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**Author:** Kistemaker, H.A.V  
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Chapter 6
Synthesis of Oligonucleotides Interconnected Through Pyrophosphate Linkages

Abstract: In this chapter a synthetic methodology for oligonucleotides interconnected via pyrophosphates is described and in doing so another method that relies on reactivity of phosphorodiamidous anhydrides is revisited. The latter method, previously reported by Ahmadibeni and Parang¹, could not be reproduced. Therefore, three pyrophosphate interconnected thymidine oligonucleotides, identical to the claimed structures in their article, were synthesized using the method described in chapter 4. Evaluation of the spectroscopic data of the newly synthesized products casts doubts on the viability of the Ahmadibeni–Parang approach. Consequently, this chapter describes the first successful synthesis of DNA oligomers interconnected via pyrophosphate linkages.

Part of this chapter has been published:
Introduction

The synthesis of nucleic acid oligomers in which the monomeric nucleotides are interconnected through pyrophosphate linkages poses considerable challenges to synthetic organic chemists. Such analogues might be useful in the search for improved antisense oligonucleotides\(^2\) and in the field of prebiotic chemistry.\(^3\) Whereas a variety of robust methods for the construction of phosphodiester linkages for the preparation of DNA and RNA oligomers exist\(^4\), methodology for the effective construction of pyrophosphate linkages has only emerged in recent years.\(^5\) The work in this thesis has focused on the synthesis of adenosine diphosphate ribose (ADPr) oligomers, this for the dual reason of their synthetic challenge and biological relevance. Adenosine diphosphate ribosylation (ADP-ribosylation) is a post-translational protein modification, of which relatively little is known. Synthetic, well-defined material is deemed necessary for unravelling the biological consequences of this post-translational modification. For this reason a synthesis program was started and the results are described in chapters 2 - 5. The results on the synthesis of ADPr-oligomers were published early 2015\(^6\) and a little later the Hergenrother laboratory reported independently another strategy with which they obtained a dimeric ADPr fragment.\(^7\) The synthetic strategy described in chapter 4 is summarized in Figure 1A and hinges on the condensation of a phosphoramidite (2) with an *in situ* generated, immobilized, phosphate monoester (1) followed by oxidation of the P(III)-P(V) intermediate. Unmasking of the phosphate triester intermediate allowed for repetition of the cycle either with amidite (2) to extend the ADPr oligomer or with amidite (3) to finalize the synthesis.
Figure 1. A) Schematic of the synthesis of ADPr oligomers as described in chapter 4. B) The strategy reported by Ahmadibeni and Parang for the construction of synthetic DNA oligomers in which the interconnecting phosphodiester are substituted with pyrophosphates.

Predating the synthesis of ADPr-oligomers described in chapter 4, and that of the Hergenrother laboratory, Ahmadibeni and Parang reported a remarkable synthesis of unnatural DNA oligonucleotides in which the nucleosides are interconnected through pyrophosphate (diphosphate) linkages. Their strategy, published in Angewandte Chemie in 2007, is summarized in Figure 1B. Central to their phosphorylation approach, also featured in successive articles co-authored by these researchers, is the use of phosphitylating reagent 4, the synthesis of which they reported in 2005 in Organic Letters. This reagent was made to react with solid support and subsequently with an unprotected mononucleoside. Condensation of the resulting secondary alcohol in 7 with another equivalent of 4 and reiteration of the procedure allowed the synthesis of an impressive array of pyrophosphate-connected oligonucleotides, composed of up to as many as twelve pyrophosphate linkages.

At first glance, this methodology appears the more facile of the two strategies and one would suspect that phosphitylating agent 4 would be equally effective in the construction of ADPr oligomers. Realizing this at the time, in 2005, an ADPr synthesis program was started with the intention to adopt the aforementioned strategy. This, however, failed because the reported synthesis of phosphitylating agent 4 could not be reproduced. Nevertheless, reagent 4 could be synthesized via a method reported...
by Foss and co-workers.\textsuperscript{16-18} In course of the synthesis of 4 inconsistencies were found in the reports of Ahmadibeni and Parang with respect to the preparation method and characterisation of reagent 4 as claimed by them. This raised the suspicion that there might be more irregularities in the work of Ahmadibeni and Parang, more specifically concerning the structural integrity of the oligomers reported in 2007 and following papers. It was decided to synthesize a number of deoxythymidine oligomers interconnected via pyrophosphate linkages. This chapter describes attempts to reproduce the synthesis of phosphitylating agent 4 and successful syntheses of three thymidine oligomers, interconnected through pyrophosphate linkages, using the methodology from chapter 4. Next the spectroscopic data of the prepared thymidine oligomers, were compared with the data of the same structures as reported by Ahmadibeni and Parang.

