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Deoxynucleoside triphosphates bearing histamine, carboxylic acid, and hydroxyl residues – synthesis and biochemical characterization†

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Modified nucleoside triphosphates (dA^{Hs}TP, dU^{POH}TP, and dC^{Val}TP) bearing imidazole, hydroxyl, and carboxylic acid residues connected to the purine and pyrimidine bases through alkyne linkers were prepared. These modified dN*TPs were excellent substrates for various DNA polymerases in primer extension reactions. Moreover, the combined use of terminal deoxynucleotidyl transferase (TdT) and the modified dNTPs led to efficient tailing reactions that rival those of natural counterparts. Finally, the triphosphates were tolerated by polymerases under PCR conditions, and the ensuing modified oligonucleotides served as templates for the regeneration of unmodified DNA. Thus, these modified dN*TPs are fully compatible with *in vitro* selection methods and can be used to develop artificial peptidases based on DNA.

Introduction

The selective scission of the amide bonds of proteins and peptides is of crucial importance for numerous biochemical and biotechnological applications[1] and plays a decisive role in physiological processes.[2,3] Considering the inertness of the peptide bond ($t_{1/2\ nonenz} \sim 500$ years),[4,5] proficient proteolytic enzymes with high catalytic efficiencies (k_{cat}/K_M) need to be used in order to degrade proteins or peptides into smaller fragments or for promoting site-specific cleavage. However, in terms of practical applications, proteolytic enzymes are not always applicable since they often lead to the generation of rather short fragmentation products, lack sequence-specificity, and cleave at multiple sites. Thus, artificial proteases based on synthetic reagents that could favor the selective cleavage of proteins under mild conditions are highly sought after. In this context, transition metal complexes were suggested as prime candidates to serve as biomimetic chemical proteases since they are often more effective than organic residues at promoting the scission of inert bonds.[1,6,7] Moreover, metal centers are often combined with reagents such as β-cyclodextrins and porphyrins that facilitate the recognition and the binding to the target site and thus reach appreciable catalytic efficiencies for the scission of amide linkages in peptides and even proteins.[8–11] However, even though some of these artificial, transition metal-based proteases achieve cleavage with multiple turnover[12] and with some site selectivity,[8,13] they suffer from severe drawbacks such as side-reactions, low k_{cat} values, and in general perform rather poorly when compared to protein enzymes.

DNA enzymes or DNAzymes have emerged as a new and prominent class of biomolecular catalysts, and have been selected to accelerate an increasing number of chemical processes.[14–18] Indeed, application of SELEX and related combinatorial methods of *in vitro* selection[19,20] resulted in the identification of nucleic acid enzymes catalyzing an abundance of reactions ranging from the formation of C–C,[21–24] C–N,[25] C–S,[26] and P–O,[27,28] bonds to the scission of ribophosphodiester linkages.[29,30] Surprisingly, the scission of amide linkages is a reaction that has eluded catalytic nucleic acids so far.[31] Indeed, in an early effort towards this goal, Joyce *et al.* initially reported on the selection of a ribozyme capable of cleaving a substrate containing an embedded 3′-NH–C(O)–CH_2-5′ linkage[32] but later realized that the reaction proceeded through a different mechanism leading to scission of a phosphodiester bond adjacent to the target site rather than the intended amide bond.[33] Similarly, Silverman *et al.* set out to select a DNAzyme capable of the selective hydrolysis of amide bonds of a tripeptide sequence,[34] which culminated in the serendipitous discovery of a class of very potent DNA-cleaving DNAzymes.[35,36]

Recently, blending of the chemical functionalities found in the active sites of enzymes together with tools of *in vitro* selection resulted in the identification of potent DNAzymes.[37,38] In particular, this approach allowed for the development of highly functionalized DNA-based RNase A mimics

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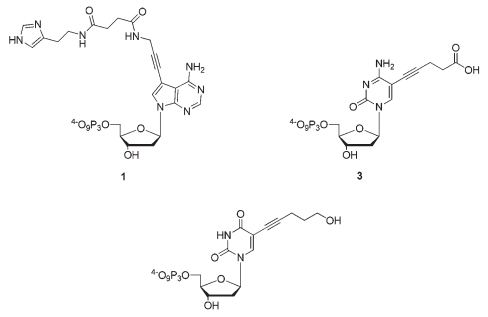
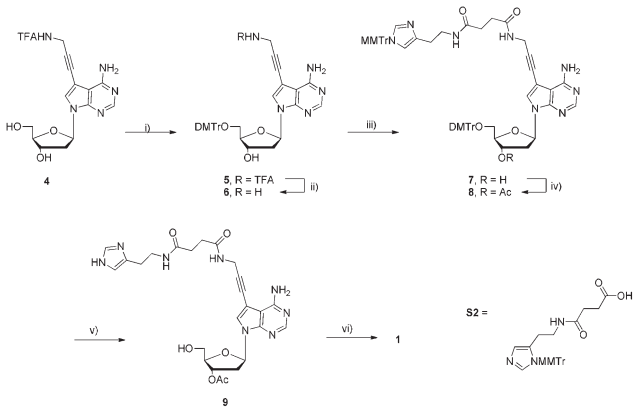


Fig. 1 Chemical structures of $dA^{Hs}TP$ **1**, $dU^{POH}TP$ **2**, and $dC^{Val}TP$ **3**.



Scheme 1 Synthesis of modified $dA^{Hs}TP$ **1**. Reagents and conditions: (i) DMTrCl, pyridine, rt, 15 h, 75%; (ii) NaOH, MeOH, H_2O, rt, 12 h, 84%; (iii) **S2**, EDC, HOBt, NMM, DMF, rt, 12 h, 64%; (iv) Ac_2O, DMAP, NEt_3, pyridine, 0 °C, 2.5 h, 86%; (v) DCAA, CH_2Cl_2, rt, 40 min, quant.; (iv) 1. 2-chloro-1,3,2-benzodioxaphosphorin-4-one, pyridine, dioxane, rt, 45 min; 2. $(nBu_3NH)_2H_2P_2O_7$, DMF, nBu_3N, rt, 45 min; 3. I_2, pyridine, H_2O, rt, 30 min; 4. NH_3(aq.), rt, 1.5 h, 17% (4 steps).

that operate either in the presence of cofactors[39–41] or in an M^{2+}-independent regime.[42–47] Moreover, all the functional groups encompassed in these modified DNAzymes seem to act in synergy and in a cooperative manner, one of the fundaments in the design of *de novo* enzyme mimics and catalysts in general.[48] Finally, this methodology is applicable to other reactions than the cleavage of ribophosphodiester bonds[25,49] provided that the functional groups can easily be introduced into DNA, *i.e.* that the modified nucleoside triphosphates (dN*TPs) are compatible with methods of *in vitro* selection.

In this context, the enzymatic polymerization of dN*TPs has advanced as a versatile platform for the inclusion of functional groups into nucleic acids.[50] Indeed, dN*TPs have been used to introduce a myriad of functionalities including amino acids,[51–57] boronic acids,[58,59] ligands for transition metals,[60–65] thiols,[66–68] diamondoid-like residues,[69] bile acids,[70] side chains capable of organocatalysis,[71] and even oligonucleotides.[72]

Herein, I report on the synthesis and biochemical characterization of modified dN*TPs bearing the side chains found in the active site of serine proteases.[31,73,74] Indeed, $dA^{Hs}TP$ (7-propargylamido-histamine-dA) **1**, $dU^{POH}TP$ (5-pentynol-dU) **2**, and $dC^{Val}TP$ (5-valeric acid-dC) **3** (Fig. 1) are equipped with imidazole, hydroxyl, and carboxylic acid residues, respectively, that are reminiscent of the side chains of the amino acids His, Ser, and Asp that form the catalytic triad of serine proteases. These modified dN*TPs are good substrates for a variety of DNA polymerases in the context of primer extension reactions. In addition, when used in conjunction with the terminal deoxynucleotidyl transferase (TdT), efficient tailing reactions that rival those of the natural counterparts could be observed. Finally, these modified dN*TPs were readily incorporated into DNA by the polymerase chain reaction (PCR), further underscoring their compatibility with *in vitro* selection techniques.

