Enzymatic synthesis of perfluoroalkylated DNA

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Thymidine analogues 5 trifluoromethyl, 5 pentafluoroethyl and 5 (heptafluoro n propyl) 2 deoxyuridines were synthesised and converted into the corresponding 5 triphosphates 1a-c. Performing DNA polymerase catalyzed primer extension reactions these modified nucleotides were incorporated into DNA to create perfluoroalkylated nucleic acids. Although single modified nucleotides were enzymatically incorporated and further elongated quite similar to the natural TTP, the enzymatic synthesis of multi modified nucleic acids was initially only feasible with modifications at every fourth base. Nevertheless, as the effects of the modified dUTPs on DNA polymerases varied significantly with the used enzyme, Therminator DNA polymerase was proficient in incorporating 11 adjacent 5 trifluoromethyl 2 deoxyuridine moieties.

1. Introduction

Aside from being the fundamental genetic material for the stor age and transfer of information, DNA has recently gained signifi cant attention as building block in nanotechnology.1 The introduction of chemically modified DNA building blocks may offer the possibility to modify the physical and geometrical properties of DNA. The generation of new DNA based materials through enzy matic synthesis is particularly promising, since this approach has the potential to introduce non nucleic acid like functionalities through usage of modified building blocks and their incorporation into long DNA stretches by DNA polymerases.2 Although this approach has only sparsely been exploited so far,2 several different modifications were successfully incorporated to create highly functionalized DNA double strands.3 Aside from amino acid groups, aromatic, basic, acidic and lipophilic modifications, functionalized dNTPs were also used to introduce Ru(II) and Os(II) complexes into DNA double strands.4 Furthermore, alkyne and azido labelled oligonucleotides offer the possibility to post synthetically modify DNA oligonucleotides by ‘Click’ chemistry or Staudinger ligation.5 Recently, we used DNA polymerases for the construction of DNA based networks6 and site specific multi spinlabelled DNA.7 In addition, modified DNA has been employed in the generation of aptamers and catalytically active DNA.8,9 These entities were obtained by SELEX that requires that the respective modified nucleotides are substrates for DNA polymerases.

Due to its small van der Waals radius and the stability of the highly polarized bond formed with carbon, fluorine is used in var ious approaches in bioorganic and medicinal chemistry.10 Fluorine is highly electronegative and shows unique nuclear magnetic res onance (NMR) spectroscopic parameters. The substitution of hydrogen by fluorine leads to significant electronic effects and can modify properties of a molecule like pKa values, dipole moments or chemical reactivities and stabilities of adjacent functional groups.11 Although fluorine is used as an isosteric replacement for hydrogen, the introduction of fluorine into a molecule leads to ste ric changes as well. This is caused by different van der Waals radii of fluorine (1.47 Å) and hydrogen (1.2 Å) and different bond order.12 The substitution of hydrogen by fluorine leads to significant electronic effects and can modify properties of a molecule like pKa values, dipole moments or chemical reactivities and stabilities of adjacent functional groups.11 Although fluorine is used as an isosteric replacement for hydrogen, the introduction of fluorine into a molecule leads to steric changes as well. This is caused by different van der Waals radii of fluorine (1.47 Å) and hydrogen (1.2 Å) and different bond order.12 The introduction of fluorine into a molecule leads to steric changes as well. This is caused by different van der Waals radii of fluorine (1.47 Å) and hydrogen (1.2 Å) and different bond order.12

Thymidine analogue 5 trifluoromethyl 2 deoxyuridine (CF3dU) has been shown to form regular base pairs with uridine DNA double helices are formed that still adopt the B form conformation.13 Thereby, the substitution of a thymidine residue by CF3dU causes a slight decrease in DNA duplex stability.14 Apart from chemical methods to introduce modified nucleosides into DNA oligonucleotides by using suitable building blocks in automated DNA synthesis, enzymatic incorporation of modified nucleoside 5' triphosphates by DNA polymerases allows the synthesis of long multi modified DNA oligonucleotides.2 Albeit it was claimed that CF3dU 5' triphosphate is a substrate for DNA polymerases in vitro,15 experimental results have not been documented. In addition, the effect of extended C5 perfluoro modifications in 2' dU 5' triphosphates has not been explored so far. Here we present the introduction of 5 trifluoromethyl, 5 penta...
fluoroethyl and 5 (heptafluoro n propyl) dU residues into DNA by exploiting DNA polymerase catalyzed template directed reactions and modified triphosphates 1a-c.

