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Enzymatic Polymerization of Phosphonate Nucleosides

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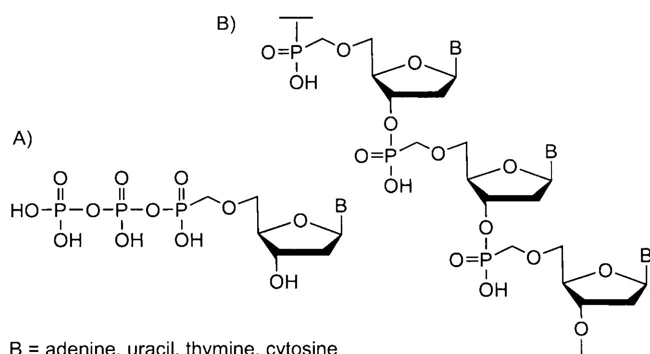
5′-O-Phosphonomethyl-2′-deoxyadenosine (PMdA) proved to be a good substrate of the Therminator polymerase. In this article, we investigated whether the A, C, T and U analogues of this phosphonate nucleoside (PMdN) series can function as substrates of natural DNA polymerases. PMdT and PMdU could only be polymerized enzymatically to a limited extent. Nevertheless, PMdA and PMdC could be incorporated into a DNA duplex with complete chain elongation by all the DNA polymerases tested. A mixed sequence of four nucleotides containing modified C, T and A residues could be obtained with the Vent(exo$^-$) and Therminator polymerases. The kinetic values for the incorporation of PMdA by Vent(exo$^-$) polymerase were determined; a reduced K_M value was found for the incorporation of PMdA compared to the natural substrate. Future polymerase directed evolution studies will allow us to select an enzyme with a heightened capacity to process these modified DNA building blocks into modified strands.

Introduction

We have recently described the efficient enzymatic synthesis of phosphonate oligonucleotides by a mutant of the 9°N DNA polymerase, Therminator (EC 2.7.7.7).[1] A DNA duplex with a 20-mer dT overhang could be elongated to completion with 5′-*O*-phosphonomethyl-2′-deoxyadenosine (PMdA) by this enzyme. In an effort to further explore the potential of PMdN oligomers as biologically active compounds (in infections, cancer or immunology) and their possible use in biotechnological applications, the substrate capacities of three other bases (PMdC, PMdT and PMdU) of this phosphonate nucleoside (PMdN; Scheme 1) series were analyzed with natural DNA polymerases as the catalyzing agents. The substrate capacity for DNA polymerases of PMdG, which is much more difficult to synthesize, was not investigated here. A much-studied application of nucleoside analogues with an altered phosphate backbone is the phosphorothioate DNA,[2,3] the dATP analogue of which can be copolymerized with dTTP opposite a poly d(AT) template by the Klenow fragment and Taq polymerase (EC 2.7.7.7).[4,5] Modifications at the β and γ positions of deoxynucleoside triphosphates led to different results[6–9] with respect to the recognition of modified pyrophosphate groups by polymerases.



Scheme 1. Structure of a PMdN A) nucleotise triphosphate analgues and B) oligomer.

Although the recognition of nucleosides with a phosphonate bond in the α position by DNA polymerases has been described, oligomers of substantial length with a stable phosphonate bond have not been synthesized successfully. An enzyme-catalyzed synthesis would create opportunities for new applications, especially for these modified oligomers which are difficult to synthesize chemically.[10–15] Modified DNA analogues could be used as therapeutic oligonucleotides[3] or in synthetic biology.[16] A new genetic system with seven instead of six intramolecular bonds that connect two successive phosphorous atoms and a nuclease-resistant phosphonate backbone could potentially display new properties, which could lead to substantial progress in biotechnological research.[17]

The advantage of PMdNs for the synthesis of modified oligonucleotides is that, once incorporated, these nucleotide analogues are not easily removed enzymatically from the 3′ end by depolymerisation.[18] In addition, the evaluation of the impact of the phosphonate bond on the replication efficiency of a natural enzyme could reveal essential information about this linchpin of the cellular replication machinery.