Results and discussion

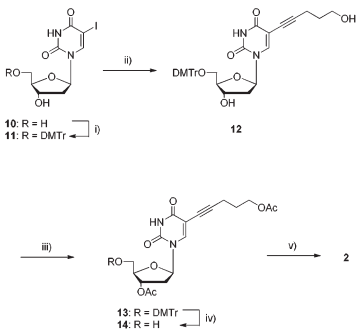
Synthesis of the modified nucleoside triphosphates

The nucleoside analogues considered herein all bear modifications either at the C5 position of the pyrimidine nucleobase or at the *N*7 of 7-deaza-2′-deoxyadenosine. Indeed, these anchoring sites were chosen for synthetic purposes (the modifications can easily be introduced *via* palladium-catalyzed coupling reactions) and because of their minimal disturbance and impact on substrate acceptance and duplex stability.[53,71,75–77]

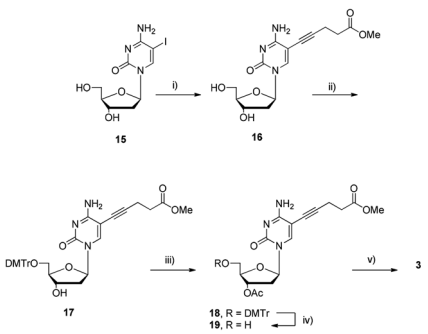
The adenosine analogue **1** adorned with a histamine residue was obtained in a 6-step sequence that started with the DMTr-protection of the known propargylamino modified 7-deaza-2′-deoxyadenosine **4** (Scheme 1).[53,75,78] After removal of the TFA masking group, nucleoside **6** was converted into derivative **7** in 64% yield under standard 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)-mediated amide bond-formation conditions using the suitably protected carboxylic acid S2 (ESI†). The resulting intermediate **7** was then 3′-*O*-acetylated under standard conditions and in good yield (84%). Following a global acid-mediated deprotection step (quantitative yield), nucleoside analogue **9** could be converted into the corresponding triphosphate $dA^{Hs}TP$ **1** in acceptable yields (17%) by application of the one-pot four-step triphosphorylation method developed by Ludwig and Eckstein.[79]

The synthetic path leading to $dU^{POH}TP$ **2** commenced with the Sonogashira cross-coupling reaction of the DMTr-protected deoxyuridine analogue **11** (which was easily obtained by tritylation of the commercially available 5-iodo-2′-deoxyuridine **10**, ESI†) and free 4-pentyn-1-ol as outlined in Scheme 2. After an uneventful acetylation–detritylation sequence, precursor **14** could be converted to the corresponding triphosphate **2** by application of the Ludwig and Eckstein conditions and was obtained in 14% yield after a thorough RP-HPLC purification step.[79]

The synthesis of the modified dC*TP analogue **3** proceeded in an analogous manner as highlighted in Scheme 3. Indeed, commercially available 5-iodo-2′-deoxycytidine **15** was converted into the ester functionalized nucleoside **16** in good yield (90%) using a Sonogashira cross-coupling reaction with 4-pentynoic acid methyl ester.[80] The free 5′-hydroxyl residue of



Scheme 2 Synthesis of modified $dU^{POH}TP$ **2**. Reagents and conditions: (i) DMTrCl, pyridine, rt, 12 h, 93%; (ii) 4-pentyn-1-ol, $Pd(PPh_3)_4$, CuI, NEt_3, DMF, rt, 12 h, 91%; (iii) Ac_2O, DMAP, NEt_3, pyridine, rt, 12 h, 86%; (iv) DCAA, CH_2Cl_2, rt, 40 min, 87%; (v) 1. 2-chloro-1,3,2-benzodioxaphosphorin-4-one, pyridine, dioxane, rt, 45 min; 2. $(n Bu_3NH)_2H_2P_2O_7$, DMF, $n Bu_3N$, rt, 45 min; 3. I_2, pyridine, H_2O, rt, 30 min; 4. NH_3(aq.), rt, 1.5 h, 14% (4 steps).



Scheme 3 Synthesis of triphosphate $dC^{Val}TP$ **3**. Reagents and conditions: (i) 4-pentynoic acid methyl ester, $Pd(PPh_3)_4$, CuI, NEt_3, DMF, rt, 12 h, 90%; (ii) DMTrCl, DMAP, NEt_3, pyridine, rt, 18 h, 45%; (iii) Ac_2O, DMAP, NEt_3, pyridine, 0 °C, 1 h, 61%; (iv) DCAA, CH_2Cl_2, rt, 40 min, 92%; (v) 1. 2-chloro-1,3,2-benzodioxaphosphorin-4-one, pyridine, dioxane, rt, 45 min; 2. $(n Bu_3NH)_2H_2P_2O_7$, DMF, $n Bu_3N$, rt, 45 min; 3. I_2, pyridine, H_2O, rt, 30 min; 4. NaOH, H_2O, 0 °C, 5 min, 9% (4 steps).

16 was then protected with a 4,4′-dimethoxytrityl group in moderate yields. The ensuing protected nucleoside **17** was then acetylated yielding a mixture of the desired 3′-*O*-monoacetylated **18** (61%) and diacetylated product S4 (ESI†). Unfortunately, all attempts at *N*-4-deacetylating S4 using $ZnBr_2$ [67] only led to detritylation (data not shown). Finally, following removal of the DMTr masking group under acidic conditions, intermediate **19** could be converted to the corresponding triphosphate **3** in 9% yield (4 steps), again by application of the Ludwig–Eckstein protocol.[79]

Primer extension reactions

An important prerequisite for modified dN*TPs to be applicable in selection experiments is that they serve as substrates for DNA polymerases in primer extension reactions.[71] Furthermore, not only do primer extension reactions constitute the first step of an *in vitro* selection experiment, but they represent the main tool for gauging the enzymatic recognition of modified dN*TPs in general. Consequently, I explored the substrate

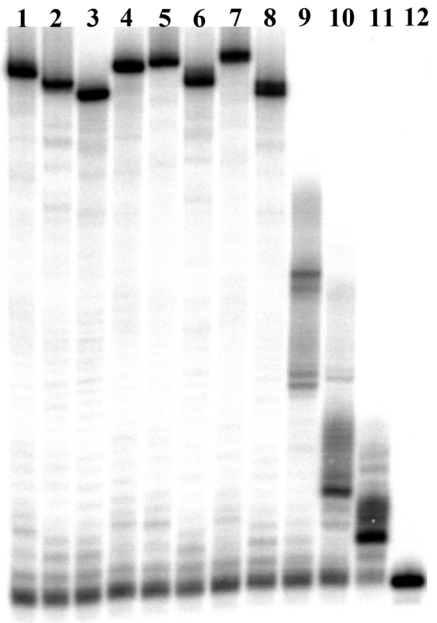


Fig. 2 Gel image (PAGE 15%) of primer extension reactions with primer **P1** and template **T1** using Vent (*exo*⁻) DNA polymerase. Lane 1: $dA^{Hs}TP$ **1**; lane 2: $dU^{POH}TP$ **2**; lane 3: $dC^{Val}TP$ **3**; lane 4: $dA^{Hs}TP$ **1** and $dC^{Val}TP$ **3**; lane 5: $dA^{Hs}TP$ **1** and $dU^{POH}TP$ **2**; lane 6: $dU^{POH}TP$ **2** and $dC^{Val}TP$ **3**; lane 7: $dA^{Hs}TP$ **1**, $dU^{POH}TP$ **2**, and $dC^{Val}TP$ **3**; lane 8: natural dNTPs; lane 9: natural dNTPs without dATP; lane 10: natural dNTPs without dTTP; lane 11: natural dNTPs without dCTP; lane 12: primer **P1**.

