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Enzymatic Synthesis of Organic-Polymer-Grafted DNA

Anna Baccaro and Andreas Marx*[a]

Abstract: To create bioorganic hybrid materials, interdisciplinary work in the fields of chemistry, biology and materials science is conducted. DNA block copolymers are promising hybrid materials due to the combination of properties intrinsic to both the polymer and the nucleic acid blocks. Until now, the coupling of DNA and organic polymers has been exercised post-synthetically in solution or on solid support. Herein, we report the first enzyme-catalysed synthesis of DNA–organic polymer chimeras. For this purpose, four novel 2′-deoxyuridine triphosphates carrying polymer-like moieties linked to the nucleobase were synthesised. Linear polyethylene glycol monomethyl ethers of different sizes (**1**) and branched polyamido dendrons with varying terminal groups (**2**) were chosen as building blocks. We investigated the ability of DNA polymerases to accept the copolymers in comparison to the natural substrate and showed, through primer extensions, polymerase chain reactions and rolling circle amplification, that these building blocks could serve as a surrogate for the natural thymidine. By this method, DNA hybrid materials with high molecular weight, modification density, and defined structure are accessible.

Keywords: dendrimers · DNA copolymers · enzyme catalysis · nucleotides · polyethylene glycol

Introduction

The development of hybrid materials is of high interest because it may allow the creation of new materials with extraordinary properties. To this end, materials from different areas of chemistry, biology and materials science are combined to generate new materials with novel characteristics. DNA block copolymers consisting of oligonucleotides and organic polymers are one of the most promising hybrid materials. Such materials show combined characteristics of both DNA and polymers. Due to hybridisation specificity and the ability of oligonucleotides to self-assemble, they have been used as tools to arrange precise structures in the nanoscale range.[1–3] Many different methods have been developed that use DNA as a scaffold to orientate and assemble inorganic[4–10] as well as organic[11–13] compounds on nanometer scales. DNA block copolymers with both a DNA segment and an organic polymer unit were reported recently.[14–17] The synthesis of DNA block copolymers have been performed in solution[18] and on solid support.[19] For coupling of DNA and organic polymers in solution, amide and disulfide bond formation, and Michael addition reactions have been used.[20,21] Both coupling strategies require modification of the employed compounds with appropriate functional groups and must ensure the solubility of both the polymer and the oligonucleotide in the required solvent. The synthesis of DNA block copolymers in solution can be performed in high yields employing water-soluble polymers such as polyethylene glycol.[21,22] Reactions involving hydrophobic polymers show low coupling efficiencies in solution because of the incompatibility of the polymer and DNA in the solvent. Thus, amphiphilic block copolymers consisting of hydrophobic polymers and DNA were synthesised on solid support,[14] employing a DNA synthesiser.

Whereas short DNA strands (50–60 nucleotides) are available through chemical synthesis on solid support, the synthesis of longer DNA strands is challenging. To overcome the synthetic restrictions of solid-supported synthesis, DNA polymerase catalysed reactions hold great promise since they allow the synthesis of long DNA fragments. For this reason, the development of enzymatic transformations that lead to polymer-modified DNA is of high interest. However, these approaches are only rarely exploited. Along these lines, Herrmann et al. employed primer strands bearing a polymer moiety at their 5′-end in polymerase chain reac-

[a] Dipl.-Chem. A. Baccaro, Prof. Dr. A. Marx
Department of Chemistry
and Konstanz Research School Chemical Biology
University of Konstanz, Universitätsstrasse 10
78457 Konstanz (Germany)
Fax: (+49) 7531-88-5140
E-mail: Andreas.Marx@uni-konstanz.de

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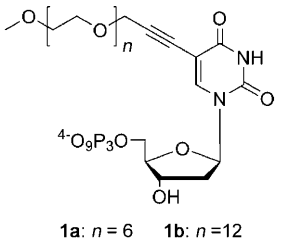
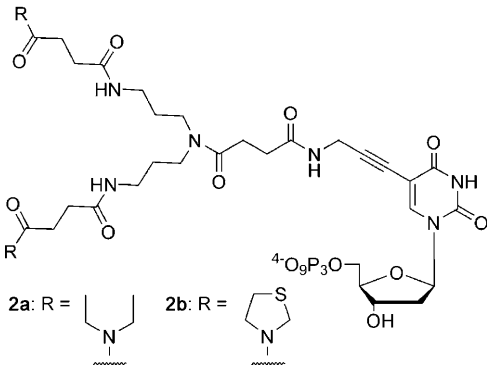
tions (PCR) to synthesise DNA block copolymers with extended DNA fragments.[15]

Numerous DNA block copolymers[18–26] with polymeric block-type architecture are known, however, there are only a few examples of grafted DNA copolymers[27,28] built out of an oligonucleotide backbone and organic polymer side chains.

Herein, we present the first enzymatic synthesis of DNA–polymer hybrids built on a DNA backbone equipped with organic polymer side chains. For the enzymatic generation of these hybrids we synthesised four novel modified thymidine analogues bearing either linear or branched modifications at their nucleobases (Scheme 1). It has been reported





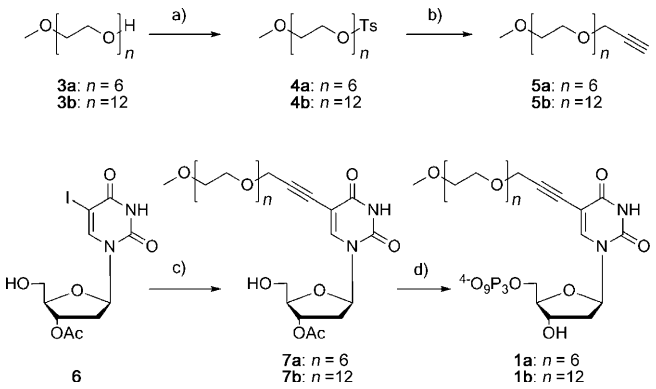
Scheme 1. Modified triphosphates used in enzyme-catalysed reactions.

that the acceptance of nucleotide analogues by DNA polymerases heavily depends on the position and the structure of the modifications.[29–31] Therefore, we chose to introduce the polymer moiety at the C5-position of the pyrimidine, since modifications at this position do not significantly interfere with Watson–Crick base pairing. In addition, it has been reported that modified 2′-deoxyuridine triphosphates bearing a C5-substituent are accepted as a substrate by DNA polymerases in several cases.[29–34] Since the type of modification has a significant influence on the acceptance of the nucleotide,[35] we synthesised triphosphates with linear as well as branched moieties. We chose linear polyethylene glycol monomethyl ethers with a range of lengths because of their ubiquitous use.[36] It is also reported that polyethylene glycol can serve as an enhancer in PCR.[37] Furthermore, we used branched polyamido dendrons bearing two terminal diethylamine or thiazolidine moieties to generate thymidine analogues with varied steric demand. If incorporation of these branched DNA analogues by DNA polymerases is successful, two new functionalities will be generated with a single incorporation step. We are not aware of any reported examples of such dendron-like modified nucleotides as substrates for DNA polymerases.

After their synthesis, the nucleosides were converted into triphosphates and subsequently tested in DNA polymerase-promoted primer extension reactions, polymerase chain reactions and rolling circle amplification. By these methods, DNA hybrid materials with high molecular weight, modified density and defined structure are accessible.

