Bioorganic & Medicinal Chemistry 17 (2009) 3653–3658

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Enzymatic synthesis of perfluoroalkylated DNA

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ARTICLE INFO

Article history:
Received 27 February 2009
Revised 23 March 2009
Accepted 29 March 2009
Available online 7 April 2009

Keywords:
DNA
Modified nucleotide
DNA polymerase
Fluor
SELEX

ABSTRACT

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 initial 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 storage and transfer of information, DNA has recently gained significant 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 enzymatic 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 networks[6] 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 various approaches in bioorganic and medicinal chemistry.[10] Fluorine is highly electronegative and shows unique nuclear magnetic resonance (NMR) spectroscopic parameters. The substitution of hydrogen by fluorine leads to significant electronic effects and can modify properties of a molecule like pK_a-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 lengths of C–H (1.09 Å) and C–F (1.35 Å).[11] However, fluorinated substrate analogues are often used as mechanistic probes to study enzyme catalysis.[10c] Furthermore, fluorine is applied in monitoring functional important transitions in biological systems via ^{19}F NMR spectroscopic methods.[12]

The fluorinated thymidine analogue 5-trifluoromethyl-2′-deoxyuridine (CF_3dU) has been shown to form regular base pairs with adenosine. By replacing an internal thymidine residue in DNA oligonucleotides with CF_3dU DNA double helices are formed that still adopt the B-form conformation.[13] Thereby, the substitution of a thymidine residue by CF_3dU 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 CF_3dU 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-

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doi:10.1016/j.bmc.2009.03.063

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

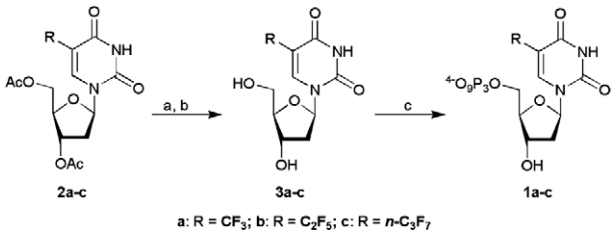
First, we synthesized the 5-perfluoroalkyl-2′-deoxyuridine-5′-triphosphates **1a–c** (Scheme 1).

The synthesis started with **2a–c** that were synthesized following published procedures.[16] **2a** was deacetylated according to the reported procedure by using a 7 M ammonia solution of methanol.[16] 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,[17] 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. Therefore, we used a ^{32}P-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 polyacrylamide 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 monitored 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, CF_3dUTP, C_2F_5dUTP, C_3F_7dUTP). 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 ^{32}P-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



Scheme 1. Synthesis of 5-perfluoroalkylated dUTP analogues **1a–c**. Reagents and yields: (a) NH_3/MeOH (7 M), 76% (**3a**); (b) p-TsOH·H_2O, DCM/MeOH (9:1), 74% (**3b**), 66% (**3c**); (c) 1,8-bis-(dimethylamino)-naphthalene, $POCl_3$, $(CH_3O)_3PO$ then (n-$Bu_3NH)_2H_2P_2O_7$, Et_3N, 8% (**1a**), 10% (**1b**), 16% (**1c**).

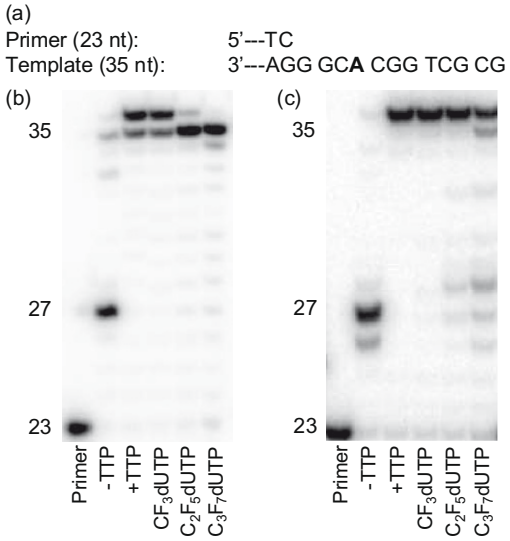


Figure 1. Primer extension studies: acceptance of **1a–c** by KF(*exo*-) (b) and KlenTaq (c). (a) Partial primer template sequences employed. (b) and (c) Primer: primer only; −TTP: primer extension in the presence of dATP, dGTP and dCTP; +TTP: as −TTP but in the presence of TTP; CF_3dUTP: as −TTP but in the presence of **1a**; C_2F_5dUTP: as −TTP but in the presence of **1b**; C_3F_7dUTP: as −TTP but in the presence of **1c**.

