Synthetic Study on ADP-ribosylation

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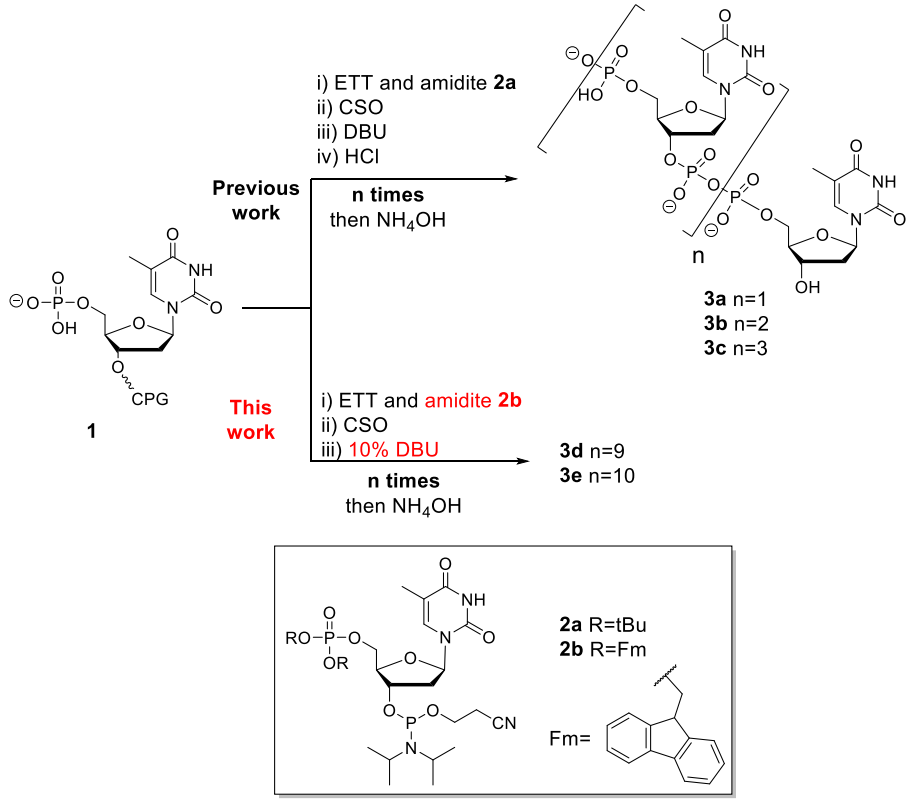
3 Synthesis of 3',5'-pyrophosphate-linked thymidine oligomers

Introduction

Pyrophosphate functions can be present in many biomolecules, ranging from lipids and inositols to nucleic acids, proteins and carbohydrates and these pyrophosphate containing molecules play an important role in a variety of biological processes.[1-4] Their broad occurrence in nature and the synthetic challenge that pyrophosphates represent have led to a row of synthetic methods for the construction of pyrophosphate esters.[5-7] This is illustrated by the continuous appearance of new or modified procedures for the synthesis of nucleoside diphosphate sugars, that are donors in the glycosyltransferase mediated biosynthesis of carbohydrates and glycoproteins. Gold *et al*[5] reported P(V)-P(III) coupling method for the formation of pyrophosphate linkages and prepared three UDP-N-acetylglucosamine derivatives. This procedure was not only adopted for the synthesis of other sugar-nucleotides[8] and glycolipids[9] but also to the synthesis of a cADPR antagonist[10] and ADP-ribosylated oligopeptides.[11, 12] Whereas these molecules contain one pyrophosphate function, poly-ADP-ribose is a polymer of repeating pyrophosphates and therefor the assembly of fragments of poly-ADP-ribose presents an additional challenge. Using the P(V)-P(III) methodology, Kistemaker *et al* reported the first synthesis of an ADPr trimer that was successfully applied in a biophysical study.[13, 14] In a comparative model study the same authors prepared pyrophosphate-linked thymidine oligomers, up to a length of a tetramer.[9] Guided by a hypothesis in prebiotic chemistry that pyrophosphate-linked DNA oligomers have played a role in the origin of oligonucleotides, Anderson and Krishnamurthyused used the same P(V)-P(III) methodology for the synthesis of pyrophosphate-linked thymidine oligomers and dertermined their binding to DNA and RNA.[10] Despite the impressive achievements attained in the

above mentioned syntheses, it is clear that the length and the associated quality of ADP-ribose oligomers and pyrophosphate-linked thymidine oligomers are restricted. It is therefore necessary to optimize the P(V)-P(III) methodology of pyrophosphate synthesis.



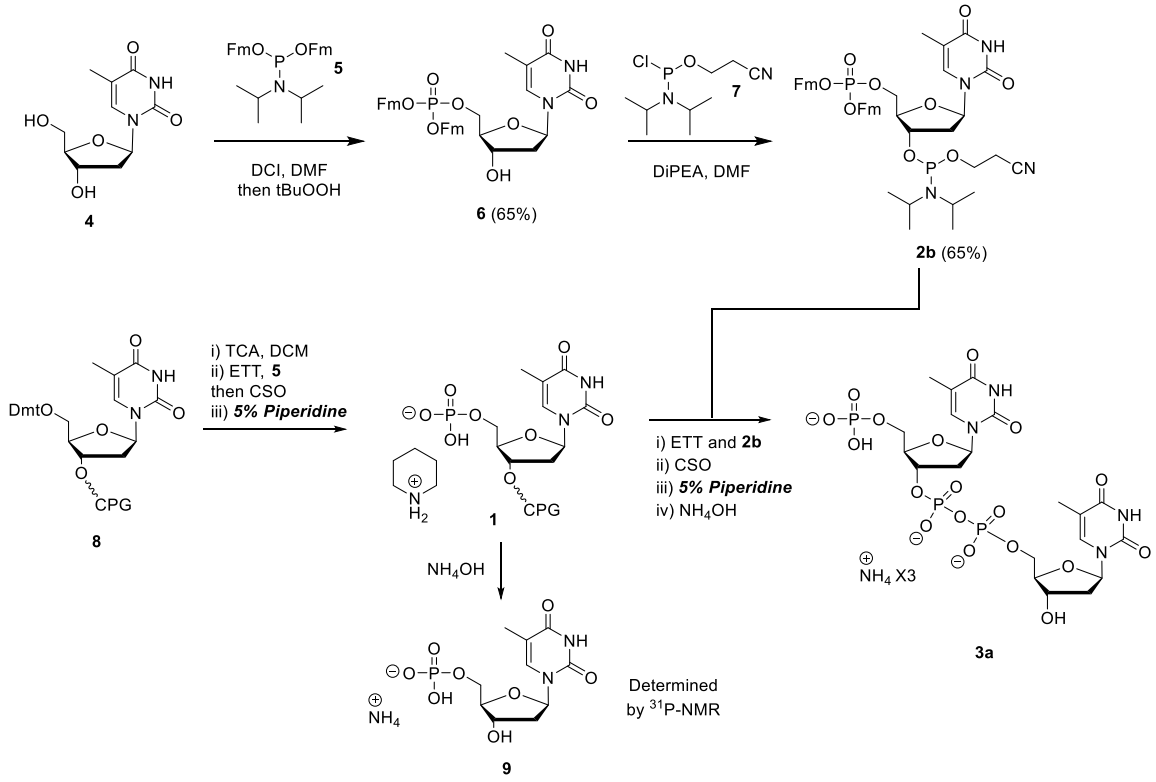
Scheme 1. Previous work and this work on the synthesis of pyrophosphate linked thymindine oligomers.