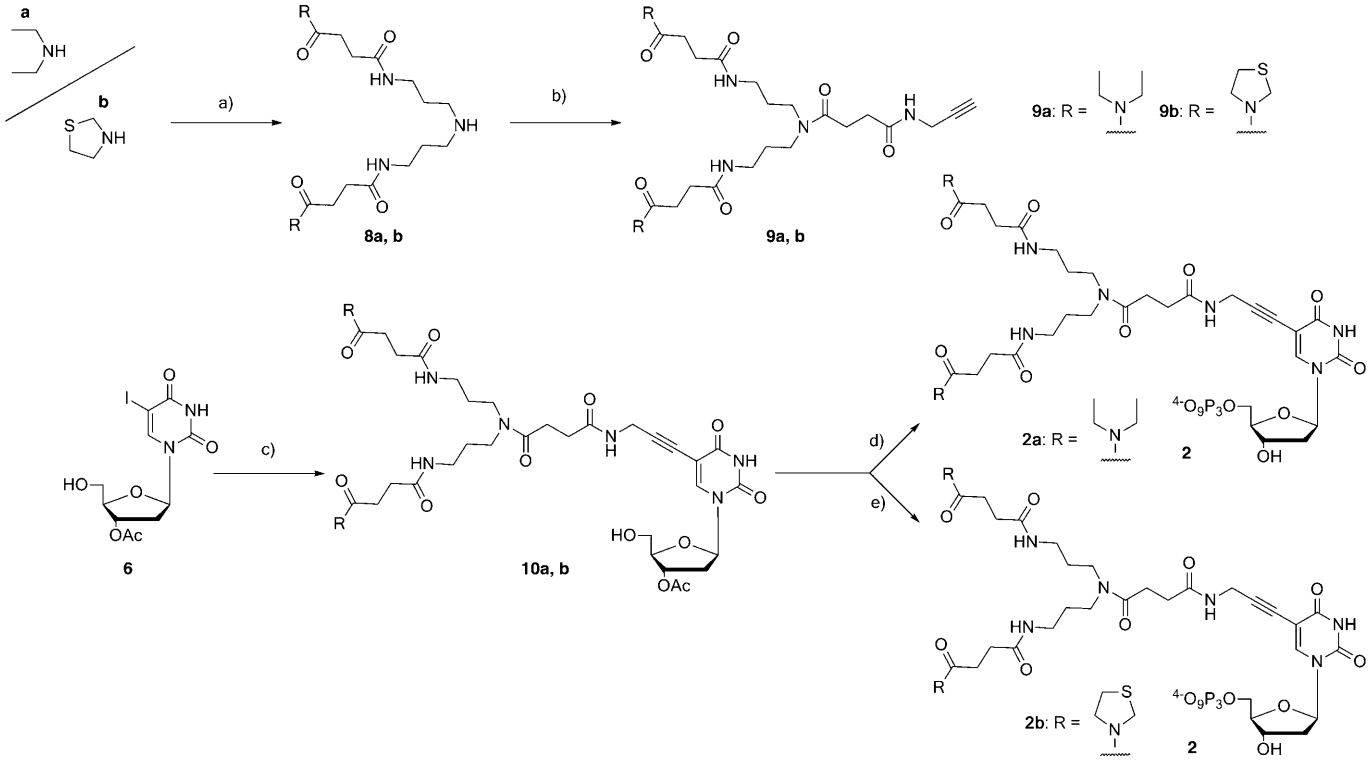
Results and Discussion

Synthesis of modified nucleotides: For the reasons discussed above, we envisioned the synthesis of nucleotides harbouring a polymer moiety at the C5-position of the nucleobase. For all four functionalised nucleosides, Sonogashira cross-coupling[38–41] and subsequent phosphorylation to form the triphosphates of the nucleosides[42–44] was followed. For this purpose we required the alkyne functionalised compounds **5a**,**b** and **9a**,**b** (Schemes 2 and 3). Thus, the alcohols **3a**,**b**



Scheme 2. a) Tosyl chloride (Ts-Cl), Et_3N, CH_2Cl_2, RT; **4a**, **4b**: quant.; b) propargyl alcohol, NaH, THF, 0 °C, **5a**: 98 %, **5b**: 95 %; c) $[Pd(PPh_3)_4]$, CuI, Et_3N, alkyne **5**, DMF, RT, **7a**,**b**: 65 %; d) proton sponge (1,8-bis(dimethylaminonaphthalene), $POCl_3$, $PO(OMe)_3$, 0 °C, then $(Bu_3NH)_2H_2P_2O_7$, nBu_3N, then triethylammonium bicarbonate (TEAB) buffer, then 33 % NH_3, **1a**: 40 %, **1b**: 7 %.

were converted into the respective tosylates **4a**,**b** and subsequently treated with sodium hydride and propargyl alcohol to yield compounds **5a**,**b** (Scheme 2). For the synthesis of alkynes **9a**,**b**, the compounds **8a**,**b** were prepared according to the literature.[45] The resulting polyamido dendron **8** was then treated with succinic anhydride, 1,1′-carbonyldiimidazole (CDI) and propargyl amine to yield the alkynes **9a**,**b** (Scheme 3). The alkynes **5a**,**b** and **9a**,**b** were then coupled to nucleoside **6** by using a standard protocol for the Sonogashira reaction[33] to yield nucleosides **7a**,**b** and **10a**,**b**. These nucleosides were then converted into the corresponding triphosphates (**1a**,**b** and **2a**,**b**) by using an appropriate method for phosphorylation.[42–44,46]



Scheme 3. a) Diethylamine, succinic anhydride, toluene, 70 °C, CDI, bis(3-aminopropyl)amine, **8a**: 25 %, **8b**: 16 %; b) succinic anhydride, THF, 70 °C, CDI, then propargyl amine, **9a**: 63 %, **9b**: 31 %; c) $[Pd(PPh_3)_4]$, CuI, Et_3N, alkyne **9**, DMF, RT, **10a**: 89 %, **10b**: 63 %; d) proton sponge, $POCl_3$, $PO(OMe)_3$, 0 °C, then $(Bu_3NH)_2H_2P_2O_7$, $n$$Bu_3N$, then TEAB buffer, then 33 % NH_3, **2a**: 6 %; e) 2-chloro-4*H*-1,2,3-dioxaphosphorin-4-one, pyridine, dioxane, RT, then $(Bu_3NH)_2H_2P_2O_7$, $n$$Bu_3N$, then I_2, then 5 % aq. $NaHSO_3$, then 33 % NH_3, **2b**: 18 %.

Incorporation of polymer-functionalised building blocks: Primer extension: To test the ability of DNA polymerases to accept the modified triphosphate and to incorporate the respective nucleotide into a nascent DNA strand, we set up a primer extension reaction. We used a 23-nucleotide primer with a ^{32}P label at the 5′ end and diverse templates. The reactions were analysed by denaturing polyacrylamide gel electrophoresis (PAGE) followed by autoradiography.

First, we used the primer and 35-nucleotide template complex with a single **A** residue, coding for the insertion of a TTP after extending the 23-nucleotide primer strand by three residues. Reactions lacking TTP predominantly abort before the template **A**, whereas reactions including dATP, dCTP, dGTP and TTP gave full-length products.

We tested *Pyrococcus woesei* (*Pwo*) and 9°N$_m$ DNA polymerases, since these enzymes have shown promising results with other modified nucleotides.[29–35,47–50] Both enzymes were able to extend the nascent DNA strand to full length when natural TTP was replaced by one of the modified triphosphates (data not shown). Encouraged by these results, we designed two different templates (69 nucleotides) to code for a modified nucleotide every fourth and every second position (Figure 1 A and B, respectively). These templates, with a more challenging sequence, should shed light on the ability of the enzymes to promote multiple incorporations of functionalised nucleotides. For these templates we found that 9°N$_m$ DNA polymerase is more proficient in extending both templates (Figure 1 A, B), while *Pwo* DNA polymerase was not able to incorporate a polyamido dendron modified nucleotide on every second position (data not shown). Therefore, we used 9°N$_m$ DNA polymerase for further experiments.

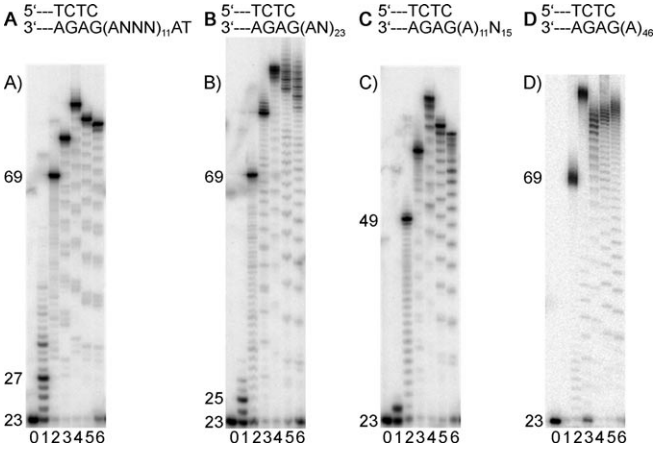


Figure 1. Incorporation experiments under 9°N$_m$ DNA polymerase catalysis for residues **A** (A), **B** (B), **C** (C) and **D** (D). Lane 0: 5′-^{32}P-labeled 23-nucleotide primer strand; lane 1: primer template complex including dATP, dCTP, dGTP; lane 2: same as lane 1 including TTP; lane 3: same as lane 1 including **1a**; lane 4: same as lane 1 including **1b**; lane 5: same as lane 1 including **2a**; lane 6: same as lane 1 including **2b**.

Upon substitution of natural TTP by **1a**, **1b**, **2a** or **2b**, the full-length products showed lower mobility on the gel in comparison with the unmodified full-length product (Figure 1). We suppose that this property is based on the increased steric demand and molecular weight of the modified entities. Similar effects have been reported before.[30]

To form one entire modified DNA helix turn in the nascent DNA strand, we used a template with eleven adjacent **A** residues. For all four thymidine analogues, full-length product formation was observed (Figure 1C). Interestingly, comparing the mobility shifts of the full-length products, the fragment containing **1b** was observed to migrate slower than the corresponding fragment containing the polyamido dendron **2b** (Figure 1A–C), even though building block **2b** has a slightly higher molecular weight than **1b**, indicating that the incorporation of a linear polymer causes a lower mobility in the polyacrylamide gel than a branched one with higher molecular weight. Another template with 46 **A** residues in a row was designed to investigate the limits of enzymatic incorporation of the modified nucleotides. In this experiment, the full-length product was formed with **1a**, whereas shorter fragments were obtained for all other modifications (Figure 1D).

