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On the Synthesis of Oligonucleotides Interconnected through Pyrophosphate Linkages

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In this article we revisit the synthetic methodology for oligonucleotides interconnected via pyrophosphates that relies on reactivity of phosphorodiamidous anhydrides. We have found this method, previously published by Ahmadibeni and Parang (2007), to be irreproducible in our hands. We synthesized successfully three pyrophosphate interconnected thymidine oligonucleotides, identical to the claimed structures in their article, via a method independently developed by us. Evaluation of the spectroscopic data of the newly synthesized products casts doubts on the viability of the Ahmadibeni–Parang approach. Therefore, we believe to report here the first successful synthesis of DNA oligomers interconnected via pyrophosphate linkages.

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[1] and in the field of prebiotic chemistry.[2] Whereas a variety of robust methods for the construction of phosphodiester linkages for the preparation of DNA and RNA oligomers exist,[3] methodology for the effective construction of pyrophosphate linkages has only emerged in recent years.[4] Our work in this context 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

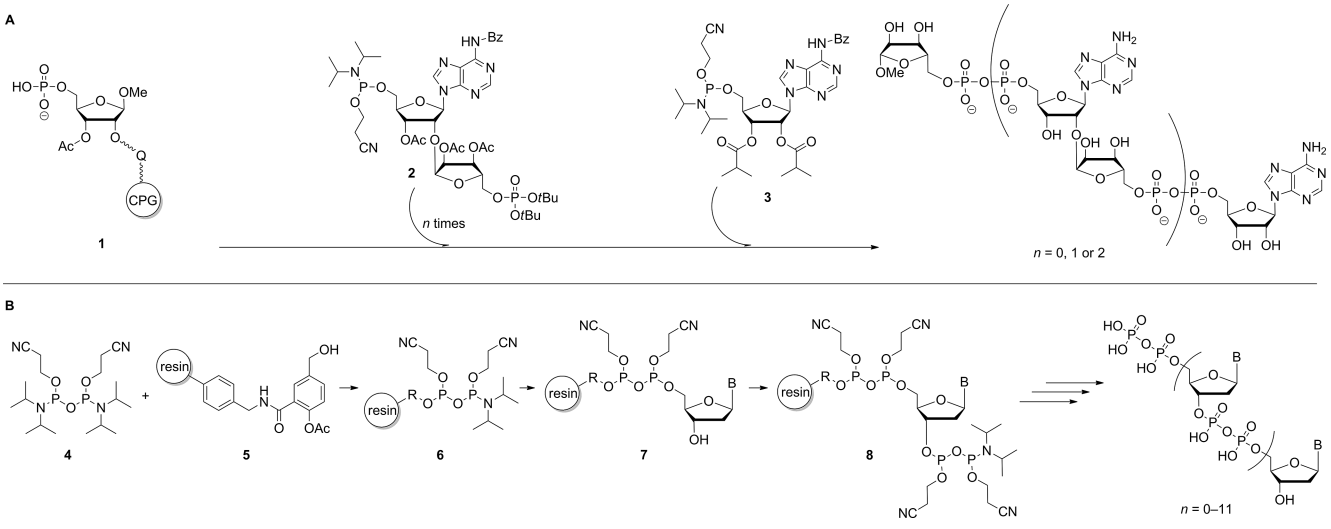

Figure 1. A) Our strategy for the synthesis of ADPr oligomers. B) The strategy reported by Ahmadibeni and Parang for the construction of synthetic DNA oligomers in which the interconnecting phosphodiesters are substituted for pyrophosphates.

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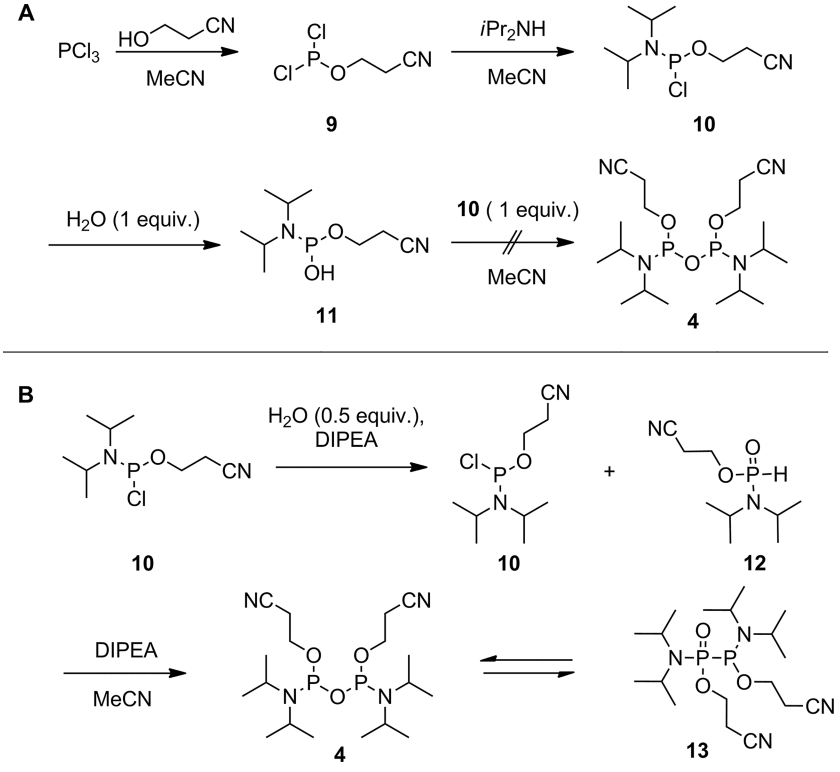
necessary for unraveling the biological consequences of this post-translational modification. For this reason we,[5] as well as the Hergenrother laboratory,[6] embarked on a synthesis program and the first results were published independently by the two laboratories at the beginning of this year. Our synthetic strategy 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.

