis of DNA molecules containing the useful dAVP(SMe) by PCR with Deep vent or Bst DNA polymerase. Further study for applying dAVP(SMe) triphosphate substrate in the PCR amplification process is ongoing.

4. Experimental

4.1. General

Unmodified oligodeoxynucleotides (ODNs) were purchased from Integrated DNA Technologies (IDT) and purified by denaturing polyacrylamide gel electrophoresis (PAGE). The dAVP(SMe) modified ODN was synthesized in our lab by conventional phosphoramidite chemistry and purified by high-performance liquid chromatography (HPLC) using a JASCO apparatus that consisted of a UV–vis detector (UV-2075 plus intelligent UV–vis detector), a quaternary gradient pump (PU-2089 plus), and a column oven (CO-2065 plus). HPLC was performed using nacalai tesque COSMOSIL 5C18-MS-II (4.6 × 250 mm) and a MonoQ column. The structure was confirmed by MALDI-TOF MS spectrometry (BRUKER REFLEX-III). The ^{1}H NMR (400 MHz) spectra were recorded on a JEOL LAMBDA 400. Chemical shift values (δ) are reported relative to H_3PO_4 (85%) for the ^{31}P NMR (external standard). ESIMSs were recorded using a BioTOF II mass spectrometer (Bruker Daltonics). The ultraviolet–visible (UV–vis) absorption spectra were recorded by a JASCO V-550 UV–vis or GENESIS 10uv scanning system (Thermo Electron Corporation). [γ-^{32}P] ATP (370 Mbq/L) was obtained from Perkin Elmer.

A gel electrophoresis apparatus (CBS Scientific, Model # 400) was used to conduct PAGE analyses. T4 polynucleotide kinase (PNK), T4 DNA ligase, and the Klenow Fragment (exo$^-$) were purchased from MBI Fermentas. The dNTP mix (PCR grade) was also purchased from MBI Fermentas. The Deep Vent (exo$^-$) and Bst DNA polymerases were purchased from New England BioLabs. Taq DNA polymerase was obtained from Invitrogen and Tth DNA polymerase was purchased from Interscience Inc. The corresponding buffers came along with the enzymes from the suppliers.

4.2. Preparation of AVP(SMe)-TP

To a solution of 2-phenoxyacetylamino-9-(2′-deoxy-D-ribofranosyl)-6-(2-methylthioethyl)purine (**1**) (52 mg, 0.13 mmol) and 1,8-bis-(dimethylamino)naphthalene (49 mg, 0.23 mmol) in trimethyl phosphate (1.0 mL), phosphorous oxytrichloride (17 μL, 0.18 mmol) was added dropwise at 0 °C. After the mixture was stirred at 0 °C for 1 h, a solution of tributylammonium pyrophosphate (360 mg, 0.76 mmol) and tributylamine (153 μL, 0.64 mmol) in anhydrous DMF (1.0 mL) was added. The reaction was stirred at room temperature for 2 h, then 1 M triethylammonium bicarbonate buffer (5 mL, pH 7.5) was added. After stirring at room temperature for 14 h, the mixture was concentrated under vacuum. The residue was dissolved in an ammonium hydroxide solution and stirred for 3 h at room temperature. Lyophilization produced the crude product, which was applied to a MonoQ column [buffer A: 20 mM TEAB buffer (pH 7.5); buffer B: 1 M TEAB buffer (pH 7.5); linear gradient: 0–35% of buffer B in 40 min; flow rate: 1 mL/min] and the fractions containing dAVP(SMe)-TP (t_R = 13.4 min) were collected and lyophilized. The residue was further purified by RP-HPLC (HPLC conditions: nacalai tesque COSMOSIL 5C18-MS; buffer A: 0.1 M TEAB (pH 7.5), solvent B: CH_3CN; linear gradient: 0–30% of B in 40 min; flow rate: 3.5 mL/min) and the fractions containing dAVP(SMe) (t_R = 21.3 min) were collected and lyophilized. The sodium salt of the triphosphate was prepared for the biological experiments: To a solution of the triphosphate dissolved in aqueous MeOH was added a solution of 0.5 M $NaClO_4$ in acetone (0.1 mL). After standing at room temperature for 5 min, acetone (5 mL) was added and the mixture was cooled to −80 °C for 30 min. After spinning down, the solvent was decanted and the pellet was resuspended in acetone (5 mL) and centrifuged as above, and the solvent decanted to remove the excess $NaClO_4$. This acetone-washing process was repeated twice. Evaporation of the remaining acetone under vacuum gave the final sodium salt of dAVP(SMe)-TP (5.6 mg, 6% yield). ^{1}H NMR (400 MHz, D_2O) δ 8.37 (s, 1H), 6.42 (t, J = 6.8 Hz, 1H), 4.25–4.12 (m, 3H), 3.26 (t, J = 6.8 Hz, 1H), 2.99 (t, J = 7.3 Hz, 2H), 2.86–2.79 (m, 1H), 2.51 (ddd, J = 13.9, 6.3, 3.4 Hz, 1H), 2.07 (s, 3H); ^{31}P NMR (D_2O) δ −7.9 (d, J = 19.7 Hz), −9.9 (d, J = 19.6 Hz), −21.4 (t, J = 19.9 Hz); ESIMS (M−H) for $C_{13}H_{21}N_5O_{12}P_3S$: calcd 564.0, found 563.8; δ_{max} (H_2O) = 303 nm, ε_{303} = 4.83 (cm^{-1} mM^{-1}).