Polymerase chain reaction (PCR): Next, we investigated the suitability of using building blocks **1a**,**b** and **2a**,**b** for substituting TTP in PCR. In this case we tested 9°N$_m$ DNA polymerase with a template of 304 nucleotides (Figure 2A, B). As with the primer extension tests, we conducted one control reaction with dATP, dCTP, dGTP and TTP, which led to PCR products with the desired length (Figure 2A, B; lane 1). In another control, we performed the same reaction in the absence of TTP, which led to no observable PCR product formation (Figure 2A, B; lane 2). The reactions were analysed by agarose gel and stained with ethidium bromide. By using standard PCR conditions, the DNA polymerase was able to substitute compounds **1a**, **2a** and **2b** (Figure 2A; lane 3 and Figure 2B; lanes 3, 4) for TTP and amplify the modified DNA in a similar fashion to those with all four natural dNTPs. Again, a mobility shift of the product bands to lower mobility was observed for the modified DNA.

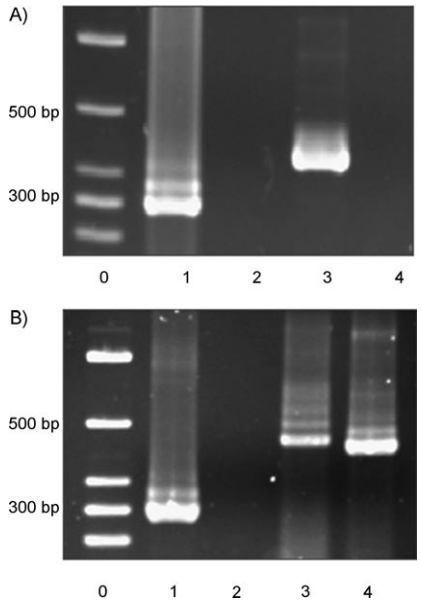


Figure 2. 2.5% agarose gel stained with ethidium bromide showing PCR products of a template 304 base pairs (bp) in length. A) Lane 0: marker; lane 1: PCR with all natural dNTPs; lane 2: PCR with dATP, dCTP, dGTP; lane 3: PCR with **1a** instead of TTP; lane 4: PCR with **1b** instead of TTP. B) Lane 0: marker; lane 1: PCR with all natural dNTPs; lane 2: PCR with dATP, dCTP, dGTP; lane 3: PCR with **2a** instead of TTP; lane 4: PCR with **2b** instead of TTP.

The use of **1b** instead of TTP in PCR resulted in no amplification product. Even after changing the reaction conditions for compound **1b**, no product band was observed. Presumably, the larger modification of **1b** compared with **1a** interfered with the DNA polymerase. Since one reason for the reduced amplification efficiency when using **1b** might be the polyethylene glycol moiety itself, we performed control reactions with added polyethylene glycol monomethyl ether of the respective molecular weight. With this experiment we could show that the PCR is more sensitive to the addition of polyethylene glycol monomethyl ether with an average molecular weight of 550 g mol^{-1} than for 350 g mol^{-1} (see the Supporting Information, Figure S1). We noted that at a certain concentration no more PCR product was formed in the presence of polyethylene glycol monomethyl ether.

Since it has been shown that 9°N$_m$ DNA polymerase is able to accept the unnatural thymidine derivatives and is able to use them as a template (**1a**, **2a**, **2b**), we switched to a genomic DNA fragment as template for the PCR (Figure 3A, B). Here, the use of **1a** resulted in higher yield of PCR product than the PCR including **2a** and **2b**, which produced less, but still observable, product, which is consistent with previous observations.

Circular dichroism (CD) and thermal denaturation measurements: The 304 nucleotide PCR product bands of compounds **1a**, **2a** and **2b** were purified by gel extraction and further characterised by thermal denaturation (T_m) measurements as well as by CD spectroscopy. To determine the influence of the modifications on the DNA conformation, CD spectra were measured. The CD spectrum of all modifications adopts an overall B-form conformation similar to the unmodified DNA (see the Supporting Information, Figure S2). We assume that the modifications of the tested thymidine analogues are, on one hand, well accommodated in the major groove and, on the other hand, flexible enough without interfering with the overall B-DNA conformation. In addition, the T_m measurements indicate that modification with **1a** causes a stabilisation of the DNA strands. The T_m value of this modified double strand is 6.5 °C higher than for the corresponding unmodified DNA strand (73.5 °C). In case of incorporated nucleotide **2a**, a destabilisation takes place. The measured T_m value was 9.5 °C lower than for the reference. Interestingly, incorporation of **2b** had only a small influence on the T_m value (72.5 °C).

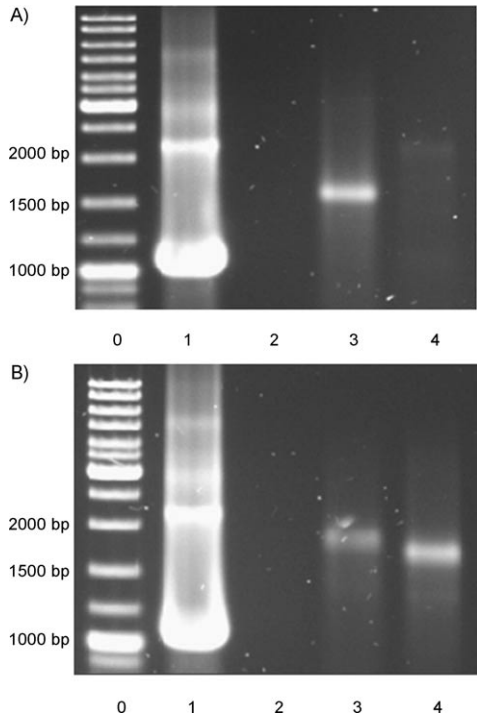


Figure 3. 0.8 % agarose gel stained with ethidium bromide showing PCR products of a template 1062 bp in length. A) Lane 0: marker; lane 1: PCR with all natural dNTPs; lane 2: PCR with dATP, dCTP, dGTP; lane 3: PCR with **1a** instead of TTP; lane 4: PCR with **1b** instead of TTP. B) Lane 0: marker; lane 1: PCR with all natural dNTPs; lane 2: PCR with dATP, dCTP, dGTP; lane 3: PCR with **2a** instead of TTP; lane 4: PCR with **2b** instead of TTP.

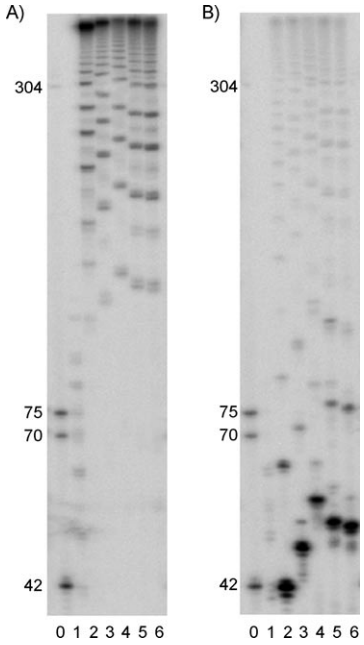


Figure 4. A) Rolling circle amplification; lane 0: marker; lane 1: primer template complex including α-dATP, dATP, dCTP, dGTP; lane 2: same as lane 1 including TTP; lane 3: same as lane 1 including **1a**; lane 4: same as lane 1 including **1b**; lane 5: same as lane 1 including **2a**; lane 6: same as lane 1 including **2b**. B) Restriction enzyme digestion; lane 0: marker; lane 1: digestion of RCA product lacking dTTP; lane 2: digest RCA product with all natural dNTPs; lane 3: digest RCA product with **1a**; lane 4: digest RCA product with **1b**; lane 5: digest RCA product with **2a**; lane 6: digest RCA product with **2b**.

Rolling circle amplification (RCA):[51–55] A method to generate long, single DNA strands is rolling circle amplification. Therefore, we tested the acceptance of the thymidine analogues **1a**,**b** and **2a**,**b** using this method. For this reaction, a template with 42 nucleotides containing an **A** residue on every fourteenth position and a restriction site for restriction endonuclease from *Haemophilus haemolyticus* (HhaI) was designed. This linear precursor was circularised enzymatically using T4 DNA ligase and a "splint" oligonucleotide with 16 nucleotides that aligns both ends of the precursor by hybridisation.[56] This DNA circle serves as a virtually never-ending template for the primer extension reaction.