Predating our synthesis of ADPr, 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 2007, is summarized in Figure 1 (B).[7] Central to their phosphorylation approach, also featured in successive articles co-authored by these researchers,[8] is the use of phosphitylating reagent **4**, the synthesis of which they reported in 2005.[9] This reagent was made to react with solid support **5** 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 a first glance, the Parang 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. This in fact also occurred to us at the time, in 2005, when we embarked on our ADPr synthesis program. Indeed, at the onset of our research on pyrophosphate-interconnected nucleosides, we set out to adopt the chemistry reported by Ahmadibeni and Parang for the synthesis of ADPr-oligomers. We failed in this, in the first instance, because we could not reproduce the reported synthesis of phosphitylating agent **4**. Reagent **4**, however, could be synthesized via a method reported by Foss and co-workers.[10] In doing so, we found inconsistencies in the work of Ahmadibeni and Parang for the synthesis and characterization of reagent **4** and this raised our suspicion that there might be more irregularities in their work, more specifically concerning the structural integrity of the oligomers reported in 2007. To put this suspicion to the test we decided to synthesize a number of deoxythymidine oligomers using the methodology available to us thanks to our work on ADPr. In this work we describe first our attempts to reproduce the synthesis of phosphitylating agent **4**. Next, we reveal that the structural data of our thymidine oligomers, interconnected through pyrophosphate linkages, are at odds with the data provided and described by Parang and co-workers.

Results and Discussion

Our attempts to synthesize phosphitylating agent **4** are summarized in Scheme 1. In the first instance we attempted

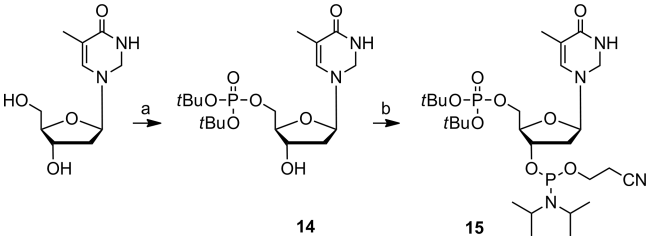


Scheme 1. A) Route towards phosphitylating agent **4** as reported by Ahmadibeni and Parang. B) Our synthetic route in attempting to synthesize pure phosphitylating agent **4**. MeCN = acetonitrile; *i*Pr_2NH = diisopropylamine; DIPEA = *N*,*N*-diisopropylethylamine.

to prepare **4** via the reported one-pot four-step protocol reported by Ahmadibeni and Parang (Scheme 1, A).[9] 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*-diisopropylchlorophosphoramidite **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 Parang protocol. To this reaction vessel, the remaining mixture containing **10** is added which should give the phosphitylating agent **4**. This procedure failed repeatedly in our hands and monitoring the reactions by ^{31}P-NMR spectroscopy analysis revealed the existence of a mixture of products in each stage. Thus, we not only did not succeed in preparing target compound **4**, we also were not able to generate 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (**10**) following the first two steps from the Ahmadibeni–Parang protocol. Compound **10** is a well-known reagent in phosphorus chemistry and is commercially available.[11] In order to further probe the merits of the Parang protocol to prepare compound **4**, we took pure compound **10** and started the protocol from there. Treatment of **10**, in MeCN, with one equivalent of water resulted in the formation of a white precipitate. ^{31}P-NMR spectroscopic analysis showed the presence of starting material **10** (^{31}P signal at δ = 181 ppm), together with H-phosphonate amide **12** (δ = 15 ppm), the putative H-phosphonate monoester (δ = 0 ppm) and some unknown product(s) (113 and 114 ppm) (see Supporting Information). Continuation of the protocol by the addition of one further equivalent of **10** gave a similar ^{31}P-NMR spectrum (see Supporting Information). From these experiments we conclude that phosphitylating agent **4** cannot be synthesized following the published protocol.[9] We should add that we find it highly unlikely that Ahmadibeni and Parang were able to observe intermediate hydroxyl phosphite **11**. The H-phosphonate **12** we observed after reacting *N*,*N*-diisopropylchlorophosphoramidite **10** with water is the expected product. The literature on phosphorus chemistry[12] is also very clear on the formation of such H-phosphonate tautomers from in situ generated phosphites such as **11** through an Arbuzov-type rearrangement.[13] We note that two groups, independently from us, have reported about the intractability of the protocol[14] reported by Ahmadibeni and Parang to produce phosphitylating agent **4**. These groups also discussed inconsistencies in the reported ^{31}P-NMR spectra.[15]