The most frequent method of investigating the interaction of a DNA polymerase with a modified substrate is the primer extension reaction. The DNA elongation properties of the

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PMdNs were studied by using the method described extensively by Creighton and Goodman.[19,20] To compare the incorporation efficiency of both the natural dATP and its phosphonate counterpart, the kinetic values were determined by means of steady-state kinetic measurements with the single completed hit model, as described by Creighton and co-workers.[19,21]

This study is aimed at finding a useful, DNA-dependent, PMdN polymerase. If phosphonate oligonucleotides of substantial length could be obtained enzymatically, then the directed evolution of a phosphonate-nucleic-acid-dependent DNA polymerase would be possible.[22,23] A third type of nucleic acid could then be propagated in vivo.[24]

Results

Elongation experiments

Earlier research showed that PMdA was a good substrate of Therminator polymerase,[25] an enzyme specifically designed to incorporate modified building blocks into a DNA duplex.[26] In this study PMdA and the pyrimidine derivatives (PMdC, PMdT and PMdU) of the series are evaluated as substrates for DNA polymerases.

We measured the incorporation of the PMdA, PMdT, PMdC and PMdU by Therminator (a mutant of the 9°N (*exo*$^-$) polymerase, a family B polymerase, EC 2.7.7.7), HIV RT (reverse transcriptase family, EC 2.7.7.49), Vent(*exo*$^-$) polymerase (a family B polymerase, EC 2.7.7.7), Taq polymerase (a family A polymerase, EC 2.7.7.7) and Tth polymerase (a family A polymerase, EC 2.7.7.7). The incorporation capacities were evaluated with the primer–template complexes shown in Table 1. The building blocks were incubated at the appropriate temperature at 1 mM with the primer–template complex and 0.2 U μL^{-1} of enzyme. Samples were taken after one or two hours and analyzed by polyacrylamide gel electrophoresis. The polymerization of PMdU resulted only with Vent(*exo*$^-$) polymerase in the elongation of the primer with two consecutive modified nucleotides (data not shown). Primer elongation could not be determined with the other polymerases. Results from the enzymatic incorporations of PMdT and PMdA are shown in Figure 1.

Table 1. Overview of the primer–template complexes used in the DNA polymerase reactions. Bold letters indicate the template overhang in the hybridized primer–template duplex.

Kinetic experiments		
A	P_1	5′-AGGAAACAGCTATGACTG-3′
	T_1	3′-GTCCTTTGTCGATACTGAC**TGAAAAA**-5′
Elongation experiments		
A	P_2	5′-GGGTACGACTCACTATAGGGAGAGG-3′
	T_2	3′-CCCATGCTGAGTGATATCCCTCTCC**TTTTTTTTTTT-TTTTTTTTT**-5′
T, U	P_2	5′-GGGTACGACTCACTATAGGGAGAGG-3′
	T_3	3′-CCCATGCTGAGTGATATCCCTCTCC**AAAAAAAAAAA-AAAAAAAAA**-5′
C+A	P_2	5′-GGGTACGACTCACTATAGGGAGAGG-3′
	T_4	3′-CCCATGCTGAGTGATATCCCTCTCC**TGTGTGTGTGT-GTGTGTGTG**-5′
A+T	P_2	5′-GGGTACGACTCACTATAGGGAGAGG-3′
	T_5	3′-CCCATGCTGAGTGATATCCCTCTCC**TATATATATAT-ATATATATA**-′
C+M+A+T	P_2	5′-GGGTACGACTCACTATAGGGAGAGG-3′
	T_6	3′-CCCATGCTGAGTGATATCCCTCTCC**AGTTGAGTAAG-TATGAGTGA**-5′