Results and discussion

The attempts to synthesize phosphitylating agent 4 are summarized in Scheme 1. In first instance it was attempted to prepare 4 via the previously reported one-pot four-step protocol (Scheme 1A).\textsuperscript{15} According to this protocol, phosphorus trichloride is reacted with 2-cyanoethanol in acetonitrile to yield dichlorophosphite 9, which is treated with diisopropylamine to provide 2-cyanoethyl-\(N,N\)-diisopropylchloro phosphoramidite 10. Addition of half of the reaction mixture, containing 10, to a vessel containing one equivalent of water should give phosphite 11 according to the protocol. To this reaction vessel, the remaining mixture containing 10 is added which should give the phosphitylating agent 4. This procedure failed repeatedly and monitoring the reactions by \(^{31}\text{P}\)-NMR spectroscopy analysis revealed the existence of a mixture of products in each stage. Thus, preparation of target compound 4 was unsuccessful but also 2-cyanoethyl \(N,N\)-diisopropylchlorophosphoramidite 10 could not be generated following the first two steps from the reported protocol. Compound 10 is a well-known reagent in phosphorous chemistry and is commercially available.\textsuperscript{19} In order to further probe the merits of the Parang protocol to prepare compound 4, the protocol was started with pure compound 10. Treatment of 10, in MeCN, with one equivalent of water resulted in the formation of a white precipitate. \(^{31}\text{P}\)-NMR spectroscopic analysis showed the presence of starting material 10 (\(^{31}\text{P}\) signal at 181 ppm), together with H-phosphonate amide 12 (15 ppm), the putative H-phosphonate monoester (0 ppm) and some unknown product(s) (113 and 114 ppm). Continuation of the protocol by the addition of one further equivalent of 10 gave a similar \(^{31}\text{P}\)-NMR spectrum. From these experiments it was concluded that phosphitylating agent 4 cannot be synthesized following the published protocol.\textsuperscript{15} Furthermore, it is highly unlikely that Ahmadibeni and Parang were able to observe intermediate hydroxyl phosphite 11. The aforementioned H-phosphonate 12 observed after reacting \(N,N\)-diisopropylchlorophosphoramidite 10 with water is the expected product. The literature on phosphorous chemistry\textsuperscript{20} is also very clear on the formation of such H-phosphonate tautomers from \textit{in situ} generated phosphites such as 11 through an Arbuzov-type rearrangement.\textsuperscript{21} Two other research groups have reported about the intractability of the protocol\textsuperscript{22} to produce phosphitylating agent 4 and discussed inconsistencies in the reported \(^{31}\text{P}\)-NMR spectra.\textsuperscript{23}
compounds they call phosphorodiamidous anhydrides is described\textsuperscript{16-18}. In line with their procedure, N,N-diisopropylchlorophosphoramidite 10 (Scheme 1B) was treated with 0.5 equivalent of water and 2 equivalents of DIPEA. This led to the initial formation of H-phosphonate amide.

**Scheme 1.** A) Route towards phosphitylating agent 4 as reported by Ahmadibeni and Parang. B) New synthetic route in attempting to synthesize pure phosphitylating agent 4.

Reagents: MeCN = acetonitrile; iPr\textsubscript{2}NH = diisopropylamine; DIPEA = N,N-diisopropylethylamine

12 (\textsuperscript{31}P signal at 15 ppm), which reacted immediately and irreversibly with remaining 10. This resulted in a mixture composed of compound 13 (32-36 ppm (P\textsuperscript{v}) and 110-121 ppm (P\textsuperscript{III})) and compound 4 (\textsuperscript{31}P signals at 139.8 and 139.6 ppm). The observed \textsuperscript{31}P-NMR chemical shifts agree well with the data of Foss and co-workers and do not match with the chemical shift reported by Ahmadibeni and Parang for compound 4 (a single peak at 118.6 ppm). It is noted that the \textsuperscript{31}P-NMR data of 4 and 13 described above are also consistent with the presence of two chiral phosphorus atoms in both isomers. In particular, compound 4 consists of three stereoisomers, namely the meso-compound and two enantiomers of the chiral diastereomer of 4 and the observed two \textsuperscript{31}P signals at 139.8 and 139.6 correlate well to the existence of such a mixture. Compound 13 is devoid of any elements of symmetry and exists as a mixture of two chiral diastereomers, each present as a racemic mixture, which is reflected in the \textsuperscript{31}P signals attributed to this P(III)-P(V) species.

Because of the lack of success in synthesizing phosphitylating agent 4, research performed nine years ago, an alternative method for the construction of...
pyrophosphate linkages in between nucleotides had to be developed. Such a method developed initially for ADPr oligomers, as described in chapter 4, opens a way to prepare DNA oligonucleotides in which the nucleosides are interconnected through pyrophosphate (diphosphate) linkages. Modified oligothymidylic acids 27, 28 and 29 (Scheme 3) were selected as target compounds because these exact structures were claimed in the Angewandte Chemie paper by Ahmadibeni and Parang. The required building block 15 for the pyrophosphate protocol was obtained by selective phosphitylation of thymidine and subsequent oxidation (P\text{III} to P\text{V}) to yield phosphotriester 14, which was uneventfully converted into 3’-phosphoramidite 15 via a base assisted phosphitylation (Scheme 2).

**Scheme 2. Synthesis of compound 15.**

Reagents and conditions: a) i. di-tert-butyl-\(N,N\)-diisopropylphosphoramidite, Pyr.HCl, pyridine; ii. tBuOOH; b) 2-cyanoethyl-\(N,N\)-diisopropylchlorophosphoramidite, DIPEA, DCM.