On the basis of the accessibility of the building blocks, the optimization of the synthesis of pyrophosphate-linked thymidine oligomers is first executed. The reported solid phase synthesis[7, 8, 15] used CPG immobilized 5'-phosphothymidine **1** together with an elongation cycle entailing the following steps. The coupling of monophosphate (e.g. **1**) with the phosphoramidite **2a** under influence of activator ETT that generates an intermediate with a P(V)-P(III) bond was followed by oxidation of the P(III) center with CSO to provide a P(V)-P(V) pyrophosphate bond. The subsequent removal of the cyanoethyl (CE) group with DBU and the deprotection of *tert*-butyl groups at the terminal phosphotriester with a catalytical amount of HCl in HFIP[11, 13, 16] allow the next elongation cycle (Scheme 1). The formation of the side products and shorter oligomers, found in the crude mixture at the end of the synthesis, is possibly caused by the repeated acidic removal of *tert*-butyl groups in the fourth step of the elongation cycle (Scheme 1).[15] The amount of side products increases with the length of the oligomers (such as decamer) making the purification of longer oligomers impossible.[8] Based on these considerations, replacement of the *t*Bu group by the base labile fluorenylmethyl (Fm)[17, 18] group that

can be easily removed using DBU or piperidine will improve the synthesis and making the longer pyrophosphate-linked thymidine oligomers available.

This Chapter describes the synthesis and application of the new phosphoramidite building block **2b** with a Fm protected 5'-phosphotriester for the assembly of pyrophosphate-linked thymidine oligomers (Scheme 1). The implementation of building block **2b** reduces the number of steps in the coupling cycle from four to three: phosphitylation, oxidation and base deprotection. The usefulness of building block **2b** for the synthesis of molecules with multi-pyrophosphates is demonstrated by the synthesis of pyrophosphate linked thymidine decamer.

Results and discussion



Scheme 2. Synthesis of key phosphoramidite monomer **2b** and oligonucleotide **3a**

The synthesis of the key phosphoramidite building blocks **2b** commenced with selective phosphitylation of thymidine **4** with known $(FmO)_2PN(iPr)_2$ (**5**)[13, 14, 15] using DCI as an activator, followed by oxidation of *t*BuOOH of the intermediate phosphite triester to give **6** (Scheme 2). The di-phosphorylated byproduct was also observed and could be separated by column chromatography. The ensuing introduction of cyanoethyl phosphoramidite on 3'-OH using 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite **7** in DCM/DMF and TEA as a base failed.[13] TLC and LC-MS analysis showed the presence of side-products, originating from the cleavage of one of the Fm groups by TEA.

Switching to the more hindered Hünig's base (DIPEA) went without Fm cleavage. Of note is that **2b** is sensitive to both mild base and acid because of the presence of a phosphoramidite and the Fm groups, making normal TEA neutralized silica gel column unsuitable for purification. Therefore, a special neutralized silica gel column was used for the purification of **2b** (see experimental section). Additionally, the H-phosphonate generated from the hydrolysis of excessive amount of reagent **7** could not be easily separated (around 14 ppm on ^{31}P-NMR). This byproduct was also observed in previous studies and did not interfere with the pyrophosphate coupling.[13, 15]