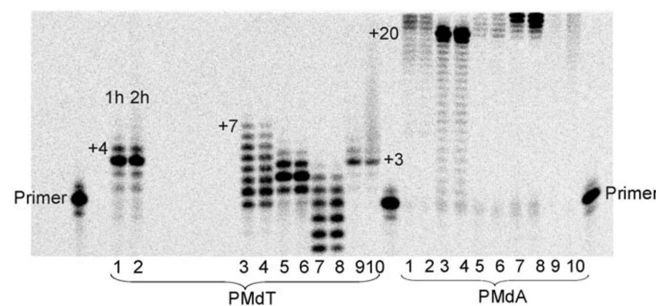

Figure 1. Incorporation of PMdT and PMdA into P_2T_3 and P_2T_2, respectively, after one or two hours by Therminator (lanes 1 and 2), HIV RT (lanes 3 and 4), Vent(*exo*$^-$; lanes 5 and 6), Taq (lanes 7 and 8) and Tth polymerase (lanes 9 and 10).

The difference in incorporation efficiency between the analogues was very striking. PMdA facilitated the elongation of the primer to completion. Due to residual terminal transferase activity, some of the enzymes could elongate the primer with even more building blocks. With PMdT, only the Vent(*exo*$^-$) and Therminator polymerases (both family B polymerases) could extend the primer with two building blocks. Therminator polymerase could extend the primer with three building blocks. HIV RT could elongate the primer up to the primer +7 position. However, the low intensity of the spots on the gel indicates that this condensation is only moderately efficient.

As a poly dG template strand forms unwanted secondary structures it was not possible to determine the incorporation of a series of consecutive PMdCs. Therefore, the P_2T_4 complex (Table 1) was used to determine whether a mixed AC sequence could be obtained. PMdA alone did not incorporate opposite the P_2T_4 poly d(TG) overhang (data not shown), but complete elongation was observed with an equimolar mixture of PMdA and PMdC; this demonstrates comparable incorporation capacity for PMdA and PMdC opposite a d(TG) alternating template (a neighbouring group effect is assumed to play a role in the incorporation efficiency of modified nucleotides).

The formation of mixed sequences containing modified T residues in the DNA duplexes P_2T_5 and P_2T_6 clearly showed the different capacities of the family B polymerases compared to those of family A and RT members to incorporate PMdNs into a DNA duplex. Taq polymerase and HIV RT could only incorporate one building block in the case of the poly d(TA) overhang, and zero or one molecule, respectively, in the case of the mixed d(AGT) overhang. The Therminator polymerase showed the incorporation of four residues in both cases (poly-d(TA) and poly-d(AGT) overhang). Vent(*exo*$^-$) polymerase incorporated two to four alternating A and T residues and showed a weak incorporation of up to four residues opposite the poly-d(AGT) overhang (data not shown).

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As family B polymerases had a higher capacity to incorporate the PMdNs than the polymerases of the other families, the concentration-dependent elongation of the primer with the modified building blocks was studied with Vent(*exo*$^-$) polymerase. The incorporation of PMdA, PMdT and PMdC separately or in combination at a concentration of 1 mM, 100 μM or 10 μM of PMdN is shown in Figure 2. A decrease in incorporation with decreasing concentration of the building blocks (from 1 mM–10 μM) was observed.

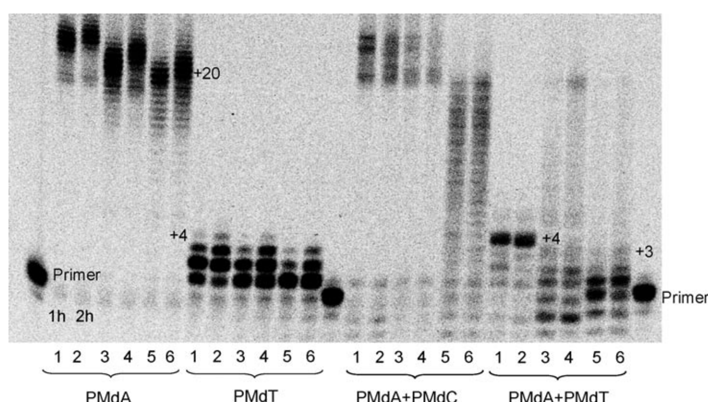

Figure 2. Incorporation of PMdA, PMdT, PMdA + PMdC and PMdA + PMdT into P_2T_2, P_2T_3, P_2T_4 and P_2T_5, respectively, after one or two hours by Vent(*exo*$^-$) DNA polymerase. The concentration of the building blocks was decreased from 1 mM (lanes 1 and 2) to 100 μM (lanes 3 and 4) to 10 μM (lanes 5 and 6).