Assembly of the target compounds proceeded through detritylation of dimethoxytrityl-deoxythymidine-controlled pore glass (DMT-dT-CPG) particles. Subsequently, reaction with di-tert-butyl-\(N,N\)-diisopropylphosphoramidite, oxidation of the intermediate phosphite and removal of the tert-butyl protective group gave immobilized 16 (Scheme 3). The modified CPG 16 (10 µmol) was loaded into an automated oligonucleotide synthesizer and the pyrophosphorylation approach, reported in chapter 4, was applied to provide immobilized oligomers 18, 19 and 20 (Scheme 3).

Reagents and conditions: a) i. compound 2, ETT, MeCN, (2x); ii. CSO, MeCN, (2x); iii. DBU, DMF, (2x); iv. HCl, HFIP, (4x); v. pyridine (10% v/v), acetonitrile, (2x); b) NH₄OH; c) i. di(PMB)-N,N-diisopropyl phosphoramidite, 1-Me-Im.HCl (0.3M), 1-Me-Im (0.2M), DMF; ii. CSO, MeCN; iii. DCM/TFA (95/5 v/v%), iv. NH₄OH (35%).

Test samples of these oligomers (≈1-2 µmol) were treated with NH₄OH to yield 22-24 as crude products, which were subsequently purified by strong anion exchange chromatography. The efficiency of the coupling reactions was determined by integration of the peak areas of the UV spectra of the chromatographic separation, which showed moderate to good yields for compounds 22 (71%), 23 (53%) and 24 (40%) (Figure 2 and Table 1).
Table 1. Yield (%) based on the peak areas of the anion exchange chromatography UV spectra.

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^a) The isolated yield after purification was determined by measuring the UV absorbance at 260 nm of the combined fractions from the three syntheses.

To obtain target compounds 27-29 the terminal phosphate at the 5-OH position in 18-20 needed to be converted into a pyrophosphate. This was achieved by treating immobilized 18-20 with di-PMB phosphoramidite 25 (Scheme 3). ^24 Subsequent oxidation of the intermediate phosphite and treatment with DCM/TFA, to remove the PMB groups, afforded 27-29. Treatment with aqueous ammonia released the crude products from the solid support after which purification by strong anion exchange chromatography and lyophilization yielded target compounds 27-29 (Figure 3 and Table 2).

Table 2. Yield (%) based on the peak areas of the anion exchange chromatography UV spectra.

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^a) The isolated yield after purification was determined by measuring the UV absorbance at 260 nm of the combined fractions from the three syntheses.
Figure 3. Anion exchange chromatography UV spectra for the synthesis of compounds 27 (blue), 28 (red) and 29 (green). a The ppTppT compound in the synthesis of 27 and 28/29 elute at different retention times because of the different gradient used.

Both terminal phosphates 21-24 and pyrophosphates 26-29 were analyzed using $^1$H-, $^{31}$P-, COSY- and J-resolved $^{31}$P-NMR spectroscopy (Figures 4 and 5). The observed chemical shifts are as expected and all proton and phosphorous atoms could be assigned (see Experimental section).

Figure 4. Stacked $^1$H-NMR spectra of compounds 21 - 24 and 26 - 29.
Figure 5. Stacked $^{31}$P-NMR spectra (top) and $^{31}$P-NMR J-Resolved spectra (bottom) of compounds 22–24 and 27–29.
The J-Resolved $^{31}$P-NMR spectrum for compound 29 is also shown in Figure 6A and compared with the spectrum for the identical compound reported by Ahmadibeni and Parang, which is depicted in Figure 6B. However, a direct comparison with a $^{31}$P-NMR from the report could not be made because all the NMR spectra are reported as protonated pyrophosphates in deuterated DMSO. The solubility of phosphates and pyrophosphates in DMSO is poor, as was observed for compounds 26-29, making DMSO an inappropriate solvent for NMR analysis of these compounds. Furthermore, the stability of pyrophosphate(s) under acidic conditions is questionable, it is therefore a common practice to isolate and store these as salts of alkali metals, ammonia or tertiary amines. Therefore, conventional practice was applied to the synthesized compounds and the isolated pyrophosphate oligonucleotides were handled as ammonium salts, except in the cases in which a direct spectroscopic comparison of the products with those reported by Parang was attempted. For this, the reported procedure was repeated to convert compounds 26-29 from the ammonium form to the protonated species by stirring with $H^+$ resin in water/dioxane for 30 min at room temperature (not at -20 °C as reported since this causes the mixture to become a frozen solid) and lyophilization resulted in deterioration of the material as seen from NMR spectra taken from these.

Comparing the $^{1}$H- and $^{31}$P-NMR spectra of 27-29 (ammonium form) with the reported protonated material reveals some telling differences (compare Figure 6A and B for structure 29). Firstly, Ahmadibeni and Parang report only J-resolved $^{31}$P-NMR spectra (labelled as fully decoupled) and the chemical shifts of the β-phosphorus of terminal pyrophosphates in their spectra are consistently at around 3 ppm. This is contrast with the recorded chemical shift of the β-phosphorus of the terminal pyrophosphate for 27-29 ranging from -6 to -10 ppm which is also backed up by literature data on related compounds.23 Secondly, the internal pyrophosphates for all the compounds show chemical shifts consistently between -10 and -13 ppm while Ahmadibeni and Parang report for these compounds shifts between -8 and -16 ppm. Moreover, some of the reported “peak-pickings” do not correspond with the values on the x-axis (Figure 6B).