acceptance of the modified dN*TPs **1–3** with different DNA polymerases under primer extension reaction conditions (Fig. 2 and ESI†). Therefore, a variety of DNA polymerases (Vent (*exo*⁻), *Pwo*, 9°N_m, and the Klenow fragment of *E. coli* DNA polymerase I) were used in conjunction with the 98-mer long template **T1** and the 25-mer primer **P1** (see ESI† for the sequence compositions).[70,71] The modified dN*TPs were then investigated as to whether they could act as surrogates for their natural counterparts, either as lone modifications (lanes 1–3, Fig. 2), as combinations of two modified and two natural dNTPs (lanes 4–6, Fig. 2), or in conjunction with the lone natural dGTP (lane 7, Fig. 2). Vent (*exo*⁻) was revealed to be highly proficient at extending primer **P1** to full length since no faster running bands were apparent in all the combinations that were investigated. Moreover, 16 dA^{Hs} **1** and 21 dU^{POH} **2** that were incorporated in the nascent chain led to a significant and expected gel retardation as compared to the natural control sequence (compare lanes 1 and 2 with lane 8, Fig. 2). Surprisingly, the inclusion of 18 dC^{Val} **3** had little impact on the gel mobility of the resulting modified DNA (lane 3 *vs.* lane 8, Fig. 2).

Furthermore, besides Vent (*exo*⁻), all the other DNA polymerases that were tested in primer extension reactions showed very high substrate tolerance for all the dN*TPs and led to fully extended products (Fig. S1–S3, ESI†). Interestingly, the Klenow fragment, a prominent member of the family A polymerases, was as efficient at incorporating the modified dN*TPs as the more tolerant family B polymerases (Fig. S2, ESI†).[53,81,82]

TdT-mediated polymerization

The terminal deoxynucleotidyl transferase (TdT) is a Co^{2+}-dependent family X DNA polymerase that catalyzes the random and template-independent polymerization of nucleoside triphosphates at the 3′-OH termini of single-stranded oligonucleotides.[83,84] Recently, TdT-mediated reactions using modified dN*TPs have been employed for the generation of 3′-fluorescently labelled oligonucleotides,[85,86] to confer exonuclease stability to specific sequences,[87] and to synthesize ssDNAs containing stretches of nucleotides bearing redox active tags.[88] However, the tailing reaction was never used for the incorporation of nucleotides bearing side chains that potentially could confer catalytic activity to the ensuing modified ssDNA. Consequently, the modified dN*TPs **1–3** were assayed for their capacity to act as substrates in TdT-mediated tailing reactions. Thus, the 5′-^{32}P-labelled 15 nt long primer **P3** (ESI†) was incubated at 37 °C for 60 min in the presence of TdT and varying concentrations of the modified dN*TPs (Fig. 3).

The 7-substituted 7-deaza-deoxyadenosine derivative $dA^{Hs}TP$ **1** was revealed to be the best substrate for TdT, since the tailing efficiencies are better than those of its natural counterpart (compare lanes 1 and 4, Fig. 3). Indeed, at a concentration as low as 10 μM, mainly 7–15 additional dA^{Hs} nucleotides were incorporated along with some longer tailed sequences, while the natural dATP led to the appendage of a rather narrow 4–17 nucleotide distribution with little polydisperse longer-sized oligonucleotides. Furthermore, at a concentration of 50 μM several tens of both the dA^{Hs} and dA nucleotides were appended. Moreover, $dU^{POH}TP$ **2** (lanes 2 in Fig. 3) led to the best tailing efficiencies amongst the pyrimidine triphosphates, a tendency that is opposite to that observed with natural dNTPs (*vide infra*). Indeed, the TdT-mediated homopolymerization was rather ineffective at 10 μM since only 2–5 additional dU^{POH} units were appended, but at higher concentrations (>50 μM) only highly modified slow-running products could be observed. Unlike its natural counterpart, $dC^{Val}TP$ **3** stifles the TdT and only a few additional residues (~3 to 15 $dC^{Val}MPs$) are added, even at 200 μM (lanes 3 in Fig. 3).

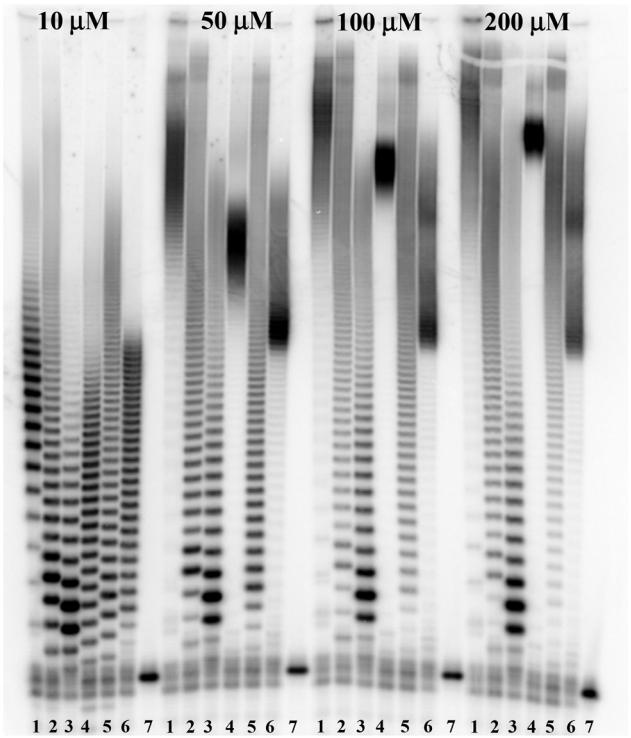


Fig. 3 Gel image (PAGE 15%) of the TdT-catalyzed extension reactions. Lane 1: $dA^{Hs}TP$ **1**; lane 2: $dU^{POH}TP$ **2**; lane 3: $dC^{Val}TP$ **3**; lane 4: dATP; lane 5: dTTP; lane 6: dCTP; lane 7: primer **P3**.

Polymerase chain reactions (PCR)

An additional and important part of the biochemical characterization of modified dN*TPs is the assessment of their substrate acceptance by DNA polymerases under PCR conditions. Consequently, the dN*TPs **1–3** were evaluated for their capacity to amplify the 98-mer template **T1**[70,71] flanked by a 20 nt long forward and a 25 nt reverse primer using four different DNA polymerases (Fig. 4 and ESI†).

Surprisingly, unlike what had been observed for primer extension reactions, under standard PCR conditions (2 mM Mg^{2+}, 200 μM dNTPs, 30 PCR cycles), only the Vent (*exo*$^-$) DNA polymerase was capable of faithfully amplifying the template, albeit with a slightly reduced efficiency as compared to the control experiment with natural dNTPs (lane 2 *versus* lane 8, Fig. 4). Indeed, the 9°N_m polymerase led to a marked smearing pattern (lane 3), the *Pwo* polymerase only partially accepted the dN*TPs under these conditions and yielded a mixture of a full length product and a shorter product (lane 4), and no amplicon could be observed in the case of the Klenow fragment (lane 5). However, when the dNTP concentration was raised from 200 to 500 μM, an efficient amplification could be observed with Vent (*exo*$^-$) (lane 6). Furthermore, additional Mg^{2+} (lane 7) had no positive impact on the amplification efficiency. Good amplifications were observed as well when only one of the natural dNTPs was substituted with a modified one (data not shown).