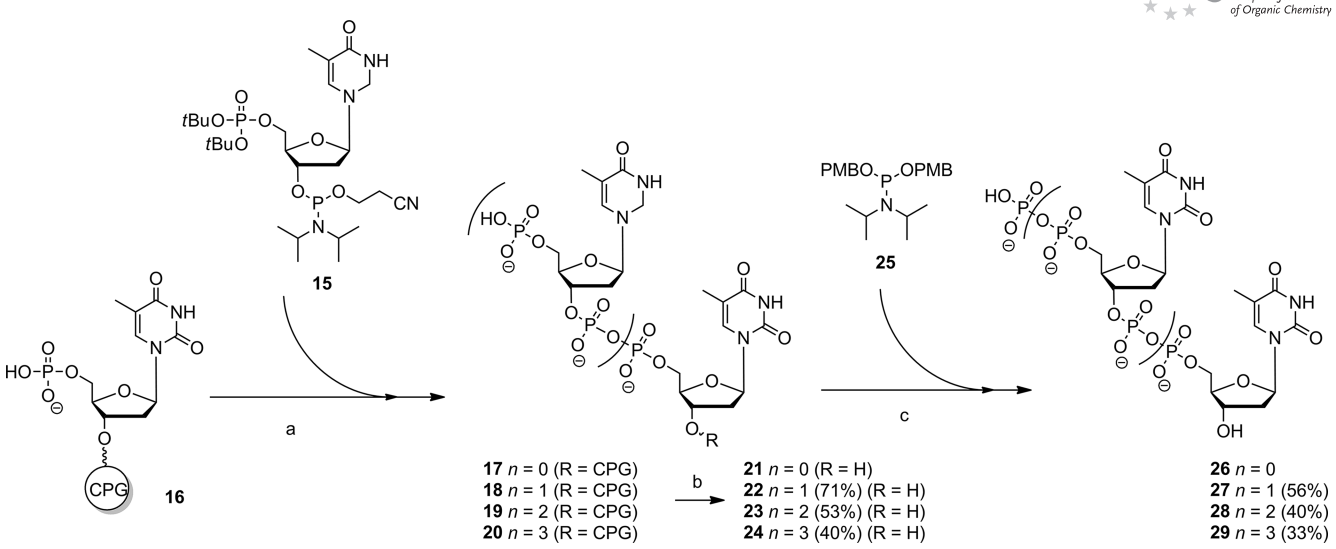
In searching for an alternative synthesis of "pyrophosphite" **4**, we encountered the work of Foss and co-workers who have studied the synthesis and behaviour of related compounds they call phosphorodiamidous anhydrides. In line with their procedure, *N*,*N*-diisopropylchlorophosphoramidite **10** (Scheme 1, B) was treated with 0.5 equiv. of water and 2 equiv. of DIPEA. This led to the initial formation of H-phosphonate amide **12** (^{31}P signal at δ = 15 ppm), which reacted immediately and irreversibly with remaining **10**. This resulted in a mixture composed of compound **13** [32–36 ppm (P^v) and 110–121 ppm (P^{III})] and compound **4** (^{31}P signals at δ = 139.8 and 139.6 ppm) (see Supporting Information). Our observed ^{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 δ = 118.6 ppm). We note that the ^{31}P-NMR spectroscopic data of **4** and **13** obtained by us are also consistent with the presence of two chiral phosphorus atoms in both isomers. In particular compound **4** which consist of three stereoisomers, namely the *meso*-compound and two enantiomers of the chiral diastereomer of **4**. Our observed two ^{31}P signals at 139.8 and 139.6, on the other hand, correlate well to the existence of such a mixture. Compound **13** is devoid of any elements of symmetry and exist as a mixture of two chiral diastereomers, each present as a racemic mixture, which is reflected in the ^{31}P signals we attribute to this P^{III}-P^V species.

Our lack of success in synthesizing phosphitylating agent **4**, research performed nine years ago, forced us to develop an alternative method for the construction of pyrophosphate linkages in between nucleotides and thus to prepare ADPr oligomers. This endeavour has, as noted before, recently been concluded successfully and we realized that by doing so we created the means to prepare DNA oligonucleotides in which the nucleosides are interconnected through pyrophosphate (diphosphate) linkages. Thus we did, and we selected modified oligothymidylic acids **27**, **28** and **29** (Scheme 3), reported as part of the targets by Ahmadibeni and Parang,[7] as target compounds. The required building block **15**, needed for the assembly of these oligomers according to our protocol, was obtained by selective phosphitylation of thymidine and subsequent oxidation (P^{III} to P^V) to yield phosphotriester **14**.[16] This was followed by the introduction of the phosphoramidite at the 3-OH to give **15** (Scheme 2).



Scheme 2. Synthesis of compound **15**. a) i. di-*tert*-butyl *N*,*N*-diisopropylphosphoramidite, Pyr·HCl, pyridine; ii. *t*BuOOH, 88%; b) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, DIPEA, DCM, 86%. Pyr·HCl = pyridinium chloride; *t*BuOOH = *tert*-butyl hydroperoxide.

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; for the synthesis see Supporting Information). We loaded the modified CPG (10 μmol) into an automated oligonucleotide synthesizer and we applied our reported pyrophosphorylation approach as summarized in Figure 1 (A)



Scheme 3. Synthesis of terminal phosphate dT-oligonucleotides **22**–**24** and of terminal pyrophosphate dT-oligonucleotides **27**–**29**. a) i. compound 2, ETT, MeCN, (2×); ii. CSO, MeCN, (2×); iii. DBU, DMF, (2×); iv. HCl, HFIP, (4×); v. pyridine (10% v/v), acetonitrile, (2×); b) NH_4OH; c) i. bis(PMB)-*N*,*N*-diisopropyl phosphoramidite, 1-Me-Im·HCl (0.3 M), 1-Me-Im (0.2 M), DMF; ii. CSO, MeCN; iii. DCM/TFA (95:5 v/v%), iv. NH_4OH (35%). ETT = 5-ethylthiotetrazole; CSO = (1*S*)-(+)-(10-camphorsulfonyl)oxaziridine; DBU = 1,8-diazabicycloundec-7-ene; HFIP = hexafluoro-2-propanol; PMB = *p*-methoxybenzyl; 1-Me-Im·HCl = 1-methylimidazolium chloride; 1-Me-Im = 1-methylimidazole; TFA = trifluoroacetic acid.

to provide immobilized oligomers **18**, **19** and **20** (Scheme 3). Test samples of these oligomers (≈ 1–2 μmol) were treated with NH_4OH 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 to show moderate to good yields for compounds **22** (71%), **23** (53%) and **24** (40%).

To obtain target compounds **27**–**29** the terminal phosphate at the 5-OH position in **18**–**20** needs to be converted into a pyrophosphate. To achieve this, we treated immobilized **18**–**20** with di-PMB phosphoramidite **25**.[17] 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** (Scheme 3).

