DOI: 10.1002/ejoc.200800381

Synthesis and Enzymatic Incorporation of Modified Deoxyadenosine Triphosphates

Curtis Lam,[a] Christopher Hipolito,[a] and David M. Perrin*[a]

Keywords: Nucleotides / Heterocycles / SELEX / Bioorganic chemistry / DNA / Enzymes

Several deoxyadenosine triphosphates containing modifications at the 8-position have been synthesized. Suitably protected 8-bromodeoxyadenosines were coupled with five imidazole-containing moieties by nucleophilic aromatic substitution or Sonagashira coupling to give modified nucleosides that were then triphosphorylated. Incorporation assays were performed for these modified residues with many commercially available DNA polymerases, and it was found that two of the modified dATPs could be effectively taken up as substrates by Sequenase V2.0. These two residues are candidates for substrates in combinatorial selections in the search for improved catalysis from DNAzymes.

Introduction

In vitro selection techniques[1–3] (e.g., SELEX) have enabled the systematic screening of libraries of up to 10^{15} random nucleic acid sequences for binding and catalysis. In the past 15 years, catalytic oligonucleotides have been selected for reactions ranging from the sequence-specific cleavage of RNA[4,5] to the formation of peptide bonds.[6,7] Aptamers to a broad collection of targets have been discovered as well, and these have been reviewed.[8,9]

Compared with proteins, nucleic acids lack many of the functional groups that protein enzymes employ for catalysis. This deficiency has in part limited the types of reactions that can be catalyzed and the catalytic rates that can be observed for nucleic acid catalysts. To overcome this apparent shortcoming, several groups have synthesized modified deoxyribonucleoside triphosphates (dNTPs) that can be incorporated into oligonucleotides by polymerases. The functionalities that have been synthetically attached include fluorescent groups,[10] boronic acids[11] and nearly every one of the amino acid side-chains.[12–16] Most of these modified nucleosides are substrates for at least one polymerase and many of them have also been successfully used in exponential amplification (PCR). Successful incorporation is a necessary precondition for combinatorial selections using modified dNTPs in place of their natural counterparts, but not a sufficient one for obtaining activity, which will depend on the modified dNTPs.

Despite a plethora of reports detailing the substrate properties of various modified dNTPs, far fewer reports have identified binding or catalytic activities that address a quantitative improvement in activity that cannot be identified by an unmodified selection. One case in which modification has demonstrably improved catalysis is the case of divalent metal-independent ribonuclease activity.

Appending extra chemical functionality to nucleic acids has led to the discovery of a sequence-specific RNase A mimic, 9_{25}-11.[12] RNase A is an endonuclease that cleaves RNA to afford a 2′–3′ cyclic phosphate intermediate product. Two histidine residues in the active site catalyze this reaction and several nearby lysine residues help to stabilize the reaction intermediate.[17] The combinatorially selected DNAzyme 9_{25}-11 contains two modified residues: 8-[2-(4-imidazoyl)ethylamino]-2′-deoxyadenosine and 5-allylamino-2′-deoxyuridine. The added imidazoles appear to facilitate general acid–general base catalysis, whereas the amines provide electrostatic stabilization. Together, these two modifications gave rise to a DNAzyme that could facilitate the cleavage of a phosphodiester bond downstream of an embedded ribocytidine in the absence of a divalent metal ion. The observed catalytic rate was substantially superior to divalent metal-independent RNA-cleaving DNAzymes that contain only unmodified nucleotides.[18–20]

Although triphosphate **1** (Figure 1) has been successfully used in a combinatorial selection,[12] the achieved rate for self-cleavage was still significantly less than what is typically observed for several divalent metal-dependent, unmodified, self-cleaving catalysts, not to mention protein enzymes. One hypothesis to account for this observation is that the linker between the imidazole and the nucleotide that is part of the catalytic scaffold was not ideal. Because nucleic acids are much more flexible polymers than proteins, a more rigid and perhaps shorter linker may allow the catalyst to orient the substrate in a more ordered fashion. In addition, the exact composition of the linker may also be very important.

[a] Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, BC V6T 1Z1, Canada
Fax: +1-604-822-2847
E-mail: dperrin@chem.ubc.ca
Supporting information for this article is available on the WWW under http://www.eurjoc.org/ or from the author.

Triphosphate **1** contains an amine at the 8-position that could participate in enhanced Hoogsteen base-pairing.[21] The presence or absence of this interaction might have an effect on the three-dimensional structure of a DNAzyme.

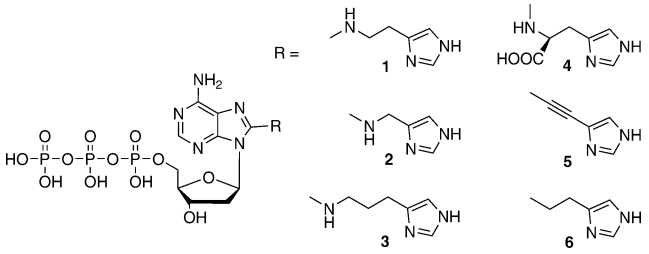


Figure 1. Imidazole-containing 8-modified deoxyadenosine triphosphates.

Thus far, most efforts towards modified nucleotides have been focused on tethering the functional groups using longer linkers, which for reasons that remain unclear enhance polymerase uptake.[11,13] Such nucleotides have for the most part been good substrates for polymerases in both primer extensions and PCR. When short linkers are used, the functional groups may clash with the polymerase scaffold that makes contacts with the growing strand such that incorporation of these modified residues becomes much more difficult. In an attempt to assess the effect of the linker length/composition on the substrate properties of these triphosphates with the long-term goal of testing the hypothesis that shorter linkers provide better catalysts, we have synthesized five imidazole-modified deoxyadenosine triphosphates containing linkers of various lengths, compositions, and flexibilities. These triphosphates have been studied for their relative ease of enzymatic incorporation.

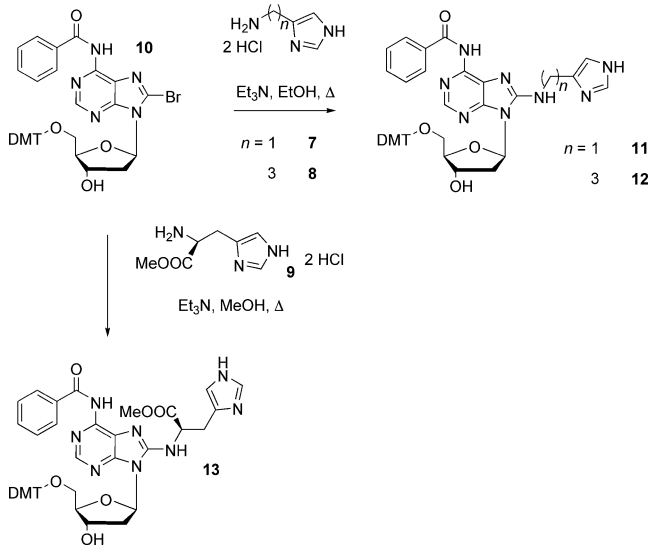
Recently, an investigation of the enzymatic incorporation of dATPs modified at the 7- and 8-positions has been published.[22] This study found that 8-substituted dATPs could not be readily incorporated by polymerases. However, when the same modifications were tethered from the 7-position of 7-deazadATP, the modified nucleotides could be taken up as substrates. The results of Capek et al.,[22] along with studies from other groups who have synthesized 7-deaza-dATPs,[23,24] have all shown that modifications at this position can be tolerated very well by polymerases. In light of these findings, it is important to note that 8-histaminyl-dATP (**1**) is a poor substrate that cannot be readily incorporated more than three times in a row at best. Nevertheless, despite poor substrate properties, triphosphate **1** was selected in the context of the 9_{25}-11 DNAzyme and gave rise to the first M^{2+}-independent RNaseA-like catalyst. This would suggest that although incorporation is necessary for selection, highly efficient incorporation may not be. Thus, the modified dATPs in this study were chosen largely because they are somewhat analogous to **1** and will provide a more comprehensive assessment of the limitation of incorporating 8-modified adenosines with short linkers.

The five linkages that were synthesized (Figure 1) are aminomethyl (**2**), aminopropyl (**3**), (*S*)-1-amino-1-carboxyethyl (**4**), ethynyl (**5**), and ethyl (**6**). Triphosphates **1** and **2** will enable the evaluation of the dependence of catalytic rate on linker length. Triphosphate **4** is the same length as **1**, but contains an extra carboxylate group. This carboxylate group could participate in catalysis or chelate metal ions. The remaining two linkages (**5** and **6**) do not possess an amino moiety and have been designed to assess the extent of hydrogen-bonding of the 8-amino functionality. Triphosphate **6** contains a flexible linker similar to **2**, and **5** contains a rigid alkynyl unit.