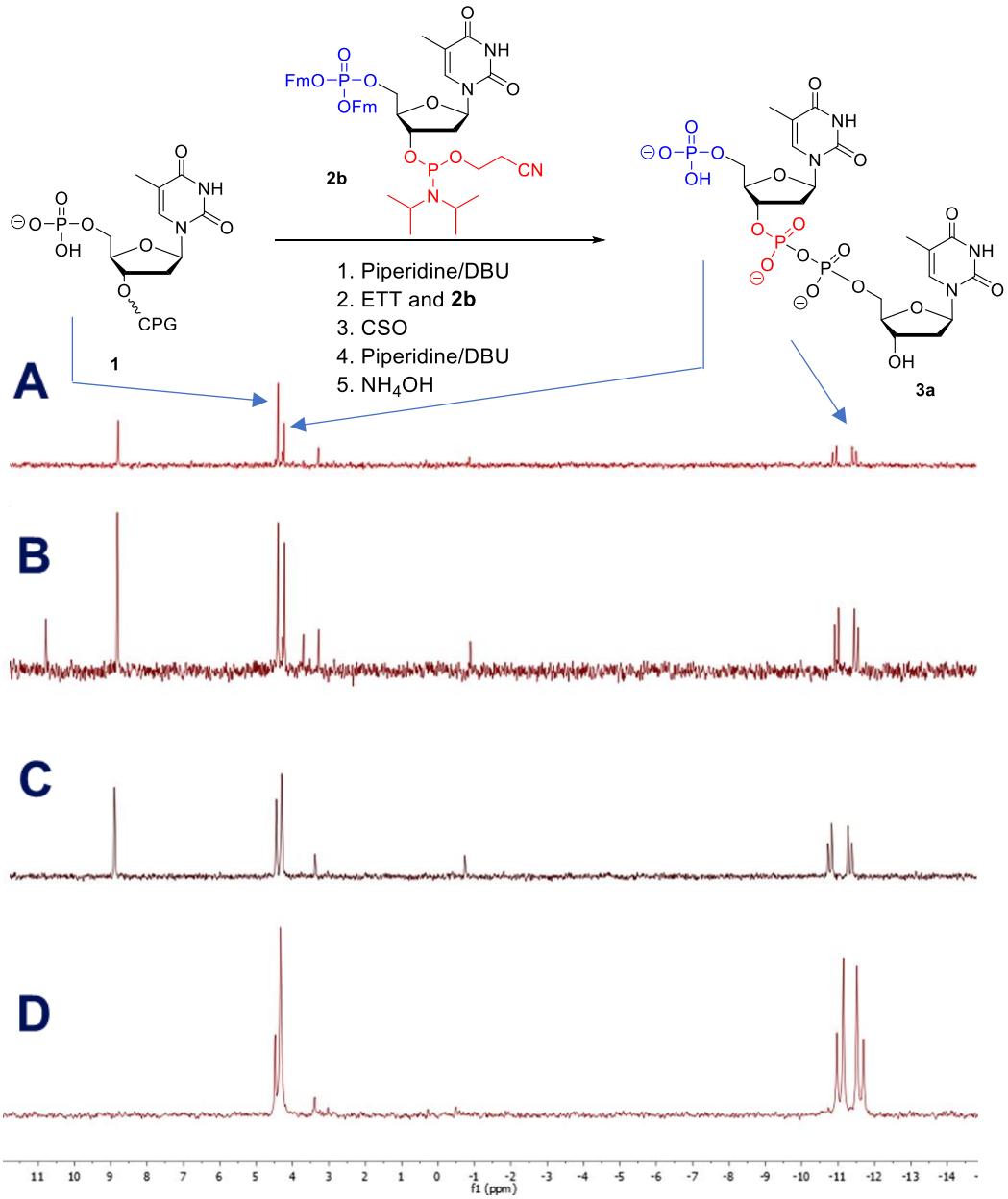
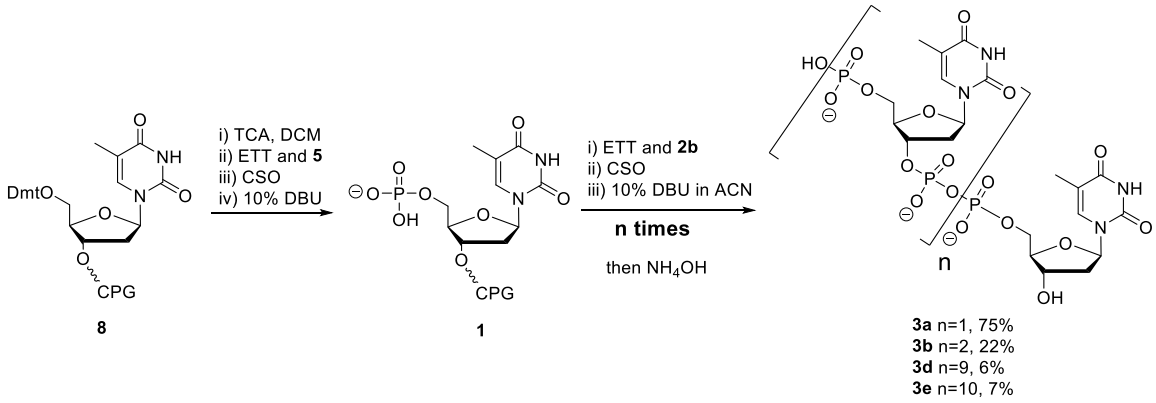


Figure 1. ^{31}P-NMR spectrums of the crude sample of pTppT cleaved from the solid phase. **A**: without step 1, step 4 = 5% piperidine in DMF; **B**: Step 1 = step 4 = 5% piperidine in DMF; **C**: Step 1 = step 4 = 20% piperidine in DMF; **D**: Step 1 = step 4 = 5% DBU in DMF

To test the viability of building block **2b**, the solid phase synthesis of monomer **3a**, having one pyrophosphate moiety, was undertaken (Scheme 2). First, commercially available 3'-dT CPG **8** was detritylated with TCA in DCM and converted into phosphate monoester **1** by treatment with phosphoramidite **5** and activator ETT, followed by oxidation of the intermediate phosphite triester by CSO and finally removal of both Fm groups in the obtained phosphate triester with 5% piperidine[19] in DMF (10 min). A small amount of resin **1** was cleaved by NH_4OH, obtaining **9** for characterization by ^{31}P-NMR. Then, resin **1** was transformed to the pyrophosphate linked nucleotide **3a** via 4 steps: a) P(V)-P(III) bond formation by coupling with **2b** under influence of ETT; b) CSO mediated oxidation; c) removal of the CE and both terminal Fm groups by piperidine (10 min); d) NH_4OH treatment to cleave **3a** from resin. The crude **3a** was analyzed by ^{31}P-NMR spectroscopy and spectrum A (Figure 1) shows two doublets at around -11 ppm which indicated the successful construction of the pyrophosphate bond. The phosphate monoester of **3a** appeared at about 4.3 ppm, accompanying with a bigger unknown peak at around 4.5 ppm which could be ascribed to the starting material, that is cleaved from CPG. It is assumed that the incomplete conversion from **1** to **3a** resulted from the incomplete Fm deprotection of the 5' phosphate of **1**, as mono Fm protected **1** would be inert in the P(V)-P(III) coupling step. Thus, additional piperidine treatment (5% in DMF, step 1) for 10 minutes before the pyrophosphates coupling showed that less amount of **9** was generated, originating from starting material **1** (Spectra B). Switching to a higher concentration of piperidine (20% in DMF) resulted in a better conversion (Spectra C). Next DBU, a stronger base (5% in DMF, 10 minutes) was used, furnishing **3a** as the dominant product with only a small amount of remaining starting material. A prolongation of the DBU treatment to 30 minutes gave, however the same result. It is notable that the DBU treatment did not cleave **1** from the resin, indicating the use of DBU is safe with respect to the preservation of the integrity of the linker. These data collectively showed that Fm protection of the intermediate phosphate triester and the ensuing DBU mediated Fm deprotection is suitable for the projected P(V)-P(III) coupling procedure to pyrophosphate formation on solid support.