Both terminal phosphates **21**–**24** and pyrophosphates **26**–**29** were analyzed using ^{1}H-, ^{31}P-, COSY- and J-resolved ^{31}P-NMR spectroscopy[18] (see Supporting information). The observed chemical shifts are as expected and we could conclusively assign all proton and phosphorus atoms in our compounds. As a representative example, the J-Resolved ^{31}P-NMR spectrum for compound **29** is shown in Figure 2 (left). A direct comparison with a ^{31}P-NMR from the Ahmadibeni/Parang report could not be made, because in this report NMR spectra of protonated pyrophosphates in

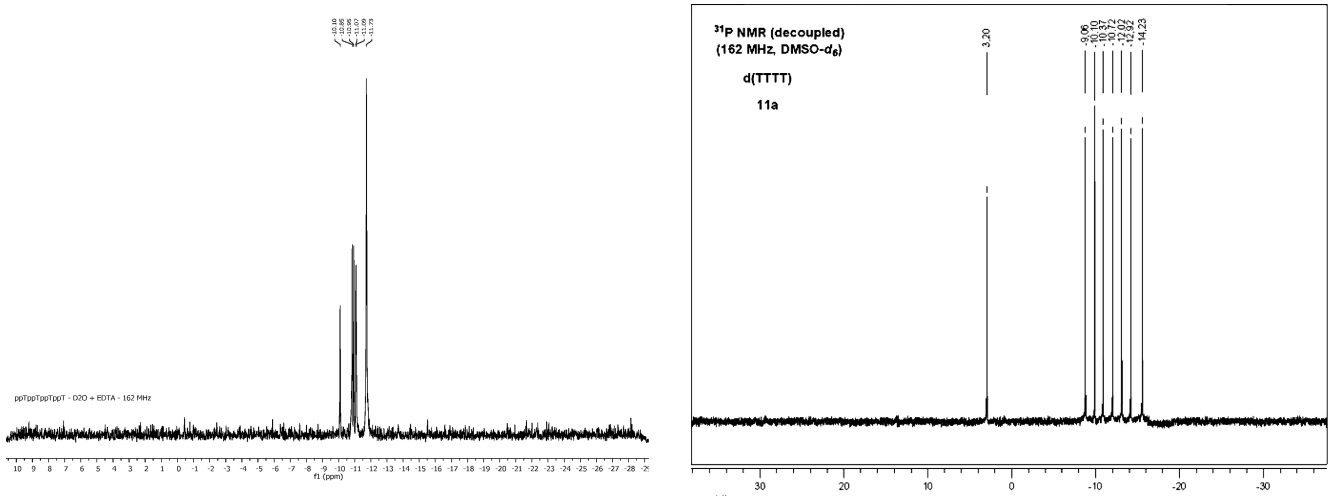


Figure 2. J-Resolved ^{31}P-NMR spectra for; Left: Compound **2**. Right: The identical compound as reported by Ahmadibeni and Parang (note that the reported "peak-pickings" do not correspond with the values on the *x*-axis for the spectrum on the right).

deuterated DMSO are presented.[19] Repeating the reported procedure 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 – we note that at this temperature water/dioxane mixtures are frozen solid) and lyophilization resulted in deterioration of the material as seen from NMR spectra taken from these (see Supporting Information for a relevant example).[20]

Comparison of our ^{1}H- and ^{31}P-NMR spectra (ammonium form) with the reported protonated material reveals some telling differences (compare Figure 2, left and right, 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. In contrast, in our experience – backed up by literature data on related compounds – the chemical shift of the β-phosphorus of the terminal pyrophosphate ranges from –6 to –10 ppm.[15] Secondly, we find the internal pyrophosphates for all the compounds to have shifts between –10 and –13 ppm while Ahmadibeni and Parang report for these compounds shifts between –13 and –20 ppm.

Thirdly, Ahmadibeni and Parang reported for the 4′-H and 5′-H protons, of the deoxyribose moiety in (pyro)phosphorylated thymidine, shifts of around 3.5 ppm. This shift in our opinion corresponds with 4′-H and 5′-H protons of thymidine having a free hydroxyl at the 5′-position. In our spectra the proton shifts for 4′-H and 5′-H of **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 Ahmadibeni and Parang.

Conclusions

In summary, artificial thymidine oligonucleotides with only pyrophosphate linkages were assembled by the method we developed for the synthesis of ADPr oligomers.[5] 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, we could not recapitulate the synthesis of phosphitylating agent **4** that features as the key reagent in various papers of the Parang group.[7–9] We are convinced that the ^{31}P-NMR spectrum of **4**, as reported in 2005, is erroneous as well. Obviously, the authors have overlooked the fact that compound **4** can exist in three stereoisomeric forms and that compound **4** has two – not one – phosphorus signals as the expected result. We witness such a double signal within a mixture of compounds containing **4** and P^V-P^{III} species **13**, a compound mixture we prepared analogous to a procedure reported by Foss and colleagues.[10] One could argue that Ahmadibeni and Parang have obtained the same mixture of compounds and that this mixture is a *bona fide* phosphitylating agent. Though we cannot exclude this – we have not attempted this ourselves – we consider this unlikely, not least because the analytical data of the artificial thymidine oligonucleotides we did make are very different from what is reported about the same compounds by Ahmadibeni and Parang. In our view, we have reported here the first successful synthesis of DNA oligomers interconnected via pyrophosphate linkages. The further development of our method might allow the synthesis of longer pyrophosphate linked oligonucleotides containing different bases. These compounds are potentially useful for the research in biophysics and biochemistry of nucleic acids.