Kinetics experiments

The kinetic parameters for the incorporation of both the natural and the PMdNs were determined on the basis of the single completed hit model;[21] P_1 and T_1 were used as the priming and templating DNA strand, respectively. Vent(*exo*$^-$) DNA polymerase was used as the polymerizing agent. The kinetic values K_M and V_{max} and the derived values k_{cat} and k_{cat}/K_M for the incorporation of the nucleotides are given in Table 2.

Although the V_{max} values obtained with the natural and modified substrate were similar, a large increase in the K_M value for the incorporation of PMdA was observed. The affinity of the enzyme for the modified substrate was greatly reduced upon substitution of the natural P–O bond with the more stable phosphonate bond or by increasing the number of bonds between two successive phosphorous atoms from six to seven. The k_{cat} value (catalytic efficiency) was reduced by a factor of 2.25; this indicates that the incorporation of the modified substrate was 2.25 times more difficult than the incorporation of the natural substrate. The specificity constant k_{cat}/K_M was decreased by a factor of 16.6; this indicates that the enzyme prefers the natural substrate to its phosphonate analogue for incorporation into a DNA strand when a mixture of both molecules is present. These results show that although the PMdNs were incorporated rather efficiently into a growing DNA strand, the affinity of the enzyme for these substrates was substantially reduced, and the enzyme preferred the natural dATP as a substrate when given a choice.

Table 2. The kinetic parameters of the incorporation of the natural nucleotide (dAMP) and the phosphonate nucleoside (PMdA) into P_1T_1 by Vent(*exo*$^-$) DNA polymerase.

	K_M [μM]	V_{max} [nM min^{-1}]	k_{cat} [min^{-1}]	k_{cat}/K_M
dAMP	1.51 ± 0.24	16.175 [Vent(*exo*$^-$)pol] = 0.001 U μL^{-1} = 0.4 nM	40.425	26.77
PMdA	43.92 ± 0.69	17.925 ± 1.35 [Vent(*exo*$^-$)pol] = 0.0025 U μL^{-1} = 1 nM	17.925 ± 1.35	0.4081

Discussion

The general mechanism, by which DNA replication fidelity is established is relatively well known.[27] Structural studies of DNA polymerase complexes[28–32] in combination with extensive enzyme kinetic studies[27,33,34] have revealed the dominant mechanistic and structural features that contribute to accurate DNA replication, which are largely shared by all polymerases. The minor groove amino-acid residues involved in the binding of the nucleotide substrate and the conformational changes of the polymerase, which moves from an "open" to a "closed" form, provide a series of checkpoints that stall the replication cycle upon incorporation of a mismatch, are well described.[35–37] Nevertheless, the mechanistic and geometric constraints that result in the preference of the enzyme for one misinsertion over another are not well understood. Moreover, variable behaviour of polymerases in relation to a specific mismatch is observed.[38,39] More data are needed in order to elucidate the complete and detailed mechanism of the polymerase action.

In this article, we investigated whether PMdA, PMdC, PMdT and PMdU can function as substrates of natural DNA polymerases. The various polymerases showed a marked difference in PMdN recognition. The reasons for the divergent acceptance of this specific modification by the polymerases remain obscure. However, the observed discrepancy warrants a few comments.

PMdA and PMdC could be incorporated into a DNA duplex with complete chain elongation by all the DNA polymerases tested. PMdT and PMdU could only be polymerized enzymatically to a limited extent. These results are rather surprising and might be explained by a weaker binding of PMdT and PMdU to the enzyme, caused by a poorer fit of the nucleotide analogues in the active site compared to that of the natural substrates, dATP and dCTP. This poorer fit could result in the stalling of the catalytic replication cycle at the active site or the misalignment of the incoming nucleotide with the terminal 3′ OH group of the primer strand, and thereby inhibit the formation of the phosphodiester bond.[27,36,37,40–44] Polypurine single strands are known to stack better than polypyrimidine

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strands,[45] which results in a more efficient incorporation of adenine opposite thymine than thymine opposite adenine by polymerases into a DNA duplex.[46] Therefore, the influence of stacking on the efficiency of the replication process should not be disregarded. However, we do not know whether the difference in stacking can explain the results described above.