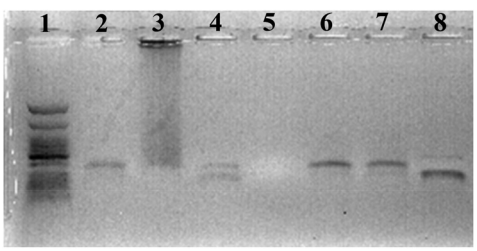


Fig. 4 Agarose gel (2%) stained with ethidium bromide, showing the PCR products with the 98-mer template **T1**, a dNTP mixture composed of $dA^{Hs}TP$ **1**, $dU^{POH}TP$ **2**, $dC^{Val}TP$ **3**, and natural dGTP and different enzymes and conditions. Lane 1: ladder; lane 2: Vent (*exo*$^-$); lane 3: 9°N_m polymerase; lane 4: *Pwo* polymerase; lane 5: Klenow fragment of *E. coli* DNA polymerase I; lane 6: Vent (*exo*$^-$) with 500 μM dN*TPs; lane 7: Vent (*exo*$^-$) with 500 μM dN*TPs and 7 mM Mg^{2+}; lane 8: natural dNTPs and Vent (*exo*$^-$).

In terms of *in vitro* selections, it is of primordial importance that single-stranded modified DNAs can be transformed into wild-type oligonucleotides that can serve as templates for subsequent rounds of selection.[30,44] Thus, in a different PCR experiment a 5′-phosphorylated 79-mer long template **T2** (ESI†) was amplified using the 19- and 20-mer primers **P4** and **P5** in the presence of Vent (*exo*$^-$) and 500 μM dNTPs. The resulting modified dsDNA was then λ-exonuclease digested, and used as a template for a subsequent PCR using natural dNTPs, the same set of primers, and Vent (*exo*$^-$) (Fig. S5, ESI†).[71] This experiment clearly underscores the compatibility of the modified dN*TPs **1–3** with methods of *in vitro* selection since a modified oligonucleotide could serve as a template to regenerate natural DNA (Fig. S5, ESI†).

Finally, in order to assess the fidelity of the PCR amplification with the modified dN*TPs **1–3**, a sequencing experiment based on the dideoxynucleoside triphosphate (ddNTP) method developed by Sanger *et al.* was carried out (Fig. S6 and S7, respectively, ESI†).[52,89] Briefly, the 5′-phosphorylated template **T2** and primer **P4** were used in a primer extension reaction with Vent (*exo*$^-$). The unmodified template was then λ-exonuclease digested, and the resulting modified ssDNA was used as a template for PCR with each one of the 5′-biotinylated primers **P4B** and **P5B**. Following immobilization of the duplexes on streptavidin coated magnetic beads, the unbiotinylated oligonucleotides were eluted with hydroxide washes and then sequenced. Comparison of the sequencing patterns of both the forward and reverse directions (Fig. S6 and S7, ESI†) clearly reveals that no loss of sequence information occurs upon converting a heavily modified ssDNA into its complementary natural sequence, and that the fidelity of PCR with the modified dN*TPs is high.

Conclusions

Three modified dN*TPs adorned with side-chains reminiscent of the Ser, His, and Asp residues found in the active site of serine proteases could easily be accessed in 5 or 6 steps starting from known and/or commercially available precursors. The functionalities were anchored on the C5 of pyrimidines and the *N*-7 of 7-deaza-adenosine in order to minimize the destabilization of duplexes and disruption of Watson–Crick base pairing, and concomitantly, to maximize the substrate acceptance. All of the modified dN*TPs were found to be excellent substrates for all four DNA polymerases, including the Klenow fragment of DNA polymerase I, in primer extension reactions. It is noteworthy that the polymerases readily accept and tolerate the presence of the negative charge of the carboxylic acid of dC^{Val}TP **3** and allow for the incorporation of up to 3 consecutive dC^{Val} residues.

Moreover, the substrate acceptance for TdT is heavily dependent on the nature and position of the substituent and followed the order dA^{Hs}TP > dU^{POH}TP > dC^{Val}TP. This trend is opposite to what is observed for natural dNTPs, since TdT utilizes dGTP and dCTP much more efficiently than dTTP and more markedly, dATP.[90] The additional negative charge on dC^{Val}TP might account for its limited substrate acceptance by TdT.

Finally, these modified dN*TPs were shown to be good substrates for the Vent (*exo*$^-$) DNA polymerase under PCR conditions. However, unlike what was observed for primer extension reactions, only optimized conditions and the sole Vent (*exo*$^-$) DNA polymerase led to clean and robust amplifications. The resulting modified oligonucleotides could serve as a template to regenerate natural DNA, which is an important prerequisite for the use of these dN*TPs in selection experiments. Moreover, PCR amplification using the modified dN*TPs and the subsequent conversion into natural DNA was shown to proceed with high fidelity.

Thus, these three nucleoside triphosphate analogues, equipped with functionalities that are reminiscent of the residues found in the active site of serine proteases, could be shown to be fully compatible with methods of *in vitro* selection. Consequently, their simultaneous polymerization will considerably increase the chemical landscape that can be covered in the course of a selection experiment for the generation of DNAzymes that can promote the catalytic scission of amide bond linkages, which is a long-standing goal in the field of nucleic acid enzymes. Such an artificial peptidase would be an invaluable synthetic and biochemical tool, and selection for such an enzyme mimic is currently under way.

Experimental part

General method for the Sonogashira coupling reactions

The nucleoside was dissolved in dry DMF (5 mL mmol^{-1}). CuI (0.1 eq.) and NEt_3 (2 eq.) were then added and the reaction mixture was stirred for 10 min at room temperature. The alkyne (4 eq.) and $Pd(PPh_3)_4$ (0.05 eq.) were then added in turn. The reaction mixture was cooled down to −78 °C and thoroughly degassed. After stirring at room temperature overnight, the DMF was removed under reduced pressure. The blackened crude product was then dissolved in CH_2Cl_2 (50 mL) and washed with brine (50 mL). Following drying ($MgSO_4$) and removal of the solvent *in vacuo*, the residue was purified by flash column chromatography.

General method for detritylation

The nucleoside analogue was dissolved in dry CH_2Cl_2 (10 mL) and dichloroacetic acid (0.1%, 0.1 mL) was added. The orange-coloured reaction mixture was stirred at room temperature for 40 min. MeOH (3 mL) was added and the solution was stirred for another 5 min at room temperature. Following removal of the solvent under reduced pressure, the residue was purified by flash column chromatography.

Synthesis of 7-[3-(trifluoroacetamido)-propynyl]-5′-*O*-(4,4′-dimethoxytrityl)-7-deaza-2′-deoxyadenosine (5). The nucleoside analogue **4** (0.35 g, 0.9 mmol) was co-evaporated twice with dry pyridine (5 mL). After dissolving **4** in dry pyridine (5 mL), DMTrCl (0.36 g, 1.1 mmol) was added and the resulting

solution was stirred at rt for 15 h. After completion of the reaction, 5 mL of MeOH were added and the mixture was stirred at rt for 5 min. After removal of the solvent under reduced pressure, the residue was purified by flash column chromatography (CH_2Cl_2–MeOH 95 : 5), yielding **5** as a pale yellow foam (0.46 g, 75%). R_f: 0.46 (CH_2Cl_2–MeOH 9 : 1 +1% NEt_3). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.41–2.52 (m, 2H), 3.37 (d, 2H, J = 4.4 Hz), 3.76 (s, 6H), 4.05 (q, 1H, J = 4.0 Hz), 4.35–4.39 (m, 2H), 4.58 (q, 1H, J = 4.9 Hz), 5.64 (br s, 2H), 6.62 (t, 1H, J = 6.2 Hz), 6.81 (dd, 4H, J = 2.0, 8.8 Hz), 7.20–7.32 (m, 7H), 7.39–7.46 (m, 3H), 8.25 (s, 1H). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 30.9, 41.2, 53.6, 63.7, 72.1, 78.3, 83.6, 85.3, 85.7, 86.9, 94.9, 103.6, 113.6, 126.7, 127.2, 128.2, 130.3, 136.2, 136.3, 144.4, 149.8, 153.3, 157.5, 158.7. ^{19}F-NMR (376.5 MHz, $CDCl_3$): δ −75.67. HR MS: m/z: calcd for $C_{37}H_{35}O_6N_5F_3$ ($[M + H]^+$): 702.2534, found: 702.2524.