Scheme 3. The synthesis of thymidine pyrophosphate oligomer

At this stage the effort was made to prepare an oligomer synthesis using an automated oligonucleotide synthesizer. The synthesis of immobilized phosphomonoester **1** was repeated with a few adaptations. After detritylation of resin **8** using TCA, the coupling with amidite **5** and activator ETT was repeated three times, ensuring the full conversion of all the hydroxyl groups (Scheme 3). After CSO oxidation, the Fm groups were cleaved by 10% DBU, to accomplish a complete deprotection. Next, phosphomonoester **1** was elongated with one pyrophosphate via above mentioned elongation cycle of three steps: phosphitylation (ETT and **2b**), oxidation (CSO), and deprotection (10% DBU). Repetition of this procedure n times should introduce n immobilized pyrophosphate linkages that upon NH_4OH treatment would result in the cleavage from the resin of the target pyrophosphate-linked thymidine oligomers, the quality of which can be established by anion exchange HPLC (Figure 2). First monomer **3a** (n=1) and dimer **3b** (n=2), were prepared and the crude mixtures were analyzed by HPLC. Purification resulted in the isolation of monomer **3a** (n=1) and dimer **3b** (n=2) in 75% yield and 22% yield respectively. Encouraged by this result the synthesis of decamer **3e** (n=10) was undertaken. Surprisingly, analytical anion exchange HPLC showed not only the presence of target decamer **3e** (n=10) but also shorter oligomers ranging from dimer to nonamer (Figure 2). Purification using anion exchange column chromatography gave pure decamer **3e** and nonamer **3d** in 7% and 6% yield respectively, which was an improvement compared with earlier synthesis.[8] The generation of these shorter oligomers during decamer synthesis could be attributed to an incomplete P(V)-P(III) coupling, thus unreacted P(V) species could still react with **2b** in the next cycle, generating n-1 oligomers. In the standard DNA oligomer synthesis, 5'-OH was capped by acetyls to suppress the side reaction, however, in our case, capping a 5'-phosphomonoester is difficult. An additional source of the shorter fragments might be an unwanted reaction of the nucleophilic pyrophosphates present in the growing chain with the incoming **2b**. Such a side reaction would lead to branched triphosphate upon the oxidation that would break under the alkaline conditions in the final deprotection step and generate the truncated sequences.

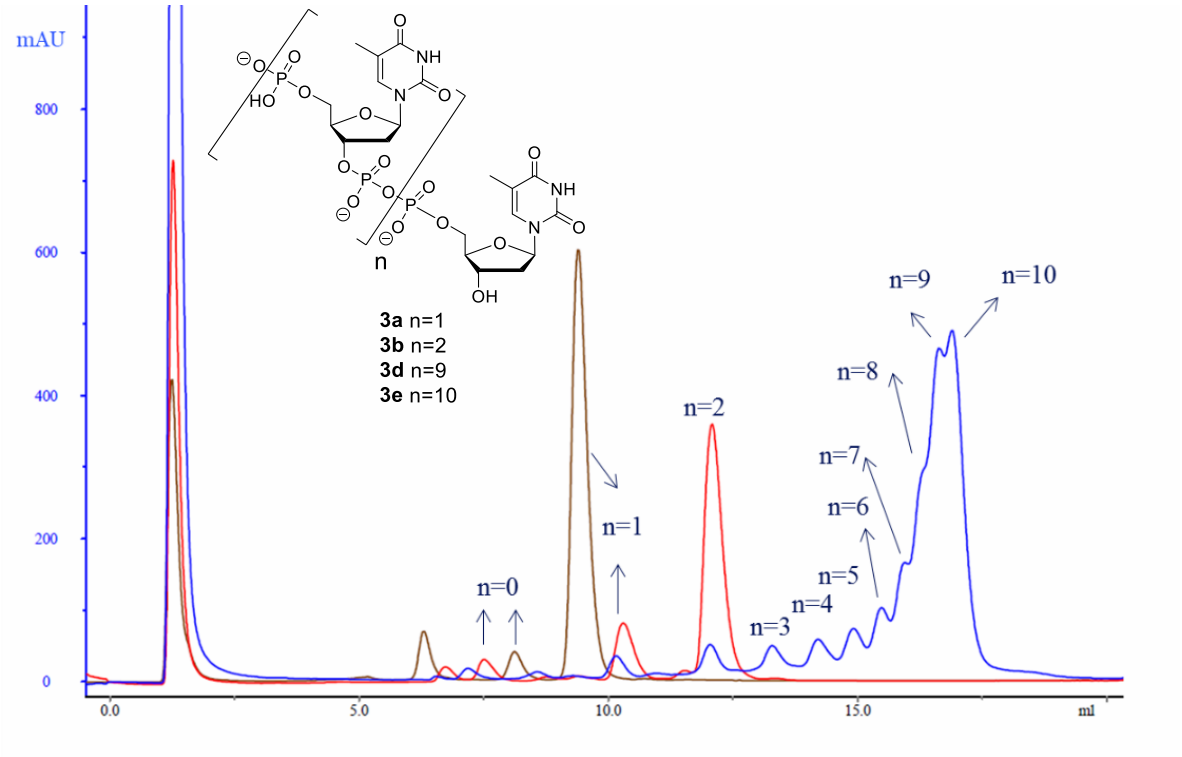


Figure 2. Analytical IE-HPLC traces of crude **3a** (brown), **3b** (red) and **3e** (blue) taken with UV detection. Column: DNAPac PA-100, 4*250 mm. Gradient: pH 7 buffer, 0%-70%. In 20 min. A: 10 Mm NaOAc, 10 mM $NaClO_4$; B:10mM NaOAc 500mM $NaClO_4$