Radioactive-labelled RCA product could be obtained by incorporation of radioactive-labelled nucleotides. Therefore, α-^{32}P-dATP was added to the reaction mixture. We tested 9°N$_m$ DNA polymerase to determine its ability to extend a short primer to a long ssDNA strand. The reactions were analysed by denaturing PAGE. First, we performed one control reaction with dATP, dCTP, dGTP, TTP and a second lacking TTP. Then all four nucleotide analogues were tested as surrogates for natural TTP. With all the thymidine analogues **1a**,**b** and **2a**,**b**, the nascent DNA strand was elongated, indicating that all modified nucleotides were accepted (Figure 4 A). To ensure that the observed product results from RCA, we purified the radioactive-labelled RCA product with a G 25-column then hybridised the RCA product with restriction helpers and carried out a digestion with restriction endonuclease HhaI; the reactions were analysed by PAGE and the resulting gel is depicted in Figure 4 B. The whole RCA product was digested by the enzyme to 42-nucleotide fragments. The shifts observed in fragments obtained from the modified RCA products (Figure 4 B; lanes 3–6) is caused by higher molecular weight and steric demand, as mentioned before. By means of this experiment, we showed that the restriction enzyme was not significantly inhibited by the DNA modifications.

Conclusion

A straightforward synthesis of polymer-modified nucleoside triphosphates has been developed. The corresponding triphosphates were tested with different DNA polymerases, indicating that 9°N$_m$ DNA polymerase was most suited for our endeavour. All four thymidine analogues were accepted by 9°N$_m$ DNA polymerase in primer extension experiments despite their different constitution. PCR amplification was possible with templates 304 and 1062 bp in length using the modified triphosphates **1a** and **2a**,**b**, and the DNA polymerase mentioned above. This provides the possibility of synthesising long DNA strands by enzyme-catalysed reactions. In CD experiments, we showed that the polymer moieties of the incorporated, modified nucleotides had a negli-

gible influence on the conformation of the product strand. Using RCA, we demonstrated that large ssDNA fragments could be synthesised enzymatically. Thus, we could show that enzyme-catalysed reactions are able to synthesise DNA polymers that can be grafted with short polymer blocks leading to entities of high molecular weight, modification density and defined structure. Future endeavours will focus on the development of synthetic systems that are able to generate long DNA backbones grafted with larger functional polymer side chains. To shed light on the processes involved in the incorporation of modified nucleotides in general, crystal structures of DNA polymerases complexed with these modified building blocks would be highly beneficial. Efforts along these lines are in progress and will be reported in due course.

Experimental Section

General: All temperatures quoted are uncorrected. All synthetic reactions were performed under an inert atmosphere. Anhydrous solvents were purchased from Fluka and were stored over molecular sieves and used without further purification. 5-Iodo-2′-deoxyuridine was purchased from Pharma Waldhof. Nucleoside **6** was prepared according to the literature.[57] NMR spectra were recorded by using Bruker Avance 400 (^{1}H: 400, ^{13}C: 101, ^{32}P: 162 MHz) and Bruker DRX 600 (^{1}H: 600, ^{13}C: 151, ^{32}P: 243 MHz) spectrometers. Chemical shifts are given in parts per million and tetramethylsilane was used as external standard. Electrospray ionisation ion trap (ESI-IT) mass spectra were recorded by using a Bruker Daltonics esquire 3000+ instrument in positive or negative mode with a flow rate of 3 μL min^{-1}. Flash chromatography was performed by using Merck silica gel G60 (230–400 mesh), and Merck precoated plates (silica gel 60 F_{254}) were used for TLC. Technical solvents were distilled prior to use. Petroleum ether, where used, refers to the fraction boiling in the range 35–80 °C. For medium-pressure liquid chromatography, a Büchi unit with a Büchi controller C-620, two pumps C-605, a UV monitor C-630 (λ = 254 nm) and fraction collector C-660 was used. For reversed-phase chromatography of polyamido dendrons or nucleosides, a Büchi RP-18 ready-for-use column was employed; for nucleotides, a LiChroprep RP-18 Lobar ready-for-use column was used. Purification of triphosphates was performed on a Bio-Rad system (BioLogic DuoFlow) with DEAE Sephadex A-25 (Pharmacia LKB) column using a linear gradient of TEAB-buffer. Oligonucleotides were obtained from Metabion (purified twice by HPLC). The *Pwo* DNA polymerase was purchased from PeqLab. 9°N$_m$ DNA polymerase and restriction endonuclease HhaI were purchased from New England Biolabs. T4 DNA ligase and T4 polynucleotide kinase were purchased from Fermentas.

General procedure for compound 4: *p*-Toluenesulfonic acid chloride (2 equiv) and Et_3N (2 equiv) were added to a solution of polyethylene glycol monomethyl ether in CH_2Cl_2 at 20 °C while stirring. The reaction mixture was stirred for 16 h and then concentrated in vacuo. The crude product was purified by flash chromatography.

Compound 4a: The reaction was carried out with hexaethylene glycol monomethyl ether (400 mg, 1.4 mmol), CH_2Cl_2 (2 mL), *p*-toluenesulfonic acid chloride (515 mg, 2.7 mmol), and Et_3N (380 μL, 2.7 mmol). Purification by silica gel (CH_2Cl_2/MeOH 100:1 to 9:1) gave compound **4a** (384 mg, 0.9 mmol, 63 %). R_f = 0.56 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.73 (d, ^{3}J = 8.3 Hz, 2H; Ar-H), 7.27 (d, ^{3}J = 8.3 Hz, 2H; Ar-H), 4.15–4.02 (m, 2H; OCH_2CH_2OTs), 3.64–3.45 (m, 22H; OCH_2CH_2O), 3.31 (s, 3H; OCH_3), 2.38 ppm (s, 3H; CH_3); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 144.8, 133.0, 129.8, 128.0, 71.9, 70.7, 70.6, 70.5, 70.4, 69.2, 68.7, 59.0, 21.7 ppm; MS (ESI): *m*/*z*: 473.1 $[M+Na]^+$.

Compound 4b: The reaction was carried out with dodecaethylene glycol monomethyl ether (100 mg, 178 μmol), CH_2Cl_2 (1 mL), *p*-toluenesulfonic acid chloride (68 mg, 357 μmol), and Et_3N (50 μL, 357 μmol). Purification by silica gel (CH_2Cl_2/MeOH 100:1 to 9:1) gave compound **4b** (121 mg, 169 μmol, 95 %). R_f = 0.65 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.79 (d, ^{3}J = 8.2 Hz, 2H; Ar-H), 7.33 (d, ^{3}J = 8.2 Hz, 2H; Ar-H), 4.19–4.11 (m, 2H; OCH_2CH_2OTs), 3.71–3.52 (m, 46H; OCH_2CH_2O), 3.37 (s, 3H; OCH_3), 2.44 ppm (s, 3H; CH_3); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 144.7, 133.0, 129.8, 127.9, 71.9, 70.7, 70.6, 70.5, 70.4, 69.2, 68.6, 59.0, 21.6 ppm; MS (ESI): *m*/*z*: 715.4 $[M]^+$, 737.4 $[M+Na]^+$.

General procedure for compound 5: Propargyl alcohol (10 equiv) was added to a suspension of NaH (95 %, 10 equiv) in THF (5 mL) at 0 °C. After 30 min, a solution of *O*-tosyl polyethylene glycol monomethyl ether (1.0 equiv) in THF (10 mL) was added and the mixture was allowed to warm to 20 °C and stirred for 12 h. Afterwards, the mixture was concentrated in vacuo and the residue was purified by flash chromatography.

Compound 5a: The reaction was carried out with compound **4a** (540 mg, 1.2 mmol), THF (5 mL), NaH (288 mg, 12.0 mmol), and propargyl alcohol (693 μL, 12.0 mmol). Purification by silica gel (EtOAc/MeOH 100:1 to 9:1) gave compound **5a** (370 mg, 1.1 mmol, 92 %). R_f = 0.55 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 4.20 (d, ^{4}J = 2.3 Hz, 2H; $CH_2C{\equiv}C$), 3.72–3.61 (m, 22H; OCH_2CH_2O), 3.56–3.54 (m, 2H; OCH_2CH_2O), 3.38 (s, 3H; OCH_3), 2.43 ppm (t, ^{4}J = 2.3 Hz, 1H; C≡CH); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 79.7, 74.5, 71.9, 70.6, 70.5, 70.4, 69.1, 59.0, 58.4 ppm; MS (ESI): *m*/*z*: 357.1 $[M+Na]^+$.

Compound 5b: The reaction was carried out with compound **4b** (120 mg, 168 μmol), THF (5 mL), NaH (43 mg, 1.7 mmol) and propargyl alcohol (103 μL, 1.7 mmol). Purification by silica gel (CH_2Cl_2/MeOH 100:1 to 10:1) gave compound **5b** (95 mg, 160 μmol, 93 %). R_f = 0.64 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.14 (d, ^{4}J = 2.3 Hz, 2H; $CH_2C{\equiv}C$), 3.65–3.54 (m, 46H; OCH_2CH_2O), 3.50–3.46 (m, 2H; OCH_2CH_2O), 3.31 (s, 3H; OCH_3), 2.37 ppm (t, ^{4}J = 2.3 Hz, 1H; C≡CH); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 79.7, 74.5, 72.0, 70.7, 70.6, 70.5, 70.4, 69.1, 59.1, 58.4 ppm; MS (ESI): *m*/*z*: 621.3 $[M+Na]^+$.