2. Results and discussion

First, we synthesized the 5 perfluoroalkyl 2'-deoxyuridine 5'-triphosphates 1a-c (Scheme 1).

The synthesis started with 2a-c that were synthesized following published procedures. 2a was deacetylated according to the reported procedure by using a 7 M ammonia solution of methanol. However, this method gave unsatisfactory results for 2b and c due to the formation of side products. Due to the known reactivity and instability of the trifluoromethyl group of 5 trifluoromethyluracil towards nucleophiles and under basic conditions, we applied a different method for deacetylation of 2b and 2c. By use of toluene 4 sulfonic acid monohydrate in dichloromethane and methanol, we were able to synthesize 3b and 3c. Compounds 3a-c were subsequently transformed to the nucleoside 5'-triphosphates 1a-c. 18

Next, we explored the action of 1a-c on DNA polymerases and investigated the ability of the Klenow fragment of Escherichia coli DNA polymerase I (3'-5' exonuclease deficient variant, KF(exo-)) and the Klenow fragment of Thermus aquaticus DNA polymerase (KlenTaq) to accept 1a-c in primer extension experiments. There fore, we used a 32P 5' end labelled primer (23 nt)/template (35 nt) complex with an adenosine residue at position 27 of the template to direct the usage of TTP and the thymidine analogues 1a-c, respectively, after incorporation of three natural nucleotides (Fig. 1a).

Subsequently all reactions were analyzed by denaturing poly acrylamide gel electrophoresis (PAGE) and phosphor imaging. In the presence of dGTP, dCTP and dATP and the absence of any TTP analogue, primer elongation was paused at position 27 when using KF(exo-) or KlenTaq (Fig. 1b and c). TTP. When all four natural dNTPs were present, formation of full length products was moni tored with both enzymes (Fig. 1b and c). TTP. Substitution of TTP by 1a-c led with both enzymes to full length products as well (Fig. 1b and c). TTP. The incorporation of an additional nucleotide in a non templated manner led to the 36 nt long products. This has been observed before using 3'-5' exonuclease deficient DNA polymerases. 19

To get first insights into the efficiency and selectivity of the enzymatic incorporation step in particular, we examined the acceptance of 1a-c by KF(exo-) catalysis performing single incorporation experiments in the presence of different concentrations of TTP and 1a-c, respectively. Thus, we used four different 32P 5' end labelled primer (24 nt)/template (36 nt) complexes with all four natural nucleotide residues at the first position after the 3' end of the primer (Fig. 2a). Doing so, we observed two bands after PAGE analysis, the given 24 nt long primer and the elongated one (25 nt) in most cases. In general, incorporation of the modified thymidines was comparable to that of the natural counterpart (Fig. 2). Especially 1a led to similar product yields opposite the four natural nucleotide residues (quantified by phosphor imaging).

![Scheme 1. Synthesis of 5-perfluoroalkylated dU analogues 1a-c. Reagents and yields: (a) NH3·MeOH (7 M), 76% (4a); (b) p-TsOH·H2O, DCM/MeOH (9:1), 74% (3b), 66% (3c); (c) 1,8-bis(dimethylamino)-naphthalene, POC13, (CH2)3PO then (n-Bu,N)H2P2O5, Et,N. 83% (3a), 10% (1b), 16% (1c).](image1)

![Figure 1. Primer extension studies: acceptance of 1a-c by KF(exo-) and KlenTaq (c). (a) Partial primer template sequences employed. (b) and (c) Primer: primer only; TTP: primer extension in the presence of dATP, dGTP and dCTP; +TTP: as TTP but in the presence of TTP; C5dUUTP: as TTP but in the presence of 1a; C5dFuUTP: as TTP but in the presence of 1b; C5dFbUTP: as TTP but in the presence of 1c.](image2)

![Figure 2. Single incorporation employing TTP and 1a-c by KF(exo-) with differing nucleotide concentrations. (a) Partial primer template sequences employed. (b) Incorporation opposite A (matched case, t=5 min); lanes 0: primer only, lanes 1: 1 µM TTP, lanes 2: 10 µM TTP, lanes 3: 100 µM TTP. (c-e) Incorporation opposite C/FT (mismatched cases, t=30 min); lanes 0: primer only, lanes 1: 10 µM TTP, lanes 2: 50 µM TTP, lanes 3: 200 µM TTP.](image3)
Higher concentrations of 1b and 1c inhibited the incorporation opposite the guanosine residue (Fig. 2c, right two panels, lanes 3). Even more unexpected was that TTP, 1a and 1b were not, but 1c was used as substrate for incorporation opposite the non canonical cytosine residue in the template (Fig. 2d).