PMdT could be most efficiently incorporated by family B polymerases. HIV RT could produce substantially lengthy stretches of the T nucleotide analogue, though with low processivity. It has previously been suggested that the RT enzyme might have a more flexible active site or a more open binding cleft than other polymerases; this could reduce the stringency of the substrate fit and explain this result.[47,48] The low processivity observed and the poor polymerization of the mixed AT and TCA sequences in the presence of HIV RT could be the result of a distorted conformation of the enzyme–DNA duplex substrate complex after the first "wrong" incorporation, which prevents further synthesis.[29,36,37] In the mixed AT and TCA sequences, the superior ability of family B polymerases to incorporate PMdNs into a growing DNA duplex in comparison to that of family A members could be due to the presence of more hydrophilic residues in the active site of the family A polymerases compared to those of the family B polymerases.[49–51]

Another interesting observation is the difference in substrate recognition between the Taq and the Tth polymerase. Although they are enzymes from the same family and share about 87% sequence identity, the Tth polymerase accepts the PMdNs much better as a substrate. Tth has a higher capability of mismatch extension than does Taq polymerase and incorporates ribonucleotide molecules more easily in the presence of manganese ions.[52] Further research needs to be carried out in order to explain the discrepancy in substrate acceptance between the two polymerases.

Kinetic evaluation demonstrated that PMdA can be incorporated by the Vent(*exo*$^-$) polymerase with a similar maximum velocity as that observed with the natural substrate; however, decreased affinity of the enzyme for the modified nucleoside led to a lower catalytic efficiency of incorporation. The decreased specificity constant indicates that the enzyme prefers the natural substrate to the PMdN with a factor of 16.6. The large increase in K_M for the incorporation of the unnatural substrate might be explained by a lower binding affinity of the substrate for the active site, a poorer fit in the enzyme pocket, a decreased stacking ability with the template base or the incorrect positioning of the incoming nucleotide. Even a slight deviation from the required geometry for nucleotide incorporation could lead to a decreased efficiency of the DNA replication process.[50,53]

Conclusions

We can conclude that the polymerases we tested were not capable of catalyzing the synthesis of more than four phosphonate residues of a mixed ACT sequence. In order to establish a new nucleic acid replicating system, it is necessary to select and develop polymerases with new and better PMdN recognition functions. A new information system with enhanced stability for in vivo use could widen and improve the possible functional applications in synthetic biology.

Experimental Section

Synthesis of 5′-*O*-phosphonomethyl-2′-deoxyribosyl nucleoside triethylammonium salts: The PMdNs were synthesized by following the procedure described by Krečmerová et al.[54,55]

Synthesis of 5′-*O*-diphosphorylphosphonomethyl-2′-deoxyribosyl nucleoside triethylammonium salts: The diphosphate derivatives of the PMdNs were prepared by using the CDI approach as described in.[25] Purification was carried out by using a Source 15Q ion-exchange column (Amersham Biosciences) with a TEAB concentration gradually increasing from 0–0.5 M over 30 min. Additional purification to remove inorganic pyrophosphate residues was carried out on a reversed-phase polystyrene-divinylbenzene column (PLRP-S 100 Å, 8 μM, Achrom, Machelen–Zulte, Belgium) with a gradient from 7 to 22% CH_3CN over 30 min in the presence of TEAA (50 mM).

5′-O-Diphosphorylphosphonomethyl-2′-deoxyadenosine triethylammonium salt: ^{31}P NMR δ (ppm; D_2O): 8.7177 (d, α-P, $J_{\alpha,\beta}$=26.0485 Hz), −10.8510 (d, γ-P, $J_{\beta,\gamma}$=19.8766 Hz), −23.3483, (t, β-P, $J_{\alpha,\beta}$=26.3887; $J_{\beta,\gamma}$=20.0345 Hz). Exact mass calcd for $C_{11}H_{17}N_5O_{12}P_3$ [*M*−H]=504.0092; found 504.0093.