Results and Discussion

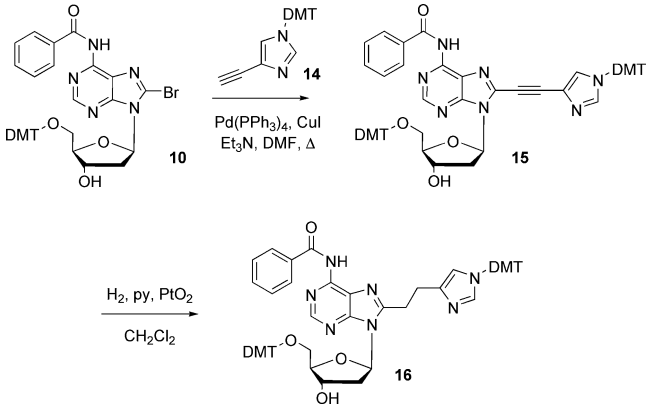
Design and Synthesis of Modified dATPs

The synthesis of the modified nucleosides is outlined in Scheme 1 and Scheme 2. To synthesize the nucleosides containing amine linkers, N^6-benzoyl-8-bromo-5′-(dimethoxytrityl)deoxyadenosine[25,26] (**10**) was heated with the appropriate imidazole-containing dihydrochloride salt in triethylamine and ethanol. Both **11** and **12** were readily prepared in this fashion. Displacement of the bromine substituent with histidine methyl ester, however, required prolonged heating. By using methanol as solvent to prevent transesterification, the desired product was isolated in low yield, giving rise to several spots in the TLC analysis. The most significant byproduct was isolated and found to be debenzoylated **10**. Attempts with other solvents such as DMF, DMSO, or pyridine showed little improvement.



Scheme 1.

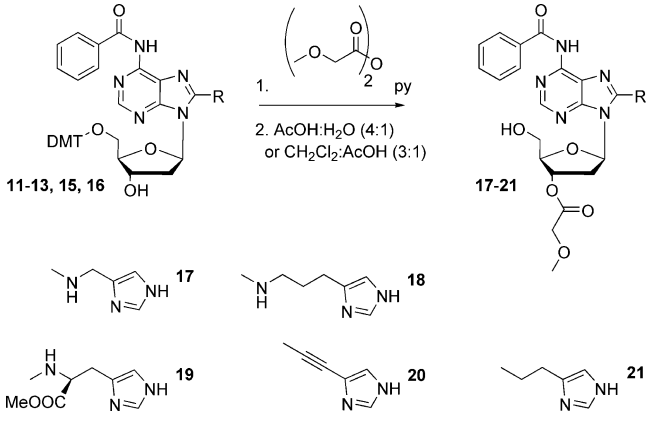
The synthesis of **15** was initially attempted unsuccessfully with 4-ethynylimidazole[27] under Sonagashira conditions.[28] It was found that protected [1-(4,4′-dimethoxytrityl)-1*H*-4-imidazolyl]ethyne (**14**) would undergo this coup-



Scheme 2.

ling much more efficiently. As the dimethoxytrityl group was compatible with our synthesis, no additional deprotection steps were required. Nucleoside **16** was obtained by the hydrogenation of **15** in the presence of platinum oxide, pyridine, and CH_2Cl_2. The pyridine/CH_2Cl_2 ratio of 1:60 was found to be quite important. When the amount of pyridine was increased, the reaction became sluggish, and when this ratio was decreased to 1:1000, significant deprotection of the dimethoxytrityl group was observed. Attempts to reduce the alkyne to the *cis*-alkene under various conditions, including the use of Lindlar's catalyst and palladium over barium sulfate, were unsuccessful and led to either unreacted starting materials or complete reduction to **16**.

The dimethoxytrityl- and benzoyl-protected nucleosides were then methoxyacetylated with methoxyacetic anhydride in pyridine and detritylated in the presence of acetic acid and water (4:1) (Scheme 3). This protocol was effective for all of the modifications except for **15**, for which the deprotection step gave poor yields. It was later found that a mixture of dichloromethane and acetic acid (3:1) gave much better yields.



Scheme 3.

The nucleosides were then converted into triphosphates by applying the multistep "one-pot" synthesis of Ludwig and Eckstein.[29] Briefly, this procedure involved treating the 3′-protected nucleoside with salicyl phosphorochloridite. The addition of pyrophosphate displaces the salicylate and the phosphite is oxidized to the phosphate with I_2. Aqueous ammonia is used for global deprotection. Triphosphates **2**, **3**, **5**, and **6** were successfully isolated in this fashion. For **4**, the methyl ester of compound **19** was first removed with lithium carbonate at slightly elevated temperatures before deprotection with aqueous ammonia. The crude product mixtures were first purified by preparative TLC and then by HPLC to afford the final products. MALDI-TOF MS was used to verify the identity of each modified dATP.

The final yields of the modified dATPs were quite low (3–23%). The initial steps of the reaction were monitored by ^{31}P NMR, and the spectra obtained did not reveal any irregularities. Possible reasons for the low yields include interference from the unprotected imidazoles and incomplete removal of the benzoyl protecting group. As the quantities isolated were more than what was required for enzymatic incorporation and combinatorial selection, the reactions were only carried out once or twice with little optimization.

Enzymatic Incorporation of Modified dATPs

Incorporation assays of the unnatural nucleotides (including **1**) were attempted with several DNA polymerases. The previously studied triphosphate **1** was included both as a control and to study its incorporation more extensively. The incorporations were carried out by extending a 30 nucleotide primer along several 55 nucleotide template oligonucleotides (Figure 2). The enzyme Sequenase V2.0 has previously been used successfully with **1** as a substrate, so initial incorporation assays were performed with this enzyme. For the most part, the incorporation gels did not include **4** as this nucleoside triphosphate was found to photodecompose during HPLC purification to give 8-amino-dATP and a fragment that appears to be urocanic acid (data not shown).[30]

T1	3′ CCGGTCATTGATTCTAGATGTCGAGG 5′
T2	3′ TTTTTCATTGATTCTACCTGTCGAGG 5′
T3	3′ TTCCGGACCGACTTTAGATGTCGACG 5′
T4	3′ TTTTTTTTTTATTCTACCTGTCGAGG 5′

Figure 2. Template sequence regions used for the primer extension experiments (the primer binding region has been omitted).

Figure 3 shows a Sequenase V2.0 primer extension using the **T1** template. This template required a total of eight dATPs to be incorporated, and in two locations along the template, two dATPs must be incorporated consecutively.

Under these conditions, both **1** and **2** were taken up efficiently. Their corresponding full extension products migrated somewhat more slowly than the product band corresponding to unmodified DNA, probably due to the extra molecular bulk/size of the modifications. In contrast, Sequenase V2.0 had difficulty incorporating both **6** and **3** as substrates. The bands corresponding to fully extended material were very faint. Their intensities suggest that **3** was tolerated slightly more than **6**. Lane 10, which contains substrate **5**, looked identical to lane 5 in which no dATP was used and hence **5** is not a substrate for Sequenase V2.0.

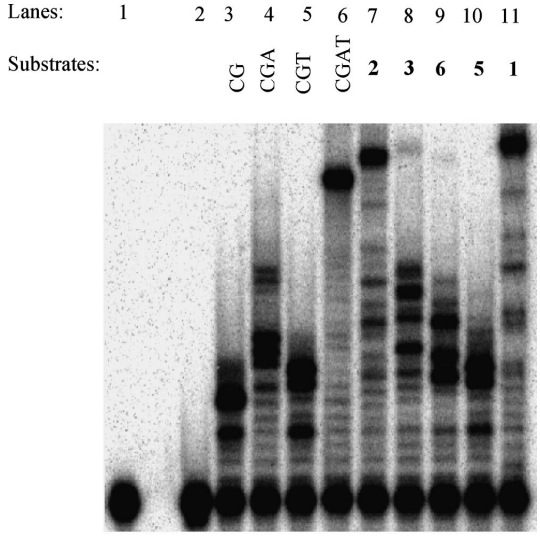


Figure 3. Sequenase V2.0 primer extensions of modified dATPs at 37 °C using the **T1** template. Lane 1: primer only; lane 2: primer and enzyme; lane 3: dCTP and dGTP; lane 4: dCTP, dGTP, and dATP; lane 5: dCTP, dGTP, and dTTP; lanes 6–11 all contain dCTP, dGTP, and dTTP; lane 6: dATP; lane 7: **2**; lane 8: **3**; lane 9: **6**; lane 10: **5**; lane 11: **1**. All nucleotides were used at a concentration of 50 μM.

In an effort to improve the incorporation of the modified dATPs, the pH of the buffers that were used was raised or lowered by one pH unit. When a different template, **T2**, was used, which requires five dATPs to be incorporated one after another, the enzymatic uptake of **3** was somewhat improved and the enzymatic uptake of the other modified dATPs was unaffected. This observation initially looked promising. However, when the **T1** template was used under full extension conditions at the elevated pH, **5**, **6**, **2**, and **3** produced no fully extended product. Modified dATP **1** produced some fully extended product, but the band was very faint.