Conclusion

This Chapter describes the design and synthesis of the new Fm protected phosphoramidite **2b** and its application in the solid phase synthesis of 3',5'-pyrophosphate-linked thymidine oligomers. Application of the base labile fluorenylmethyl (Fm) as a phosphate protecting group avoid intermediate acid treatment of the immobilized 3',5'-pyrophosphate-linked thymidine oligomers, thereby preventing possible acid mediated pyrophosphate cleavage. Optimization of the deprotection conditions of Fm groups to 10% DBU in ACN and its subsequent implementation in the solid phase synthesis led to the successful isolation of the 3' 5'-pyrophosphates linked thymidine decamer **3e**. The use of fluorenylmethyl (Fm) instead of the *t*Bu group improved the synthesis of 3',5'-pyrophosphate-linked thymidine oligomers, but was also accompanied by the formation of substantial amounts of shorter oligomers. Although it must be investigated whether this side reaction(s) can be suppressed, the improvement of method described here is sufficient to evaluate its applicability to the synthesis of the biologically relevant ADPr oligomers.

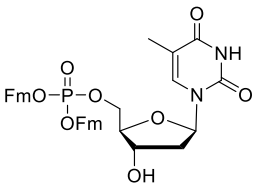
Acknowledgement

Nico Meeuwenoord is kindly acknowledged for his help in oligomer synthesis and final product purification.

Experimental section

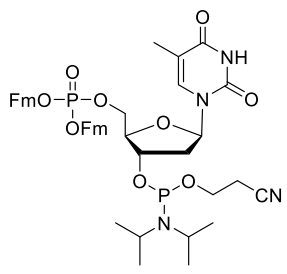
General procedure

All chemicals and solvents were used as described in Chapter 2. TLC, NMR, LCMS, anion exchange, HRMS, IR, optical rotation facilities were used as described in Chapter 2. dT-CPG **8** was purchased from Sigma-Aldrich. The synthesis of compound **5** will be discussed in Chapter 4. The solid phase synthesis of the thymidine oligomers was performed on a Mermade-6 oligonucleotide synthesizer (Bioautomation corporation).



5'-O-(di-O-flourenylmethyl)-phosphoryl-2'-deoxylthymidine (6)

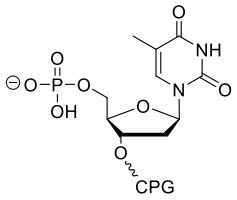
DCI (12 mL, 3.0 mmol) and thymidine **4** (242 mg, 1.0 mmol) were coevaporated with ACN (3 x) and dissolved in DMF (7 mL) with 3 Å molecular sieves under N_2. To this solution, $(FmO)_2PN(iPr)_2$ **5** (625 mg, 1.2 mmol, in 3 mL DMF) was added and the reaction was stirred for 30 minutes. The reaction mixture was cooled to 0 °C and *t*BuOOH (0.55 mL, 3 mmol, 5.5 M in nonane) was added. The mixture was allowed to reach to room temperature and was stirred for 1 hour and quenched by aq. $NaHCO_3$ (sat.). EtOAc extracted this mixture (1 x) and the organic layer was extracted with H_2O (1 x) and brine (1 x) and dried ($MgSO_4$). The mixture was filtered, concentrated and purified by silica gel column chromatography (DCM : MeOH = 99/1 – 98/2 – 95/5) to furnish **6** (439 mg, 0.65 mmol, 65%) and 3',5'-diphosphorylated byproduct (100 mg, 0.09 mmol, 9%) as a white foam. ^{1}H NMR (500 MHz, Methanol-*d*$_4$) δ 7.71 (dddt, *J* = 14.8, 12.0, 7.6, 1.0 Hz, 4H, arom.), 7.54 – 7.17 (m, 12H, arom.), 7.11 (q, *J* = 1.2 Hz, 1H, H6), 6.15 (dd, *J* = 7.8, 6.1 Hz, 1H, H1'), 4.37 – 4.26 (m, 4H, CH_2 Fm), 4.12 (dt, *J* = 6.2, 3.0 Hz, 1H, H3'), 4.05 (q, *J* = 6.1 Hz, 2H, CH Fm), 3.85 – 3.66 (m, 3H, H4', H5'), 2.11 (AB, *J* = 13.7, 6.1, 3.0 Hz, 1H, H2'), 1.82 (AB, *J* = 13.9, 7.8, 6.3 Hz, 1H, H2'), 1.48 (d, *J* = 1.2 Hz, 3H, CH_3). ^{13}C NMR (126 MHz, MeOD) δ 164.02 (C4), 149.98 (C2), 142.21, 142.19, 142.16, 140.74, 140.69, 140.66 (Cq. arom.), 134.89 (C6), 126.90, 126.88, 126.87, 126.85, 126.10, 126.09, 123.81, 123.79, 123.74, 123.69, 119.00, 118.94, 118.91 (arom.), 109.59 (C5), 84.14 (C1'), 84.02, 83.96 (C4'), 69.78 (C3'), 68.22, 68.18, 68.14, 68.09 (CH_2 Fm), 66.26, 66.21 (C5'), 46.99 (CH Fm), 38.74 (C2'), 10.37 (CH_3). ^{31}P NMR (202 MHz, MeOD) δ -2.05. IR (film): 3065, 1686, 1450, 1274, 1104, 1017, 758, 741, 554, 515 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{38}H_{36}N_2O_8P$ (M+H) 679.2204. Found 679.2201. $[\alpha]_D^{20}$ +2.6 (c = 1, in DCM)



3'-*O*-(*N*,*N*-diisopropylamino-2-cyanoethylphosphinyl)-5'-O-(di-O-flourenylmethyl)-phosphoryl-2'-deoxylthymidine (2b)