Synthesis of 7-[3-amino-propynyl]-5′-*O*-(4,4′-dimethoxytrityl)-7-deaza-2′-deoxyadenosine (6). Nucleoside **5** (0.95 g, 1.4 mmol) was dissolved in MeOH (8 mL). A solution of NaOH (0.16 g, 4.1 mmol) in H_2O (0.78 mL) was then added and the resulting mixture was stirred at rt for 12 h. The solvent was removed under reduced pressure and the residue dissolved in 50 mL of CH_2Cl_2. After washing with brine (1 × 50 mL) and extracting the aqueous phase with CH_2Cl_2 (2 × 50 mL), the combined organic layers were dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by flash column chromatography (CH_2Cl_2–MeOH 9 : 1) yielded **6** as a white foam (0.69 g, 84%). R_f: 0.33 (CH_2Cl_2–MeOH 9 : 1). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.29–2.51 (m, 2H), 3.30–3.40 (m, 2H), 3.64 (s, 2H), 3.77 (s, 6H), 4.03 (q, 1H, J = 3.1 Hz), 4.56 (q, 1H, J = 4.9 Hz), 5.57 (br s, 2H), 6.62 (t, 1H, J = 6.4 Hz), 6.81 (d, 4H, J = 8.7 Hz), 7.17–7.32 (m, 8H), 7.38–7.43 (m, 2H), 8.22 (s, 1H). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 32.6, 40.9, 55.5, 64.0, 72.6, 76.0, 83.5, 85.5, 86.8, 92.7, 96.2, 103.7, 113.5, 125.6, 127.1, 128.1, 128.4, 130.2, 130.3, 136.0, 144.8, 149.9, 153.2, 157.5, 158.8. HR MS: m/z: calcd for $C_{35}H_{36}O_5N_5$ ($[M + H]^+$): 606.2711, found: 606.2704.

Synthesis of 7-[*N*-4-(methoxytrityl)-1*H*-imidazol-5-yl-ethylamino-3-(carbamido)-propynyl]-5′-*O*-(4,4′-dimethoxytrityl)-7-deaza-2′-deoxyadenosine (7). Analogue **6** (0.35 g, 0.58 mmol) was dissolved in dry DMF (10 mL). *N*-Methylmorpholine (0.13 mL, 1.2 mmol) and derivative **S2** (0.34 g, 0.69 mmol) were then added and the resulting solution was cooled down to 0 °C. At this stage, HOBt (0.086 g, 0.64 mmol) and EDC·HCl (0.12 g, 0.64 mmol) were added in turn. After allowing the reaction mixture to warm up to room temperature, the solution was stirred at rt for 12 h. The solvent was removed under reduced pressure and the crude mixture was purified by flash column chromatography by eluting with a gradual gradient of CH_2Cl_2–MeOH from 95 : 5 to 9 : 1. The histamine modified derivative **7** was obtained as a white foam (0.40 g, 64%). R_f: 0.55 (CH_2Cl_2–MeOH 9 : 1). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.39–2.49 (m, 7H), 2.64 (t, 2H, J = 6.4 Hz), 3.30–3.36 (m, 2H), 3.45 (q, 2H, J = 6.2 Hz), 3.75 (s, 6H), 3.77 (s, 3H), 4.03 (q, 1H, J = 3.7 Hz), 4.12 (d, 1H, J = 5.2 Hz), 5.55 (br s, 2H), 6.55 (d, 1H, J = 0.8 Hz), 6.59 (t, 1H, J = 6.4 Hz), 6.77–6.83 (m, 7H), 7.00–7.02 (m, 3H), 7.07–7.13 (m, 2H), 7.18–7.32 (m, 12H), 7.35–7.40 (m, 3H), 8.18 (s, 1H). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 27.6, 30.5, 31.9, 39.8, 40.9, 55.5, 64.0, 72.3, 83.4, 85.5, 86.8, 88.1, 95.9, 103.8, 113.5, 118.8, 125.2, 128.1, 128.2, 128.4, 129.8, 130.3, 131.3, 134.6, 136.1, 138.8, 139.2, 142.9, 144.8, 149.9, 153.3, 157.7, 158.7, 159.3, 172.1, 172.9. HR MS: m/z: calcd for $C_{64}H_{63}O_8N_8$ ($[M + H]^+$): 1071.4763, found: 1071.4750.

Synthesis of 7-[*N*-4-(methoxytrityl)-1*H*-imidazol-5-yl-ethylamino-3-(carbamido)-propynyl]-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-acetyl-7-deaza-2′-deoxyadenosine (8). Compound **7** (0.39 g, 0.36 mmol) was dissolved in dry pyridine (3 mL) and the resulting solution was cooled down to 0 °C. DMAP (0.011 g, 0.09 mmol), NEt_3 (0.1 mL, 0.73 mmol), and acetic anhydride (0.09 mL, 0.91 mmol) were then added in turn. The resulting reaction mixture was then stirred at 0 °C for 2.5 h, at which stage MeOH (5 mL) was added and the mixture stirred for an additional 5 min. The solvent was then removed under reduced pressure and the residue was purified by flash column chromatography (CH_2Cl_2–MeOH 96 : 4). The fully protected analogue **8** was obtained as a white foam (0.35 g, 86%). R_f: 0.63 (CH_2Cl_2–MeOH 95 : 5 +1% NEt_3). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.06 (s, 3H), 2.45–2.53 (m, 5H), 2.56–2.62 (m, 1H), 2.65 (t, 2H, J = 6.0 Hz), 3.35 (t, 2H, J = 3.2 Hz), 3.46 (q, 2H, J = 6.0 Hz), 3.76 (s, 6H), 3.78 (s, 3H), 4.12 (d, 2H, J = 5.6 Hz), 4.16 (q, 1H, J = 3.2 Hz), 5.37–5.40 (m, 1H), 5.87 (br s, 2H), 6.55 (d, 1H, J = 1.2 Hz), 6.62 (q, 1H, J = 4.7 Hz), 6.78–6.83 (m, 6H), 7.01 (d, 2H, J = 8.8 Hz), 7.07–7.11 (m, 4H), 7.17–7.22 (m, 2H), 7.23–7.26 (m, 3H), 7.27–7.31 (m, 10H), 7.37–7.40 (m, 2H), 8.18 (s, 1H). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 21.3, 27.6, 30.5, 31.9, 38.4, 39.8, 55.5, 64.0, 75.3, 83.4, 83.7, 96.3, 103.8, 113.5, 118.8, 124.9, 127.14, 128.1, 128.2, 128.3, 128.5, 129.8, 130.3, 130.4, 131.3, 134.6, 135.9, 136.0, 138.9, 139.3, 142.9, 144.7, 150.2, 153.5, 157.7, 158.8, 159.3, 170.6, 172.1, 172.9. HR MS: m/z: calcd for $C_{66}H_{65}O_9N_8$ ($[M + H]^+$): 1113.4869, found: 1113.4872.