(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).

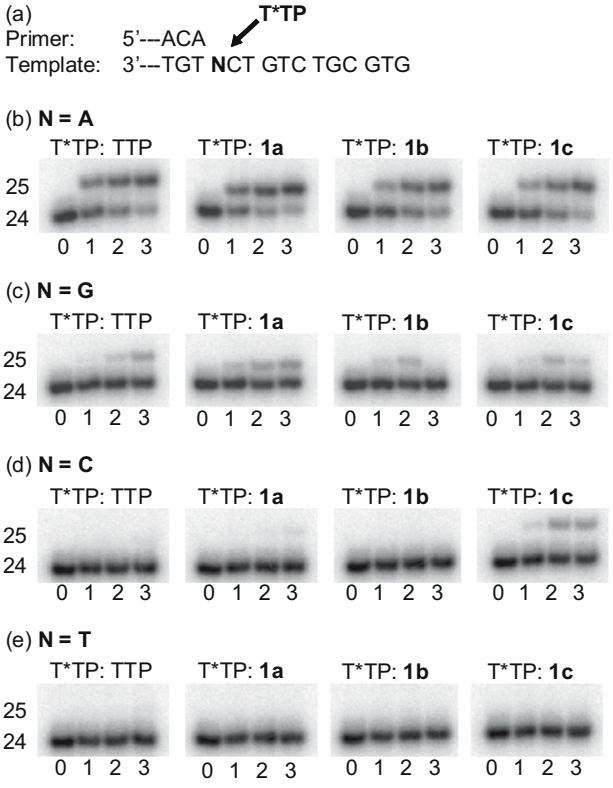


Figure 2. Single incorporation employing TTP and **1a–c** by KF(*exo*-) with differing nucleotide concentrations. (a) Partial primer template sequences employed. (b) Incorporation opposite A (matched case, t = 5 min); lanes 0: primer only, lanes 1: 1 μM T*TP, lanes 2: 10 μM T*TP, lanes 3: 100 μM T*TP. (c–e) Incorporation opposite G/C/T (mismatched cases, t = 30 min); lanes 0: primer only, lanes 1: 10 μM T*TP, lanes 2: 50 μM T*TP, lanes 3: 200 μM T*TP.

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 **1a–c**, 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, CF_3dUTP, C_2F_5dUTP, C_3F_7dUTP). The acceptance of **1a–c** at every second position led only in the case of **1a** to the full length product (Fig. 3b, CF_3dUTP). The template with an adenosine residue at every position could not be extended to full length with any of the TTP analogues (Fig. 3c, CF_3dUTP, C_2F_5dUTP, C_3F_7dUTP). 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, CF_3dUTP, C_2F_5dUTP, C_3F_7dUTP).

However, we found that the effects of **1a–c** on DNA polymerases significantly vary with the used enzyme. It was found that Therminator DNA polymerase (A485L mutant of *Thermococcus species* 9°N DNA polymerase) is most proficient in processing multiple incorporations 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 internal CF_3dU residues,[14] we examined the multiple incorporation experiments at temperatures between 37 and 70 °C. Interestingly, Therminator DNA polymerase is proficient in incorporating eleven adjacent CF_3dU 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 pentafluoroethyl- and heptafluoro-*n*-propyl-modified residues exhibit enhanced inhibition of DNA synthesis in comparison to the CF_3dU 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.