General procedure for compound 8: Succinic anhydride (1.1 equiv) was added to a solution of secondary amine in THF at 70 °C and the mixture was stirred until the solid anhydride had completely dissolved. CDI (1.1 equiv) was added slowly such that the CO_2 evolution was controlled. After complete addition of CDI, the solution was allowed to stir at 70 °C for 1 h. An argon purge was conducted by bubbling argon through the solution then, after 1 h, a solution of dipropylene triamine (0.5 equiv) in THF was added slowly. The reaction mixture was stirred for a further 2 h at 70 °C and then allowed to cool to 20 °C and stirred for 12 h. The reaction mixture was concentrated in vacuo and the remaining residue was purified by reversed-phase medium-pressure liquid chromatography (RP-MPLC).

Compound 8a: The reaction was carried out with diethylamine (5.0 g, 68.4 mmol) in THF (250 mL), succinic anhydride (7.5 g, 75.2 mmol), CDI (12.2 g, 75.2 mmol), and dipropylene triamine (4.8 mL, 34.2 mmol) in THF (20 mL). Purification: RP-MPLC (RP-18, 40–63 μm) using a linear gradient of water/MeCN (5 to 100 % MeCN, 2000 mL). The product eluted at 20 % MeCN to yield compound **8a** (10.0 g, 22.6 mmol, 66 %). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.02 (s, 1H; NH), 3.40–3.22 (m, 8H; CH_2CH_3), 2.72–2.58 (m, 8H; $CONHCH_2CH_2CH_2$, $COCH_2CH_2CO$), 2.49 (t, ^{3}J = 6.6 Hz, 4H; $COCH_2CH_2CO$), 1.72–1.59 (m, 4H; $CONHCH_2CH_2CH_2$), 1.16 (t, ^{3}J = 7.1 Hz, 6H; CH_2CH_3), 1.07 ppm (t, ^{3}J = 7.1 Hz, 6H; CH_2CH_3); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 172.7, 171.1, 47.5, 42.0, 40.4, 37.9, 31.7, 29.2, 28.8, 14.2, 13.1 ppm; MS (ESI): *m*/*z*: 442.2 $[M]^+$.

Compound 8b: The reaction was carried out with thiazolidine (1.8 mL, 22.4 mmol) in THF (50 mL), succinic anhydride (2.5 g, 24.7 mmol), CDI (3.9 g, 24.7 mmol) and dipropylene triamine (1.6 mL, 11.2 mmol) in THF (5 mL). Purification: RP-MPLC (RP-18, 40–63 μm) using a linear gradient of water/MeCN (5 to 100 % MeCN, 2000 mL). The product eluted at 20 % MeCN to yield compound **8b** (1.7 g, 3.5 mmol, 32 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.69 (s, 1H; NH), 4.47–4.40 (m, 4H; NCH_2S), 3.78–3.64 (m, 4H; SCH_2CH_2N), 3.35–3.13 (m, 4H; $CONHCH_2CH_2CH_2$), 3.03 (t, ^{3}J = 6.0 Hz, 2H; SCH_2CH_2N), 2.95–2.87 (m, 2H; SCH_2CH_2N), 2.69–2.39 (m, 12H; $CONHCH_2CH_2CH_2$, $COCH_2CH_2CO$), 1.92–1.64 ppm (m, 4H; $CONHCH_2CH_2CH_2$); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 177.5,

172.6, 172.5, 170.6, 49.0, 48.8, 48.6, 48.3, 46.4, 46.1, 36.6, 36.3, 31.3, 29.6, 28.2 ppm; MS (ESI): *m/z*: 473.8 $[M]^+$.

General procedure for compound 9: Compound **8** was dissolved in THF, succinic anhydride (1.5 equiv) was added and the reaction mixture was heated to 70 °C. After 3 h, CDI (1.2 equiv) was added slowly such that the CO_2 evolution was controlled. An argon purge was then conducted by bubbling argon through the solution and, after 30 min, propargyl amine (2 equiv) was injected whilst stirring. The reaction mixture was stirred for 16 h and then concentrated in vacuo. The crude product was purified by RP-MPLC.

Compound 9a: The reaction was carried out with compound **8a** (1.5 g, 3.4 mmol) in THF (30 mL), succinic anhydride (510 mg, 5.1 mmol), CDI (660 mg, 4.1 mmol) and propargyl amine (440 μL, 6.8 mmol). Purification: RP-MPLC (RP-18, 40–63 μm) using a linear gradient of water/MeCN (5 to 100% MeCN, 1000 mL). The product eluted at 35% MeCN to yield compound **9a** (1.4 g, 72%, 2.4 mmol). ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 7.65$–7.55 (m, 2H; NH), 6.72 (t, $^3J = 4.8$ Hz, 1H, NH), 4.03 (dd, $^3J = 4.8$, $^4J = 2.5$ Hz, 2H; $CH_2C{\equiv}C$), 3.42–3.28 (m, 14H, CH_2CH_3; $NHCH_2CH_2CH_2N$), 3.27–3.22 (m, 2H; $NHCH_2CH_2CH_2N$), 2.76–2.68 (m, 6H; $COCH_2CH_2CO$), 2.61–2.53 (m, 6H; $COCH_2CH_2CO$), 2.25 (t, $^4J = 2.5$ Hz, 1H; C≡CH), 1.86–1.80 (m, 2H; $NHCH_2CH_2CH_2N$), 1.80–1.73 (m, 2H; $NHCH_2CH_2CH_2N$), 1.25 (t, $^3J = 7.1$ Hz, 6H; CH_2CH_3), 1.13 ppm (t, $^3J = 5.5$ Hz, 6H; CH_2CH_3); ^{13}C NMR (151 MHz, $CDCl_3$): $\delta = 173.0$, 172.6, 172.4, 171.9, 171.3, 171.2, 100.0, 79.8, 71.3, 45.1, 43.1, 42.1, 40.6, 40.5, 36.3, 36.2, 31.6, 31.2, 29.1, 28.5, 28.5, 28.3, 28.2, 26.9, 14.2, 14.2, 13.1 ppm; MS (ESI): *m/z*: 577.5 $[M]^-$.

Compound 9b: The reaction was carried out with compound **8b** (617 mg, 1.3 mmol) in THF (20 mL), succinic anhydride (200 mg, 2.0 mmol), CDI (270 mg, 1.6 mmol) and propargyl amine (170 μL, 2.6 mmol). Purification: RP-MPLC (RP-18, 40–63 μm) using a linear gradient of water/MeCN (5 to 100% MeCN, 1000 mL). The product eluted at 20% MeCN to yield compound **9b** (241 mg, 31%, 0.4 mmol). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 6.73$–6.52 (m, 2H; NH), 4.60–4.45 (m, 4H; NCH_2S), 4.00–3.92 (m, 2H; $CH_2C{\equiv}C$), 3.80–3.70 (m, 4H; SCH_2CH_2N), 3.37–3.20 (m, 6H; $CONHCH_2CH_2CH_2$), 3.20–3.11 (m, 2H; $CONHCH_2CH_2CH_2$), 3.08 (t, $^3J = 6.2$ Hz, 2H; SCH_2CH_2N), 2.97 (t, $^3J = 6.3$ Hz, 2H; SCH_2CH_2N), 2.75–2.59 (m, 6H; $COCH_2CH_2CO$), 2.58–2.46 (m, 6H; $COCH_2CH_2CO$), 2.20 (t, $^4J = 2.5$ Hz, 1H; C≡CH), 1.85–1.72 (m, 2H; $NHCH_2CH_2CH_2N$), 1.72–1.58 ppm (m, 2H; $NHCH_2CH_2CH_2N$); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 172.6$, 172.3, 172.1, 170.6, 170.5, 170.2, 99.0, 79.8, 71.4, 49.0, 48.7, 48.6, 48.3, 45.2, 43.0, 36.6, 36.3, 31.5, 31.3, 30.8, 30.3, 30.3, 30.0, 29.6, 29.2, 28.5, 28.3, 27.1 ppm; MS (ESI): *m/z*: 610.9 $[M]^+$, 632.9 $[M+Na]^+$.

General procedure for compounds 7a,b and 10a,b: The corresponding alkyne, $[Pd(PPh_3)_4]$ (0.1 equiv) and Et_3N (2.0 equiv) were added with stirring to a solution of 3′-*O*-acetyl-5-iodo-2′-deoxyuridine (1 equiv) and CuI (0.2 equiv) in degassed DMF. The mixture was stirred at 20 °C. After completion of the reaction, the mixture was concentrated in vacuo and purified by flash chromatography.