Following the examination of Sequenase V2.0, other polymerases were investigated. We opted for enzymes that lacked 3′–5′ exonuclease activity. The enzymes tested included *Taq*, Vent exo-, *Bst* large fragment, Phusion, and Dpo4. Of these enzymes, only Dpo4 was able to incorporate the modified dATPs. Dpo4 is a DNA polymerase that belongs to the Y family, a group of enzymes that are capable of bypassing lesions. Isolated from *Sulfolobus solfataricus*, the enzyme is thermostable, but is also active at physiological temperatures.[31]

The enzymatic uptake of the modified dATPs by Dpo4 in the absence of other natural nucleotides is shown in Figure 4. By using the **T2** template, the enzyme was able to incorporate **1** and **3** with the extension going to completion for **1**. For the other three modified dATPs, Dpo4 was only able to incorporate one to two residues before stopping. These results initially looked very promising as Dpo4 seemed to be more capable of tolerating the modified dATPs than Sequenase V2.0. However, when the other three natural dNTPs were included in the incorporation experiments, the results were very different; the extension went no further than what was observed in Figure 4. In other words, the enzyme was able to incorporate up to five modified dATPs in a row, but could not accept any natural substrates afterwards.

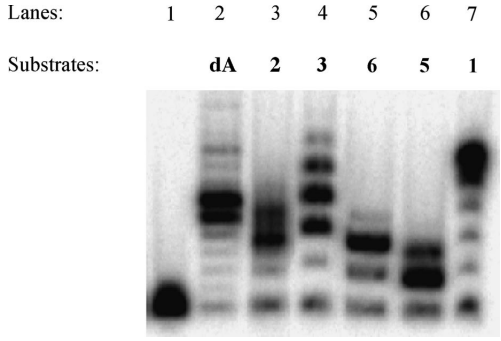


Figure 4. Dpo4 primer extensions of modified dATPs at 37 °C using the **T2** template. Lane 1: primer; lane 2: dATP (5 μM); lane 3: **2**; lane 4: **3**; lane 5: **6**; lane 6: **5**; lane 7: **1**. Modified dATPs were used at a concentration of 100 μM.

With this result in mind, full extensions using Dpo4 and the **T1** template were attempted (Figure 5). As this template only had regions that required up to two modified dATPs to be incorporated in a row, it was thought that **1** and **3** would both be easily fully extended. Unfortunately, no bands corresponding to the fully extended products could be identified.

To understand this result, the incorporation of **1** using Dpo4 was attempted with two other templates (Figure 6). The **T3** template contains the same base composition as **T1**. It also requires up to two modified dATPs to be incorporated in a row, but the locations of the modifications are somewhat more spaced apart. The **T4** template, which required 10 modified dATPs to be incorporated sequentially, was also used to gauge how many modified nucleotides could be incorporated sequentially one after another. The full extension with **T3** was successful at both 37 and 70 °C. In the case of the reaction at 70 °C, a slightly longer product was produced as well. Use of **T4** under the same conditions gave products with five to six modified nucleotides in a row.

Through extensive incorporation studies, it was found that the 8-modified dATPs studied were somewhat tolerated as substrates for DNA polymerases. Sequenase V2.0 was found to be the enzyme that could most tolerate these modifications and could consecutively incorporate up to three residues of **1**. When the linker region between the

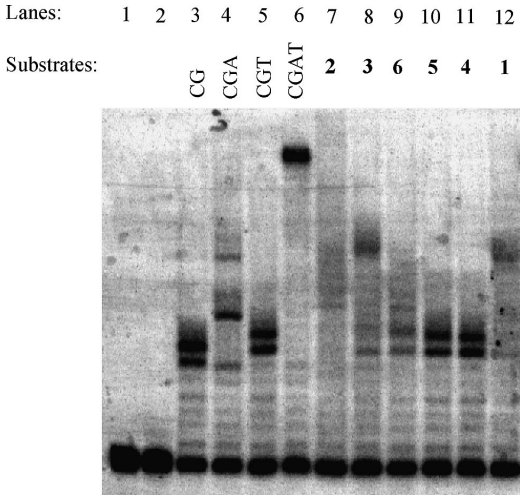


Figure 5. Dpo4 primer extension with the **T1** template at 37 °C. Lane 1: primer only; lane 2: primer and Dpo4; lane 3: dCTP and dGTP; lane 4: dCTP, dGTP, and dATP; lane 5: dCTP, dGTP, and dTTP; lanes 6–12 all contain dCTP, dGTP, and dTTP; lane 6: dATP; lane 7: **2**; lane 8: **3**; lane 9: **6**; lane 10: **5**; lane 11: **4**; lane 12: **1**. Nucleotides were used at a concentration of 50 μM.

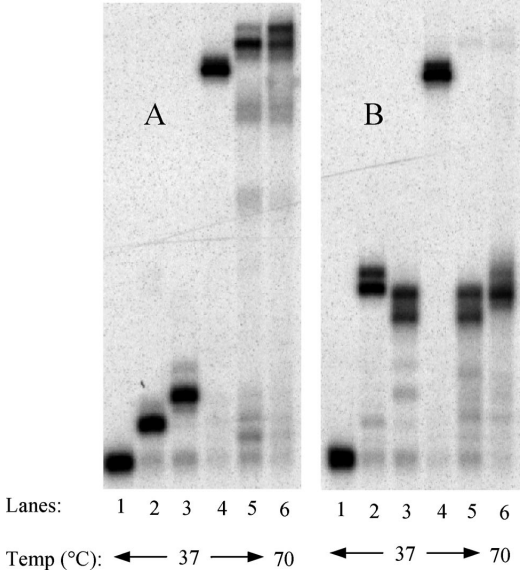


Figure 6. Dpo4 primer extension of **1** using the **T3** (A) and **T4** (B) templates. Samples used in the incorporation experiments were incubated for 2 h at 37 °C (lanes 1–5) or at 70 °C (lane 6). Lane 1: primer and enzyme; lane 2: dATP; lane 3: **1**; lane 4: dATP, dGTP, dTTP, and dCTP; lanes 5 and 6: **1**, dGTP, dTTP, and dCTP. Natural nucleotides were used at a concentration of 50 μM and modified nucleotides were used at a concentration of 100 μM.

imidazole and adenine was elongated or shortened by one methylene unit, only two modified residues in a row could be incorporated. Similarly, Dpo4 also preferred **1** as a substrate over **2** and **3**. These observations suggest that the linker length in **1** may be optimal for incorporation. For the two nucleotides that did not contain an amine in the linker, incorporation was found to be much more difficult. This observation was probably not due to linker length as the linkers of both **5** and **6** contained the same number of atoms as the linker of **2**. A more plausible explanation for this would be that the amine can potentially hydrogen bond to the 5′-oxygen on the sugar. In double-stranded DNA, nucleotides adopt an *anti* conformation in which the 8-position is on the same side as the phosphates. When modifications were added to this position, the extra steric bulk would tend to favor the *syn* conformation. The presence of a hydrogen bond between the linker and the sugar would have helped to stabilize the *anti* conformation and allow the amine containing modified dATPs to be relatively better substrates for polymerases.

Even though the 8-modified dATPs were to some extent difficult to incorporate, several of them may still be used for in vitro selection. Earlier, Roth and Breaker carried out a selection that employed free histidine as a cofactor for the self-cleavage of an embedded RNA residue.[32] Their results show that imidazoles are probably not essential to the folding of a catalytic motif, and hence a DNAzyme should only require a very small number of properly positioned catalytic residues within the random region of an oligonucleotide library. As long as this requirement can be met, the modified nucleotide will be a useful substrate. The DNAzyme 9_{25}-11 was the most active sequence that arose from a selection using Sequenase V2.0 and **1**, and this sequence only required up to two residues of **1** to be incorporated in a row. Consequently, both **2** and **3** will serve as candidates for the selection of more catalytically active DNAzymes. The use of **6** may be possible as well.

Conclusions

Five imidazole-containing modified dATPs have been successfully synthesized. The process involved coupling of the imidazole-containing moieties to the 8-position of adenosine followed by triphosphorylation. In general, the modified dATPs that contain alkyl, aminomethyl, and aminopropyl linkages could be incorporated by Sequenase V2.0 sufficiently when the template did not require very many modified residues to be incorporated. As a DNAzyme will only require a small number of imidazoles for catalysis, at least two of these nucleotides are candidates for the in vitro selection of improved catalytic systems.

This work was undertaken with a view to developing novel DNA polymerase substrates for use in combinatorial selections. Some 8-substituted dATPs are not competent as substrates for most DNA polymerases and thus they will not find use in such selections. It is nevertheless essential to report these findings in order to characterize the current limitations of enzymatic incorporation for those who are pursuing the use of modified dNTPs in combinatorial selections and for those who are seeking to evolve new DNA polymerases with relaxed substrate specificities.