5′-O-Diphosphorylphosphonomethyl-2′-deoxythymidine triethylammonium salt: ^{31}P NMR δ (ppm; D_2O): 8.4654 (d, α-P, $J_{\alpha,\beta}$=26.7167 Hz), −11.0138 (d, γ-P, $J_{\beta,\gamma}$=19.8523 Hz), −23.4766 (t, β-P, $J_{\alpha,\beta}$=26.5406; $J_{\beta,\gamma}$=20.2593 Hz). Exact mass calcd for $C_{11}H_{18}N_2O_{14}P_3$ [*M*−H]=494.9976; found 494.9972.

5′-O-Diphosphorylphosphonomethyl-2′-deoxycytidine triethylammonium salt: ^{31}P NMR δ (ppm; D_2O): 8.4051 (d, α-P, $J_{\alpha,\beta}$=26.1578 Hz), −10.6542 (d, γ-P, $J_{\beta,\gamma}$=19.9616 Hz), −23.4074 (t, β-P, $J_{\alpha,\beta}$=25.9331; $J_{\beta,\gamma}$=20.1985 Hz). Exact mass calcd for $C_{10}H_{17}N_3O_{13}P_3$ [*M*−H]=479.9980; found 479.9976.

5′-O-Diphosphorylphosphonomethyl-2′-deoxyuridine triethylammonium salt: ^{31}P NMR δ (ppm; D_2O): 8.3862 (d, α-P, $J_{\alpha,\beta}$=26.4373 Hz), −10.8598 (d, γ-P, $J_{\beta,\gamma}$=20.1074 Hz), −23.4282 (t, β-P, $J_{\alpha,\beta}$=26.4373; $J_{\beta,\gamma}$=20.1074 Hz). Exact mass calcd for $C_{10}H_{16}N_2O_{14}P_3$ [*M*−H]=480.9820; found 480.9811.

DNA polymerase reactions: Primers and templates were purchased from Sigma–Aldrich. The sequences are given in Table 1.

^{33}P-Labelling of the primer and hybridisation with template: Unlabelled primer (100 pmol) was added to a reaction mixture containing ^{33}P-γ-ATP (2.5 μL of a 370 MBq mL^{-1} (10 mCi mL^{-1}) solution, Perkin–Elmer), T4 polynucleotide kinase (45 U; Amersham Biosciences, EC 2.7.1.78), T4 kinase buffer and water (total volume of 25 μL). After incubation of the mixture (37 °C for 1 h), the kinase was inactivated by heating the solution (95 °C for 5 min). The solution was loaded onto a prespun microspin G-25 column (Amersham Biosciences) and centrifuged (2 min at 3000 rpm). A 5′-^{33}P-labelled solution of primer (4 μM) in water was obtained.

Annealing of the 5′-^{33}P-γ-labelled primer to the template (in a mole ratio of 1:2.5 primer/template) was carried out by adding labelled primer (27 μL of the 4 μM solution) to a template strand (2.7 μL of a 100 μM solution) in a total volume of 72 μL and heating the mixture (95 °C for 10 min). The mixture was then allowed to slowly cool to room temperature. The final concentration of the primer–template hybrid was 1.5 μM. When elongation reactions were car-

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ried out, this solution was mixed with water and a 10× concentrated solution of reaction buffer to obtain a hybrid mix with a concentration of 250 nM primer–template complex (5× concentrate). The final concentration of the primer–template mix for the kinetic experiments was 625 nM (2.5× concentrate).

Elongation experiments: Therminator™ DNA polymerase (EC 2.7.7.7), Vent(*exo*⁻) DNA polymerase (EC 2.7.7.7) and Taq DNA polymerase (EC 2.7.7.7) were purchased from New England BioLabs. HIV RT (EC 2.7.7.49) was purchased from Amersham Biosciences and Tth Polymerase (EC 2.7.7.7) was purchased from Eurogentec.