Compound 7a: The reaction was carried out with 3′-*O*-acetyl-5-iodo-2′-deoxyuridine (97 mg, 0.24 mmol), CuI (9.3 mg, 0.05 mmol), alkyne **5a** (164 mg, 0.49 mmol), $[Pd(PPh_3)_4]$ (25 mg, 0.02 mmol) and Et_3N (69 μL, 0.49 mmol) in DMF (3 mL). The reaction mixture was stirred for 18 h. Purification by silica gel (EtOAc/MeOH 100:1 to 4:1) gave compound **7a** (96 mg, 0.16 mmol, 65%). $R_f = 0.32$ (EtOAc/MeOH 5:1); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 8.53$ (s, 1H; NH), 8.42 (s, 1H; H-6), 6.34–6.28 (m, 1H; H-1′), 5.35 (m, 1H; H-3′), 4.39 (s, 2H; $CH_2C{\equiv}C$), 4.13 (s, 1H; H-4′), 3.96–3.87 (m, 2H; H-5′), 3.75–3.59 (m, 22H; OCH_2CH_2O), 3.57–3.50 (m, 2H; OCH_2CH_2O), 3.36 (s, 3H; OCH_3), 2.42 (dd, $J = 14.0$ Hz, 5.8 Hz, 1H; H-2′a), 2.38–2.31 (m, 1H; H-2′b), 2.07 ppm (s, 3H; OAc); ^{13}C NMR (151 MHz, $CDCl_3$): $\delta = 170.6$, 161.5, 149.5, 144.1, 99.7, 89.4, 85.9, 85.7, 77.8, 75.4, 71.9–70.3 (m), 69.1, 62.3, 59.2, 59.0, 44.8, 38.3, 21.0 ppm; HRMS: *m/z*: calcd for $C_{27}H_{41}N_2O_{13}^-$: 601.2614; found: 601.2556.

Compound 7b: The reaction was carried out with 3′-*O*-acetyl-5-iodo-2′-deoxyuridine (33 mg, 0.08 mmol), CuI (3.2 mg, 0.02 mmol), alkyne **5b** (100 mg, 0.17 mmol), $[Pd(PPh_3)_4]$ (9.7 mg, 8.4 μmol), and Et_3N (23 μL, 0.17 mmol) in DMF (1 mL). The reaction mixture was stirred for 18 h. Purification by silica gel (CH_2Cl_2/MeOH 100:1 to 9:1) gave compound **7b** (47 mg, 54 μmol, 65%). $R_f = 0.34$ (CH_2Cl_2/MeOH 10:1); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 8.37$ (s, 1H; NH), 8.31 (s, 1H; H-6′), 6.33 (dd, $^3J = 8.3$ Hz, 5.8 Hz, 1H; H-1′), 5.36 (m, 1H; H-3′), 4.39 (s, 2H; $CH_2C{\equiv}C$), 4.16–4.13 (m, 1H; H-4′), 3.95 (dd, $^2J = 11.4$, $^3J = 2.2$ Hz, 1H; H-5′a), 3.90 (dd, $^2J = 11.4$, $^3J = 1.9$ Hz, 1H; H-5′b), 3.74–3.62 (m, 46H; OCH_2CH_2O), 3.56–3.53 (m, 2H; OCH_2CH_2O), 3.38 (s, 3H; OCH_3), 2.45 (ddd, $^2J = 14.0$, $^3J = 5.8$, 1.3 Hz, 1H; H-2′a), 2.35 (ddd, $^2J = 14.0$, $^3J = 8.3$, 6.2 Hz, 1H; H-2′b), 2.10 ppm (s, 3H; OAc); ^{13}C NMR (151 MHz, $CDCl_3$): $\delta = 171.5$, 161.8, 150.1, 145.0, 100.8, 90.5, 86.9, 86.7, 78.7, 76.3, 73.0, 71.6–71.4 (m), 70.1, 63.3, 60.2, 60.0, 39.3, 22.0 ppm; HRMS: *m/z*: calcd for $C_{39}H_{65}N_2O_{19}^-$: 865.4187; found: 865.4193.

Compound 10a: The reaction was carried out with 3′-*O*-acetyl-5-iodo-2′-deoxyuridine (87 mg, 0.22 mmol), CuI (8.4 mg, 0.04 mmol), alkyne **9a** (256 mg, 0.44 mmol), $[Pd(PPh_3)_4]$ (25 mg, 0.02 mmol), and Et_3N (62 μL, 0.44 mmol) in DMF (3 mL). The reaction mixture was stirred for 4 h. Purification by silica gel (CH_2Cl_2/MeOH 100:1 to 4:1) gave compound **10a** (167 mg, 0.2 mmol, 90%). $R_f = 0.38$ (CH_2Cl_2/MeOH 10:1); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 9.99$ (s, 1H; NH), 8.37 (s, 1H; H-6), 7.53 (brs, 1H; NH), 7.45 (brs, 1H; NH), 7.36 (brs, 1H; NH), 6.30 (dd, $^3J = 8.2$, 5.9 Hz, 1H; H-1′), 5.36 (d, $^3J = 6.0$ Hz, 1H; H-3′), 4.18–4.08 (m, 3H; H-4′, $CH_2C{\equiv}C$), 3.94–3.81 (m, 2H; H-5′), 3.40–3.25 (m, 12H; CH_2CH_3, $NCH_2CH_2CH_2$), 3.24–3.12 (m, 4H; $CH_2CH_2CH_2NH$), 2.71–2.62 (m, 6H; $COCH_2CH_2CO$), 2.61–2.49 (m, 6H; $COCH_2CH_2CO$), 2.42 (dd, $^2J = 14.0$, $^3J = 5.9$ Hz, 1H; H-2′a), 2.31 (ddd, $^2J = 14.0$, $^3J = 8.2$, 6.0 Hz, 1H; H-2′b), 2.06 (s, 3H; OAc), 1.82–1.71 (m, 2H; $CH_2CH_2CH_2$), 1.71–1.61 (m, 2H; $CH_2CH_2CH_2$), 1.18 (t, $^3J = 7.1$ Hz, 6H; CH_3), 1.07 ppm (t, $^3J = 7.1$ Hz, 6H; CH_3); ^{13}C NMR (151 MHz, $CDCl_3$): $\delta = 173.1$, 172.9, 172.7, 172.3, 171.4, 170.5, 162.3, 149.7, 144.2, 99.5, 89.7, 85.9, 85.7, 75.4, 74.0, 62.0, 45.5, 43.4, 42.1, 40.6, 40.5, 38.4, 36.6, 36.4, 31.5, 31.3, 29.9, 28.5, 28.4, 28.3, 27.1, 21.0, 14.1, 13.1 ppm; HRMS: *m/z*: calcd for $C_{40}H_{61}N_8O_{12}^-$: 845.4414; found: 845.4427.

Compound 10b: The reaction was carried out with 3′-*O*-acetyl-5-iodo-2′-deoxyuridine (205 mg, 0.518 mmol), CuI (19.8 mg, 0.1 mmol), alkyne **9b** (946 mg, 1.6 mmol), $[Pd(PPh_3)_4]$ (60 mg, 0.05 mmol), and Et_3N (146 μL, 1.04 mmol) in DMF (4 mL). The reaction mixture was stirred for 5 h. Purification by silica gel (CH_2Cl_2/MeOH 100:0 to 85:5) gave compound **10b** (286 mg, 0.32 mmol, 63%). $R_f = 0.47$ (CH_2Cl_2/MeOH 10:1); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.26$ (s, 1H; H-6), 7.70–7.60 (m, 1H; NH), 7.39–7.26 (m, 2H; NH), 6.25 (dd, $^3J = 7.9$, 5.9 Hz, 1H; H-1′), 5.35–5.30 (m, 1H; H-3′), 4.50 (s, 4H; SCH_2N), 4.16–4.03 (m, 3H; H-4′, $CH_2C{\equiv}C$), 3.90–3.70 (m, 6H; H-5′, SCH_2CH_2N), 3.39–3.09 (m, 8H; $CH_2CH_2CH_2NH$), 3.08–3.01 (m, 2H; SCH_2CH_2N), 2.99–2.90 (m, 2H; SCH_2CH_2N), 2.73–2.48 (m, 12H; $COCH_2CH_2CO$), 2.40 (dd, $^2J = 13.2$, $^3J = 5.9$ Hz, 1H; H-2′a), 2.36–2.25 (m, 1H; H-2′b), 2.05 (s, 3H; OAc), 1.82–1.69 (m, 2H; $CH_2CH_2CH_2$), 1.69–1.57 ppm (m, 2H; $CH_2CH_2CH_2$); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 172.9$, 172.6, 172.6, 170.8, 170.7, 170.5, 162.5, 149.9, 144.3, 99.6, 89.8, 86.0, 85.9, 75.4, 74.2, 62.2, 53.6, 49.2, 48.9, 48.8, 48.5, 45.7, 43.5, 38.5, 36.9, 36.7, 31.4, 31.0, 30.5, 30.1, 29.7, 28.6, 28.5, 27.3, 21.2 ppm; HRMS: *m/z*: calcd for $C_{38}H_{54}N_8O_{12}S_2Na^+$: 901.3195; found: 901.3156.