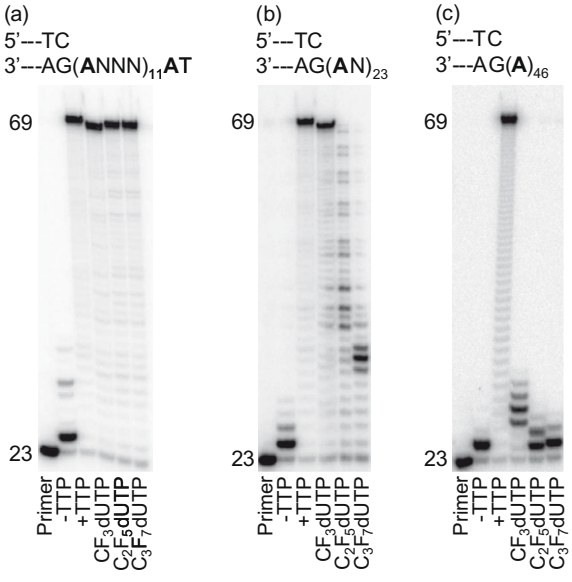


Figure 3. Multiple incorporation of modified nucleotides. Primer template sequences are indicated on the top of every panel. (a–c) Incorporation by KF(*exo*-) catalysis; *Primer*: primer only; *−TTP*: primer extension in the presence of dATP, dGTP and dCTP; *+TTP*: as - *TTP* but in the presence of TTP; *CF_3dUTP*: as *−TTP* but in the presence of **1a**; *C_2F_5dUTP*: as *−TTP* but in the presence of **1b**; *C_3F_7dUTP*: as *−TTP* but in the presence of **1c**.

3. Conclusions

In summary, we report the synthesis of 5-trifluoromethyl-, 5-pentafluoroethyl- 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' proficiencies 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 ECX-400 spectrometer. ESI-IT mass spectra were recorded on a Bruker Daltonics esquire 3000+. ESI-TOF mass spectra were recorded on a Bruker micrOTOFII. 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) R_f 0.34. ^{1}H NMR (600 MHz; MeOH-d_4) δ (ppm) 2.25–2.29 (m, 1H, H2′a), 2.35–2.39 (m, 1H, H2′b), 3.75 (dd, 1H, H5′a, $^2J_{H5'b}$ = 12 Hz, $^3J_{H4'}$ = 3 Hz), 3.84 (dd, 1H, H5′b, $^2J_{H5'a}$ = 12 Hz, $^3J_{H4'}$ = 3 Hz), 3.97 (m, 1H, H4′), 4.41 (m, 1H, H3′), 6.24 (dd, 1H, H1′, $^3J_{H2'}$ = 6 Hz), 8.79 (s, 1H, H6). ^{13}C NMR (151 MHz; MeOH-d_4) δ (ppm) 42.15, 62.17, 71.73, 87.58, 89.34, 105.35 (q, 1C, C5, ^2J_F = 33 Hz), 123.98 (q, 1C, CF_3, ^1J_F = 269 Hz), 143.82 (q, 1C, C6, ^3J_F = 6 Hz), 151.37, 161.27. ^{19}F NMR (376 MHz; MeOH-d_4) δ (ppm) −64.4 (s, 3F, CF_3). 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-Pentafluoroethyl-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)

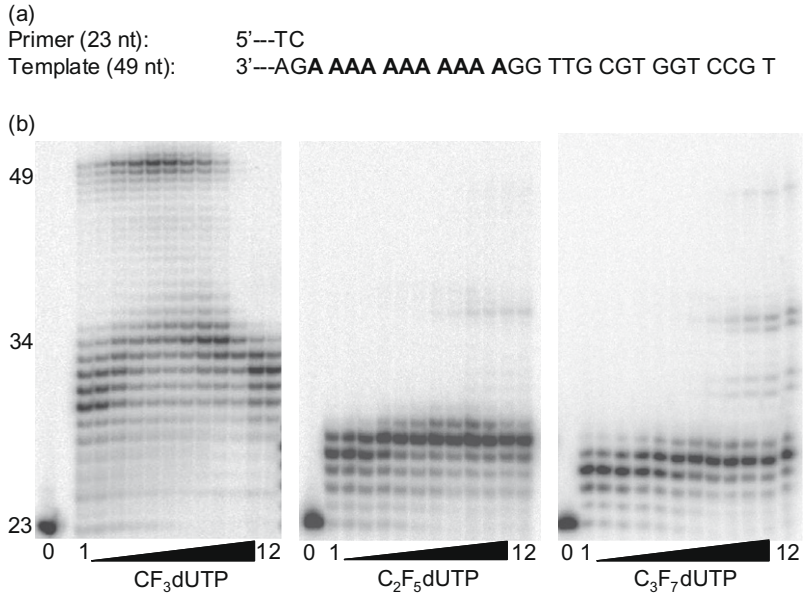


Figure 4. Temperature dependent polymerization of **1a–c** by Therminator DNA polymerase; lanes 0: primer only, lanes 1–12: primer extension in the presence of dATP, dGTP, dCTP and **1a, 1b**, or **1c**, respectively; incubation temperatures: 37.0, 37.8, 40.1, 43.7, 47.6, 51.5, 55.4, 59.4, 63.3, 66.9, 69.2 and 70.0 °C.