Experimental Section

General: All solvents used under anhydrous conditions were stored over molecular sieves (4 Å) except for methanol which was stored over molecular sieves (3 Å). Solvents used for workup and column chromatography were of technical grade from Sigma Aldrich and used directly. Unless stated otherwise, solvents were removed by rotary evaporation under reduced pressure at 40 °C. Reactions were monitored by TLC-analysis using Merck 25 DC plastikfolien 60 F254 with detection by spraying with 20% H_2SO_4 in EtOH, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) in 10% sulfuric acid or by spraying with a solution of ninhydrin (3 g/L) in EtOH/AcOH (20:1 v/v), followed by charring at approx. 150 °C. Column chromatography was performed by automation using a Biotage® Isolera™ Spektra Four machine. For LC-MS analysis a JASCO HPLC-system (detection simultaneously at 214 and 254 nm) equipped with an analytical C18 column (4.6 mmD × 50 mmL, 3μ particle size) in combination with buffers A: H_2O, B: MeCN and C: 0.5% aq. TFA and coupled to a PE/SCIEX API 165 single quadruple mass spectrometer (Perkin–Elmer) was used, unless stated otherwise. Alternatively a Thermo Finnigan LCQ Advantage MAX ion-trap mass spectrometer with an electrospray ion source coupled to Surveyor HPLC system (Thermo Finnegan) was used with the same analytical column. High resolution mass spectra were recorded by direct injection (2 μL of a 2 μM solution in water/acetonitrile; 50:50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60000 at m/z 400 (mass range: m/z = 150–2000) and dioctyl phthalate (m/z = 391.2842) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). ^{1}H-, ^{13}C- and ^{31}P-NMR spectra were measured on a Bruker AV-400 (400 MHz), Bruker AV-500 (500 MHz), AVIII-Bruker DMX-600 (600 MHz) or a Bruker Ascend 850 (850 MHz) and all individual signal were assigned using 2D-NMR spectroscopy. Chemical shifts are given in ppm (δ) and directly referenced to TMS (δ = 0.00 ppm) in $CDCl_3$ or indirectly referenced to H_3PO_4 (δ = 0.00 ppm) in D_2O via the solvent residual signal and coupling constants are given in Hz.

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 (3×) 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 min. The reaction mixture was cooled to 0 oC and *t*BuOOH (3 equiv., 5.5 M in nonane) was added. The mixture was allowed to reach room temperature and was left to stir for 16 h. DCM (50 mL) was added and the mixture was washed with aq. $NaHCO_3$ (satd.) and the aqueous layer was extracted with DCM. The combined organic layers were washed with brine, dried with $MgSO_4$ 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. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.52 (d, 1 H, 6-H), 6.41 (t, J = 6.8 Hz, 1 H, 1′-H), 4.51 (dt, J = 6.4, 3.2 Hz, 1 H, 3′-H), 4.26–4.13 (m, 2 H, 4′-H, 5′-H), 4.13–4.07 (m, 1 H, 5′-H), 2.41 (ddd, J = 13.5, 6.0, 3.3 Hz, 1 H, 2′-H), 2.16 (dt, J = 13.7, 6.8 Hz, 1 H, 2′-H), 1.94 (s, 3 H, CH_3), 1.49 (d, J = 2.5 Hz, 18 H, *t*Bu CH_3) ppm. ^{31}P NMR (162 MHz, $CDCl_3$): δ = –9.79 ppm.

5′-*O*-(Di-*tert*-butyl)phosphate-3′-*O*-(*N*,*N*-diisopropylamino-*O*-cyanoethyl) Phosphoramidite-2′-deoxythymidine (15): Compound **7** (750 mg, 1.73 mmol) was co-evaporated with anhydrous MeCN (3×) 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 min. The reaction mixture was diluted with DCM, washed with aq. $NaHCO_3$ (satd.) and the aqueous layer was extracted with DCM. The combined organic layers were washed with brine, dried ($MgSO_4$), 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%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.55 (d, J = 1.1 Hz, 1 H, 6-H), 7.53 (d, J = 1.1 Hz, 1 H, 6-H), 6.45–6.39 (m, 2 H, 1′-H), 4.62–4.53 (m, 2 H, 3′-H), 4.27–4.22 (m, 1 H, 4′-H), 4.22–4.16 (m, 3 H, 4′-H, 5′-H), 4.16–4.12 (m, 2 H, 5′-H), 3.91–3.82 (m, 2 H, CH_2 OCNE), 3.81–3.72 (m, 2 H, CH_2 OCNE), 3.68–3.56 (m, 4 H, C*H* *i*Pr), 2.67 (td, J = 6.3, 2.5 Hz, 4 H, CH_2 OCNE), 2.49 (ddd, J = 13.7, 5.8, 2.3 Hz, 1 H, 2′-H), 2.40 (ddd, J = 13.5, 5.8, 2.3 Hz, 1 H, 2′-H), 2.17 (dt, J = 14.1, 6.8 Hz, 2 H, 2′-H), 1.96 (s, 6 H, CH_3), 1.53–1.47 (m, 36 H, *t*Bu CH_3), 1.23–1.16 (m, 24 H, CH_3 *i*Pr) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): 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 (*C*CH$_3$ *t*Bu), 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 (*C*H$_2$ OCNE), 43.39, 43.29 (*C*H *i*Pr), 39.57, 39.55, 39.53 (C2′), 29.93, 29.91, 29.88 (*C*H$_3$ *t*Bu), 24.66, 24.63, 24.61, 24.58, 24.55, 24.50 (*C*H$_3$ *i*Pr), 20.44, 20.42, 20.39, 20.36 (*C*H$_2$ OCNE), 12.50 (*C*H$_3$) ppm. ^{31}P NMR (122 MHz, $CDCl_3$): δ = 148.29, 147.97, –11.00 ppm.

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 (1×) with a mixture of 1-methylimidazolium chloride (0.3 M) and 1-methylimidazole (0.2 M) 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 min under an atmosphere of argon. The reaction mixture was drained and the resin washed with MeCN (5×). (1S)-(+)-(10-camphorsulfonyl)oxaziridine (CSO) (0.5 M) in MeCN (5 mL) was added and the mixture was shaken for 30 min under an argon atmosphere. The reaction mixture was drained and the resin washed with MeCN (3×) and DCM (3×). Pyridine (10 mL), Ac_2O (50 equiv.) and DMAP (cat.) were added and the mixture was shaken for 30 min to cap any remaining free hydroxyl functionality. The reaction mixture was drained and the resin washed with DMF (3×) and DCM (3×). A sample was analyzed with ^{31}P NMR upon cleavage with NH_4OH (35%) for 1 h. ^{31}P NMR (162 MHz, D_2O): δ = –3.08 (mono-*t*Bu), –9.88 (di-*t*Bu) ppm. A solution of DCM/TFA (9:1 $^v/_v$, 8 mL) was added to the resin and shaken at room temperature for 30 min. The reaction mixture was drained and the resin washed with DCM (2×), pyridine/H_2O (9:1 $^v/_v$, 2× 10 mL), DMF (3×) and DCM (3×). The resin was dried under reduced pressure and a sample was analyzed with ^{31}P NMR upon cleavage with NH_4OH (35%) for 1 h. ^{1}H NMR (400 MHz, D_2O): δ = 7.77 (s, 1 H, 6-H), 6.27 (t, J = 7.0 Hz, 1 H, 1′-H), 4.51 (dt, J = 5.6, 2.6 Hz, 1 H, 3′-H), 4.08–4.00 (m, 1 H, 4′-H), 3.93–3.81 (m, 2 H, 5′-H), 2.33 (dt, J = 14.1, 7.0 Hz, 1 H, 2′-H), 2.23 (ddd, J = 14.0, 6.1, 3.1 Hz, 1 H, 2′-H), 1.87 (s, 3 H, CH_3) ppm. ^{31}P NMR (162 MHz, D_2O): δ = 4.14 ppm.