Thirdly, Ahmadiben and Parang reported for the H4’ and H5’ protons of the deoxyribose moiety in (pyro)phosphorylated thymidine shifts of around 3.5 ppm. This shift is more likely to correspond with H4’ and H5’ protons of thymidine having a free hydroxyl at the 5’-position. The observed proton shifts for H4’ and H5’ of compounds 21-24 and 26-29 are around 4 to 4.5 ppm, as expected for (pyro) phosphorylated thymidine. Furthermore, the shifts and splitting patterns for the majority of the other protons in 21-24 and 26-29 are as expected and they differ from the data presented by Ahmadiben and Parang.
Figure 6. J-Resolved $^{31}$P-NMR spectra for; A) compound 29, B) the identical compound as reported by Ahmadibeni and Parang.

Conclusion

In summary, artificial thymidine oligonucleotides with only pyrophosphate linkages were assembled using the method described in chapter 4 for the synthesis of ADPr oligomers. The three target thymidine oligonucleotides 27, 28 and 29 were obtained in moderate to good yields and their identity was ascertained by spectroscopic analysis. The spectroscopic evidence collected for the synthesized compounds contradicts the data of the same artificial thymidine oligonucleotides as reported by Ahmadibeni and Parang. In addition, the synthesis of phosphitylating agent 4 that features as the key reagent in various papers of the Parang group could not be repeated. Moreover, the $^{31}$P-NMR spectroscopical assignment of 4, as reported in 2005, is incorrect. The authors have overlooked the fact that compound 4 can exist in three stereoisomeric forms and that compound 4 has two - not one - phosphorous signals as the expected result. Such a double signal was observed within a mixture of compounds containing 4 and P(V)-P(III)-species 13, a compound
mixture prepared analogous to a procedure reported by Foss and colleagues.\textsuperscript{16-18} One could argue that Ahmadibeni and Parang have obtained the same mixture of compounds and that this mixture is a bona vide phosphitylating agent. Though it cannot be excluded, this - which was not further attempted during this research - is considered unlikely, not least because the analytical data of the artificial thymidine oligonucleotides described in this chapter are very different from what is reported about the same compounds by Ahmadibeni and Parang.

**Experimental section**

**General procedures**

All chemicals were used as received unless stated otherwise. All solvents used under anhydrous conditions were stored over 4Å molecular sieves except for methanol which was stored over 3Å molecular sieves. Unless stated otherwise, solvents were removed by rotary evaporation under reduced pressure at 40 °C. TLC analysis, silica gel chromatography, NMR, LCMS, HPLC and HRMS techniques were used as described in chapter 2. The solid phase synthesis of the thymidine oligomers was performed on a Mermade-6 oligonucleotide synthesizer (Bioautomation corporation).

5’-O-(di-tert-butyl)phosphate-2’-deoxythymidine (14)

Pyridinium chloride (690 mg, 6.0 mmol) and thymidine (480 mg, 2.0 mmol) were coevaporated with dry pyridine (3x) and finally dissolved in dry pyridine (20 mL) under an argon atmosphere. To this solution di-tert-butyl-\(N,N\)-diisopropylphosphoramidite (600 mg, 2.4 mmol) was added and the reaction was stirred for 30 minutes. The reaction mixture was cooled to 0 °C and tBuOOH (3 eq., 5.5 M in nonane) was added. The mixture was allowed to reach room temperature and was left to stir for 16 hours. DCM (50 mL) was added and the mixture was washed with 

\[\text{aq. NaHCO}_3\] (sat.) and the aqueous layer was extracted with DCM. The combined organic layers were washed with brine, dried over MgSO\(_4\) and concentrated under reduced pressure. The mixture was purified by silica gel chromatography (DCM/MeOH, 100/0 - 95/5) to afford the title compound as a white foam (764 mg, 1.76 mmol, 88%) and double phosphorylated thymidine (71 mg, 0.16 mmol, 8%) was obtained separately as a byproduct. \(\textsuperscript{1}H\text{-NMR} (500 MHz, CDCl\textsubscript{3}) \delta 7.52 (d, 1H, H6), 6.41 (t, J = 6.8 Hz, 1H, H1’), 4.51 (dt, J = 6.4, 3.2 Hz, 1H, H3’), 4.26 - 4.13 (m, 2H, H4’, H5’), 4.13 - 4.07 (m, 1H, H5’), 2.41 (ddd, J = 13.5, 6.0, 3.3 Hz, 1H, H2’), 2.16 (dt, J = 13.7, 6.8 Hz, 1H, H2’), 1.94 (s, 3H, CH\textsubscript{3}), 1.49 (d, J = 2.5 Hz, 18H, tBu CH\textsubscript{3}). \(\textsuperscript{31}P\text{-NMR} (162 MHz, CDCl\textsubscript{3}) \delta -9.79.