Nucleotide building-block solution was added to a preheated reaction mixture containing water, buffer (Thermopolbuffer 10× concentrate: 200 mM Tris-HCl, pH 8.8 at 25 °C, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 100 mM KCl, 1% Triton X-100 for Therminator, Tth, Vent(*exo*⁻), or Taq polymerizations and AMV RT reaction buffer 5× concentrate: 250 mM Tris-HCl, pH 8.3, 40 mM $MgCl_2$, 250 mM NaCl, 5 mM DTT for reactions with HIV RT), DNA polymerase and primer–template complex (final volume 10 μL). Nucleotide building blocks were used in a final concentration of 1 mM, 100 μM or 10 μM. Final concentrations were 50 nM and 0.2 U μL^{-1} for the primer–template complex and the DNA polymerases, respectively. The reactions were performed at 75 °C for Therminator, Tth, Taq and Vent(*exo*⁻) DNA polymerase, and at 37 °C for HIV RT. Reactions were quenched after one or two hours by mixing 1 μL with quenching buffer (2 μL) containing formamide (80%), EDTA (2 mM) and TBE buffer (1×). The samples were heated (95 °C for 5 min) prior to analysis by electrophoresis. Reactions carried out with Tth polymerase contained Mn^{2+} ions (1 mM final concentration).

Kinetic experiments: To determine the kinetic parameters for the incorporation of both the natural and modified building blocks into a DNA duplex by DNA polymerase, the single completed hit model was used.[19,21] Pseudo-first-order conditions were created by determining the constants for the nucleotide substrates in the presence of excess primer–template complex. The experiments were carried out with Vent(*exo*⁻) DNA polymerase. The 5′-^{33}P-γ-labelled primer P_1, which was annealed to the temple strand T_1, was used to evaluate the natural and modified nucleotide as a substrate of the polymerase. A range of building block concentrations between 1 mM and 5 μM for the PMdN derivative, and between 50 μM and 0.5 μM for the natural building block were used for extension. The final concentrations of primer–template complex and DNA polymerase were 250 nM and 0.005 (for PMdA) or 0.001 U μL^{-1} (for dATP), respectively. Thermopolbuffer (for the composition see above) was used as the reaction buffer. Reactions were initiated by the addition of the preheated building block solution to the assay mixtures preheated at 55 °C. At various time intervals (between 1 and 10 min) a sample (1 μL) was removed from the reactions for analysis and mixed with quenching buffer (2 μL) containing formamide (80%), EDTA (2 mM) and 1×TBE buffer. The samples were heated (95 °C for 5 min) prior to analysis by electrophoresis. Because of the use of shorter primers and templates in this series, the reactions were carried out at 55 °C to ensure complete hybridisation of the primer and template strands during polymerizations. The reactions were carried out at a suboptimal temperature for the polymerase. Nevertheless, the results allowed us to compare the kinetic parameters of the enzyme for both substrates, as all experiments were carried out under the same conditions.

Plots of the reaction time courses were analyzed for the various concentrations to determine the initial reaction velocities. The plotting of these velocities against the substrate concentrations allowed the fitting of a Michaelis–Menten curve. Data analysis was carried out by regression analysis with the help of GraphPad Prism 5 software. The kinetic values shown were determined as a mean of three independent experiments. The k_{cat} was determined from V_{max} through normalization by the enzyme concentration.

Polyacrylamide gel electrophoresis: Quenched reactions were analyzed and visualized by loading the reaction sample (2 μL) onto a 12% denaturing polyacrylamide gel (acrylamide/bisacrylamide 19:1, 7 M urea, 0.4 mm×30 cm×40 cm) run in a 1×TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.3) at 60 W. The products were visualized by means of phosphorimaging. The relative intensity of the bands corresponding to the products of the enzymatic reactions was determined by means of the OptiQuant image analysis software (Perkin–Elmer).

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Keywords: chain elongation · enzymes · nucleic acids · phosphonate nucleosides · substrate specificity

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