Synthesis of 7-[1*H*-imidazol-5-yl-ethylamino-3-(carbamido)-propynyl]-3′-*O*-acetyl-7-deaza-2′-deoxyadenosine (9). The reaction was carried out with **8** (0.34 g, 0.3 mmol) by application of the general detritylation method. The residue was purified by flash column chromatography on silica gel by eluting with CH_2Cl_2–MeOH 9 : 1 to give **9** as a white foam (0.17 g, quant.). R_f: 0.16 (CH_2Cl_2–MeOH 9 : 1). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.09 (s, 3H), 2.30–2.38 (m, 5H), 2.45–2.50 (m, 2H), 2.60 (t, 2H, J = 7.4 Hz), 2.65–2.72 (m, 1H), 3.23 (q, 2H, J = 6.8 Hz), 3.60 (d, 2H, J = 1.6 Hz), 4.02 (q, 1H, J = 1.9 Hz), 4.09 (d, 2H, J = 5.2 Hz), 5.31 (d, 1H, J = 6.0 Hz), 6.46 (dd, 1H, J = 5.6, 9.2 Hz), 6.75 (br s, 1H), 7.50 (s, 1H), 7.69 (s, 1H), 7.88 (t, 1H, J = 3.6 Hz), 8.10 (s, 1H), 8.44 (t, 1H, J = 5.2 Hz). ^{13}C-NMR (101 MHz, DMSO-d_6): δ 20.9, 29.2, 30.5, 30.6, 36.9, 54.9, 61.7, 74.8, 75.1, 83.3, 84.8, 89.3, 95.0, 102.3, 125.7, 134.5, 149.3, 152.7, 157.5, 170.0, 171.0, 171.6. HR MS: m/z: calcd for $C_{25}H_{31}O_6N_8$ ($[M + H]^+$): 539.2361, found: 539.2375.

Synthesis of 5-[5-hydroxy-pentynyl]-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyuridine (12). The reaction was carried out with **11** (0.6 g, 0.9 mmol) by application of the general method for Sonogashira coupling reactions. However, slightly larger quantities of CuI (0.4 eq.) and $Pd(PPh_3)_4$ (0.2 eq.) were used. The crude product was purified by flash column chromatography on silica gel (gradual gradient from CH_2Cl_2–MeOH 100 : 0 +1%

NEt_3 to 95 : 5) to give **12** as a white foam (0.51 g, 91%). R_f: 0.32 (CH_2Cl_2–MeOH 95 : 5 +1% NEt_3). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.43–1.49 (m, 2H), 2.19 (t, 2H, J = 6.8 Hz), 2.23–2.28 (m, 1H), 2.48 (ddd, 1H, J = 2.8, 5.6, 13.6 Hz), 3.28 (dd, 1H, J = 3.4, 10.6 Hz), 3.40 (dd, 1H, J = 3.0, 10.6 Hz), 3.47 (t, 2H, J = 6.0 Hz), 3.77 (s, 6H), 4.06 (q, 1H, J = 2.9 Hz), 4.49–4.53 (m, 1H), 6.31 (dd, 1H, J = 5.8, 7.4 Hz), 6.82 (d, 4H, J = 9.2 Hz), 7.24–7.33 (m, 7H), 7.41 (d, 2H, J = 7.2 Hz), 8.00 (s, 1H). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 16.3, 30.9, 41.7, 55.5, 61.5, 63.7, 71.5, 72.5, 85.8, 86.7, 87.2, 95.0, 101.1, 113.6, 127.2, 128.2, 128.3, 130.1, 130.2, 135.8, 135.9, 141.7, 144.7, 149.4, 158.8, 162.2. HR MS: m/z: calcd for $C_{35}H_{37}O_8N_2$ ($[M + H]^+$): 613.2544, found: 613.2537.

Synthesis of 5-[5-acetoxy-pentynyl]-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-acetyl-2′-deoxyuridine (13). Compound **12** (0.3 g, 0.49 mmol) was dissolved in pyridine (3 mL) and the solution cooled down to 0 °C. NEt_3 (0.31 mL, 2.2 mmol), acetic anhydride (0.12 mL, 1.2 mmol), and DMAP (0.015 g, 0.12 mmol) were then added in turn and the reaction mixture was stirred at rt for 5 h. MeOH (3 mL) was then added and after an additional 5 min of stirring, the solvent was removed under reduced pressure and the residue purified by flash column chromatography on silica (EtOAc–hexanes 1 : 1). The fully protected compound **13** was obtained as a white foam (0.30 g, 86%). R_f: 0.34 (CH_2Cl_2–MeOH 95 : 5 +1% NEt_3). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.34–1.41 (m, 2H), 1.91 (s. 3H), 1.99 (s, 3H), 2.06 (t, 2H, J = 7.0 Hz), 2.25–2.33 (m, 1H), 2.43–2.48 (m, 1H), 3.28–3.37 (m, 2H), 3.70 (s, 6H), 3.81 (t, 2H, J = 6.4 Hz), 4.05 (d, 1H, J = 1.6 Hz), 5.30 (d, 1H, J = 6.0 Hz), 6.25 (dd, 1H, J = 5.6, 8.8 Hz), 6.75 (d, 4H, J = 8.8 Hz), 7.11–7.27 (m, 7H), 7.32–7.36 (m, 2H), 7.98 (s, 1H). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 16.5, 21.1, 21.2, 27.6, 29.9, 38.8, 55.4, 63.3, 63.8, 71.3, 75.2, 84.7, 85.3, 87.5, 94.1, 101.4, 113.6, 127.2, 128.1, 128.3, 130.2, 135.6, 135.7, 136.2, 141.7, 144.6, 149.4, 149.7, 158.9, 161.7, 170.5, 171.1. HR MS: m/z: calcd for $C_{39}H_{41}O_{10}N_2$ ($[M + H]^+$): 697.2756, found: 697.2763.

Synthesis of 5-[5-acetoxy-pentynyl]-3′-*O*-acetyl-2′-deoxyuridine (14). The reaction was carried out with **13** (0.43 g, 0.6 mmol) by application of the general detritylation method. The residue was purified by flash column chromatography on silica gel by eluting with CH_2Cl_2–MeOH 9 : 1 to give **9** as a pale yellow solid (0.21 g, 87%). R_f: 0.16 (CH_2Cl_2–MeOH 9 : 1). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 1.76–1.83 (m, 2H), 2.01 (s, 3H), 2.06 (s, 3H), 2.25–2.32 (m, 2H), 2.45 (t, 2H, J = 7.2 Hz), 3.63 (d, 2H, J = 2.8 Hz), 4.01 (q, 1H, J = 2.8 Hz), 4.09 (t, 2H, J = 6.4 Hz), 5.20–5.22 (m, 1H), 6.14 (apparent t, 1H, J = 7.2 Hz), 8.13 (s, 1H), 11.72 (s, 1H). ^{13}C-NMR (101 MHz, DMSO-d_6): δ 15.6, 20.7, 20.8, 27.3, 37.1, 61.1, 62.7, 63.4, 73.1, 74.6, 84.4, 84.9, 92.3, 99.2, 142.6, 149.5, 161.6, 165.5, 170.0, 170.4. HR MS: m/z: calcd for $C_{18}H_{23}O_8N_2$ ($[M + H]^+$): 395.1449, found: 395.1441.

Synthesis of 5-[5-(pent-1-ynylic acid methyl ester)]-2′-deoxycytidine (16). Nucleoside analogue **16** (pale yellow solid, 0.52 g, 90%) was obtained by application of the general method for Sonogashira coupling reactions starting from **15** (0.6 g, 1.7 mmol) and after purification by flash column chromatography on silica gel (gradient from CH_2Cl_2–MeOH 96 : 4 to 9 : 1). R_f: 0.20 (CH_2Cl_2–MeOH 9 : 1). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 1.93–2.00 (m, 1H), 2.10–2.16 (m, 1H), 2.60–2.67 (m, 4H), 3.52–3.61 (m, 2H), 3.62 (s, 3H), 3.78 (q, 1H. J = 3.3 Hz), 4.17–4.22 (m, 1H), 5.04 (t, 1H, J = 5.2 Hz), 5.18 (d, 1H, J = 4.0 Hz), 6.10 (t, 1H, J = 6.6 Hz), 6.68 (br s, 1H), 7.71 (br s, 1H), 8.06 (s, 1H). ^{13}C-NMR (101 MHz, DMSO-d_6): δ 15.0, 32.4, 45.7, 51.9, 61.0, 70.2, 72.5, 85.3, 87.4, 90.0, 94.3, 143.5, 153.5, 164.4, 172.2. HR MS: m/z: calcd for $C_{15}H_{20}O_6N_3$ ($[M + H]^+$): 338.1347, found: 338.1344.