5’-O-(di-tert-butyl)phosphate-3’-O-\(N,N\)-di-isopropylamino-O-cyanoethyl phosphoramidite-2’-deoxythymidine (15)

Compound 7 (750 mg, 1.73 mmol) was co-evaporated with anhydrous MeCN (3x) and dissolved in anhydrous DCM (10 mL) under an atmosphere of argon. DIPEA (0.75 mL, 4.33 mmol) and 2-cyanoethyl \(N,N\)-diisopropylchlorophosphoramidite (0.40 mL, 1.82 mmol) were consecutively added and the mixture was
stirred at room temperature for 15 minutes. The reaction mixture was diluted with DCM, washed with aq. NaHCO₃ (sat.) and the water layer was extracted with DCM. The combined organic layers were washed with brine, dried (MgSO₄), concentrated in vacuo and purified by silica gel chromatography (Pentane/EtOAc with 1% MeOH, 55/45) to obtain the title compound as a white foam (946 mg, 1.49 mmol, 86%).

1H-NMR (500 MHz, CDCl₃) δ 7.55 (d, J = 1.1 Hz, 1H, H₆), 7.53 (d, J = 1.1 Hz, 1H, H₆), 6.45 – 6.39 (m, 2H, H₁'), 4.62 – 4.53 (m, 2H, H₃'), 4.27 – 4.22 (m, 1H, H₄'), 4.22 – 4.16 (m, 3H, H₄', H₅'), 4.16 – 4.12 (m, 2H, H₅'), 3.91 – 3.82 (m, 2H, C₆H₂OCNE), 3.81 – 3.72 (m, 2H, C₆H₂OCNE), 3.68 – 3.56 (m, 4H, C₆H₃tBu), 2.67 (td, J = 6.3, 2.5 Hz, 4H, CH₂OCNE), 2.49 (dd, J = 13.7, 5.8, 2.3 Hz, 1H, H₂'), 2.40 (dd, J = 13.5, 5.8, 2.3 Hz, 1H, H₂'), 2.17 (dt, J = 14.1, 6.8 Hz, 2H, H₂'), 1.96 (s, 6H, C₆H₃), 1.53 – 1.47 (m, 36H, tBu C₆H₃), 1.23 – 1.16 (m, 24H, C₆H₃iPr).

13C-NMR (126 MHz, CDCl₃) δ 164.07, 164.04 (C₄), 150.68, 150.62 (C₂), 135.58, 135.56 (C₆), 117.59 (C≡N), 111.48, 111.41 (C₅), 84.70, 84.66 (C₁'), 84.41, 84.36, 84.33, 84.29 (C₄'), 83.12, 83.10, 83.06, 83.04, 82.92, 82.86 (C₆H₃tBu), 73.72, 73.64, 73.58, 73.51 (C₃'), 66.25, 66.20, 66.08, 66.03 (C₅'), 58.40, 58.37, 58.25, 58.22 (CH₂OCNE), 43.39, 43.29 (CH₂iPr), 39.57, 39.55, 39.53 (C₂'), 29.93, 29.91, 29.88 (CH₃tBu), 24.66, 24.63, 24.61, 24.58, 24.55, 24.50 (CH₃iPr), 20.44, 20.42, 20.39, 20.36 (CH₂OCNE), 12.50 (CH₃).

31P-NMR (122 MHz, CDCl₃) δ 148.29, 147.97, -11.00.

5′-O-phosphate-dT-CPG (16)

DMT-dT-CPG (2.6 g, 29 µmol/g) was loaded in a plastic fritted syringe (20 mL) and repeatedly washed with a solution of TFA (10% v/v) in DCM until no longer an orange color was observed. The resin was extensively washed with DCM, placed under an argon atmosphere and extensively washed with MeCN to remove traces of water. The resin was washed (1x) with a mixture of 1-methylimidazolium chloride (0.3M) and 1-methylimidazole (0.2M) in DMF and the aforementioned activator mixture (8 mL) was added. Di-tert-butyl-N,N-diisopropylphosphoramidite (40 µL, 120 µmol) was added and the mixture was shaken at room temperature for 15 minutes under an atmosphere of argon. The reaction mixture was drained and the resin washed with MeCN (5x). (1S)-(+)-(10-camphorsulfonyl)-oxaziridine (CSO) (0.5 M) in MeCN (5 mL) was added and the mixture was shaken for 30 minutes under an argon atmosphere. The reaction mixture was drained and the resin washed with MeCN (3x) and DCM (3x). Pyridine (10 mL), Ac₂O (50 eq.) and DMAP (cat.) were added and the mixture was shaken for 30 minutes to cap any remaining free hydroxyl functionality. The reaction mixture was drained and the resin washed with DMF (3x) and DCM (3x). A sample was analyzed with 31P NMR upon cleavage with NH₄OH (35 %) for 1 hour: 31P-NMR (162 MHz, D₂O) δ -3.08 (mono-tBu), -9.88 (di-tBu).