Next, we investigated the multiple incorporations of the modified nucleotides into DNA by using different templates that call for usage of TTP and 1c, respectively, at every fourth, every second or every singular nucleotide position. Figure 3 shows the results obtained in multiple incorporation experiments by using KF(exo).

Using the template with an adenosine residue at every fourth position, we obtained full length products when natural TTP was substituted by 1a c (Fig. 3a, CF3dUTP, C2F5dUTP, C3F7dUTP). The acceptance of 1a c at every second position led only in the case of 1a to the full length product (Fig. 3b, CF3dUTP). The template with an adenosine residue at every position could not be extended to full length with any of the TTP analogues (Fig. 3c, CF3dUTP, C2F5dUTP, C3F7dUTP). Although the modified nucleotides were incorporated opposite the adenosine residue in comparable yields to the natural counterpart (Fig. 2b), the multiple incorporation of additional nucleotide analogues was inhibited. With employed conditions we obtained reaction products that were only elongated by one or two (1b, 1c) or four incorporated thymidine analogues in the case of 1a (Fig. 3c, CF3dUTP, C2F5dUTP, C3F7dUTP).

However, we found that the effects of 1a c on DNA polymerase assays significantly vary with the used enzyme. It was found that Therminator DNA polymerase (A845L mutant of Thermococcus species 9’N DNA polymerase) is most proficient in processing multiple modifications of these modified nucleotides. In order to form one entire DNA helix turn of 5 perfluoroalkyl deoxyuridines, we examined the multiple incorporation of these modified nucleotides. In order to form one entire DNA helix turn of 5 perfluoroalkyl 2 deoxyuridines, we used a template with eleven adjacent adenosine residues (Fig. 4a).

Due to the known decrease in stability of DNA helices with adjacent CF3dU nucleotides,14 we examined the multiple incorporation experiments at temperatures between 37 and 70 °C. Interestingly, Therminator DNA polymerase is proficient in incorporating eleven adjacent CF3dU nucleotides at about 40 to 60 °C (Fig. 4b, left panel, lanes 3 8). In case of 1b and 1c Therminator DNA polymerase incorporates only about 4 6 adjacent nucleotides under the same conditions (Fig. 4b, middle and right panel). This inhibition of DNA synthesis is in part surprising since it has been shown that bulkier nucleotides are accepted by the same DNA polymerase.2 3 However, the nucleotide perfluoroalkyl modification hampers catalysis to a greater extent. Thereby, the pentfluoroethanol and heptafluoro n propyl modified residues exhibit enhanced inhibition of DNA synthesis in comparison to the CF3dU residue. Perturbation of DNA polymerase/DNA contacts, for example, H bonds between amino acid side chains and the nucleobases which are required for binding and activity of the enzyme might be the origin of this effect.

3. Conclusions

In summary, we report the synthesis of 5 trifluoromethyl, 5 pentfluoroethoxy and 5 (heptafluoro n propyl) 2 deoxyuridine 5 triphosphates 1a c and their usage for site specific introduction of perfluorinated residues into DNA in DNA polymerase catalyzed template directed reactions. Since fluorine has properties that are distinct from other polar or non polar modifications the herein depicted approach presents new routes for the generation of highly modified nucleic acids for future DNA biotechnological applications like in SELEX. Directed evolution of DNA polymerases might be suited for the improvement of enzymes’ efficiencies to process non natural substrates.20

4. Experimental

4.1. General

NMR spectra were recorded on a Bruker AC 250 Cryospec, a Bruker DRX 600 and a Jeol ECOX 400 spectrometer. ESI IT mass spectra were recorded on a Bruker Daltonics esquire 3000+. ESI TOF mass spectra were recorded on a Bruker microTOFQII. All reagents are commercially available and were used without further purification. Solvents were stored over molecular sieves (Fluka) and used directly without further purification.