General procedure for compounds 1a,b and 2a: The nucleosides (**7a**,**b** or **10a**, 1.0 equiv) and proton sponge (1.5 equiv) were dried overnight in vacuo, dissolved in trimethyl phosphate, and cooled to 0 °C. $POCl_3$ (**7a**: 1.8 equiv, **7b**: 6.2 equiv, **10a**: 4 equiv) was added to the mixture and stirred (**4a** and **4b**: 2 h, **9a**: 2.5 h). A 0.5 M solution of $(Bu_3NH)_2H_2P_2O_7$ in anhydrous DMF (**7a** and **10a**: 5 equiv, **7b**: 10 equiv) and nBu_3N (**7a** and **10a**: 5 equiv, **7b**: 10 equiv) were added simultaneously to the mixture. After 5 min, 1 M aqueous TEAB buffer (pH 7.5) was added and the aqueous layer was washed with EtOAc (3×2 mL). The aqueous layer was lyophilised and the residue was dissolved in water (5 mL), concentrated NH_3 (2.5 mL) was added and the reaction was stirred slowly at RT. The mixture was concentrated in vacuo and the resulting residue was purified by ion-exchange chromatography (DEAE-Sephadex A-25, linear gradient of TEAB buffer (0.1 M to 1 M, 1000 mL), flow rate: 2 mL min^{-1}) and further purified by RP-MPLC (RP-18, 40–63 μm) using a gradient of 5 (200 mL), 20 (200 mL) and 40% (200 mL) MeCN in 50 mM aqueous triethylammonium acetate (TEAA buffer, pH 7.0). The triphosphates **1a** and **1b** eluted with 20% MeCN, **2a** with 40% MeCN in 50 mM aqueous TEAA buffer.

Compound 1a: Prepared from **7a** (17.0 mg, 28 μmol), proton sponge (9.0 mg, 42 μmol), $POCl_3$ (3 μL, 34 μmol), $(Bu_3NH)_2H_2P_2O_7$ solution (0.28 mL, 0.14 mmol), $n$$Bu_3N$ (75 μL, 0.28 mmol) and trimethyl phosphate (1 mL) to give **1a** (40% estimated by UV, 11 μmol (14.0 mg of triethylammonium salt)). ^{1}H NMR (600 MHz, D_2O): δ = 8.10 (s, 1H; H-6), 6.21 (t, ^3J = 6.6 Hz, 1H; H-1′), 4.56 (br s, 1H; H-3′), 4.40 (s, 2H; CH_2C≡C), 4.18–4.10 (m, 3H; H-4′, H-5′), 3.77–3.73 (m, 2H; OCH_2CH$_2$O), 3.70–3.66 (m, 2H; OCH$_2$CH_2O), 3.66–3.61 (m, 18H; OCH_2CH_2O), 3.58–3.54 (m, 2H; OCH_2CH$_2$O), 3.32 (s, 3H; OCH_3), 3.14 (q, ^3J = 7.3 Hz, 16H; Et_3N), 2.38–2.29 (m, 2H; H-2′a, H-2′b), 1.22 ppm (t, ^3J = 7.3 Hz, 24H; Et_3N); ^{13}C NMR (151 MHz, D_2O): δ = 164.5, 150.4, 144.9, 98.8, 89.5, 85.7, 85.5, 77.2, 70.8, 70.5, 69.4, 69.3, 68.5, 65.2, 58.3, 57.9, 46.4, 38.5, 8.0 ppm; ^{31}P NMR (243 MHz, D_2O): δ = −9.99–−10.46 (m, 1P; P_γ), −10.78 (d, ^2J = 17.3 Hz, 1P; P_α), −22.31–−22.91 ppm (m, 1P; P_β); HRMS: m/z: calcd for $C_{25}H_{42}N_2O_{21}P_3^-$: 799.1498; found: 799.1498.

Compound 1b: Prepared from **7b** (23.0 mg, 26 μmol), proton sponge (8.5 mg, 42 μmol), $POCl_3$ (3 μL, 34 μmol), $(Bu_3NH)_2H_2P_2O_7$ solution (0.26 mL, 0.13 mmol), $n$$Bu_3N$ (70 μL, 0.27 mmol) and trimethyl phosphate (1 mL) to give **1b** (7% estimated by UV, 2 μmol (0.8 mg of triethylammonium salt)). ^{1}H NMR (600 MHz, D_2O): δ = 8.07 (s, 1H; H-6), 6.18 (t, ^3J = 6.8 Hz, 1H; H-1′), 4.56–4.53 (m, 1H; H-3′), 4.37 (s, 2H; CH_2C≡C), 4.14–4.07 (m, 3H; H-4′, H-5′), 3.74–3.71 (m, 2H; OCH_2CH$_2$O), 3.67–3.64 (m, J = 5.5, J = 3.2 Hz, 2H; OCH$_2$CH_2O), 3.61 (br s, 42H; OCH_2CH_2O), 3.55–3.51 (m, 2H; OCH_2CH$_2$O), 3.29 (s, 3H; OCH_3), 3.11 (q, ^3J = 7.3 Hz, 24H; Et_3N), 2.36–2.27 (m, 2H; H-2′a, H-2′b), 1.19 ppm (t, ^3J = 7.3 Hz, 36H; Et_3N); ^{13}C NMR (151 MHz, D_2O): δ = 164.5, 152.8, 145.1, 98.9, 89.8, 85.9, 77.4, 71.0, 70.6, 69.6, 69.4, 68.7, 58.9, 58.5, 58.0, 46.7, 38.7, 8.2 ppm; ^{31}P NMR (243 MHz, D_2O): δ = −9.75–−10.15 (m, 1P; P_γ), −10.74 (d, ^2J = 19.0 Hz, 1P; P_α), −22.07–−22.66 ppm (m, 1P; P_β); HRMS: m/z: calcd for $C_{37}H_{66}N_2O_{27}P_3^-$:1063.3071; found: 1063.3071.

Compound 2a: Prepared from **10a** (30.9 mg, 36 μmol), proton sponge (11.7 mg, 55 μmol), $POCl_3$ (4 μL, 44 μmol), $(Bu_3NH)_2H_2P_2O_7$ solution (0.37 mL, 0.18 mmol), $n$$Bu_3N$ (97 μL, 0.36 mmol) and trimethyl phosphate (1 mL) to give **2a** (6% estimated by UV, 2 μmol (8.1 mg of triethylammonium salt)). ^{1}H NMR (600 MHz, D_2O): δ = 8.08 (s, 1H; H-6), 6.17 (t, ^3J = 6.6 Hz, 1H; H-1′), 4.55 (br s, 1H; H-3′), 4.17–4.05 (m, 5H; H-4′, H-5′, CH$_2$C≡C), 3.34–3.26 (m, 6H; CH_2CH$_3$, NCH_2CH$_2$CH$_2$), 3.25–3.19 (m, 6H; CH_2CH$_3$, NCH_2CH$_2$CH$_2$), 3.15–3.06 (m, 26H; Et_3N, CH$_2$CH$_2$CH_2NH), 3.02 (t, ^3J = 6.9 Hz, 2H; CH$_2$CH$_2$CH_2NH), 2.64–2.55 (m, 6H; COCH$_2$CH_2CO), 2.48 (t, ^3J = 7.0 Hz, 2H; COCH_2CH$_2$CO), 2.41 (q, ^3J = 7.0 Hz, 4H; COCH_2CH$_2$CO), 2.34–2.24 (m, 2H; H-2′a, H-2′b), 1.79–1.69 (m, 2H; CH$_2$CH_2CH$_2$), 1.65–1.55 (m, 2H; CH$_2$CH_2CH$_2$), 1.22–1.14 (m, 36H; Et_3N), 1.08 (t, ^3J = 7.2 Hz, 6H; CH$_3$), 0.96 ppm (t, ^3J = 7.1 Hz, 6H; CH$_3$); ^{13}C NMR (151 MHz, D_2O): δ = 175.0, 174.8, 174.6, 173.9, 173.0, 172.9, 144.8, 98.9, 89.7, 85.6, 73.1, 70.0, 65.0, 46.5, 45.5, 43.5, 42.6, 40.7, 38.6, 36.7, 36.6, 30.9, 30.8, 30.6, 29.5, 27.9, 27.9, 27.3, 26.3, 12.9, 12.0, 8.0 ppm; ^{31}P NMR (243 MHz, D_2O): δ = −8.27–−9.06 (m, 1P; P_γ), −10.29 (d, ^2J = 19.9 Hz, 1P; P_α), −20.93–−22.71 ppm (m, 1P; P_β); HRMS: m/z: calcd for $C_{38}H_{62}N_8O_{20}P_3^-$: 1043.3299; found: 1043.3299.