Experimental Section

General: Starting materials were purchased from Sigma–Aldrich and Fisher Scientific. 4-Aminomethylimidazole dihydrochloride (**7**)

was prepared according to the literature.[33] Flash chromatography was performed on silica gel (230–400 mesh) from Silicycle. Thin-layer chromatography (TLC) and preparatory TLC were performed on precoated glass-backed plates of silica gel 60 F_{254} from EMD Chemicals. Natural dNTPs and Klenow Fragment exo– were purchased from Fermentas Life Sciences. T4 polynucleotide kinase, Therminator DNA polymerase, *Bst* DNA polymerase, large fragment, Vent exo–, *Taq* DNA polymerase, and Phusion DNA polymerase were purchased from New England Biolabs. Sequenase Version 2.0 T7 DNA polymerase, inorganic pyrophosphatase and single-stranded DNA binding protein (SSB) were purchased from GE Healthcare. DNA polymerase IV was purchased from Trevigen. NMR spectra were obtained by using a Bruker AV-300 or AV-400 instrument. ^{13}C, ^{31}P, and ^{1}H NMR spectra were calibrated to the signal of the deuterated solvent. ESI-MS were obtained with a Micromass LCT, Bruker Esquire-LC or Waters LC/MS instrument in positive ion mode. MALDI-TOF MS were obtained with a Bruker Biflex instrument in positive ion mode. Solvents were dried by distillation or with molecular sieves (4 Å).

HPLC of Triphosphates: HPLC purification was carried out with an Agilent 1100 system using a Phenomenex Jupiter 10μ C4 300A column. The flow rate was set at 1 mL/min and the eluents contained 0.05 M ammonium acetate (pH 7). Because the absorption of imidazole is very far from the absorbance maxima of the modified adenosines, the ε_{max} value for dATP (15400 $M^{-1}cm^{-1}$) was used to quantify the synthesized triphosphates. The HPLC gradient programs used are shown in Table 1.

Table 1. HPLC gradients.

Time [min]	Program A: ACN/H_2O [%]	Program B: ACN/H_2O [%]
0	1	0
10	5	4
18	25	
19	50	
24	50	
25	0	
30	0	

MALDI-TOF MS of Triphosphate Products: The matrix solution was prepared by suspending 3-hydroxypicolinic acid (30 mg) in acetonitrile (0.25 mL) and H_2O (0.25 mL) and mixing this solution with aq. ammonium citrate (2.4%) in a ratio of 4:1. Prior to sample loading, a few cation-exchange beads (Bio-Rad AG50W-X8 resin converted into the ammonium form) were added to the nucleoside triphosphate samples (2 μL, 100 μM) and left at room temperature for at least 15 min. Matrix solution (1 μL) was first applied to the MALDI target followed by the nucleoside triphosphate sample (1 μL) and the solutions were mixed thoroughly. Care was taken to ensure that a portion of the cation-exchange beads were transferred as well. The samples were analyzed in the positive ion mode with a reflectron detector.

4-(3-Aminopropyl)imidazole Dihydrochloride (8): 3-(*N*-Trityl-4-imidazolyl)acrylonitrile[34] (1.81 g, 5.0 mmol) was converted into **8** according to the procedure of Adger and Surtees.[35] However, recrystallization of the product could not be performed as described. The crude product and trityl chloride (2.79 g, 10 mmol) were suspended in Et_3N (5.6 mL, 40 mmol) and DMF (50 mL). After stirring at room temperature for 12 h, the solvent was removed. The crude product was redissolved in CH_2Cl_2, washed with aq. $NaHCO_3$ (5%), and dried with sodium sulfate (anhydrous). The bis-tritylated intermediate was purified on silica gel. A white solid (1.03 g, 34%) was eluted with EtOAc/hexanes (3:2). This product was then suspended in aq. HCl (25 mL, 3 M) and acetone was added until all solid components dissolved. The contents were refluxed for 1 h. After removal of the acid, the product was recrystallized with EtOH/EtOAc to give 216 mg of white crystals. ^{1}H NMR (300 MHz, D_2O, 25 °C): δ = 8.44 (s, 1 H, 2-H), 7.12 (s, 1 H, 5-H), 2.91 (t, J = 7.8 Hz, 2 H, 8-H), 2.70 (t, J = 7.6 Hz, 2 H, 6-H), 1.91 (dt, J = 7.8, 7.6 Hz, 2 H, 7-H) ppm.

[1-(4,4′-Dimethoxytrityl)-1*H*-4-imidazolyl]ethyne (14): 4-(2,2-Dibromovinyl)-1-(4,4′-dimethoxytrityl)imidazole (2.68 g, 4.8 mmol) was suspended in THF (100 mL) and cooled to –78 °C. *tert*-Butyllithium (20 mL, 34 mmol) was added to this solution. The cold bath was then removed. After 5 min of stirring, MeOH (10 mL) was slowly added followed by the addition of H_2O (30 mL). The THF was evaporated. The resulting crude product was extracted with CH_2Cl_2 (60 mL), dried with sodium sulfate (anhydrous), and purified by flash chromatography. An off-white solid (1.53 g, 81%) was eluted with EtOAc/hexanes (2:3). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 7.36 (s, 1 H, 2-H Imid), 7.36–7.24 (m, 3 H, 3-H Phe, 4-H Phe), 7.20–7.15 (m, 3 H, 5-H Imid, 2-H Phe), 7.00 (d, J = 9.0 Hz, 4 H, 2-H PheOMe), 6.82 (d, J = 9 Hz, 4 H, 3-H PheOMe), 3.79 (s, 6 H, OMe), 3.03 (s, 1 H, HCC) ppm. LRMS (ESI^+): calcd. for $C_{26}H_{22}N_2O_2Na^+$: 417.2; found 417.1.

***N*^{6}-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-8-[(4-imidazolyl)methylamino]deoxyadenosine (11):** 4-(Aminomethyl)imidazole dihydrochloride (**7**) (460 mg, 2.72 mmol), was suspended in Et_3N (1.52 mL, 10.9 mmol) and EtOH (10 mL). This mixture was added to a solution of *N*^{6}-benzoyl-8-bromo-5′-*O*-(4,4′-dimethoxytrityl)-2-deoxyadenosine (**10**) (1.00 g, 1.36 mmol) in EtOH (10 mL). After 4.5 h of stirring at 65 °C, the solvent was removed and the reaction was redissolved in CH_2Cl_2 and washed with aq. $NaHCO_3$ (5%). The organic extracts were combined and dried with Na_2SO_4 (anhydrous) and purified on silica gel. The desired product was eluted with MeOH/$CHCl_3$ (5:95) to give 650 mg (63%) of a slightly off-white solid. ^{1}H NMR (400 MHz, CD_2Cl_2, 25 °C): δ = 8.38 (s, 1 H, 2-H), 8.05 (d, J = 7.3 Hz, 2 H, 2-H Bz), 7.61 (s, 1 H, 1-H Imid), 7.61 (t, J = 7.4 Hz, 1 H, 4-H Bz), 7.53 (dd, J = 7.4, 7.3 Hz, 2 H, 3-H Bz), 7.40 (d, J = 6.7 Hz, 2 H, 2-H Phe), 7.35–7.25 (m, 7 H, 3-H Phe, 4-H Phe, 2-H PheOMe), 6.82 (d, J = 8.8 Hz, 4 H, 3-H PheOMe), 6.47 (dd, J = 8.9, 5.7 Hz, 1 H, 1′-H), 6.39 (s, 1 H, 5-H Imid), 4.75–4.70 (m, 1 H, 3′-H), 4.15–4.00 (m, 2 H, CH_2-Imid, 4′-H), 3.77 (s, 6 H, OMe), 3.60–3.50 (m, 1 H, CH_2-Imid), 3.50 (dd, J = 10.6, 2.5 Hz, 1 H, 5′-H), 3.41 (dd, J = 10.6, 3.0 Hz, 1 H, 5′-H), 2.88 (ddd, J = 13.5, 8.9, 6.3 Hz, 1 H, 2′-H), 2.30 (ddd, J = 13.5, 5.7, 1.9 Hz, 1 H, 2′-H) ppm. ^{13}C NMR (100 MHz, CD_2Cl_2, 25 °C): δ = 166.5, 159.5, 154.7 (C-8), 154.4 (C-4), 148.8 (C-2), 144.7, 143.9 (C-6), 135.9 (C-2 Imid), 135.7, 134.1 (C-4 Imid), 133.2, 130.9, 130.8, 129.3, 129.0, 128.6, 128.6, 127.9, 124.1 (C-5), 113.8, 87.6, 86.8 (C-4′), 84.1 (C-1′), 71.9 (C-3′), 63.5 (C-5′), 55.8, 39.3 (C-2′), 37.3 (CH_2-Imid) ppm. HRMS (ESI^+): calcd. for $C_{42}H_{41}N_8O_6^+$: 753.3149; found 753.3160.