4.1.1. 5-Trifluoromethyl-2-deoxyuridine 3a

To cleave the acetyl protecting groups, compound 2a (445 mg, 1.17 mmol) was treated with a 7 M ammonia solution of methanol (4.5 mL) for 19 h at room temperature (TLC analysis ETOAc/petroleum ether 5/1). The solvent was removed in vacuo and the crude product was purified by silica gel chromatography (eluent: ETOAc/petroleum ether 5/1) to yield 3a (264 mg, 76%). TLC (ETOAc/petroleum ether 5/1) Rf 0.34. 1H NMR (600 MHz; MeOH d4) δ (ppm) 2.25 2.29 (m, 1H, H2’a), 2.35 2.39 (m, 1H, H2’b), 3.75 (dd, 1H, H5’a, JH5a = 12 Hz, JH5 = 3 Hz), 3.84 (dd, 1H, H5’d, JH5b = 12 Hz, JH5 = 3 Hz), 3.97 (m, 1H, H4’), 4.41 (m, 1H, H3’), 6.24 (dd, 1H, H1’), JH12 = 6 Hz), 8.79 (s, 1H, H6). 13C NMR (151 MHz; MeOH d4) δ (ppm) 42.15, 42.67, 71.73, 87.58, 89.34, 105.35 (q, 1C, C5), JF = 33 Hz), 123.98 (q, 1C, C5), JF = 269 Hz), 143.82 (q, 1C, C6, JF = 6 Hz), 151.37, 161.27. 19F NMR (376 MHz; MeOH d4) δ (ppm) -64.4 (3F, CF3). ESI IT MS calcd for (M H) 295.1, found 294.8. ESI TOF MS calcd for (M H) 295.0542, found 295.0536.

4.1.2. 5-Pentafluoroethoxy-2-deoxyuridine 3b

The deacetylation of compound 2b (140 mg, 0.33 mmol) was performed in dichloromethane and methanol (9:1; 10 mL) with toluene 4 sulfonic acid monohydrate (309 mg, 1.63 mmol) for 3 days at room temperature to get full turnover of the reactant (TLC analysis ETOAc/petroleum ether 5/1). The solvent was removed in vacuo and the crude product was purified by RP MPLC (linear grade from water with 5% acetonitrile to 100% acetonitrile)
to yield 3b (84 mg, 74%). TLC (dichloromethane/methanol 9:1) \( R_f \) 0.30. 1H NMR (600 MHz; MeOH \( d_4 \)) \( \delta \) (ppm) 2.23 2.29 (m, 1H, \( H^2_a \)), 2.37 2.41 (m, 1H, \( H^2_b \)), 3.74 (dd, 1H, \( H^5_a \), \( j_{H^5_H^2} = 12 \) Hz, \( j_{H^5_H^6} = 3 \) Hz), 3.83 (dd, 1H, \( H^6_b \), \( j_{H^6_H^5} = 12 \) Hz, \( j_{H^6_H^7} = 3 \) Hz), 3.98 (m, 1H, \( H^4 \)), 4.31 (m, 1H, \( H^3 \)), 6.24 (dd, 1H, \( H^1 \), \( H^6 \)), 8.80 (s, 1H, \( H^6 \)). 13C NMR (151 MHz; MeOH \( d_4 \)) \( \delta \) (ppm) 22.38, 26.17, 71.85, 79.89, 80.67, 103.21 (t, 1H, \( C^5 \), \( j_{H^5_H^4} = 24 \) Hz), 110.36 (t, 1H, \( C^3 \)), 115.69 (t, 1H, \( C^2 \)), \( j_{H^2_C^3} = 256 \) Hz, \( j_{H^2_H^3} = 33 \) Hz), 119.49 (t, 1H, \( C^1 \)), \( j_{H^1_H^2} = 287 \) Hz, \( j_{H^1_H^6} = 34 \) Hz), 146.19 (t, 1H, \( C^6 \)), \( j_{H^6_H^7} = 12 \) Hz), 151.26, 160.95. 31P NMR (376 MHz; MeOH \( d_4 \)) \( \delta \) (ppm) 127.5 (s, 2F, \( C^5 \)), 111.5 (m, 1F, \( C^2 \)), 82.5 (t, 3F, \( C^3 \)), 70.8 (t, 1H, \( H^6 \)).