A solution of DCM/TFA (9/1 v/v, 8 mL) was added to the resin and shaken at room temperature for 30 minutes. The reaction mixture was drained and the resin washed with DCM (2x), pyridine/H₂O (9/1 v/v, 2 x 10 mL), DMF (3x) and DCM (3x). The resin was dried under reduced pressure and a sample was analyzed with 31P NMR upon cleavage with NH₄OH (35 %) for 1 hour. 1H-NMR (400 MHz, D₂O) δ 7.77 (s, 1H, H₆), 6.27 (t, J = 7.0 Hz, 1H, H₁'), 4.51 (dt, J = 5.6, 2.6 Hz, 1H, H₃'), 4.08 - 4.00 (m, 1H, H₄'), 3.93 - 3.81 (m, 2H, H₅'), 2.33 (dt, J = 14.1, 7.0 Hz, 1H, H₂'), 2.23 (ddd, J = 14.0, 6.1, 3.1 Hz, 1H, H₂'), 1.87 (s, 3H, CH₃). 31P-NMR (162 MHz, D₂O) δ 4.14.
General procedure A; solid support synthesis poly-pyrophosphate
The pre-loaded CPG resin 16 (400 mg, ~10 µmol) was loaded in a Mermade 6 oligonucleotide synthesizer and washed with MeCN (3x) and the complete synthesis was performed under an argon atmosphere. The resin was rinsed with MeCN (3x) and compound 15 (0.10 M in MeCN; 300 µL) and ETT (0.25 M in MeCN; 400 µL) were added. The mixture was left to stand for 5 minutes, drained and followed by a second addition of compound 15 and ETT. The reaction mixture was drained and the resin rinsed with MeCN (3x). The intermediate phosphate-phosphite was oxidized with CSO (2 mL; 0.5 M in MeCN) for 5 minutes (2x) and washed with MeCN (3x). The resin was rinsed with MeCN (3x) and treated with HCl (1 mL; 50 mM in hexafluoroisopropanol (HFIP)) for 1 minute (4x) to remove the tert-Butyl groups (tBu). The resin was rinsed with MeCN (4x), pyridine (10 v/v % in MeCN; 2x 30 seconds) and MeCN (3x). Test samples were taken and the products were cleaved from the resin upon treatment with NH₄OH (35 %) for 1 hour.

Compound 21; pT
The general procedure A was applied to synthesize compound 22-24. The crude material was purified by strong anion exchange chromatography on Source 15Q (10mm x 10cm) using gradient elution with NH₄OAc (20 mM to 1.0 M) and repeated lyophilization afforded byproduct 21 as a white solid (29% based on UV integration, 33% based on ³¹P NMR integration for the synthesis of 22). ¹H-NMR (500 MHz, D₂O) δ 7.78 (d, 1H, H₆), 6.36 (t, J = 7.0 Hz, 1H, H₁'), 4.60 - 4.56 (m, 1H, H₃'), 4.21 - 4.16 (m, 1H, H₄'), 4.11 - 4.02 (m, 2H, H₅'), 2.40 - 2.35 (m, 2H, H₂'), 1.93 (d, J = 1.2 Hz, 3H, CH₃). ³¹P-NMR (162 MHz, D₂O) δ 1.11. HRMS [C₁₀H₁₅N₂O₈P₁ + H]⁺: 323.0639 found, 323.0639 calculated.

Compound 22; pTppT
The general procedure A was applied to synthesize compound 22. The crude material was purified by strong anion exchange chromatography on Source 15Q (10mm x 10cm) using gradient elution with NH₄OAc (20 mM to 1.0 M) and repeated lyophilization afforded 22 as a white solid (71% based on UV integration, 67% based on ³¹P NMR integration for the synthesis of 22). ¹H-NMR (500 MHz, D₂O) δ 7.77 (d, J = 1.3 Hz, 1H, H₆), 7.74 (d, J = 1.3 Hz, 1H, H₁'), 6.36 (dd, J = 8.9, 5.7 Hz, 1H, H₁''), 6.32 (t, J = 6.8 Hz, 1H, H₁'), 5.04 - 4.96 (m, 1H, H₃''), 4.61 (q, J = 5.0 Hz, 1H, H₃'), 4.45 - 4.38 (m, 2H, H₄''), 4.26 - 4.14 (m, 2H, H₄', H₅''), 4.14 - 4.02 (m, 2H, H₅', H₆), 2.59 (ddd, J = 14.1, 5.8, 1.7 Hz, 1H, H₂''), 2.43 - 2.32 (m, 3H, H₂', H₂''), 1.92 (d, J = 1.1 Hz, 3H, CH₃), 1.91 (d, J = 1.2 Hz, 3H, CH₃). ³¹P-NMR (162 MHz, D₂O) δ 0.68, -10.90, -11.03, -11.65, -11.78. HRMS [C₂₀H₂₉N₄O₁₈P₃ + H]⁺: 707.0766 found, 707.0762 calculated.
Compound 23; pTppTppT
The general procedure A was applied to synthesize compound 23. The crude material was purified by strong anion exchange chromatography on Source 15Q (10mm x 10cm) using gradient elution with NH₄OAc (20 mM to 1.0 M) and repeated lyophilization afforded 23 as a white solid (53% based on UV integration). \(^1\)H-NMR (500 MHz, D₂O) δ 7.76 (d, J = 1.1 Hz, 1H, H6), 7.75 (d, J = 1.2 Hz, 1H, H6), 7.73 (d, J = 1.0 Hz, 1H, H6), 6.36 – 6.28 (m, 3H, H1'-H1''), 5.01 – 4.95 (m, 2H, H3'', H3''''), 4.63 – 4.58 (m, 1H, H3'), 4.42 – 4.37 (m, 2H, H4'', H4''''), 4.25 – 4.15 (m, 5H, H4', H5', H5'''), 2.61 – 2.52 (m, 2H, H2'', H2'''), 2.41 – 2.31 (m, 4H, H2'-H2'''), 1.91 (d, J = 1.0 Hz, 3H, CH₃), 1.90 (d, J = 1.0 Hz, 3H, CH₃), 1.90 (d, J = 1.0 Hz, 3H, CH₃).