***N*^{6}-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-8-[3-(4-imidazolyl)propylamino]-2′-deoxyadenosine (12):** Compound **12** was synthesized in the same way as **11**. 4-(3-Aminopropyl)imidazole dihydrochloride (**8**) (182 mg, 0.92 mmol) and **10** (450 mg, 0.61 mmol) were converted into 190 mg (40%) of a flaky, slightly off-white solid. ^{1}H NMR (400 MHz, CD_3OD, 25 °C): δ = 8.28 (s, 1 H, 2-H), 8.04 (d, J = 7.3 Hz, 2 H, 2-H Bz), 7.62 (t, J = 7.3 Hz, 1 H, 4-H Bz), 7.51 (t, J = 7.3 Hz, 2 H, 3-H Bz), 7.49 (s, 1 H, 2-H Imid), 7.31 (d, J = 6.8 Hz, 2 H, 2-H Phe), 7.25–7.13 (m, 7 H, 3-H Phe, 4-H Phe, 2-H PheOMe), 6.78–6.68 (m, 5 H, 3-H PheOMe, 5-H Imid), 6.26 (t, J = 6.4 Hz, 1 H, 1′-H), 4.78 (ddd, J = 6.5, 5.0, 3.9 Hz, 1 H, 3′-H), 4.15–4.05 (m, 1 H, 4′-H), 3.70 (s, 3 H, OMe), 3.69 (s, 3 H, OMe),

3.52 (ddd, J = 13.3, 6.5, 6.4 Hz, 1 H, 2′-H), 3.45–3.25 (m, 4 H, 5′-H, H_2C-NH), 2.58 (t, J = 7.3 Hz, 2 H, CH_2-Imid), 2.32 (ddd, J = 13.3, 6.4, 5.0 Hz, 1 H, 2′-H), 1.93 (q, J = 7.3 Hz, 2 H, $CH_2CH_2CH_2$-H) ppm. ^{13}C NMR (100 MHz, CD_3OD, 25 °C): δ = 168.0, 160.1, 160.0, 156.1 (C-8), 154.6 (C-4), 149.0 (C-2), 146.1, 144.4 (C-6), 137.1, 136.9, 135.7 (C-2 Imid), 135.4 (C-4 Imid), 133.7, 131.3, 131.2, 129.7, 129.4, 129.2, 128.7, 127.8, 124.9 (C-5), 118.0 (C-5 Imid), 114.0, 87.6 (C-4′), 87.5 (C-1′), 85.2, 72.6 (C-3′), 64.7 (C-5′), 55.7, 43.4 (CH_2-NH), 38.2 (C-2′), 30.0 (CH_2-CH_2-CH_2), 24.7 (CH_2-Imid) ppm. HRMS (ESI$^+$): calcd. for $C_{44}H_{44}N_8O_6^+$: 781.3462; found 781.3459.

***N*^{6}-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-8-[(*S*)-1-methylcarboxy-2-(4-imidazolyl)ethylamino]-2′-deoxyadenosine (13):** L-Histidine methyl ester dihydrochloride (**9**) (0.48 g, 2 mmol) and Et_3N (1.1 mL, 7.9 mmol) were dissolved in MeOH (4 mL). Nucleoside **10** (735 mg, 1 mmol) was added and the mixture was stirred at 50 °C for 2 d. Following the removal of solvent, the crude product was redissolved in CH_2Cl_2 and washed with aq. $NaHCO_3$ (5%). Sodium sulfate (anhydrous) was used to dry the product. Purification of the product was carried out on silica gel. The product was eluted in MeOH/$CHCl_3$ (6:94) to give 131 mg (16%) of a slightly off-white powder. A yellow foam (212 mg, 33.6%) was isolated and was identified as the debenzoyled product of **7**. ^{1}H NMR (400 MHz, CD_2Cl_2, 25 °C): δ = 8.97 (s, 1 H, CO-NH), 8.31 (s, 1 H, 2-H), 7.96 (d, J = 7.4 Hz, 2 H, 2-H Bz), 7.59 (t, J = 7.4 Hz, 1 H, 4-H Bz), 7.50 (t, J = 7.4 Hz, 2 H, 3-H Bz), 7.45–7.15 [m, 9 H, 2-H Phe, 3-H Phe, 4-H Phe, 2-H PheOMe], 7.39 (s, 1 H, 2-H Imid), 6.85–6.70 (m, 5 H, 3-H PheOMe, 5-H Imid), 6.23 (t, J = 6.6 Hz, 1 H, 1′-H), 4.82 (dd, J = 6.2, 4.0 Hz, 1 H, CH-CO_2), 4.61 (m, 1 H, 3′-H), 4.11 (m, 1 H, 4′-H), 3.72 (2s, 6 H, OMe), 3.64 (s, 3 H, OMe), 3.4–3.1 (m, 5 H, CH_2-Imid, 5′-H, 2′-H), 2.32 (ddd, J = 13.5, 6.6, 4.0 Hz, 1 H, 2′-H) ppm. ^{13}C NMR (100 MHz, CD_2Cl_2, 25 °C): δ = 172.3 (CO_2Me), 165.7, 158.9, 153.6 (C-8), 153.3 (C-4), 148.6 (C-2), 145.3, 144.2 (C-6), 136.2, 135.5 (C-2 Imid), 134.4 (C-4 Imid), 132.9, 130.4, 130.3, 129.1, 128.4, 128.2, 128.1, 127.1, 123.2 (C-5), 118.6 (C-5 Imid), 113.3, 86.6 (C-4′), 86.4 (C-1′), 84.7, 72.4 (C-3′), 64.2 (C-5′), 56.1 (CH-CO_2Me), 55.5, 52.8, 38.0 (C-2′), 29.4 (CH_2-Imid) ppm. HRMS (ESI$^+$): calcd. for $C_{45}H_{45}N_8O_8^+$: 825.3360; found 825.3367.

***N*^{6}-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-8-{2-[*N*-(4,4′-dimethoxytrityl)-4-imidazolyl]ethynyl}deoxyadenosine (15):** This reaction was performed with the total exclusion of O_2 and H_2O. Catalysts were transferred in a glove box and solvents were degassed by three freeze–thaw cycles under vacuum. A catalyst suspension was prepared by adding cuprous iodide (92 mg, 0.48 mmol) and tetrakis(triphenylphosphane)palladium (280 mg, 0.24 mmol) to DMF (40 mL). This mixture was added to a solution of **10** (1.77 g, 2.4 mmol) and 1-[1-(4,4′-dimethoxytrityl)-1*H*-4-imidazolyl]ethyne (**14**) (2.00 g, 5.07 mmol) in DMF (80 mL) and Et_3N (20 mL). After the reaction was stirred at 45 °C for 22 h, the solvent was removed. The crude product was redissolved in CH_2Cl_2, washed with aq. $NaHCO_3$ (5%), and dried with $NaSO_4$ (anhydrous). Purification by silica gel flash chromatography with Et_3N/EtOAc (1:1000) as eluent afforded 1.98 g (78%) of compound **15** as a yellow-green foam. Excess starting material [**14**; 0.78 g (1.12 mmol)] was isolated on passing through an additional silica column using Et_3N/EtOAc/hexanes (0.1:60:40) as eluent. ^{1}H NMR (400 MHz, CD_2Cl_2, 25 °C): δ = 8.94 (s, 1 H, 17-H), 8.53 (s, 1 H, 2-H), 7.98 (d, J = 7.3 Hz, 2 H, 2-H Bz), 7.70–7.12 [m, 15 H, 2-H Phe, 3-H Phe, 4-H Phe, 4-H Bz, 3-H Bz, 5-H Imid, 2-H Imid], 7.25 (d, J = 8.9 Hz, 4 H, 2-H PheOMe), 7.06 (d, J = 9.0 Hz, 4 H, 2-H PheOMe), 6.88 (d, J = 9.0 Hz, 4 H, 3-H PheOMe), 6.75 (d, J = 8.9 Hz, 2 H, 3-H PheOMe), 6.72 (d, J = 8.9 Hz, 2 H, 3-H PheOMe), 6.64 (dd, J = 7.3, 5.8 Hz, 1 H, 1′-H), 5.02–4.96 (m, 1 H, 3′-H), 4.14–4.08 (m, 1 H, 4′-H), 3.82 (s, 6 H, OMe), 3.75 (s, 3 H, OMe), 3.74 (s, 3 H, OMe), 3.52–3.35 (m, 3 H, 5′-H, 2′-H), 2.42 (ddd, J = 13.0, 7.3, 5.0 Hz, 1 H, 2′-H) ppm. ^{13}C NMR (100 MHz, CD_2Cl_2, 25 °C): δ = 164.6, 159.7, 158.8, 152.9 (C-2), 151.3 (C-4), 149.4 (C-6), 145.4, 142.7, 140.2 (C-2 Imid), 137.8 (C-8), 136.4, 136.3, 134.3, 133.0, 132.3, 131.3, 130.4, 130.2, 129.7, 129.2, 128.9, 128.5, 128.4, 128.1, 128.0, 126.9, 123.7 (C-5), 120.9 (C-5 Imid), 113.8, 113.2, 92.0 (*C*C-Imid), 86.7, 86.4 (C-4′), 85.5 (C-1′), 78.8 (C*C*-Imid), 75.9, 72.8 (C-3′), 64.5 (C-5′), 55.7, 55.5, 37.8 (C-2′) ppm. HRMS (ESI$^+$): calcd. for $C_{64}H_{55}N_7O_8^+$: 1072.4010; found 1072.4011.