4.3. Preparation of ^{32}P-5′-end-labelled DNA

DNA was radioactively labelled at the 5′ end following slightly modified procedure provided by MBI Fermentas. Briefly, 100 pmol of an ODN was placed in a 0.5 mL microcentrifuge tube containing 16 μL of ddH_2O, to which 2 μL of PNK buffer was added, followed by 1 μL of [γ-^{32}P]ATP. Ten units (1 μL) PNK was then added and the volume was adjusted to 20 μL with ddH_2O. The mixture was incubated at 37 °C for 30 min, and then treated by heating at 90 °C for 5 min. The labelled DNA was purified by 10% denaturing PAGE. After drying by a speedvac, the DNA was dissolved in 100 μL of ddH_2O and stored at −20 °C until use.

4.4. Primer extension reactions with dAVP(SMe)-TP

The typical volume of the primer extension reaction was 10 μL. The primer and template were dissolved in ddH_2O at the final concentration of 2 μM for the primer and 2.2 μM for the template. A trace amount of radioactive primer was also added to the mixture and the volume was adjusted to 7.0 μL with ddH_2O. This solution was heated at 90 °C for 1 min and then cooled to room temperature for 20 min. One microlitre of 10X polymerase reaction buffer was then added to the mixture, followed by 1 μL of dAVP(SMe)-TP (10 mM stock solution). Finally, 1 μL of the relevant DNA polymerase (diluted in 1× reaction buffer as needed) was added (Klenow Fragment (0.1 U/μL) Bst polymerase (0.16 U/μL), Taq (0.1 U/μL), Tth (0.25 U/μL) and Deep vent DNA polymerases (0.04 U/μL)). (It is to be noted here that the

concentrations shown above are the final concentrations in the reaction mixture). The mixture was gently mixed by a pipette and incubated for 15 min at 37 °C for the Klenow Fragment, 65 °C for Bst polymerase, and 70 °C for Taq, Tth and Deep vent polymerases. Each reaction was quenched by adding 10 μL of the 2× gel-loading buffer (180 mM Tris, 180 mM boric acid, 20% sucrose (w/v), 0.05% xylene cyanol and 0.05% bromophenol blue, 16 M urea and 80 mM EDTA (pH 8.0, 25 °C), followed by heating at 90 °C for 5 min. These mixtures were analyzed by 20% denaturing PAGE. The DNA bands were visualized by autoradiogram using a Storm 820 scanner.

4.5. Primer extension reactions with natural dNTPs against dAVP(SMe) modified ODN

The typical primer extension reaction was conducted in a 10 μL volume. The modified template incorporating the dAVP(SMe) at the 5′ end was synthesized by an automated DNA synthesizer following the standard phosphoramidite chemistry, and purified by RP-HPLC. The primer was radioactively labelled at the 5′ end following the same protocol as described in Section 4.3. The primer and template were mixed in equimolar concentrations, including a trace amount of radioactive primer, in a 7.0 μL volume with ddH_2O. This mixture was heated at 90 °C for 1 min and cooled to room temperature for 20 min. One microlitre of a 10× polymerase reaction buffer was added to the reaction followed by 1.0 μL of each standard deoxyribo-nucleoside 5′-triphosphate (dATP, dGTP, dCTP and dTTP, 10 mM stock solution). Finally, 1 μL of the enzyme (diluted to a concentration in the 1× reaction buffer as needed) was added. The mixture was gently mixed by a pipette and incubated for 15 min at 37 °C for the Klenow Fragment (0.1 U/μL) and at 70 °C for Deep vent DNA polymerases (0.04 U/μL) and Taq (0.1 U/μL), 65 °C for Bst polymerase (0.16 U/μL). (It is to be noted here that the concentrations shown above are the final concentrations in the reaction mixture.) The subsequent procedures (the stoppage of the reaction, gel analysis and visualization) were identical to the ones described in Section 4.4.

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