**4.1.3. 5-(Heptafluoro-n-propyl)-2'-deoxyuridine 3c**

The deacetylation of compound 2c (161 mg, 0.33 mmol) was performed in dichloromethane and methanol (9:1; 10 mL) with sodium 4 sulfonic acid monohydrate (319 mg, 1.68 mmol) for 3 days at room temperature (TLC analysis EtOAc/petroleum ether 5:1). The solvent was removed in vacuo and the crude product was purified by RP MPLC (linear grade from water with 5% acetonitrile to 100% acetonitrile) to yield 3c (87 mg, 66%). TLC (dichloromethane/methanol 9:1) \( R_f \) 0.36. 1H NMR (600 MHz; MeOH \( d_4 \)) \( \delta \) (ppm) 2.22 2.28 (m, 1H, \( H^2_a \)), 2.35 2.43 (m, 1H, \( H^2_b \)), 3.74 (dd, 1H, \( H^5_a \), \( j_{H^5_H^2} = 12 \) Hz, \( j_{H^5_H^6} = 3 \) Hz), 3.82 (dd, 1H, \( H^6_b \), \( j_{H^6_H^5} = 12 \) Hz, \( j_{H^6_H^7} = 3 \) Hz), 3.98 (m, 1H, \( H^4 \)), 4.41 (dd, 1H, \( H^3 \)), 6.25 (dd, 1H, \( H^1 \)), \( j_{H^1_H^2} = 6 \) Hz), 8.81 (s, 1H, \( H^6 \)). 13C NMR (151 MHz; MeOH \( d_4 \)) \( \delta \) (ppm) 22.38, 62.17, 71.85, 79.89, 80.67, 103.21 (t, 1H, \( C^5 \), \( j_{H^5_H^4} = 24 \) Hz), 110.36 (t, 1H, \( C^3 \)), 115.69 (t, 1H, \( C^2 \)), \( j_{H^2_C^3} = 256 \) Hz, \( j_{H^2_H^3} = 33 \) Hz), 119.49 (t, 1H, \( C^1 \)), \( j_{H^1_H^2} = 287 \) Hz, \( j_{H^1_H^6} = 34 \) Hz), 146.19 (t, 1H, \( C^6 \)), \( j_{H^6_H^7} = 12 \) Hz), 151.26, 160.95. 31P NMR (376 MHz; MeOH \( d_4 \)) \( \delta \) (ppm) 127.5 (s, 2F, \( C^5 \)), 111.5 (m, 1F, \( C^2 \)), 82.5 (t, 3F, \( C^3 \)), 70.8 (t, 1H, \( H^6 \)).

**4.1.4. 5-Trifluoromethyl-2'-deoxyuridine-5'-triphosphate 1a**

The nucleoside 3a (67 mg, 0.23 mmol) and 1.8 bis(dimethyl amino)naphthalene (72 mg, 0.34 mmol) were dried in the dark in vacuo overnight. Then the mixture was dissolved under argon in trimethyl phosphate (1.8 mL) and POCl3 (55 mL, 0.60 mmol) was added dropwise at 0°C. After full turnover of the reactant (TLC analysis 2 propanol/NH3/H2O 3:1/1) a 0.5 M bis tri n butylammonium pyrophosphate solution of DMF (2.25 mL, 1.13 mmol) and n tributylamine (540 mL, 2.27 mmol) were added simultaneously. After stirring for additional 20 min 1 M TEAB buffer (Et3NH(HCO3), pH 7.5) was added (10 mL). The aqueous layer was washed with EtOAc (2×10 mL) and the solvent was removed in vacuo. The residue was resolved in 0.1 M TEAB buffer (3 mL) and purified by sephadex ion exchange chromatography (eluent: linear grade from 0.1 M to 1 M TEAB buffer). The salts were removed by RP MPLC (eluent: grade from 50 mM TEAB buffer (Et3NH(OAc), pH 7.0) with 5% acetonitrile to 100% acetonitrile) to yield 1a (18 mg, 8%, triethyl ammonium salt of the triphosphate). 1H NMR (400 MHz; MeOH \( d_4 \)) \( \delta \) (ppm) 1.30 (t, 3H, \( Me \)), 2.32 (m, 2H, \( H^2 \)), 3.17 (q, 3H, \( CH_3 \)), 4.09 4.22 (m, 2H, \( H^5 \)), 4.31 (m, 1H, \( H^3 \)), 6.16 (dd, 1H, \( H^1 \)), \( j_{H^1_H^6} = 7 \) Hz), 8.29 (s, 1H, \( H^6 \)). 31P NMR (162 MHz; MeOH \( d_4 \)) \( \delta \) (ppm) 22.94 (dd, 1H, \( P_a \)), 21.21 (s, 1H, \( P_b \)), 9.66 (d, 1H, \( P_a \), \( j_{H^2_P_a} = 21 \) Hz), 9.56 (d, 1H, \( P_a \)). 31P NMR (376 MHz; MeOH \( d_4 \)) \( \delta \) (ppm) 64.7 (s, 3F, \( C^5 \)), 67.8 (s, 3F, \( C^2 \)). ESI TOF MS calcd for (M H) \( 534.9 \), found 534.6. ESI TOF MS calcd for (M H) \( 534.9 \), found 534.9.