***N*^{6}-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-8-{2-[*N*-(4,4′-dimethoxytrityl)-4-imidazolyl]ethyl}deoxyadenosine (16):** Compound **15** (500 mg, 0.48 mmol) and PtO_2 (250 mg, 1.10 mmol) were suspended in pyridine (2.5 mL) and CH_2Cl_2 (150 mL). This mixture was stirred at room temperature under H_2 (1 atm) for 4 h. Following the removal of solvent, the crude product was dissolved in MeOH. Filter paper was used to remove the catalyst. The MeOH was then evaporated. The resulting material was redissolved in CH_2Cl_2, washed with aq. $NaHCO_3$ (5%), and dried with Na_2SO_4 (anhydrous). Purification by silica gel flash chromatography using Et_3N/MeOH/$CHCl_3$ (1:20:980) gave 265 mg of **16** (53%). ^{1}H NMR (400 MHz, CD_2Cl_2, 25 °C): δ = 9.00 (s, 1 H, NH-CO), 8.46 (s, 1 H, 2-H), 7.93 (d, J = 7.5 Hz, 2 H, 2-H Bz), 7.60 (t, J = 7.4 Hz, 1 H, 4-H Bz), 7.51 (dd, J = 7.5, 7.4 Hz, 2 H, 3-H Bz), 7.36 (d, J = 6.7 Hz, 2 H, 2-H Phe), 7.32 (s, 1 H, 2-H Imid), 7.30–7.12 (m, 10 H, 4-H Phe, 3-H Phe, 4-H Phe, 3-H Phe, 2-H PheOMe), 7.03 (d, J = 6.9 Hz, 2 H, 2-H Phe), 6.96 (d, J = 8.1 Hz, 4 H, 2-H PheOMe), 6.80–6.68 (m, 8 H, 3-H PheOMe), 6.56 (s, 1 H, 5-H Imid), 6.39 (t, J = 6.8 Hz, 1 H, 1′-H), 4.85–4.79 (m, 1 H, 3′-H), 4.15–4.07 (m, 1 H, 4′-H), 3.76 (s, 6 H, OMe), 3.74 (s, 3 H, OMe), 3.73 (s, 3 H, OMe), 3.57–3.49 (m, 1 H, 2′-H), 3.39–3.27 (m, 4 H, CH_2CH_2-Imid, 5′-H), 3.15–3.00 (m, 2 H, CH_2-Imid), 2.23 (ddd, J = 13.4, 6.8, 4.0 Hz, 1 H, 2′-H) ppm. ^{13}C NMR (100 MHz, CD_2Cl_2, 25 °C): δ = 164.4, 159.0, 158.4, 156.1 (C-8), 152.3 (C-4), 151.3 (C-2), 148.2 (C-6), 144.7, 142.9, 139.0 (C-4 Imid), 138.4 (C-2 Imid), 136.0, 135.9, 134.6, 134.1, 132.5, 130.9, 130.0, 129.9, 129.4, 128.8, 128.2, 127.9, 127.7, 126.7, 113.2, 113.0, 86.2, 86.1 (C-4′), 84.4 (C-1′), 74.4, 73.0 (C-3′), 63.9 (C-5′), 55.3, 55.2, 36.8 (C-2′), 28.1 (CH_2CH$_2$-Imid), 26.6 (CH$_2$CH_2-Imid) ppm. HRMS (ESI$^+$): calcd. for $C_{64}H_{59}N_7O_8^+$: 1054.4503; found 1054.4507.

***N*^{6}-Benzoyl-8-[(4-imidazolyl)methylamino]-3′-*O*-(methoxyacetyl)deoxyadenosine (17):** Compound **11** (200 mg, 266 μmol) was dried by co-evaporation from pyridine under vacuum overnight. After dissolving the sample in pyridine (2.0 mL), 75% methoxyacetic anhydride[36] (234 μL, 0.98 mmol) was added. The reaction was stirred at room temperature for 1 h. The reaction contents were concentrated to give a gum and then resuspended in acetic acid/H_2O (2.0 mL, 80%). After 2.0 h at room temperature, the reaction was concentrated and purified on silica gel. The product was eluted in MeOH/$CHCl_3$ (7:93) to give 106 mg (76%) of a light, pale-yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 8.40 (s, 1 H, 2-H), 8.02 (d, J = 7.3 Hz, 2 H, 2-H Bz), 7.66 (s, 1 H, 2-H Imid), 7.58 (t, J = 7.3 Hz, 1 H, 4-H Bz), 7.48 (t, J = 7.3 Hz, 2 H, 3-H Bz), 6.99 (s, 1 H, 5-H Imid), 6.57 (dd, J = 10.1, 5.4 Hz, 1 H, 1′-H), 5.50 (d, J = 6.4 Hz, 1 H, 3′-H), 4.51 (d, J = 15.2 Hz, 1 H, 2-H Imid), 4.41 (d, J = 15.2 Hz, 1 H, 2-H Imid), 4.15–4.11 (m, 1 H, 4′-H), 4.06 (s, 2 H, OCH_2CO_2), 3.96 (d, J = 11.6 Hz, 1 H, 5′-H), 3.86 (dd, J = 11.6, 2.0 Hz, 1 H, 5′-H), 3.43 (s, 3 H, OMe), 2.75 (ddd, J = 14.2, 10.1, 6.4 Hz, 1 H, 2′-H), 2.26 (dd, J = 14.2, 5.4 Hz, 1 H, 2′-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 169.9, 166.1, 153.6 (C-4), 153.0 (C-8), 148.0 (C-2), 143.1 (C-6), (C-2 Imid), 133.4, 132.5, 132.0 (C-4 Imid), 128.5, 127.8, 123.0 (C-5), 119.4 (C-5 Imid), 85.2

(C-4′), 83.5 (C-1′), 76.3 (C-3′), 69.4, 61.4 (C-5′), 59.2, 37.1 (CH_2-Imid), 35.3 (C-2′) ppm. HRMS (ESI^+): calcd. for $C_{24}H_{27}N_8O_6^+$: 523.2054; found 523.2043.

N^6-Benzoyl-8-[3-(4-imidazolyl)propylamino]-3′-*O*-(methoxyacetyl)-2′-deoxyadenosine (18): Compound **18** was prepared in the same way as **17**. Compound **12** (150 mg, 192 μmol) was converted into 103.3 mg (98%) of a light, pale-yellow solid. ^1H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 8.47 (s, 1 H, 2-H), 8.02 (d, J = 7.2 Hz, 2 H, 2-H Bz), 7.66 (s, 1 H, 2-H Imid), 7.53 (t, J = 7.3 Hz, 1 H, 4-H Bz), 7.45 (dd, J = 7.3, 7.2 Hz, 2 H, 3-H Bz), 6.77 (s, 1 H, 5-H Imid), 6.63 (dd, J = 10.3, 5.3 Hz, 1 H, 5′-H), 5.54 (d, J = 6.5 Hz, 1 H, 3′-H), 4.20–4.16 (m, 1 H, 4′-H), 4.07 (s, 2 H, OCH_2CO_2), 3.99 (d, J = 10.9 Hz, 1 H, 5′-H), 3.90 (dd, J = 10.9, 1.7 Hz, 1 H, 5′-H), 3.55–3.35 (m, 2 H, NHCH_2), 3.44 (s, 3 H, OMe), 2.77 (ddd, J = 14.0, 10.3, 6.5 Hz, 1 H, 2′-H), 2.70–2.60 (m, 2 H, H_2-Imid), 2.26 (dd, J = 10.3, 5.3 Hz, 1 H, 2′-H), 2.12–2.00 (m, 1 H, $CH_2CH_2CH_2$), 1.90–1.80 (m, 1 H, 12-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 169.9, 165.9, 153.0 (C-8), 152.8 (C-4), 148.1 (C-2), 143.3 (C-6), 133.9, 132.3, 128.5, 127.8, 121.7 (C-5), 114.7 (C-5 Imid), 85.2 (C-4′), 83.4 (C-1′) 76.5 (C-3′), 69.5, 61.3 (C-5′), 59.3, 41.6 (CH_2NH), 35.2 (C-2′), 28.4 ($CH_2CH_2CH_2$), 22.6 (CH_2-Imid) ppm. HRMS (ESI^+): calcd. for $C_{26}H_{31}N_8O_6^+$: 551.2367; found 551.2362.