to yield **3b** (84 mg, 74%). TLC (dichloromethane/methanol 9:1) R_f 0.30. ^{1}H NMR (600 MHz; MeOH-d_4) δ (ppm) 2.23–2.29 (m, 1H, H2′a), 2.37–2.41 (m, 1H, H2′b), 3.74 (dd, 1H, H5′a, $^2J_{H5'b}$ = 12 Hz, $^3J_{H4'}$ = 3 Hz), 3.83 (dd, 1H, H5′b, $^2J_{H5'a}$ = 12 Hz, $^3J_{H4'}$ = 3 Hz), 3.98 (m, 1H, H4′), 4.41 (m, 1H, H3′), 6.24 (dd, 1H, H1′, $^3J_{H2'}$ = 6 Hz), 8.80 (s, 1H, H6). ^{13}C NMR (151 MHz; MeOH-d_4) δ (ppm) 42.30, 62.16, 71.80, 87.79, 89.45, 103.05 (t, 1C, C5, ^2J_F = 24 Hz), 113.71 (tq, 1C, CF_2, ^1J_F = 254 Hz, ^2J_F = 40 Hz), 120.49 (tq, 1C, CF_3, ^1J_F = 286 Hz, ^2J_F = 40 Hz), 145.85 (t, 1C, C6, ^3J_F = 10 Hz), 151.29, 160.97. ^{19}F NMR (376 MHz; MeOH-d_4) δ (ppm) −114.8 (s, 1F, CF_2a), −114.8 (s, 1F, CF_2b), −86.0 (s, 3F, CF_3). ESI-IT-MS calcd for $(M-H)^-$ 345.1, found: 344.8. ESI-TOF-MS calcd for $(M-H)^-$ 345.0510, found 345.0609.

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 toluene-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. ^{1}H NMR (600 MHz; MeOH-d_4) δ (ppm) 2.22–2.28 (m, 1H, H2′a), 2.35–2.43 (m, 1H, H2′b), 3.74 (dd, 1H, H5′a, $^2J_{H5'b}$ = 12 Hz, $^3J_{H4'}$ = 2 Hz), 3.82 (dd, 1H, H5′b, $^2J_{H5'a}$ = 12 Hz, $^3J_{H4'}$ = 3 Hz), 3.98 (m, 1H, H4′), 4.41 (m, 1H, H3′), 6.25 (dd, 1H, H1′, $^3J_{H2'}$ = 6 Hz), 8.81 (s, 1H, H6). ^{13}C NMR (151 MHz; MeOH-d_4) δ (ppm) 42.38, 62.17, 71.85, 87.79, 89.50, 103.21 (t, 1C, C5, ^2J_F = 24 Hz), 110.36 (m, 1C, $CF_2\beta$), 115.69 (tq, 1C, $CF_2\alpha$, ^1J_F = 256 Hz, ^2J_F = 33 Hz), 119.49 (tq, 1C, CF_3, ^1J_F = 287 Hz, ^2J_F = 34 Hz), 146.19 (t, 1C, C6, ^3J_F = 10 Hz), 151.26, 160.95. ^{19}F NMR (376 MHz; MeOH-d_4) δ (ppm) −127.5 (s, 2F, $CF_2\beta$), −111.5 (m, 1F, $CF_2\alpha$), −82.4 (t, 3F, CF_3, $^4J_{F\alpha}$ = 10 Hz). ESI-IT-MS calcd for $(M-H)^-$ 395.1, found 394.8. ESI-TOF-MS calcd for $(M-H)^-$ 395.0478, found 395.0595.