**4.1.5. 5-Pentafluoroethyl-2'-deoxyuridine-5'-phosphate 1b**

The nucleoside 3b (32 mg, 0.09 mmol) and 1.8 bis(dimethyl amino)naphthalene (30 mg, 0.14 mmol) were dried in the dark in vacuo overnight. Then the mixture was dissolved under argon in trimethyl phosphate (1 mL) and POCl3 (20 mL, 0.22 mmol) was added dropwise at 0°C. After full turnover of the reactant (TLC analysis 2 propanol/NH3/H2O 3:1/1) a 0.5 M bis tri n butylammonium pyrophosphate solution of DMF (0.92 mL, 0.46 mmol) and n tributylamine (220 mL, 0.92 mmol) were added simultaneously. After stirring for additional 20 min 1 M TEAB buffer (Et3NH(HCO3), pH 7.5) was added (10 mL). The aqueous layer was washed with EtOAc (2×10 mL) and the solvent was removed in vacuo. The residue was resolved in 0.1 M TEAB buffer (3 mL) and purified by sephadex ion exchange chromatography (linear gradient from 0.1 M to 1 M TEAB buffer). The salts were removed by RP MPLC (gradient from 50 mM TEAB buffer (Et3NH(OAc), pH 7.0) with 5% acetonitrile to 100% acetonitrile) to yield 1b (9 mg, 10%, triethyl ammonium salt of the triphosphate). 1H NMR (400 MHz; MeOH
1.4.6. 5-(Heptfluoro-n-propyl)-2-deoxyuridine-5'-triphosphate 1c

The nucleoside 3c (31 mg, 0.08 mmol) and 1,8 bis (dimethyl amino)naphthalene (25 mg, 0.12 mmol) were dried in the dark in vacuo overnight. Then the mixture was dissolved under argon in trimethyl phosphate (1 mL) and POCl₃ (29 µL, 0.32 mmol) was added dropwise at 0 °C. After full turnover of the reactant (TLC analysis 2 propanol/NH₄H₂O₂ 3/1/1) a 0.5 M bis tri butylammonium pyrophosphate solution of DMP (0.78 mL, 0.39 mmol) and n tributylamine (190 µL, 0.80 mmol) were added simultaneously. After stirring for additional 20 min 1 M TEAB buffer (Et₃NH(HCO₃), pH 7.5) was added (10 mL). The aqueous layer was washed with EtOAc (2 x 10 mL) and the solvent was removed in vacuo. The residue was dissolved in 0.1 M TEAB buffer (3 mL) and purified by sephadex ion exchange chromatography (eluent: linear grade from 0.1 M to 1 M TEAB buffer). The salts were removed by RP MPLC (eluent: grade from 50 mM TEAA buffer (Et₃NH(OAc), pH 7.0) with 5% acetonitrile to 100% acetonitrile) to yield

1H NMR (400 MHz; d$_2$-methoI) δ (ppm) 1.25 (s, 3H, H2), 1.29 (q, 2F, CF$_2$), 3.16 (m, Et$_3$N, CH$_2$), 3.20 (q, 2F, CF$_2$), 4.09 4.31 (m, 3H, H4), 4.38 (m, 1H, H3''), 6.15 (dd, 1H, H1'), J$_{HF}$ = 7 Hz), 8.15 (s, 1H, H6). 31P NMR (376 MHz; MeOH d$_4$) δ (ppm) 22.90 (dd, 1P, P, J$_{HF}$ = 21 Hz), 9.56 (d, 1P, P, J$_{HF}$ = 21 Hz). 13C NMR (90 MHz; d$_2$-methoI) δ (ppm) 58.4, 59.4, 63.3, 66.9, 69.2 and 70.0 ppm.