N^6-Benzoyl-3′-*O*-(methoxyacetyl)-8-[(*S*)-1-methoxycarbonyl-2-(4-imidazolyl)ethylamino]deoxyadenosine (19): Compound **13** (36 mg, 43 μmol) was dried by co-evaporation from pyridine under vacuum overnight. After dissolving the sample in pyridine (0.6 mL), 90% methoxyacetic anhydride (30.8 μL, 174 μmol) was added. The reaction was stirred at room temperature for 100 min. The reaction was concentrated to a gum and redissolved in CH_2Cl_2. The organic solution was washed with aq. $NaHCO_3$ (5%) and dried with sodium sulfate (anhydrous). Following removal of the solvent, the crude product was resuspended in acetic acid/H_2O (0.6 mL, 80%). After 2.25 h at room temperature, the reaction was concentrated and purified on silica gel. The product was eluted in MeOH/$CHCl_3$ (8:92) to give 20.6 mg (80%) of a white powder. ^1H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 8.74 (s, 1 H, NHCO), 8.47 (s, 1 H, 2-H), 7.88 (d, J = 7.6 Hz, 2 H, 2-H Bz), 7.54 (t, J = 7.4 Hz, 1 H, 4-H Bz), 7.50 (s, 1 H, 2-H Imid), 7.44 (dd, J = 7.6, 7.4 Hz, 2 H, 3-H Bz), 6.88 (s, 1 H, 5-H Imid), 6.60 (dd, J = 10.0, 5.4 Hz, 1 H, 1′-H), 5.63 (d, J = 6.4 Hz, 1 H, 3′-H), 4.85–4.75 (m, 1 H, NHCH-COO), 4.12 (s, 1 H, 4′-H), 4.09 (s, 2 H, OCH_2CO_2), 4.02 (d, J = 12.1 Hz, 1 H, 5′-H), 3.98 (d, J = 12.1 Hz, 1 H, 5′-H), 3.70 (s, 3 H, CO_2Me), 3.47 (s, 3 H, CH_2OCH_3), 3.27 (d, J = 13.3 Hz, 1 H, CH_2-Imid), 3.15–3.00 (m, 2 H, CH_2-Imid, 2′-H), 2.30 (dd, J = 14.2, 5.4 Hz, 1 H, 2′-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 172.4 (CO_2Me), 169.9, 165.3, 152.7 (C-4), 152.5 (C-8), 148.4 (C-2), 143.8 (C-6), 133.9, 132.3, 128.6, 127.6, 122.0 (C-5), 115.0 (C-5 Imid), 85.4 (C-1′), 83.2 (C-4′), 76.0 (C-3′), 69.7, 60.6 (C-5′), 59.5, 57.6 ($CHCO_2Me$), 52.5, 35.3 (C-2′), 29.7 (CH_2-Imid) ppm. HRMS (ESI^+): calcd. for $C_{27}H_{31}N_8O_8^+$: 595.2264; found 595.2265.

N^6-Benzoyl-8-[2-(4-imidazolyl)ethynyl]-3′-*O*-(methoxyacetyl)deoxyadenosine (20): Compound **15** (250 mg, 0.240 mmol) was dried by co-evaporating with pyridine overnight. The nucleoside was dissolved in pyridine (2.5 mL) and 90% methoxyacetic anhydride (150 μL, 625 mmol) was added to this solution. After 45 min of stirring at room temperature, the solvent was removed. The resulting gum was resuspended in CH_2Cl_2, washed with aq. $NaHCO_3$ (5%), and dried with Na_2SO_4 (anhydrous). Following the removal of the solvent, pyridine (1 mL) and H_2O (1 mL) were added to the crude product, and the mixture was left under vacuum until dry. The crude product was then dissolved in CH_2Cl_2 (5.4 mL), acetic acid (1.8 mL), and H_2O (0.2 mL). After 16 h of stirring at room temperature, the solvent was removed. The reaction contents were again resuspended in CH_2Cl_2, washed with aq. $NaHCO_3$ (5%), and dried with Na_2SO_4 (anhydrous). Purification by silica gel chromatography with MeOH/$CHCl_3$ (5:95) as eluent gave 70.5 mg (57%) of a yellow solid. ^1H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 8.64 (s, 1 H, 2-H), 7.96 (d, J = 7.4 Hz, 2 H, 2-H Bz), 7.59 (s, 1 H, 2-H Imid), 7.52 (t, J = 7.4 Hz, 1 H, 4-H Bz), 7.46 (s, 1 H, 5-H Imid), 7.42 (t, J = 7.4 Hz, 2 H, 3-H Bz), 6.60 (dd, J = 9.2, 5.6 Hz, 1 H, 1′-H), 5.59 (d, J = 6.5 Hz, 1 H, 3′-H), 4.23–4.19 (m, 1 H, 4′-H), 4.05 (s, 2 H, OCH_2CO_2), 3.90 (d, J = 12.5 Hz, 1 H, 5′-H), 3.83 (dd, J = 12.5, 1.6 Hz, 1 H, 5′-H), 3.38 (s, 3 H, OMe), 3.14 (ddd, J = 14.1, 9.2, 6.5 Hz, 1 H, 2′-H), 2.46 (dd, J = 14.1, 5.6 Hz, 1 H, 2′-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 169.9, 165.4, 152.3 (C-2), 150.3 (C-4), 149.8 (C-6), 136.9 (C-8), 133.2, 132.8, 128.6, 128.0, 123.4 (C-5), 86.9 (C-1′), (C-4′), 76.2 (C-3′), 69.5, 62.7 (C-5′), 59.2, 36.8 (C-2′) ppm. HRMS (ESI^+): calcd. for $C_{25}H_{23}N_7O_6^+$: 540.1608; found 540.1611.

N^6-Benzoyl-8-[(4-imidazolyl)ethyl]-3′-*O*-(methoxyacetyl)deoxyadenosine (21): Compound **21** was prepared in the same way as **17**. Compound **16** (150 mg, 0.14 mmol) was converted into 45.4 mg (61%) of a light orange solid that was eluted from a column of silica gel with MeOH/$CHCl_3$ (6:94) as eluent. ^1H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 8.64 (s, 1 H, 2-H), 8.09 (d, J = 7.2 Hz, 2 H, 2-H Bz), 7.72 (s, 1 H, 2-H Imid), 7.65 (t, J = 7.3 Hz, 1 H, 4-H Bz), 7.56 (dd, J = 7.3, 7.2 Hz, 2 H, 3-H Bz), 6.89 (s, 1 H, 5-H Imid), 6.39 (dd, J = 8.6, 6.1 Hz, 1 H, 1′-H), 5.63 (d, J = 6.4 Hz, 1 H, 3′-H), 4.20–4.15 (m, 1 H, 4′-H), 4.17 (s, 2 H, OCH_2CO_2), 3.89 (dd, J = 12.3, 3.1 Hz, 1 H, 5′-H), 3.82 (dd, J = 12.3, 3.7 Hz, 1 H, 5′-H), 3.46 (s, 3 H, OMe), 3.45–3.30 (m, 3 H, CH_2CH$_2$-Imid, 2′-H), 3.19 (t, J = 6.7 Hz, 2 H, CH_2-Imid), 2.37 (ddd, J = 14.0, 6.1, 1.4 Hz, 1 H, 2′-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 169.5, 165.9, 154.2 (C-4), 152.7 (C-8), 150.8 (C-2), 148.7 (C-6), 132.9, 128.6, 127.9, 123.0 (C-5), 86.6 (C-1′), 85.4 (C-4′), 76.2 (C-3′), 69.4, 62.5 (C-5′), 59.2, 36.4 (C-2′), 29.4 (CH_2CH_2-Imid), 28.1 (CH_2-Imid) ppm. HRMS (ESI^+): calcd. for $C_{25}H_{27}N_7O_6^+$: 522.2101; found 522.2103.

8-[2-(4-Imidazolyl)ethynyl]deoxyadenosine Triphosphate (5): Compound **20** (26 mg, 50 μmol) was dried by co-evaporation from pyridine under vacuum overnight in a 5 mm diameter NMR tube. The reaction was monitored by ^{31}P NMR spectroscopy. The starting material was suspended in dioxane (0.3 mL) and pyridine (0.1 mL). Following the addition of a solution of 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (11.1 mg, 55 μmol) in dioxane (55 μL), the reaction was vortexed thoroughly and then periodically over 15 min. Bis(tri-*n*-butylammonium) pyrophosphate (35.6 mg, 75 μL) in DMF (150 μL) and tri-*n*-butylamine (50 μL) were mixed thoroughly and added to the reaction. Following another 15 min of vortexing, I_2 (20 mg, 79 μmol) in H_2O (20 μL) and pyridine (980 μL) was added. After 20 min of periodic vortexing, the reaction contents were transferred to a 25 mL flask. Aq. $NaHSO_3$ (5%) was used to quench excess I_2. Once the reaction had been evaporated to dryness, it was left to stand at room temperature in H_2O (5 mL) for 30 min. Then concentrated NH_3 (10 mL) was added. The flask was stoppered and while stirring was heated at 50 °C for 1 h. Following evaporation of the reaction, the contents were purified by preparative TLC using dioxane/H_2O/NH_4OH (6:4:1) as eluent. The appropriate bands were eluted with H_2O and further purified by HPLC. Triphosphate **5** eluted at 8.0 min in program A. Yield 23%. UV: λ_{max} = 312 nm; λ_{min} = 263 nm. MS ($MALDI^+$): m/z = 582.1. ^{31}P NMR (121.5 MHz, D_2O, 25 °C): δ = –9.9 (m, 1 P, P_γ), –10.6 (m, 1 P, P_α), –22.3 (m, 1 P, P_β) ppm.

8-[(4-Imidazolyl)methylamino]deoxyadenosine Triphosphate (2): Compound **2** was synthesized in the same way as **5** and eluted at 5.4 min

in program A. Yield 8.6%. UV: λ_{max} = 276 nm; λ_{min} = 238 nm. MS (MALDI$^+$): *m*/*z* = 587.2. ^{31}P NMR (121.5 MHz, D_2O, 25 °C): δ = –8.3 (m, 1 P, P_γ), –10.6 (m, 1 P, P_α), –21.3 (m, 1 P, P_β) ppm.