Compound **6** (900 mg, 1.33 mmol), DMF (13 mL), DIPEA (0.69 mL, 3.98 mmol) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite **7** (0.33 mL, 1.46 mmol) were added into the flask under N_2. The reaction was stirred at room temperature for 10 minutes after which was quenched by 0.15 mL EtOH. Excessive amount of EtOAc was added and the mixture was washed with aq. $NaHCO_3$ (sat.) (2 x), H_2O (1 x) and brine (1 x). The organic layer

was dried (Na_2SO_4) and filtered. The filtration was co-evaporated with toluene (1 x) then purified by automatic column (pentane/EA, 100/0 – 50/50 – 45/65) to furnish **2b** as a white foam (760 mg, 0.87 mmol, 65%). **Note**: Careful wash was needed for the work-up because the DIPEA in the reaction could cleave the Fm group. Automatic column was performed on Biotage Isolera Specktra Four machine using High-quality IRR silica gel column (40-63 µm). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.76 – 7.63 (m, 4H, arom.), 7.55 – 7.13 (m, 13H, arom.), 6.26 (ddd, *J* = 8.1, 5.8, 4.8 Hz, 1H, H1'), 4.40 (ddq, *J* = 9.3, 6.0, 2.8 Hz, 1H, H3'), 4.35 – 3.43 (m, 11H, H4', H5', CH_2 Fm, CH Fm, CH *i*Pr), 2.78 – 2.70 (m, 2H, CH_2 OCE), 2.57 (dt, *J* = 19.8, 6.2 Hz, 2H, CH_2 OCE), 2.33 (AB, *J* = 42.7, 13.7, 5.9, 2.6 Hz, 1H, H2'), 1.90 (AB, *J* = 19.9, 14.0, 8.2, 6.4 Hz, 1H, H2'), 1.64 (s, 3H, CH_3), 1.18 – 1.06 (m, 12H, *i*Pr). ^{13}C NMR (126 MHz, $CDCl_3$) δ 163.80, 163.78 (C4), 150.43, 150.37 (C2), 142.84, 142.82, 142.77, 141.45, 141.42, 141.38, 141.35 (Cq. arom.), 135.00 (C6), 127.99, 127.96, 127.14, 127.12, 124.95, 124.93, 124.86, 124.81, 120.12, 120.08, 120.04 (arom.), 117.67, 117.63, 116.98 (CN), 111.34, 111.29 (C5), 84.73 (C1'), 84.15, 84.12, 84.09, 84.06, 83.82, 83.77, 83.76, 83.71 (C4'), 73.28, 73.15, 73.01 (C3'), 69.34, 69.33, 69.30, 69.21, 69.17 (CH_2 Fm), 66.75, 66.71, 66.58, 66.54 (C5'), 58.20, 58.16, 58.13, 58.07, 57.98 (CH_2 OCE), 47.90, 47.84 (CH Fm), 43.34, 43.24 (CH *i*Pr), 39.38, 39.34, 39.32 (C2'), 24.61, 24.55, 24.46 (CH3 *i*Pr), 20.39, 20.34, 20.32, 20.26, 20.11, 20.05 (CH_2 OCE), 12.30 (CH_3). ^{31}P NMR (202 MHz, $CDCl_3$) δ 149.17, 148.84, 14.17 (H-phosphonate), -1.30, -1.34. IR (film): 2967, 1688, 1450, 1365, 1275, 1183, 1021, 914, 515 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{41}H_{39}N_3O_{10}P_2Na$ ([H-phosphonate]+Na) 818.2003. Found 818.1996. $[\alpha]_D^{20}$ +6.6 (c = 1, in DCM)



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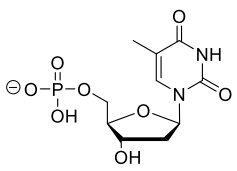
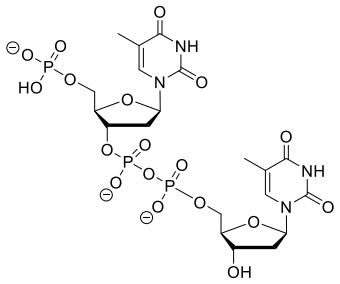


9

5'-O-Phosphoryl-dT-CPG (1) and 5'-O-phosphoryl-dT (9)

To a 20 mL reaction syringe with filter frit was added **8** (2.0 g, 60 µmol, 30 µmol/g). Dichloroacetic acid (5 %, v/v, in DCM) was added repeatedly until no yellow color was observed. The resin was extensively washed with DCM (3 x), ACN (5 x) under N_2. DCI (0.96 mL, 0.24 mmol, 0.25M in ACN) and $(FmO)_2PN(iPr)_2$ **5** (0.48 mL, 0.24 mmol, 0.5 M in ACN) were added into the resin and the mixture was shaken under N_2 for 10 minutes and drained. Repeat this coupling 1 more time and the resin was drained and washed with ACN (5 x). 3.6 mL (1*S*)-(+)-(10-camphorsulfonyl)-oxaziridine (CSO) (0.5 M in ACN) was added and the mixture was shaken for 30 minutes under N_2. The resin was drained, washed with ACN (3 x), DCM (3 x) and drained under N_2. Piperidine (8.3 mL, 4.2 mmol, 5% in DMF) was added and the mixture was shaken for 10 minutes. The resin was drained, washed with ACN (3 x) and dried under reduced pressure to obtain resin **1**. A test sample (20 mg) was added into a 2 mL reaction syringe and 1mL NH_4OH (35%) was added to cleave the molecule from resin. 1 hour later the filtration was concentrated and analyzed by ^{31}P-NMR in D_2O. ^{31}P NMR (162 MHz, D_2O): δ 4.45 (s)



pTppT (3a)

To a 5 mL reaction syringe with filter frit, **1** (200 mg, 10 µmol) was added, washed with ACN (3 x) under N_2. 1 mL DBU (5%, v/v, in ACN) was added and it was shaken for 10 minutes (2 x) to remove Fm groups on 5-phosphate after which was drained and washed with ACN (5 x). Then ETT (0.20 mL, 50 µmol, 0.25 M in ACN) and **2b** (0.1 mL, 20 µmol, 0.2M in ACN) were added into the resin and the mixture was shaken for 10 minutes (2 x). The mixture was drained and the resin was washed with ACN (5 x) under N_2. The intermediate phosphate-phosphite was oxidized with (1*S*)-(+)-(10-camphorsulfonyl)-oxaziridine (CSO) solution (1 mL, 0.5 mmol, 0.5 M in ACN) for 5 minutes (2 x) and washed with ACN (5 x). DBU solution (1 mL, 5%, v/v, in ACN) was added into the syringe and was shaken for 20 minutes to remove Fm groups on 5'-phosphate and CE group after which was drained and washed with ACN (5 x) under N_2. The resin was treated with NH_4OH (35%) overnight to cleave the product from the resin. The mixture was filtered and the filtration was concentrated. The crude material was purified by anion exchange and desalting to obtain **3a** (3.02 mg, 4.3 µmol, 43%) and pT (0.73 mg, 2.3 µmol, 23%) as white solid.