Compound 2b: Nucleoside **10b** (200 mg, 0.3 mmol) was dissolved in dioxane/pyridine (3:1). A freshly prepared solution of 2-chloro-4H-1,2,3-dioxaphosphorin-4-one in dioxane (1 M, 330 μL) was then injected into the well-stirred solution of **10b**. After 10 min, $(Bu_3NH)_2H_2P_2O_7$ (0.5 M, 1.5 equiv, 0.45 mmol, 980 μL) and $n$$Bu_3N$ (4.1 equiv, 1.2 mmol, 355 μL) were added and the reaction mixture was stirred for 10 min. A solution of 1% iodine in pyridine/water (98:2, v/v) (4 mL, 0.3 mmol) was then added. After 15 min, excess iodine was destroyed by adding a few drops of 5% aqueous $NaHSO_3$ and the reaction solution evaporated to dryness. The residue was dissolved in water (33 mL) and, after standing at RT for 30 min, concentrated ammonia (65 mL) was added. After 1 h, the mixture was concentrated in vacuo and the resulting residue was purified by ion-exchange chromatography (DEAE-Sephadex A-25, linear gradient of TEAB buffer (0.1 M to 1 M, 1000 mL), flow rate: 2 mL min^{-1}) and further purified by RP-MPLC (RP-18, 40–63 mm) using a gradient of 5 (200 mL), 20 (200 mL) and 40% (200 mL) MeCN in 50 mM aqueous triethylammonium acetate (TEAA buffer, pH 7.0). The triphosphate **2b** eluted with 40% MeCN in 50 mM aqueous TEAA buffer (18% estimated by UV, 50 μmol (150 mg of triethylammonium salt)). ^{1}H NMR (600 MHz, D_2O): δ = 8.09 (s, 1H; H-6), 6.18 (t, ^3J = 6.6 Hz, 1H; H-1′), 4.59–4.38 (m, 5H; H-3′, SCH$_2$N), 4.18–4.05 (m, 5H; H-4′, H-5′, CH$_2$C≡C), 3.73 (q, ^3J = 6.3 Hz, 2H; SCH$_2$CH_2N), 3.66–3.59 (m, 2H; SCH$_2$CH_2N), 3.34–3.28 (m, 2H; NCH_2CH$_2$CH$_2$), 3.24 (t, ^3J = 6.9 Hz, 2H; NCH_2CH$_2$CH$_2$), 3.16–3.08 (m, 24H; Et_3N), 3.07–3.01 (m, 4H; SCH_2CH$_2$N, CH$_2$CH$_2$CH_2NH), 3.00–2.92 (m, 4H; SCH_2CH$_2$N, CH$_2$CH$_2$CH_2NH), 2.68–2.57 (m, 6H; COCH_2CH$_2$CO), 2.53–2.40 (m, 6H; COCH_2CH$_2$CO), 2.37–2.24 (m, 2H; H-2′a, H-2′b), 1.79–1.71 (m, 2H; CH$_2$CH_2CH$_2$), 1.65–1.57 (m, 2H; CH$_2$CH_2CH$_2$), 1.23–1.13 ppm (m, 36H; Et_3N); ^{13}C NMR (151 MHz, D_2O): δ = 174.6, 173.9, 172.2, 171.9, 171.8, 164.5, 144.8, 98.9, 89.8, 85.6, 70.2, 49.4, 49.3, 49.2, 49.1, 48.8, 48.7, 48.5, 48.4, 48.3, 48.2, 48.1, 48.0, 46.4, 46.3, 42.6, 40.5, 38.7, 36.5, 30.4, 30.3, 30.2, 30.1, 29.9, 29.8, 29.6, 29.5, 29.4, 28.8, 27.3, 26.3, 8.1, 8.0 ppm; ^{31}P NMR (162 MHz, D_2O): δ = −8.97–−10.29 (m, 1P; P_γ), −11.45 (d, ^2J = 19.5 Hz, 1P; P_α), −22.50–−23.35 ppm (m, 1P; P_β); HRMS: m/z: calcd for $C_{36}H_{54}N_8O_{20}P_3S_2^-$: 1075.2114; found: 1075.2112.

Primer extension: DNA primer strands were purchased from Metabion, dNTPs were from Roche, 9°N$_m$ DNA polymerase, 10×ThermoPol reaction buffer (200 mM Tris·HCl (pH 8.8), 100 mM KCl, 20 mM $MgSO_4$, 100 mM $(NH_4)_2SO_4$, 1% Triton X-100) was purchased from NEB. A typical primer extension reaction (20 μL) contained: 1×ThermoPol reaction buffer, ^{32}P-labeled primer (150 nM), template (200 nM), **1a**, **1b**, **2a** or **2b** (200 μM each), dATP, dCTP, dGTP (200 μM each) and 9°N$_m$ DNA polymerase (0.4 U/20 μL). First primer and template were annealed in 1×9°N$_m$ reaction buffer by heating the probes to 95 °C and allowing to cool to 20 °C. Afterwards the primer template complex, nucleotides and 9°N$_m$ DNA polymerase were incubated at 59 °C for 30 min. The reactions were quenched by the addition of PAGE gel-loading buffer (45 μL; 80% formamide, 20 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanole FF) and the product mixtures were analysed by 12% denaturing polyacrylamide gel, and subjected to autoradiography.

Circularisation: The linear DNA precursor (10 μM) and primer (20 μM) were hybridised in Tris·HCl (50 mM, pH 7.2) and MgCl (10 mM). Afterwards, ATP (100 μM), 1,4-dithiothreitol (10 mM), bovine serum albumin (25 μg mL^{-1}) and T4 DNA Ligase (0.24 U μL^{-1}) were added. The reaction was incubated at RT for 18 h and terminated by heating to 80 °C for 10 min. The product was purified by 15% denaturing polyacrylamide gel.

Rolling circle amplification: A typical rolling circle amplification reaction (20 μL) contained: 1×ThermoPol reaction buffer, primer (75 nM, 16 nt), template (108 nM, 42 nt), **1a**, **1b**, **2a** or **2b** (200 μM each), dATP, dCTP, dGTP (200 μM each), α-^{32}P-dATP (66 nM) and 9°N$_m$ DNA polymerase (0.4 U/20 μL). First the primer and template were annealed in 1×9°N$_m$ reaction buffer by heating the probes to 95 °C and allowing to cool to 20 °C. Afterwards, the primer template complex, nucleotides and 9°N$_m$ DNA polymerase were incubated at 59 °C for 60 min. The reactions were quenched by the addition of 45 μL PAGE gel-loading buffer (80% formamide, 20 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanole FF) and the product mixtures were analysed by 6% denaturing polyacrylamide gel, and subjected to autoradiography. For digestion with restriction enzyme, the reaction was purified with G25 column without quenching.

Restriction enzyme digestion: An aliquot of the RCA product (10 μL) was hybridised with 300 nM restriction helper strand in 1×digestion buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM 1,4-dithiothreitol, pH 7.9). After annealing, bovine serum albumin (0.1 mg mL^{-1}) and HhaI (0.4 U μL^{-1}, New England Biolabs) were added. The restriction reaction (25 μL) was carried out at 37 °C for 18 h and stopped by heating at 75 °C for 20 min. The digested products were diluted with PAGE gel-loading buffer (17.5 μL), analysed using 6% denaturing polyacrylamide gel and subjected to autoradiography.

PCR: Typical PCR reactions (20 μL) contained 1×9°N$_m$ reaction buffer, **1a**, **1b**, **2a** or **2b** (200 μM each), dATP, dCTP, dGTP (200 mM each), primer I (2 μM), primer II (2 μM), template (304 bp, 400 pM), and 9°N$_m$ DNA polymerase (0.32 U/20 μL). A typical PCR cycling protocol began with an initial denaturation at 95 °C for 60 s and was followed by 35 cycles of 95 °C, 40 s; 65 °C, 30 s; 59 °C, 90 s. The reactions were quenched by the addition of agarose gel-loading buffer (4 μL; 10 mM Tris·HCl, 60 mM EDTA, 30% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanole

FF) and the product mixtures were analysed by agarose gel electrophoresis. The PCR reaction using the 1062 bp plasmid as the template was performed according to the procedure mentioned above. The PCR cycling protocol was then run at 95 °C for 120 s, followed by 30 cycles of 95 °C, 60 s; 69 °C, 60 s; and 59 °C, 120 s. The reactions were quenched by the addition of agarose gel-loading buffer (4 μL) and the product mixtures were analysed by agarose gel electrophoresis.

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