The 23 nt long primer was 5'-d(GAC CCA CTC CAT CGA GAT TTC TC) 3' (49 nt). The reactions were incubated for 5 min (matched case) or 30 min (mismatched cases) in 1x reaction buffer

\[ \text{d(GAC CCA CTC CAT CGA GAT TTC TC)} \]

for 30 min at 37 °C (KF(exo)), and 72 °C (KlenTaQ), (ii) for reactions depicted in Figure 3: for 60 min at 37 °C and (iii) for reactions depicted in Figure 4: for 60 min at 37.0, 37.8, 40.1, 47.6, 51.5, 55.4, 59.4, 63.3, 66.9, 69.2 and 70.0 °C, respectively (lanes 1 12) in a thermocycler and were stopped by addition of 40 µL stop solution [80% [v/v] formamide, 20 mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol]. After denaturing at 95 °C for 5 min the reaction mixtures were separated using a 12% denaturing PAGE gel. Visualization was performed using phosphorimaging.

The used templates were (i) for reactions depicted in Figure 1b and c: 5'-d(GCC GTG CTA CCG CGG AAA TAA TCT CGA TGG CTT GC) (35 nt), (ii) for reactions depicted in Figure 3a: 5'-d(TAG GAT TCT ATT TTA CCG ATT CAT TTA CGC AGT CAG GCA TTC AGA) 3' (50 nt) in Figure 3b: 5'-d(TAG GAT TCT ATT TTA CCG ATT CAT TTA CGC AGT CAG GCA TTC AGA) 3' (55 nt) and (iv) for reactions depicted in Figure 4: 5'-d(TAG GAT TCT ATT TTA CCG ATT CAT TTA CGC AGT CAG GCA TTC AGA) 3' (55 nt) and (v) for reactions depicted in Figures 4: 5'-d(TAG GAT TCT ATT TTA CCG ATT CAT TTA CGC AGT CAG GCA TTC AGA) 3' (55 nt) and (v) for reactions depicted in Figures 4: 5'-d(TAG GAT TCT ATT TTA CCG ATT CAT TTA CGC AGT CAG GCA TTC AGA) 3' (55 nt) and (v) for reactions depicted in Figures 4: 5'-d(TAG GAT TCT ATT TTA CCG ATT CAT TTA CGC AGT CAG GCA TTC AGA) 3' (55 nt) and (v) for reactions depicted in Figures 4: 5'-d(TAG GAT TCT ATT TTA CCG ATT CAT TTA CGC AGT CAG GCA TTC AGA) 3' (55 nt) and (v) for reactions depicted in Figures 4: 5'-d(TAG GAT TCT ATT TTA CCG ATT CAT TTA CGC AGT CAG GCA TTC AGA) 3' (55 nt) and (v) for reactions depicted in Figures 4: 5'-d(TAG GAT TCT ATT TTA CCG ATT CAT TTA CGC AGT CAG GCA TTC AGA) 3' (55 nt) and (v) for reactions depicted in Figures 4: 5'-d(TAG GAT TCT ATT TTA CCG ATT CAT TTA CGC AGT CAG GCA TTC AGA) 3' (55 nt). The reactions were incubated for 5 min (matched case) or 30 min (mismatched cases) at 37 °C in a thermocycler and were stopped by addition of 40 µL stop solution [80% [v/v] formamide, 20 mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol]. After denaturing at 95 °C for 5 min the reaction mixtures were separated using a 12% denaturing PAGE gel. Visualization was performed using phosphorimaging.

1.4.9. Single nucleotide incorporation assays (Fig. 2)

The 24 nt long primer was 5'-2^32P labelled using \([\gamma^{32}P]\) ATP according to standard techniques. The reaction mixture (20 µL) contained 2 nm DNA polymerase KF(exo), 60 nm template (5'-d(GTC GTG CTA TCA CGT CAC), 40 nm radioactively labelled primer (5'-d(GTC GTG CTA AAT TTC TCA CGA ACA) 3') and 1, 10, 100 µM dNTPs (matched case) or 10, 50, 200 µM dNTPs (mismatched cases) in 1x reaction buffer KF(exo). The reactions were incubated for 5 min (matched case) or 30 min (mismatched cases) at 37 °C in a thermocycler and were stopped by addition of 40 µL stop solution [80% [v/v] formamide, 20 mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol]. After denaturing at 95 °C for 5 min the reaction mixtures were separated using a 12% denaturing PAGE gel. Visualization was performed using phosphorimaging.