Column: Resource 15Q (10 mm x 10 cm).

Gradient: 40% - 65%. (A: 10 mM NaOH, B: 10 mM NaOH and 1 M NaCl)

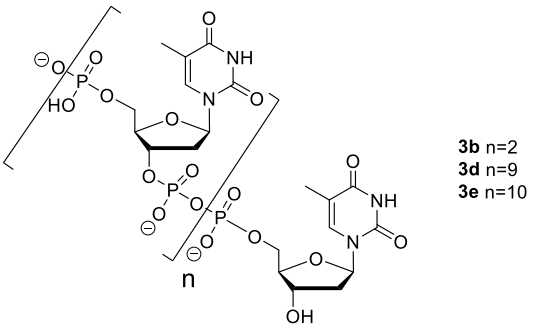
Desalting: 0.15M NH_4HCO_3

Compound 9

^{1}H NMR (500 MHz, Deuterium Oxide) δ 7.70 (q, *J* = 1.1 Hz, 1H, H6), 6.23 (dd, *J* = 7.8, 6.3 Hz, 1H, H1'), 4.46 (dt, *J* = 6.3, 3.2 Hz, 1H, H3'), 4.02 (qd, *J* = 3.8, 1.3 Hz, 1H, H4'), 3.85 (dd, *J* = 4.9, 3.8 Hz, 2H, H5'), 2.34 – 2.15 (m, 2H, H2'), 1.81 (d, *J* = 1.2 Hz, 3H, CH_3). ^{31}P NMR (162 MHz, D_2O): δ 4.45 (s)

Compound 3a

^{1}H NMR (500 MHz, Deuterium Oxide) δ 7.74 – 7.54 (m, 2H, H6), 6.30 – 6.12 (m, 2H, H1'), 4.52 (s, 1H, H3'), 4.49 (td, *J* = 4.7, 2.8 Hz, 1H, H3'), 4.35 – 4.22 (m, 1H, H4'), 4.14 – 4.00 (m, 3H, H4', H5'), 3.94 (dt, *J* = 5.0, 2.5 Hz, 2H, H5'), 2.45 (AB, *J* = 14.1, 5.8, 1.6 Hz, 1H, H2'), 2.35 – 2.19 (m, 3H, H2'), 1.79 (dd, *J* = 4.1, 1.2 Hz, 6H, CH_3). ^{31}P NMR (202 MHz, D_2O) δ 1.38 (phosphate), -11.00, -11.11, -11.72, -11.83 (pyrophosphate).



3b n=2
3d n=9
3e n=10

pTppTppT (3b), pT(ppT)$_9$ (3d) and pt(ppT)$_{10}$ (3e)

200 mg **8** (10 µmol) was added into a reaction column of a Mermade 6 oligonucleotide synthesizer, TCA solution (2 mL, 3%, m/v, in DCM) was added into the resin and drained (5 x). The resin was washed with ACN (5 x) after which ETT (600 uL, 0.25 M in ACN) and **5** (300 uL, 0.1 M in ACN) were added into the resin and the mixture was left to stand for 5 minutes and drained (3 x). The resin was rinsed by with ACN (3 x). The intermediate phosphite was oxidized with CSO solution (2 mL, 0.5 M in ACN) for 5 minutes (2 x). The resin was drained and washed with ACN (3 x). DBU solution (2 mL,

10%, v/v, in ACN) was added into the resin and was left to stand for 5 minutes (4 x) to remove Fm groups on 5'-phosphate after which was drained and washed with ACN (5 x). Next, pyrophosphate coupling cycle was performed (n times):

ETT (600 uL, 0.25 M in ACN) and **2b** (300 uL, 0.1 M in ACN) were added into the resin and the mixture was left to stand for 5 minutes, drained (2 x). The resin was rinsed by ACN (3x). The intermediate phosphate-phosphite was oxidized with CSO solution (2 mL, 0.5 M in MeCN) for 5 minutes (2 x). The resin was drained and washed with ACN (3 x). DBU solution (2 mL, 10%, v/v, in ACN) was added into the resin and was left to stand for 5 minutes (4 x) to remove Fm groups on 5'-phosphate and CE group after which was drained and washed with ACN (3 x).

After the completion of the cycle above (n times), the resin was transferred to a tube and treated with 10 mL NH_4OH (35%). The mixture was stirred overnight in a sealed condition, filtered and concentrated. The crude was purified by the same anion exchange chromatography and desalting mentioned above to obtain **3a-e** as white solid. For HRMS measurement, **3e** and **3d** were dissolved in MiliQ H**2**O (1 mg/mL) and added into a reaction syringe containing Dowex resin (NH_4^+ form). The mixture was shaken for 1 hour and filtered. The fieltration was used for HRMS mesurment.

n = 1, **3a** (5.69 mg, 7.52 µmol, 75%)

n = 2, **3b** (2.63 mg, 2.24 µmol, 22%)

n = 10, **3e** (3.15 mg, 0.70 µmol, 7%)

n = 9, **3d** (2.54 mg, 0.62 µmol, 6%)

3b

^{1}H NMR (500 MHz, Deuterium Oxide) δ 7.80 – 7.53 (m, 3H, H6), 6.35 – 6.11 (m, 3H, H1'), 4.88 (t, *J* = 6.5 Hz, 2H, H3'), 4.51 (p, *J* = 5.0, 4.4 Hz, 1H, H3'), 4.29 (d, *J* = 8.0 Hz, 2H, H4'), 4.17 – 4.05 (m, 6H, H4', H5'), 3.96 (d, *J* = 3.6 Hz, 1H, H5'), 2.46 (ddd, *J* = 15.1, 10.1, 5.9 Hz, 1H, H2'), 2.32 – 2.20 (m, 5H, H2'), 1.85 – 1.75 (m, 9H, CH_3). ^{31}P NMR (202 MHz, D_2O) δ 0.52 (phosphate), -11.63, -11.74, -11.78, -11.88, -12.36, -12.41, -12.46, -12.52 (pyrophosphates).