8-[3-(4-Imidazolyl)propylamino]deoxyadenosine Triphosphate (3): Compound **3** was synthesized in the same way as **5** and eluted at 7.1 min in program A. Yield 3.3%. UV: λ_{max} = 279 nm; λ_{min} = 239 nm. MS (MALDI$^+$): *m*/*z* = 615.1.

8-[(*S*)-1-Carboxy-2-(4-imidazolyl)ethylamino]deoxyadenosine Triphosphate (4): Compound **4** was synthesized in the same way as **5** except the deprotection was performed differently. After the reaction was left to stand in H_2O (5 mL) for 30 min, aq. Li_2CO_3 (0.2 M, 10 mL) was added. The reaction was stirred at 50 °C for 5.5 h and at room temperature overnight. The contents were once again dried. H_2O (2.5 mL) and concentrated aqueous ammonia (10 mL) were then used for deprotection. This was carried out at 50 °C for 3 h. Compound **4** eluted at 4.1 min in program B. Yield 2.6%. UV: λ_{max} = 277 nm; λ_{min} = 239 nm. MS (MALDI$^+$): *m*/*z* = 644.9. ^{31}P NMR (121.5 MHz, D_2O, 25 °C): δ = –8.7 (m, 1 P, P_γ), –10.2 (m, 1 P, P_α), –21.6 (m, 1 P, P_β) ppm.

8-[2-(4-Imidazolyl)ethyl]deoxyadenosine Triphosphate (6): Compound **6** was synthesized in the same way as **5** and eluted at 5.6 min in program A. Yield 8.3%. UV: λ_{max} = 262 nm; λ_{min} = 236 nm. MS (MALDI$^+$): *m*/*z* = 586.2.

DNA Templates: (5′ to 3′) **P1** ATTAGCCCTTCCAGTCCCCCC-TTTTCTTTT. **T1** GGAGCTGTAGATCTTAGTTACTGGCC-*AAAAGAAAAGGGGGGACTGGAAGGGCTAA*. **T2** GGAGCTGT-CCATCTTAGTTACTTTTT*AAAAGAAAAGGGGGGACTGGAAG-GGCTAA*. **T3** GCAGCTGTAGATCTTAGCCAGGCCTT*AAAA-GAAAAGGGGGGACTGGAAGGGCTAA*. **T4** GGAGCTGTCCAT-CACATTTTTTTTTT*AAAAGAAAAGGGGGGACTGGAAGGGCT-AA*.

Primer Extension Protocol: Typically, a ^{32}P-labelled primer (**P1**) was annealed with the appropriate template in the presence of enzyme buffers. Single-stranded binding protein (SSB) (0.2 μL/reaction), pyrophosphatase (0.3 μL/reaction), and dithiothreitol (DTT) (0.5–1 μL/reaction) were added to the annealed oligomers as a cocktail. SSB and pyrophosphatase were omitted at elevated incubation temperatures. The enzymes were added last; 1–6 units of enzyme were used. Reactions were prepared with the primer (ca. 1 pmol) in a final incorporation volume of 10 μL. Following incubation, formamide/aq. EDTA solution (30 μL) was added to each incorporation assay and denaturing PAGE was carried out.

Supporting Information (see also the footnote on the first page of this article): Synthesis of 4-(2,2-dibromovinyl)-1-(4,4′-dimethoxytrityl)-imidazole, ^{1}H and ^{13}C NMR spectra of the 3′-protected triphosphate precursors, and HPLC traces for the synthesized triphosphates.

Acknowledgments

This work was supported by NSERC Operating Funds. We wish to thank Dr. Richard Ting for expert synthetic advice in preparing these derivatives.

[1] A. D. Ellington, J. W. Szostak, *Nature* **1990**, *346*, 818.
[2] D. L. Robertson, G. F. Joyce, *Nature* **1990**, *344*, 467.
[3] C. Tuerk, L. Gold, *Science* **1990**, *249*, 505.
[4] S. W. Santoro, G. F. Joyce, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 4262.
[5] J. W. Liu, A. K. Brown, X. L. Meng, D. M. Cropek, J. D. Istok, D. B. Watson, Y. Lu, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 2056.
[6] B. L. Zhang, T. R. Cech, *Nature* **1997**, *390*, 96.
[7] P. A. Lohse, J. W. Szostak, *Nature* **1996**, *381*, 442.
[8] D. S. Wilson, J. W. Szostak, *Annu. Rev. Biochem.* **1999**, *68*, 611.
[9] M. Famulok, J. S. Hartig, G. Mayer, *Chem. Rev.* **2007**, *107*, 3715.
[10] G. Giller, T. Tasara, B. Angerer, K. Muhlegger, M. Amacker, H. Winter, *Nucleic Acids Res.* **2003**, *31*, 2630.
[11] N. Lin, J. Yan, Z. Huang, C. Altier, M. Y. Li, N. Carrasco, M. Suyemoto, L. Johnston, S. M. Wang, Q. Wang, H. Fang, J. Caton-Williams, B. H. Wang, *Nucleic Acids Res.* **2007**, *35*, 1222.
[12] D. M. Perrin, T. Garestier, C. Helene, *J. Am. Chem. Soc.* **2001**, *123*, 1556.
[13] M. Kuwahara, K. Hanawa, K. Ohsawa, R. Kitagata, H. Ozaki, H. Sawai, *Bioorg. Med. Chem.* **2006**, *14*, 2518.
[14] M. Kuwahara, J. Nagashima, M. Hasegawa, T. Tamura, R. Kitagata, K. Hanawa, S. Hososhima, T. Kasamatsu, H. Ozaki, H. Sawai, *Nucleic Acids Res.* **2006**, *34*, 5383.
[15] T. Gourlain, A. Sidorov, N. Mignet, S. J. Thorpe, S. E. Lee, J. A. Grasby, D. M. Williams, *Nucleic Acids Res.* **2001**, *29*, 1898.
[16] K. Sakthivel, C. F. Barbas, *Angew. Chem. Int. Ed.* **1998**, *37*, 2872.
[17] R. T. Raines, *Chem. Rev.* **1998**, *98*, 1045.
[18] C. R. Geyer, D. Sen, *Chem. Biol.* **1997**, *4*, 579.
[19] M. A. Carrigan, A. Ricardo, D. N. Ang, S. A. Benner, *Biochemistry* **2004**, *43*, 11446.
[20] D. Faulhammer, M. Famulok, *J. Mol. Biol.* **1997**, *269*, 188.
[21] E. Cubero, A. Avino, B. G. de la Torre, M. Frieden, R. Eritja, F. J. Luque, C. Gonzalez, M. Orozco, *J. Am. Chem. Soc.* **2002**, *124*, 3133.
[22] P. Capek, H. Cahova, R. Pohl, M. Hocek, C. Gloeckner, A. Marx, *Chem. Eur. J.* **2007**, *13*, 6196.
[23] S. E. Lee, A. Sidorov, T. Gourlain, N. Mignet, S. J. Thorpe, J. A. Brazier, M. J. Dickman, D. P. Hornby, J. A. Grasby, D. M. Williams, *Nucleic Acids Res.* **2001**, *29*, 1565.
[24] S. Jager, G. Rasched, H. Kornreich-Leshem, M. Engeser, O. Thum, M. Famulok, *J. Am. Chem. Soc.* **2005**, *127*, 15071.
[25] M. Ikehara, M. Kaneko, *Tetrahedron* **1970**, *26*, 4251.
[26] D. Singh, V. Kumar, K. N. Ganesh, *Nucleic Acids Res.* **1990**, *18*, 3339.
[27] J. G. Phillips, C. E. Tedford, N. C. Chaturvedi, S. M. Ali, US Patent 6166060, **2000**; *Chem. Abstr.* **2001**, *134*, 56668.
[28] M. T. Tierney, M. W. Grinstaff, *Org. Lett.* **2000**, *2*, 3413.
[29] J. Ludwig, F. Eckstein, *J. Org. Chem.* **1989**, *54*, 631.
[30] UV irradiation from the photodiode array of the HPLC resulted in the formation of a small amount of byproduct that interfered with enzymatic incorporation. The byproduct was a much better substrate for polymerases and thus incorporation of **4** could not be observed.
[31] F. Boudsocq, S. Iwai, F. Hanaoka, R. Woodgate, *Nucleic Acids Res.* **2001**, *29*, 4607.
[32] A. Roth, R. R. Breaker, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 6027.
[33] R. A. Turner, C. F. Huebner, C. R. Scholz, *J. Am. Chem. Soc.* **1949**, *71*, 2801.
[34] R. K. Griffith, R. A. Dipietro, *Synth. Commun.* **1986**, *16*, 1761.
[35] B. M. Adger, J. Surtees, *Synth. Commun.* **1987**, *17*, 223.
[36] B. V. L. Potter, F. Eckstein, B. Uznanski, *Nucleic Acids Res.* **1983**, *11*, 7087.

Received: April 16, 2008
Published Online: September 5, 2008