4.1.4. 5-Trifluoromethyl-2′-deoxyuridine-5′-triphosphate 1a

The nucleoside **3a** (67 mg, 0.23 mmol) and 1,8-bis-(dimethylamino)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 $POCl_3$ (55 μL, 0.60 mmol) was added dropwise at 0 °C. After full turnover of the reactant (TLC analysis 2-propanol/NH_3/H_2O 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 μL, 2.27 mmol) were added simultaneously. After stirring for additional 20 min 1 M TEAB-buffer ($Et_3NH(HCO_3)$, 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 TEAA-buffer ($Et_3NH(OAc)$, pH 7.0) with 5% acetonitrile to 100% acetonitrile) to yield **1a** (18 mg, 8%, triethylammonium salt of the triphosphate). ^{1}H NMR (400 MHz; MeOH-d_4) δ (ppm) 1.30 (t, Et_3N, Me), 2.32 (m, 2H, H2′), 3.17 (q, Et_3N, CH_2), 4.09–4.22 (m, 2H, H5′), 4.31 (m, 1H, H4′), 4.59 (m, 1H, H3′), 6.19 (dd, 1H, H1′, $^3J_{H2'}$ = 7 Hz), 8.29 (s, 1H, H6). ^{31}P NMR (162 MHz; MeOH-d_4) δ (ppm) −22.94 (dd, 1P, P_β, ^2J_P = 21 Hz), −10.66 (d, 1P, P_α, $^2J_{P\beta}$ = 21 Hz), −9.56 (d, 1P, P_γ, $^2J_{P\beta}$ = 21 Hz). ^{19}F NMR (376 MHz; MeOH-d_4) δ (ppm) −64.7 (s, 3F, CF_3). ESI-IT-MS calcd for $(M-H)^-$ 534.9, found 534.6. ESI-TOF-MS calcd for $(M-H)^-$ 534.9537, found 534.9651.

4.1.5. 5-Pentafluoroethyl-2′-deoxyuridine-5′-triphosphate 1b

The nucleoside **3b** (32 mg, 0.09 mmol) and 1,8-bis(dimethylamino)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 $POCl_3$ (20 μL, 0.22 mmol) was added dropwise at 0 °C. After full turnover of the reactant (TLC analysis 2-propanol/NH_3/H_2O 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 μL, 0.92 mmol) were added simultaneously. After stirring for additional 20 min 1 M TEAB-buffer ($Et_3NH(HCO_3)$, 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 TEAA-buffer ($Et_3NH(OAc)$, pH 7.0) with 5% acetonitrile to 100% acetonitrile) to yield **1b** (9 mg, 10%, triethylammonium salt of the triphosphate). ^{1}H NMR (400 MHz; MeOH-

d_4) δ (ppm) 1.24 (t, Et_3N, Me), 2.44 (m, 2H, H2′), 3.26 (q, Et_3N, CH_2), 4.11–4.32 (m, 3H, H4′, H5′), 4.66 (m, 1H, H3′), 6.24 (dd, 1H, H1′, $^3J_{H2'}$ = 7 Hz), 8.28 (s, 1H, H6). ^{31}P NMR (162 MHz; MeOH-d_4) δ (ppm) −22.43 (m, 1P, P_β), −11.04 (d, 1P, P_α, $^2J_{P\beta}$ = 19 Hz), −9.56 (d, 1P, P_γ, $^2J_{P\beta}$ = 17 Hz). ^{19}F NMR (376 MHz; MeOH-d_4) δ (ppm) −114.6 (s, 2F, CF_2), −85.5 (s, 3F, CF_3). ESI-IT-MS calcd for $(M-H)^-$ 584.9, found 584.5. ESI-TOF-MS calcd for $(M-H)^-$ 584.9505, found 584.9688.

4.1.6. 5-(Heptafluoro-*n*-propyl)-2′-deoxyuridine-5′-triphosphate 1c

The nucleoside **3c** (31 mg, 0.08 mmol) and 1,8-bis-(dimethylamino)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 $POCl_3$ (29 μL, 0.32 mmol) was added dropwise at 0 °C. After full turnover of the reactant (TLC analysis 2-propanol/NH_3/H_2O 3/1/1) a 0.5 M bis-tri-*n*-butylammonium pyrophosphate solution of DMF (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 ($Et_3NH(HCO_3)$, 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 TEAA-buffer ($Et_3NH(OAc)$, pH 7.0) with 5% acetonitrile to 100% acetonitrile) to yield **1c** (13 mg, 16%, triethylammonium salt of the triphosphate). ^{1}H NMR (400 MHz; d_4-MeOH) δ (ppm) 1.30 (m, Et_3N, Me), 2.25–2.43 (m, 2H, H2′), 3.17 (m, Et_3N, CH_2), 4.09–4.31 (m, 3H, H4′, H5′), 4.58 (m, 1H, H3′), 6.15 (dd, 1H, H1′, $^3J_{H2'}$ = 7 Hz), 8.15 (s, 1H, H6). ^{31}P NMR (162 MHz; MeOH-d_4) δ (ppm) −22.90 (dd, 1P, P_β, ^2J_P = 21 Hz), −10.60 (d, 1P, P_α, $^2J_{P\beta}$ = 21 Hz), −9.56 (d, 1P, P_γ, $^2J_{P\beta}$ = 21 Hz). ^{19}F NMR (376 MHz; MeOH-d_4) δ (ppm) −127.1 (m, 2F, $CF_{2\beta}$), −111.5 (q, 2F, $CF_2\alpha$, ^4J_F = 10 Hz), −82.2 (t, 3F, CF_3, ^4J_F = 10 Hz). ESI-IT-MS calcd for $(M-H)^-$ 634.9, found 634.5. ESI-TOF-MS calcd for $(M-H)^-$ 634.9473, found 634.9729.