\(^{31}\)P-NMR (162 MHz, D₂O) δ 0.67, -10.90, -11.04, -11.18, -11.64, -11.69, -11.77, -11.82.

\(^{31}\)P-NMR (162 MHz, D₂O, J-Resolved) δ 0.67, -10.96, -11.09, -11.12, -11.69, -11.75.

HRMS [C₃₀H₄₃N₆O₂₈P₅ + Na]^+: 1113.0719 found, 1113.0706 calculated.

Compound 24; pTppTppTppT
The general procedure A was applied to synthesize compound 24. The crude material was purified by strong anion exchange chromatography on Source 15Q (10mm x 10cm) using gradient elution with NH₄OAc (20 mM to 1.0 M) and repeated lyophilization afforded 24 as a white solid (40% based on UV integration). \(^1\)H-NMR (500 MHz, D₂O) δ 7.75 (d, J = 1.1 Hz, 1H, H6), 7.75 (d, J = 1.2 Hz, 1H, H6), 7.72 (d, J = 1.0 Hz, 1H, H6), 7.70 (d, J = 1.0 Hz, 1H, H6), 6.37 – 6.25 (m, 4H, H1'-H1'''), 5.02 – 4.95 (m, 4H, H3'', H3''', H3''''), 4.63 – 4.56 (m, 1H, H3'), 4.42 – 4.33 (m, 3H, H4'', H4'''', H4'''''), 4.25 – 4.14 (m, 9H, H4', H5', H5'', H5'''), 4.11 – 4.05 (m, 2H, H5''''), 2.61 – 2.51 (m, 2H, H2''', H2''''), 2.39 – 2.30 (m, 5H, H2'-H2'''''), 1.91 (d, J = 1.0 Hz, 3H, CH₃), 1.90 (d, J = 1.0 Hz, 3H, CH₃), 1.89 (s, 6H, CH₃). \(^{31}\)P-NMR (162 MHz, D₂O) δ 0.67, -10.90, -11.04, -11.18, -11.64, -11.69, -11.77, -11.82.

\(^{31}\)P-NMR (162 MHz, D₂O, J-Resolved) δ 0.67, -10.96, -11.09, -11.12, -11.69, -11.75.

HRMS [C₄₀H₅₇N₈O₃₈P₇ + Na]^+: 1497.0846 found, 1497.0829 calculated.
General procedure B; solid support synthesis terminal pyrophosphate

The intermediate products 18 - 20 were loaded in a plastic fritted syringe and washed with MeCN (3x) and the complete synthesis was performed under an argon atmosphere. The resin was rinsed with DMF (3x) and an activator mixture of 1-methylimidazolium chloride (0.3M) and 1-methylimidazole (0.2M) in DMF. The activator mixture (0.5 mL) was added followed by di-(para-methoxybenzyl)-N,N-diisopropylphosphoramidite (20 mg, 50 µmol). The mixture was shaken for 30 minutes, drained and rinsed with MeCN (3x). The intermediate phosphate-phosphite was oxidized with CSO (0.5 M) in MeCN (2 mL) for 15 minutes (two times) and washed with MeCN (3x). The resin was rinsed with DCM (3x) and the para-methoxybenzyl (PMB) groups were removed by treating the resin with a solution of DCM/TFA (95/5 v/v, 2 mL) for 30 minutes. The reaction mixture was drained and the resin washed with DCM (2x), pyridine/H₂O (9/1 v/v, 2 x 10 mL), DMF (3x) and DCM (3x). The products were cleaved from the resin upon treatment with NH₄OH (35 %) for 1 hour.

Compound 26; ppT

The general procedure B was applied to synthesize compounds 27-29. The crude material was purified by strong anion exchange chromatography on Source 15Q (10mm x 10cm) using gradient elution with NH₄OAc (20 mM to 1.0 M) and repeated lyophilization afforded byproduct 26 as a white solid (25% based on UV integration). ¹H-NMR (500 MHz, D₂O) δ 7.75 (d, J = 1.4 Hz, 1H, H6), 6.35 (t, J = 3.3 Hz, 1H, H3'), 4.64 (dt, J = 6.2, 3.3 Hz, 1H, H3'), 4.20 - 4.13 (m, 3H, H4', H5'), 2.45 - 2.28 (m, 2H, H2'), 1.91 (d, J = 1.0 Hz, 3H, CH₃). ³¹P-NMR (162 MHz, D₂O) δ -9.38, -9.51, -10.62, -10.75. HRMS [C₁₀H₁₆N₂O₁₁P₂ + Na⁺]: 425.0121 found, 425.0122 calculated.