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 (3×) and the complete synthesis was performed under an argon atmosphere. The resin was rinsed with MeCN (3×) 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 min, drained and followed by a second addition of compound **15** and ETT. The reaction mixture was drained and the resin rinsed with MeCN (3×). The intermediate phosphate-phosphite was oxidized with CSO (2 mL; 0.5 M in MeCN) for 5 min (2×) and washed with MeCN (3×). The resin was rinsed with MeCN (3×) and the cyanoethyl (CNE) was removed by treating the resin with (DRY!) 1,8-diazabicycloundec-7-ene (DBU) (1 mL; 1.0 M in DMF) for 5 min (2×) and washed with MeCN (3×). The resin was rinsed with MeCN (3×) and treated with HCl [1 mL; 50 mM in hexafluoro-2-propanol (HFIP)] for 1 min (4×) to remove the *tert*-Butyl groups (*t*Bu). The resin was rinsed with MeCN (4×), pyridine (10 v/v% in MeCN; 2× 30 seconds) and MeCN (3×). Test samples were taken and the products were cleaved from the resin upon treatment with NH_4OH (35%) for 1 h.

pT (21): The general procedure A was applied to synthesize compounds **22**–**24**. The crude material was purified by strong anion exchange chromatography on Source 15Q (10 mm × 10 cm) using gradient elution with NH_4OAc (20 mM to 1.0 M) and repeated lyophilization afforded byproduct **21** as a white solid (29% based on UV integration, 33% based on ^{31}P NMR integration for the synthesis of 22). ^{1}H NMR (500 MHz, D_2O): δ = 7.78 (d, 1 H, 6-H), 6.36 (t, J = 7.0 Hz, 1 H, 1′-H), 4.60–4.56 (m, 1 H, 3′-H), 4.21–4.16 (m, 1 H, 4′-H), 4.11–4.02 (m, 2 H, 5′-H), 2.40–2.35 (m, 2 H, 2′-H), 1.93 (d, J = 1.2 Hz, 3 H, CH_3) ppm. ^{31}P NMR (162 MHz, D_2O): δ = 1.11 ppm. HRMS $[C_{10}H_{15}N_2O_8P_1 + H]^+$: 323.0639 found, 323.0639 calculated.

pTppT (22): The general procedure A was applied to synthesize compound **22**. The crude material was purified by strong anion exchange chromatography on Source 15Q (10 mm × 10 cm) using gradient elution with NH_4OAc (20 mM to 1.0 M) and repeated lyophilization afforded 22 as a white solid (71% based on UV integration, 67% based on ^{31}P NMR integration for the synthesis of 22). ^{1}H NMR (500 MHz, D_2O): δ = 7.77 (d, J = 1.3 Hz, 1 H, 6-H), 7.74 (d, J = 1.3 Hz, 1 H, 6-H), 6.36 (dd, J = 8.9, 5.7 Hz, 1 H, 1′′-H), 6.32 (t, J = 6.8 Hz, 1 H, 1′-H), 5.04–4.96 (m, 1 H, 3′′-H),

4.61 (q, J = 5.0 Hz, 1 H, 3′-H), 4.45–4.38 (m, 2 H, 4′′-H), 4.26–4.14 (m, 3 H, 4′-H, 5′-H), 4.14–4.02 (m, 2 H, 5′′-H), 2.59 (ddd, J = 14.1, 5.8, 1.7 Hz, 1 H, 2′′-H), 2.43–2.32 (m, 3 H, 2′-H, 2′′-H), 1.92 (d, J = 1.1 Hz, 3 H, CH_3), 1.91 (d, J = 1.2 Hz, 3 H, CH_3) ppm. ^{31}P NMR (162 MHz, D_2O): δ = 0.68, –10.90, –11.03, –11.65, –11.78 ppm. ^{31}P NMR (162 MHz, D_2O, J-Resolved): δ = 0.69, –10.96, –11.71 ppm. HRMS [$C_{20}H_{29}N_4O_{18}P_3$ + H]$^+$: 707.0766 found, 707.0762 calculated.

pTppTppT (23): The general procedure A was applied to synthesize compound **23**. The crude material was purified by strong anion exchange chromatography on Source 15Q (10 mm × 10 cm) using gradient elution with NH_4OAc (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, D_2O): δ = 7.76 (d, J = 1.1 Hz, 1 H, 6-H), 7.75 (d, J = 1.2 Hz, 1 H, 6-H), 7.73 (d, J = 1.0 Hz, 1 H, 6-H), 6.36–6.28 (m, 3 H, 1′-H–1′′′-H), 5.01–4.95 (m, 2 H, 3′′-H, 3′′′-H), 4.63–4.58 (m, 1 H, 3′-H), 4.42–4.37 (m, 2 H, 4′′-H, 4′′′-H), 4.25–4.15 (m, 5 H, 4′-H, 5′-H, 5′′-H), 4.14–4.02 (m, 2 H, 5′′′-H), 2.61–2.52 (m, 2 H, 2′′-H, 2′′′-H), 2.41–2.31 (m, 4 H, 2′-H–2′′′-H), 1.91 (d, J = 1.0 Hz, 3 H, CH_3), 1.90 (d, J = 1.0 Hz, 3 H, CH_3), 1.90 (d, J = 1.0 Hz, 3 H, CH_3) ppm. ^{31}P NMR (162 MHz, D_2O): δ = 0.65, –10.93, –11.06, –11.08, –11.21, –11.67, –11.72, –11.80, –11.85 ppm. ^{31}P NMR (162 MHz, D_2O, J-Resolved): δ = 0.66, –10.99, –11.14, –11.73, –11.78 ppm. HRMS [$C_{30}H_{43}N_6O_{28}P_5$ + Na]$^+$: 1113.0719 found, 1113.0706 calculated.