4.1.7. Materials for DNA polymerase experiments

dNTPs were purchased from Roche.

4.1.7.1. Primers and templates. Figures 1, 3 and 4: Templates were obtained from Purimex (2× HPLC purified). The 23 nt primer strand was synthesized on an Applied-Biosystems-392 DNA synthesizer and afterwards purified by preparative PAGE. Figure 2: Used primer and templates were synthesized on the DNA synthesizer and afterwards purified by HPLC and preparative PAGE.

4.1.7.2. Enzymes. The Klenow fragment of *E. coli* DNA polymerase I (KF(*exo*-)) was generated and purified as described.[21] The Klenow fragment of *Thermus aquaticus* DNA polymerase I (KlenTaq) was generated and purified as described.[20f] The Therminator DNA polymerase (A485L mutant of *Thermococcus species* 9°N DNA polymerase) was purchased from New England Biolabs.

4.1.7.3. Buffers. 10x reaction buffer KF(*exo*-): 500 mM Tris–HCl, 100 mM $MgCl_2$, 10 mM DTT, 0,5% Triton X-100, pH 7.3. 10× reaction buffer KlenTaq: 500 mM Tris–HCl, 160 mM $(NH_4)_2SO_4$, 25 mM $MgCl_2$, 1% Tween 20, pH 9.2. 10× reaction buffer Therminator: 200 mM Tris–HCl, 20 mM $MgSO_4$, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 1% Triton X-100, pH 8.8.

4.1.8. Primer extension assays (Figs. 1, 3 and 4)

The 23 nt long primer was 5′-^{32}P-labelled using [γ^{32}P]-ATP according to standard techniques. The reaction mixture (20 μL) contained DNA polymerase, 200 nM template, 150 nM radioactive labelled primer (5′-d(GAC CCA CTC CAT CGA GAT TTC TC)-3′) and 200 μM dNTPs in the according 1× reaction buffer. Used DNA polymerase concentrations were (i) for reactions depicted in Figure 1b: 30 nM DNA polymerase KF(*exo*-), (ii) for reactions depicted in Figure 1c: 30 nM DNA polymerase KlenTaq, (iii) for reactions depicted in Figure 3: 10 nM DNA polymerase KF(*exo*-) and (iv) for reactions depicted in Figure 4: 0.05 U/μL Therminator DNA polymerase. The reactions were incubated (i) for reactions depicted in Figure 1b and c: for 20 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, 43.7, 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(GCG CTG GCA CGG GAG AAA TCT CGA TGG AGT GGG TC) (35 nt), (ii) for reactions depicted in Figure 3a: 5′-d(TAG GGA TCT ATT TAC TTA CCG ATT CAT TTA CGC AGT CAG GCA TTC AGA GAA ATC TCG ATG GAG TGG GTC) (69 nt), (iii) for reactions depicted in Figure 3b: 5′-d(TAG ACA TAG ACA TAG ACA TAG ACA TAG ACA TAC AGA CAG ACA TAC AGA GAA ATC TCG ATG GAG TGG GTC) (69 nt), (iv) for reactions depicted in Figure 3c: 5′-d(AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AGA GAA ATC TCG ATG GAG TGG GTC) (69 nt) and (v) for reactions depicted in Figure 4: 5′-d(TGC CTG GTG CGT TGG AAA AAA AAA AAG AGA AAT CTC GAT GGA GTG GGT C) (49 nt).

4.1.9. Single nucleotide incorporation assays (Fig. 2)

The 24 nt long primer was 5′-^{32}P-labelled using [γ^{32}P]-ATP according to standard techniques. The reaction mixture (20 μL) contained 2 nM DNA polymerase KF(*exo*-), 60 nM template (5′-d(GTG CGT CTG TC**A/G/C/T** TGT CTG TCA GAA ATT TCG CAC CAC), 40 nM radioactive labelled primer (5′-d(GTG GTG CGA AAT TTC TGA CAG 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.

Acknowledgements

We thank M. Liebmann for her contributions to this work and Konstanz Research School Chemical Biology for financial support.

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