Compound 27; ppTppT

The general procedure B was applied to synthesize compound 27. The crude material was purified by strong anion exchange chromatography on Source 15Q (10mm x 10cm) using gradient elution with NH₄OAc (20 mM to 1.0 M) and repeated lyophilization afforded 27 as a white solid (56% based on UV integration, 82% based on 18). ¹H-NMR (500 MHz, D₂O) δ 7.76 (d, J = 1.1 Hz, 1H, H6), 7.74 (d, J = 1.2 Hz, 1H, H6), 6.35 (t, J = 5.6 Hz, 1H, H3'), 4.63 - 4.57 (m, 1H, H3'), 4.46 - 4.39 (m, 1H, H4''), 4.25 - 4.19 (m, 2H, H5', H5''), 4.19 - 4.14 (m, 3H, H5', H5'', H4''), 2.58 (ddd, J = 14.0, 5.6, 1.6 Hz, 1H, H2''), 2.41 (td, J = 8.9, 4.5 Hz, 1H, H2''), 2.36 (dd, J = 6.8, 5.1 Hz, 2H, H2'), 1.92 (d, J = 1.1 Hz, 3H, CH₃), 1.91 (d, J = 1.0 Hz, 3H, CH₃). ³¹P-NMR (162 MHz, D₂O) δ -10.06, -10.18, -10.83, -10.91, -10.95, -11.05, -11.70, -11.84, -11.84. HRMS [C₂₀H₃₀N₄O₂₁P₄ + Na⁺]: 809.0247 found, 809.0245 calculated.
**Compound 28; ppTppTppT**

The general procedure B was applied to synthesize compound 28. The crude material was purified by strong anion exchange chromatography on Source 15Q (10mm x 10cm) using gradient elution with NH₄OAc (20 mM to 1.0 M) and repeated lyophilization afforded 28 as a white solid (40% based on UV integration, 87% based on 19). ¹H-NMR (500 MHz, D₂O) δ 7.75 (d, J = 1.2 Hz, 1H, H6), 7.73 (d, J = 1.2 Hz, 1H, H6), 7.73 (d, J = 1.2 Hz, 1H, H6), 6.34 - 6.28 (m, 3H, H1'-H1'''), 5.03 - 4.93 (m, 2H, H3'', H3'''), 4.60 (q, J = 4.7 Hz, 1H, H3'), 4.42 - 4.36 (m, 2H, H4'', H4'''), 4.24 - 4.12 (m, 7H, H4', H5', H5'', H5'''), 2.62 - 2.50 (m, 2H, H2'', H2'''), 2.43 - 2.29 (m, 4H, H2', H2'', H2'''), 1.92 (d, J = 1.0 Hz, 3H, CH₃), 1.90 (d, J = 1.0 Hz, 3H, CH₃), 1.89 (d, J = 1.0 Hz, 3H, CH₃). ³¹P-NMR (162 MHz, D₂O) δ -10.08, -10.21, -10.92, -11.76. HRMS [C₃₀H₄₄N₆O₃₁P₆ + Na]+: 1193.0385 found, 1193.0369 calculated.

**Compound 29; ppTppTppTppT**

The general procedure B was applied to synthesize compound 29. The crude material was purified by strong anion exchange chromatography on Source 15Q (10mm x 10cm) using gradient elution with NH₄OAc (20 mM to 1.0 M) and repeated lyophilization afforded 29 as a white solid (33% based on UV integration, 81% based on 20). ¹H-NMR (500 MHz, D₂O) δ 7.74 (d, J = 1.4 Hz, 1H, H6), 7.73 (d, J = 1.3 Hz, 1H, H6), 7.72 (d, J = 1.4 Hz, 1H, H6), 7.69 (d, J = 1.4 Hz, 1H, H6), 6.35 - 6.23 (m, 4H, H1'-H1'''), 5.03 - 4.92 (m, 3H, H3''', H3''''), 4.60 (q, J = 4.8 Hz, 1H, H3'), 4.43 - 4.34 (m, 3H, H4'', H4''', H4'''''), 4.25 - 4.12 (m, 9H, H4', H5'-5'''''), 2.61 - 2.50 (m, 3H, H2'', H2''', H2''''), 2.42 - 2.27 (m, 5H, H2'-2'''''), 1.92 (d, J = 1.1 Hz, 3H, CH₃), 1.90 (d, J = 1.2 Hz, 3H, CH₃), 1.88 (s, 6H, CH₃). ³¹P-NMR (162 MHz, D₂O) δ -10.04, -10.17, -10.80, -10.92, -11.02, -11.14, -11.16, -11.67, -11.80. ³¹P-NMR (162 MHz, D₂O, J-Resolved) δ -10.10, -10.85, -10.95, -11.02, -11.06, -11.10, -11.73. HRMS [C₄₀H₅₈N₆O₄₁P₈ + Na]+: 1577.05 11 found, 1577.0493 calculated.

**References**

2. Bell, N. M.; Micklefield, J., *Chembiochem* 2009, 10 (17), 2691-703.