pTppTppTppT (24): The general procedure A was applied to synthesize compound **24**. The crude material was purified by strong anion exchange chromatography on Source 15Q (10 mm × 10 cm) using gradient elution with NH_4OAc (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, D_2O): δ = 7.75 (d, J = 1.1 Hz, 1 H, 6-H), 7.75 (d, J = 1.2 Hz, 1 H, 6-H), 7.72 (d, J = 1.0 Hz, 1 H, 6-H), 7.70 (d, J = 1.0 Hz, 1 H, 6-H), 6.37–6.25 (m, 4 H, 1′-H–1′′′′-H), 5.02–4.95 (m, 4 H, 3′′-H, 3′′′-H, 3′′′′-H), 4.63–4.56 (m, 1 H, 3′-H), 4.42–4.33 (m, 3 H, 4′′-H, 4′′′-H, 4′′′′-H), 4.25–4.14 (m, 9 H, 4′-H, 5′-H, 5′′-H, 5′′′-H), 4.11–4.05 (m, 2 H, 5′′′′-H), 2.61–2.51 (m, 3 H, 2′′-H, 2′′′-H, 2′′′′-H), 2.39–2.30 (m, 5 H, 2′-H–2′′′′-H), 1.91 (d, J = 1.0 Hz, 3 H, CH_3), 1.90 (d, J = 1.0 Hz, 3 H, CH_3), 1.89 (s, 6 H, CH_3) ppm. ^{31}P NMR (162 MHz, D_2O): δ = 0.67, –10.90, –11.04, –11.18, –11.64, –11.69, –11.77, –11.82 ppm. ^{31}P NMR (162 MHz, D_2O, J-Resolved): δ = 0.67, –10.96, –11.09, –11.12, –11.69, –11.75 ppm. HRMS [$C_{40}H_{57}N_8O_{38}P_7$ + 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 (3 ×) and the complete synthesis was performed under an argon atmosphere. The resin was rinsed with DMF (3 ×) and an acitvator mixture of 1-methylimidazolium chloride (0.3 M) and 1-methylimidazole (0.2 M) in DMF. The activator mixture (0.5 mL) was added followed by bis(*p*-methoxybenzyl)-*N*,*N*-diisopropylphosphoramidite (20 mg, 50 μmol). The mixture was shaken for 30 min, drained and rinsed with MeCN (3 ×). The intermediate phosphate-phosphite was oxidized with CSO (0.5 M) in MeCN (2 mL) for 15 min (two times) and washed with MeCN (3 ×). The resin was rinsed with DCM (3 ×) and the *para*-methoxybenzyl (PMB) goups were removed by treating the resin with a solution of DCM/TFA (95:5 $^v/_v$, 2 mL) for 30 min. The reaction mixture was drained and the resin washed with DCM (2 ×), pyridine/H_2O (9:1 $^v/_v$%, 2 × 10 mL), DMF (3 ×) and DCM (3 ×). The products were cleaved from the resin upon treatment with NH_4OH (35%) for 1 h.

ppT (26): The general procedure B was applied to synthesize compounds **27**–**29**. The crude material was purified by strong anion exchange chromatography on Source 15Q (10 mm × 10 cm) using gradient elution with NH_4OAc (20 mM to 1.0 M) and repeated lyophilization afforded byproduct **26** as a white solid (25% based on UV integration). ^{1}H NMR (500 MHz, D_2O): δ = 7.75 (d, J = 1.4 Hz, 1 H, 6-H), 6.35 (t, J = 7.0 Hz, 1H 1′-H), 4.64 (dt, J = 6.2, 3.3 Hz, 1 H, 3′-H), 4.20–4.13 (m, 3 H, 4′-H, 5′-H), 2.45–2.28 (m, 2 H, 2′-H), 1.91 (d, J = 1.0 Hz, 3 H, CH_3) ppm. ^{31}P NMR (162 MHz, D_2O): δ = –9.38, –9.51, –10.62, –10.75 ppm. HRMS [$C_{10}H_{16}N_2O_{11}P_2$ + Na]$^+$: 425.0121 found, 425.0122 calculated.

ppTppT (27): The general procedure B was applied to synthesize compound **27**. The crude material was purified by strong anion exchange chromatography on Source 15Q (10 mm × 10 cm) using gradient elution with NH_4OAc (20 mM to 1.0 M) and repeated lyophilization afforded 27 as a white solid (56% based on UV integration, 82% based on 22). ^{1}H NMR (500 MHz, D_2O): δ = 7.76 (d, J = 1.1 Hz, 1 H, 6-H), 7.74 (d, J = 1.2 Hz, 1 H, 6-H), 6.35 (dd, J = 9.1, 5.6 Hz, 1 H, 1′′-H), 6.32 (t, J = 6.9 Hz, 1 H, 1′-H), 5.05–4.98 (m, 1 H, 3′′-H), 4.63–4.57 (m, 1 H, 3′-H), 4.46–4.39 (m, 1 H, 4′′-H), 4.25–4.19 (m, 2 H, 5′-H, 5′′-H), 4.19–4.14 (m, 3 H, 5′-H, 5′′-H, 4′-H), 2.58 (ddd, J = 14.0, 5.6, 1.6 Hz, 1 H, 2′′-H), 2.41 (td, J = 8.9, 4.5 Hz, 1 H, 2′′-H), 2.36 (dd, J = 6.8, 5.1 Hz, 2 H, 2′-H), 1.92 (d, J = 1.1 Hz, 3 H, CH_3), 1.91 (d, J = 1.0 Hz, 3 H, CH_3) ppm. ^{31}P NMR (162 MHz, D_2O): δ = –10.06, –10.18, –10.83, –10.91, –10.95, –11.05, –11.70, –11.84 ppm. ^{31}P NMR (162 MHz, D_2O, J-Resolved): δ = –10.11, –10.88, –10.97, –11.76 ppm. HRMS [$C_{20}H_{30}N_4O_{21}P_4$ + Na]$^+$: 809.0247 found, 809.0245 calculated.