Synthesis of 5-[5-(pent-1-ynylic acid methyl ester)]-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine (17). Derivative **16** (0.27 g, 0.81 mmol) was dissolved in pyridine (4 mL). DMTrCl (0.33 g, 1 mmol), NEt_3 (0.22 mL, 1.6 mmol), and DMAP (0.024 g, 0.2 mmol) were then added to the solution and stirred for 18 h at rt. MeOH (2 mL) was added and the yellow solution was stirred for an additional 5 min. After removal of the solvent under reduced pressure, the residue was dissolved in CH_2Cl_2 (50 mL) and washed with $NaHCO_3$ (satd., 1 × 50 mL). The aqueous phase was extracted with CH_2Cl_2 (2 × 50 mL) and the combined organic layers were dried ($MgSO_4$). After removal of the solvent *in vacuo*, the residue was purified by flash column chromatography on silica gel by eluting with CH_2Cl_2–MeOH 96 : 4 to give **17** as a pale yellow solid (0.23 g, 45%). R_f: 0.45 (CH_2Cl_2–MeOH 9 : 1 +1% NEt_3). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.08–2.15 (m, 1H), 2.25–2.31 (m, 2H), 2.33–2.39 (m, 2H), 2.63–2.68 (m, 1H), 3.20–3.28 (m, 2H), 3.58 (s, 3H), 3.69 (s, 6H), 4.06 (q, 1H, J = 3.3 Hz), 4.40–4.43 (m, 1H), 5.89 (br s, 1H), 6.24 (t, 1H, J = 6.4 Hz), 6.42 (br s, 1H), 6.73 (dd, 4H, J = 1.2, 8.8 Hz), 7.09–7.20 (m, 3H), 7.23–7.27 (m, 4H), 7.35 (d, 2H, J = 7.2 Hz), 8.01 (s, 1H). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 15.5, 33.0, 46.2, 52.1, 55.4, 63.8, 72.3, 72.5, 86.6, 87.0, 87.1, 91.4, 94.5, 113.5, 127.1, 128.1, 128.2, 130.1, 130.2, 135.9, 136.0, 143.4, 144.8, 154.9, 158.8, 165.2, 172.7. HR MS: m/z: calcd for $C_{36}H_{38}O_8N_3$ ($[M + H]^+$): 640.2653, found: 640.2647.

Synthesis of 5-[5-(pent-1-ynylic acid methyl ester)]-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-acetyl-2′-deoxycytidine (18). The DMTr-protected nucleoside **17** (0.35 g, 0.55 mmol) was dissolved in dry pyridine (3 mL) and the solution was cooled down to 0 °C. DMAP (0.017 g, 0.14 mmol) and NEt_3 (0.15 mL, 1.1 mmol) were then added. Acetic anhydride (0.13 mL, 1.4 mmol) was then added dropwise to the solution. The reaction was stirred at 0 °C. TLC analysis (CH_2Cl_2–MeOH 95 : 5 +1% NEt_3, R_f (starting material): 0.38; R_f (**18**): 0.5; R_f (**S2**): 0.86) revealed the complete disappearance of the starting material. MeOH (3 mL) was then added and the solution was stirred for an additional 5 min at 0 °C. The solvents were removed under reduced pressure and the crude product was purified by flash column chromatography on silica gel (gradient from CH_2Cl_2–MeOH 98 : 2 to 95 : 5), yielding **18** (0.23 g, 61%) and **S4** (0.12 g, 30%) both as white foams. Characterization for **18**: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.04 (s, 3H), 2.20–2.28 (m, 1H), 2.35–2.40 (m, 2H), 2.43–2.48 (m, 2H), 2.69 (ddd, 1H, J = 1.5, 5.5, 14.1 Hz), 3.06–3.12 (m, 1H), 3.31–3.39 (m, 2H), 3.66 (s, 3H), 3.77 (s, 6H), 4.16 (q, 1H, J = 2.5 Hz), 5.34 (apparent d, 1H, J = 6.0 Hz), 6.10 (br s, 1H), 6.20 (br s, 1H), 6.31 (dd, 1H, J = 5.4, 8.2 Hz),

6.81 (dd, 4H, J = 1.8, 9.0 Hz), 7.16–7.33 (m, 8H), 7.40 (apparent d, 1H, J = 7.2 Hz), 8.07 (s, 1H). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 15.6, 21.2, 33.0, 39.7, 52.2, 55.5, 63.8, 72.2, 75.4, 84.6, 86.8, 87.3, 91.6, 94.8, 113.5, 127.4, 128.1, 128.2, 130.1, 130.2, 135.7, 135.8, 143.2, 144.7, 154.3, 158.9, 164.9, 170.6, 172.7. HR MS: m/z: calcd for $C_{38}H_{40}O_9N_3$ ($[M + H]^+$): 682.2759, found: 682.2750.

Synthesis of 5-[5-(pent-1-ynylic acid methyl ester)]-3′-*O*-acetyl-2′-deoxycytidine (19). The reaction was carried out with **18** (0.22 g, 0.3 mmol) by application of the general detritylation method. The residue was purified by flash column chromatography on silica gel (gradient from CH_2Cl_2–MeOH 100 : 0 to 94 : 6), to give **9** as a white solid (0.11 g, 92%). R_f: 0.31 (CH_2Cl_2–MeOH 95 : 5 +% NEt_3). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.06 (s, 3H), 2.11–2.19 (m, 1H), 2.25–2.32 (m, 1H), 2.63–2.67 (m, 4H), 3.56–3.60 (m, 1H), 3.64 (s, 3H), 4.01 (q, 1H, J = 2.8 Hz), 5.19 (apparent d, 2H, J = 6.0 Hz), 6.15 (dd, 1H, J = 5.8, 7.8 Hz), 6.83 (br s, 1H), 7.73 (br s, 1H), 8.06 (s, 1H). ^{13}C-NMR (101 MHz, DMSO-d_6): δ 15.0, 20.8, 32.4, 37.8, 51.5, 61.2, 72.3, 74.7, 84.9, 85.2, 90.5, 94.5, 143.3, 153.5, 164.4, 170.0, 172.2. HR MS: m/z: calcd for $C_{17}H_{22}O_7N_3$ ($[M + H]^+$): 380.1452, found: 380.1443.

Triphosphorylation of nucleosides

General method for the triphosphorylation of nucleosides. The nucleoside was co-evaporated twice with dry pyridine (1 mL) and then dried under vacuum overnight. After dissolving the nucleoside in dry pyridine (0.2 mL), dry dioxane (0.4 mL) was added, followed by the addition of 2-chloro-1,3,2-benzodioxaphosphorin-4-one (1.1 eq.). The reaction mixture was stirred at room temperature for 45 min. A solution of tributylammonium pyrophosphate (1.3 eq.) in dry DMF (170 μL) and tributylamine (58 μL) was then added and the resulting solution was stirred at room temperature for 45 min. Iodine (1.6 eq.) in a mixture of pyridine (980 μL) and water (20 μL) was then added and the resulting dark solution was stirred at room temperature for 30 min. Excess iodine was then quenched by addition of a solution of sodium thiosulfate (10%) and the solvents were removed under reduced pressure (water bath ≤30 °C). Water (5 mL) was then added and the mixture was allowed to stand at room temperature for 30 min. Following removal of the various protecting groups (see below) and evaporation of the solvent, the crude triphosphates were precipitated with $NaClO_4$ in acetone (2%) and then purified by RP-HPLC. The appropriate fractions were freeze-dried and co-evaporated several times with water.

HPLC of triphosphates

The purification of the various triphosphates was carried out on a Phenomenex Jupiter semi-preparative RP-HPLC column (see ESI†) at a flow rate of 3.5 mL min^{-1}, using a triethylammonium bicarbonate (TEAB) buffer system (eluent I: 50 mM TEAB, pH 7.7; eluent II: 50 mM TEAB in CH_3CN–H_2O 1 : 1), and one of the programs highlighted in Table 1.