3d

^{1}H NMR (500 MHz, Deuterium Oxide) δ 7.76 – 7.47 (m, 10H, H6), 6.21 (dtd, *J* = 11.5, 7.9, 5.6 Hz, 10H, H1'), 4.94 – 4.77 (m, 9H, H3'), 4.50 (td, *J* = 4.9, 3.1 Hz, 1H, H3'), 4.29 (dt, *J* = 10.2, 2.9 Hz, 9H, H4'), 4.20 – 4.04 (m, 19H, H4', H5'), 4.00 (q, *J* = 3.6 Hz, 2H, H5'), 2.59 – 2.34 (m, 9H, H2'), 2.34 – 2.17 (m, 11H, H2'), 1.92 – 1.70 (m, 30H, CH_3). ^{31}P NMR (202 MHz, D_2O) δ -0.11 (phosphate), -11.65, -11.75, -11.79, -11.89, -12.39, -12.43, -12.48, -12.54 (pyrophosphates). HRMS (ESI^+) calcd for $C_{100}H_{147}N_{21}O_{98}P_{19}$ ($M+3NH_4^+$)/3 1266.0721. Found 1266.0717.

3e

^{1}H NMR (500 MHz, Deuterium Oxide) δ 7.64 (d, *J* = 27.5 Hz, 11H, H6), 6.27 – 6.19 (m, 11H, H1'), 4.88 (s, 10H, H3'), 4.58 – 4.46 (m, 1H, H3'), 4.30 (s, 10H, H4'), 4.12 (s, 21H, H4', H5'), 4.01 (s, 2H, H5'), 2.50 – 2.46 (m,10H, H2'), 2.29 - 2.25 (m, 12H, H2'), 1.83 – 1.80 (m, 33H, CH_3). ^{31}P NMR (202 MHz, D_2O) δ -0.06 (phosphate), -11.62, -11.73, -11.76, -11.84, -11.88, -12.37, -12.40, -12.47, -12.51 (pyrophosphates). HRMS (ESI^+) calcd for $C_{110}H_{167}N_{25}O_{108}P_{21}$ ($M+3NH_4^+$)/3 1394.0762. Found 1394.0764.

References

1. X. Tang, M. G. K. Benesch and D. N. Brindley, *J. Lipid Res.* 2015, **56**, 2048-2060.
2. Z. Xu, *Bioorg. Med. Chem. Lett.*, 2015, **25**, 3777-3783.
3. E. Saavedra, R. Encalada, C. Vázquez, A. Olivos-García, P. A. M. Michels and R. Moreno-Sánchez, *Mol. Biochem. Parasitol.*, 2019, DOI: 10.1016/j.molbiopara.2019.02.002.
4. A. Chakraborty, *Biol. Rev.*, 2018, **93**, 1203-1227.
5. H. Gold, P. van Delft, N. Meeuwenoord, J. D. C. Codée, D. V. Filippov, G. Eggink, H. S. Overkleeft and G. A. van der Marel, *J. Org. Chem.*, 2008, **73**, 9458-9460.
6. M. Laurent, J.-L. Ricard, J.-J. Bahain, P. Voinchet and L. Rousseau, *C. R. Acad. Sci. IIA*, 2000, **330**, 581-583.
7. P. Dabrowski-Tumanski, J. Kowalska and J. Jemielity, *Eur. J. Org. Chem.*, 2013, **2013**, 2147-2154.
8. B. A. Anderson and R. Krishnamurthy, *Chemistry*, 2018, **24**, 6837-6842.
9. K. Fujikawa, S. Suzuki, R. Nagase, S. Ikeda, S. Mori, K. Nomura, K. I. Nishiyama and K. Shimamoto, *ACS Chem. Biol.*, 2018, **13**, 2719-2727.
10. N. Qi, K. Jung, M. Wang, L. X. Na, Z. J. Yang, L. R. Zhang, A. H. Guse and L. H. Zhang, *Chem. Commun.*, 2011, **47**, 9462-9464.
11. H. A. Kistemaker, A. P. Nardozza, H. S. Overkleeft, G. A. van der Marel, A. G. Ladurner and D. V. Filippov, *Angew. Chem. Int. Ed. Engl.*, 2016, **55**, 10634-10638.
12. J. Voorneveld, J. G. M. Rack, I. Ahel, H. S. Overkleeft, G. A. van der Marel and D. V. Filippov, *Org. Lett.*, 2018, **20**, 4140-4143.
13. H. A. Kistemaker, L. N. Lameijer, N. J. Meeuwenoord, H. S. Overkleeft, G. A. van der Marel and D. V. Filippov, *Angew. Chem. Int. Ed. Engl.*, 2015, **54**, 4915-4918.
14. H. R. Singh, A. P. Nardozza, I. R. Moller, G. Knobloch, H. A. V. Kistemaker, M. Hassler, N. Harrer, C. Blessing, S. Eustermann, C. Kotthoff, S. Huet, F. Mueller-Planitz, D. V. Filippov, G. Timinszky, K. D. Rand and A. G. Ladurner, *Mol. Cell*, 2017, **68**, 860-871 e867.
15. H. A. V. Kistemaker, N. J. Meeuwenoord, H. S. Overkleeft, G. A. van der Marel and D. V. Filippov, *Eur. J. Org. Chem.*, 2015, **2015**, 6084-6091.
16. A. G. Volbeda, H. A. Kistemaker, H. S. Overkleeft, G. A. van der Marel, D. V. Filippov and J. D. Codee, *J. Org. Chem.*, 2015, **80**, 8796-8806.
17. H. Jessen, *Synlett*, 2018, **29**, 699-713.
18. G. S. Cremosnik, A. Hofer and H. J. Jessen, *Angew. Chem. Int. Ed. Engl.*, 2014, **53**, 286-289.
19. R. Gerasimaite, I. Pavlovic, S. Capolicchio, A. Hofer, A. Schmidt, H. J. Jessen and A. Mayer, *ACS Chem. Biol.*, 2017, **12**, 648-653.