ppTppTppT (28): The general procedure B was applied to synthesize compound **28**. The crude material was purified by strong anion exchange chromatography on Source 15Q (10 mm × 10 cm) using gradient elution with NH_4OAc (20 mM to 1.0 M) and repeated lyophilization afforded 28 as a white solid (40% based on UV integration, 87% based on 23). ^{1}H NMR (500 MHz, D_2O): δ = 7.75 (d, J = 1.2 Hz, 1 H, 6-H), 7.73 (d, J = 1.2 Hz, 1 H, 6-H), 7.73 (d, J = 1.2 Hz, 1 H, 6-H), 6.34–6.28 (m, 3 H, 1′-H–1′′′-H), 5.03–4.93 (m, 2 H, 3′′-H, 3′′′-H), 4.60 (q, J = 4.7 Hz, 1 H, 3′-H), 4.42–4.36 (m, 2 H, 4′′-H, 4′′′-H), 4.24–4.12 (m, 7 H, 4′-H, 5′-H, 5′′-H, 5′′′-H), 2.62–2.50 (m, 2 H, 2′′-H, 2′′′-H), 2.43–2.29 (m, 4 H, 2′-H, 2′′-H, 2′′′-H), 1.92 (d, J = 1.0 Hz, 3 H, CH_3), 1.90 (d, J = 1.0 Hz, 3 H, CH_3), 1.89 (d, J = 1.0 Hz, 3 H, CH_3) ppm. ^{31}P NMR (162 MHz, D_2O): δ = –10.08, –10.21, –10.83, –10.92, –10.96, –11.05, –11.20, –11.70, –11.83 ppm. ^{31}P NMR (162 MHz, D_2O, J-Resolved): δ = –10.14, –10.88, –10.98, –11.13, –11.76 ppm. HRMS [$C_{30}H_{44}N_6O_{31}P_6$ + Na]$^+$: 1193.0385 found, 1193.0369 calculated.

ppTppTppTppT (29): The general procedure B was applied to synthesize compound **29**. The crude material was purified by strong anion exchange chromatography on Source 15Q (10 mm × 10 cm) using gradient elution with NH_4OAc (20 mM to 1.0 M) and repeated lyophilization afforded 29 as a white solid (33% based on UV integration, 81% based on 24). ^{1}H NMR (500 MHz, D_2O): δ = 7.74 (d, J = 1.4 Hz, 1 H, 6-H), 7.73 (d, J = 1.3 Hz, 1 H, 6-H), 7.72 (d, J = 1.4 Hz, 1 H, 6-H), 7.69 (d, J = 1.4 Hz, 1 H, 6-H), 6.35–6.23 (m, 4 H, 1′-H–1′′′′-H), 5.03–4.92 (m, 3 H, 3′′-H, 3′′′-H, 3′′′′-H), 4.60 (q, J = 4.8 Hz, 1 H, 3′-H), 4.43–4.34 (m, 3 H, 4′′-H, 4′′′-H, 4′′′′-H), 4.25–4.12 (m, 9 H, 4′-H, 5′–5′′′′-H), 2.61–2.50 (m, 3 H, 2′′-H, 2′′′-H, 2′′′′-H), 2.42–2.27 (m, 5 H, 2′-H–2′′′′), 1.92 (d, J = 1.1 Hz, 3 H, CH_3), 1.90 (d, J = 1.2 Hz, 3 H, CH_3), 1.88 (s, 6 H, CH_3) ppm. ^{31}P NMR (162 MHz, D_2O): δ = –10.04, –10.17, –10.80, –10.89, –10.92, –11.02, –11.14, –11.16, –11.67, –11.80 ppm. ^{31}P NMR (162 MHz, D_2O, J-Resolved): δ = –10.10, –10.85, –10.95, –11.06, –11.09, –11.73 ppm. HRMS [$C_{40}H_{58}N_8O_{41}P_8$ + Na]$^+$: 1577.0511 found, 1577.0493 calculated.

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[19] The solubility of phosphates and pyrophosphates in DMSO is poor, as was observed for our compounds **26**–**29**, making DMSO an inappropriate solvent for NMR analysis of these compounds. 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. We followed in our study the conventional practice and handled the isolated pyrophosphate oligonucleotides as ammonium salts, except in the cases when we tried to make direct spectroscopic comparison of our products with those reported by Parang.
[20] Decomposition has probably taken place in course of lyophilization of acidified pyrophosphate oligonucleotide **28** because we have started with purified homogeneous material in the ammonium form. When **28** was dissolved in D_2O, treated with strong cation exchange resin to transform **28** to the acidic (protonated) form and subjected to ^{31}P- and ^{1}H-NMR spectroscopy, without prior lyophylization, the resulting spectra (see Supporting Information) proved to be only slightly different from those of **28** in the ammonium form (see Supporting Information). The ^{31}P-NMR spectrum of **28** in acid form is clearly distinct from the one reported by Parang for the same oligonucleotide. Lyophilization of this protonated compound **28**, however, shows decomposition of the material as was observed by ^{1}H- and ^{31}P-NMR spectroscopy (see Supporting Information).

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