Table 1 HPLC programs used for the purification of the dNTPs

Time (min)	Program A (% II)	Program B (% II)	Time (min)
0	0	0	0
8	0	0	8
50	50	17	31
52	100	80	33
54	100	80	35
56	0	0	37

Modified dATP 1 (dA^{Hs}TP). This modified triphosphate was obtained by application of the general triphosphorylation procedure starting from **9** (35 mg, 0.065 mmol). Deprotection was carried out by incubation in aqueous NH_3 (10 mL) at rt for 1.5 h. RP-HPLC purification (program A, R_t = 26.9 min) yielded **1** as its triethylammonium salt (pale yellow solid, 12 mg, 17%). ^{1}H-NMR (400 MHz, D_2O): δ 1.31 (t, 27 H, J = 7.2 Hz), 2.43–2.52 (m, 1H), 2.56–2.67 (m, 5H), 2.72 (t, 2H, J = 7.0 Hz), 3.00–3.05 (m, 1H), 3.06–3.13 (m, 2H), 3.22 (q, 18H, J = 7.3 Hz), 3.29–3.56 (m, 2H), 3.52 (q, 1H, J = 7.2 Hz), 4.21 (d, 2H, J = 4.0 Hz), 4.26–4.32 (m, 2H), 6.52 (t, 1H, J = 6.8 Hz), 7.02 (s, 1H), 7.74 (s, 1H), 8.50 (s, 1H). ^{13}P-NMR (121.4 MHz, D_2O): δ −5.18 (d, 1P, J = 18.5 Hz, P_γ), −5.56 (d, 1P, J = 19.4 Hz, P_α), −20.32 (t, 1P, J = 17.3 Hz, P_β). MS (MALDI$^-$): m/z calcd for $C_{23}H_{30}N_8O_{14}P_3^-$: 735.11 $[M - H]^-$; found: 734.84; UV: λ_{max} = 282, 240 nm.

Modified dUTP 2 (dU^{POH}TP). Triphosphate **2** was synthesized by application of the general triphosphorylation procedure starting from **14** (40 mg, 0.101 mmol). Deprotection was carried out by incubation in aqueous NH_3 (10 mL) at rt for 1.5 h. RP-HPLC purification (program A, R_t = 24.7 min) yielded **1** as its triethylammonium salt (white solid, 12 mg, 14%). ^{1}H-NMR (300 MHz, D_2O): δ 1.28 (t, 27H, J = 7.5 Hz), 1.82 (t, 2H, J = 6.6 Hz), 2.34–2.41 (m, 2H), 2.51 (t, 2H, J = 7.1 Hz), 2.96–3.11 (m, 2H), 3.20 (q, 18H, J = 7.3 Hz), 3.73 (t, 2H, J = 6.3 Hz), 4.14 (m, 3H), 4.59–4.68 (m,1H), 6.29 (t, 1H, J = 6.6 Hz), 8.05 (s, 1H). ^{13}P-NMR (121.4 MHz, D_2O): δ −10.27 (d, 1P, J = 23.2 Hz, P_γ), −11.34 (d, 1P, J = 20.5 Hz, P_α), −23.11 (t, 1P, J = 18.0 Hz, P_β). MS (MALDI$^-$): m/z calcd for $C_{14}H_{20}N_2O_{15}P_3^-$: 549.01 $[M - H]^-$; found: 548.76; UV: λ_{max} = 233, 292 nm.

Modified dCTP 3 (dC^{Val}TP). Analogue **3** was obtained by application of the general triphosphorylation procedure starting from **19** (45 mg, 0.119 mmol). Deprotection: the crude reaction mixture was dissolved in H_2O (1.69 mL). After cooling to 0 °C, NaOH (380 μL, 2.5 M; 0.46 M final concentration) was added and the reaction stirred at 0 °C for 5 min. The mixture was then neutralized by adding NaH_2PO_4 buffer (925 μL; 1 M, pH 1.9) and further diluted by addition of H_2O (3 mL). The solvent was then removed *in vacuo* and the residue purified by RP-HPLC using program B (R_t = 21.8 min). dC^{Val}TP **3** was obtained as its triethylammonium salt (white solid, 10 mg, 9%). ^{1}H-NMR (300 MHz, D_2O): δ 1.28 (t, 27H, J = 7.4 Hz), 2.25–2.35 (m, 1H), 2.38–2.45 (m, 1H), 2.49 (t, 2H, J = 6.9 Hz), 2.68 (t, 2H, J = 6.9 Hz), 2.95–3.01 (m, 1H), 3.03–3.11 (m, 2H), 3.20 (q, 18H, J = 7.3 Hz), 3.39 (q, 2H, J = 7.3 Hz), 4.20 (d, 2H, J = 4.5 Hz), 4.59 (t, 1H, J = 2.7 Hz), 6.27 (t, 1H, J = 6.8 Hz), 8.03 (s, 1H). ^{13}P-NMR (121.4 MHz, D_2O): δ −10.71 (d, 1P,

J = 14.6 Hz, P_γ), −11.27 (d, 1P, J = 19.3 Hz, P_α), −23.18 (d, 1P, J = 20.6 Hz, P_β). MS (MALDI$^-$): m/z calcd for $C_{14}H_{19}N_3O_{15}P_3^-$: 562.00 [M − H]$^-$; found: 562.16; UV: λ_{max} = 209, 235, 293 nm.

Primer extension experiments. The 5′-^{32}P-labelled primer **P1** (*ca.* 1 pmol) and 10 pmol of primer **P1** were annealed to appropriate template **T1** (10 pmol) in the presence of 10× enzyme buffer (provided by the supplier of the DNA polymerase) by heating to 95 °C and then gradually cooling to room temperature (over 30 min). The appropriate DNA polymerase (1 U) was then added to the annealed oligonucleotides mixture at 4 °C. Finally, dNTPs (final concentration of 100 μM) were added for a total reaction volume of 20 μL. Following incubation at the optimal temperature for the enzyme, the reactions were quenched by adding stop solution (20 μL; formamide (70%), ethylenediaminetetraacetic acid (EDTA; 50 mM), bromophenol (0.1%), xylene cyanol (0.1%)). The reaction mixtures were subjected to gel electrophoresis in denaturing polyacrylamide gel (15%) containing trisborate-EDTA (TBE) 1× buffer (pH 8) and urea (7 M). Visualization was performed by phosphorimaging.

Standard conditions for TdT-mediated insertion of modified triphosphates into a DNA primer. A solution containing 7 pmol of primer **P3** (ESI†), ∼2 pmol of 5′-^{32}P-radio-labelled **P3**, and 4 U of TdT was added to a mixture composed of the appropriate concentration of dNTPs (10, 50, 100 or 200 μM final), reaction buffer, and H_2O (for a total reaction volume of 10 μL). The reaction mixtures were then incubated at 37 °C for 60 min and quenched by addition of 10 μL of loading buffer. The reaction products were then resolved by electrophoresis (PAGE 15%) and visualized by phosphorimager analysis.

Polymerase chain reactions. The PCR reaction mixtures (20 μL total) contained both primers (400 nM each; **P1** and **P2**, see the ESI† for the sequences), template **T1** (25 nM), dNTPs (200 μM; both natural and modified), and Vent (*exo*$^-$) (1 U) in the reaction buffer (Thermopol buffer) provided by the manufacturer. The PCRs were carried out in a Gene Q Thermal Cycler from Bioconcept and 30 cycles were performed. Each cycle included denaturation at 94 °C for 1 min, annealing for 1 min at 55 °C, and extension for 1.5 min at 72 °C. A final extension step of 5 min at 72 °C was included. After the completion of the reactions, 20 μL of 2× sucrose loading buffer was added. All PCR products were analysed by agarose gels (2%) in 1× TBE buffer, containing ethidium bromide. The gels were visualized by phosphorimager analysis.

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