Syntheses of Nucleosides Designed for Combinatorial DNA Sequencing

Mike B. Welch,[a] Carlos I. Martinez,[b] Alex J. Zhang,[a] Song Jin,[a] Richard Gibbs,[b] and Kevin Burgess*[a]

Abstract: Nucleoside triphosphates **I** with 3′-O-blocking groups that are both photolabile and fluorescent were required to investigate the viability of a strategy for sequencing DNA in a combinatorial fashion (see Figure 1). Four compounds were prepared to realize this goal. Two of them, **14a** and **14t**, had dansyl-functionalized, 3′-*O*-(2″-nitrobenzyl) ether groups, while the other two, **18a** and **18t**, had similar pendant carbonate groups. Tests for incorporation of these analogues were performed by using five different DNA replicating enzymes, but the analogues were not incorporated. These results were surprising in view of the fact that previous studies had shown that 3′-*O*-(2″-nitrobenzyl)adenosine triphosphate **II** was incorporated by *Bst* DNA polymerase I. However, molecular simulations with the coordinates of a T7 polymerase crystal structure as a model demonstrates that analogues **14a**, **14t**, **18a** and **18t** are too large to fit into the enzyme active site, whereas accommodation of the unsubstituted 2-nitrobenzyl compound **II** is much less demanding. We conclude that both the nucleoside triphosphates and the DNA polymerase enzyme must be modified if the proposed DNA sequencing scheme is to be viable.

Keywords: DNA sequencing · enzyme catalysis · nucleotides

Introduction

Complete DNA sequence analysis of the human genome is a costly and time-consuming project. Accelerated methods for sequencing large DNA strands are therefore highly desirable. Most of the current efforts to improve sequencing are technological improvements of the Sanger[1, 2] or Maxam–Gilbert[3] schemes.[4] These include adaptation of robotic systems for processing fluorescent dideoxy-terminated nucleotides on commercially available DNA sequencing machines[5, 6] coupled with ultra thin,[7] or capillary gel,[8–11] electrophoresis to improve efficiency. Conventional gel electrophoresis is not required for some of these approaches, nevertheless they are unlikely to reduce the cost and time factors to acceptable levels. Other modifications of conventional sequencing schemes focus on the primer, but still require gel electrophoresis.[6, 12–14] For instance, contiguous hexamer strings may be used in primer walking methods wherein the appropriate primers are drawn from an oligonucleotide library,[15, 16] but the feasibility of this methodology remains to be proven for large-scale projects.

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Other advances in sequencing focus on simultaneous processing of data. The simplest and most widely applied form of such multiplexing is separations of combinations of Sanger sequencing reactions in single gel lanes.[17] Schemes involving combination of two sets of Sanger sequencing reactions have also been proposed,[17] but are not used frequently. Multichannel capillary electrophoresis has also been explored, and shows considerable promise.[8, 18–20] Other forms of multiplex sequencing involve oligonucleotide probes to visualize fragments after they have been transferred to a nylon membrane.[21–23] However, efficiency enhancements from any one of these methods is unlikely to raise the throughput of sequence data by more than one or two orders of magnitude.

There are few fundamentally new approaches to sequencing. Novel methodologies include those involving scanning tunneling microscopy,[24] single molecule detection,[25, 26] methods based on detection of the pyrophosphate liberated in each addition step,[27, 28] mass spectrometry,[29] and hybridization (SBH-techniques).[30–32] These protocols may offer significant increases in efficiency over the established procedures. They generally do not require gel electrophoresis, therefore some can potentially process larger numbers of samples without concomitant increases in equipment, reagents, or time. However, at this stage these procedures are largely unproven, and some have obvious disadvantages. Scanning tunneling microscopy and other single molecule detection methods, for instance, have not evolved to the level required for reliable

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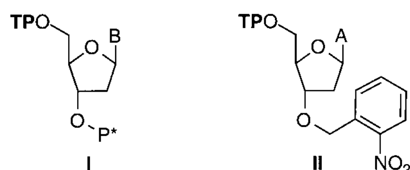
sequencing, and the techniques based on pyrophosphate liberation and hybridization cannot be used to characterize repeated sequences. The main applications of SBH-techniques appear to be for detecting mutations.[33–35] Numerous other schemes have been proposed but work on these has apparently ceased.[36, 37]

Our group and others have been exploring another way to facilitate parallel analyses of multiple samples in an array without gel electrophoresis.[38–43] We call this the Base Addition Sequencing Scheme or BASS. Central to this approach is a set of four nucleoside triphosphates **I** that have 3′-O-blocking groups that are both labile and fluorescent. The



TP = triphosphate, $P_3O_9H_4$

B = adenine, A; thymine, T; guanine, G; cytosine, C

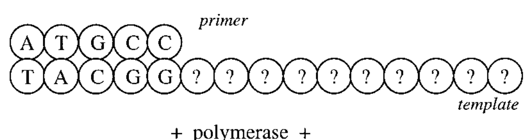
P* = photolabile blocking group that is also fluorescent and encodes for the base B (B = A, T, G, or C)

fluorescence of the protecting group should enable the parent base on the ribose skeleton (A, T, G, or C) to be identified. A cycle in the proposed sequencing scheme would consist of the following steps (Figure 1): 1) incorporation of the appropriate nucleotide triphosphate analogue **I** by a DNA replicating enzyme(s); 2) spectroscopic identification of the base analogue incorporated; and, 3) removal of the blocking group P* to regenerate a 3′-hydroxy terminus on the (now elongated) polynucleotide chain.

If realized, base addition sequencing would have several significant advantages over the methodologies currently used for sequencing DNA. First, results from each DNA sample will be distinguished by direct analysis of the array, so addition of more samples would not proportionately increase the amount of effort or materials required. This compares favorably with procedures wherein each DNA template must be handled separately, and for which every additional sample requires a new gel lane. Consequently, the proposed scheme potentially has a much greater capacity than conventional sequencing methods. Second, if the experiment could be arranged in such a way that millions of primed DNA fragments were analyzed simultaneously, then it would not be necessary to characterize each primer. Instead the primers could be generated by a combinatorial method, and the arrangement of sequences would be deduced from overlaps in the data. It is possible that the extent of multiplexing would be such that the method would be viable even if a relatively short read of DNA sequence was obtained from each individual experiment (for example 10–50 bases). Moreover, the ease of primer synthesis would represent a highly significant cost/time saving advantage. Finally, the method could have incidental benefits like circumvention of artifacts due to gel compressions (frequently associated with G,C-rich strings in the sequence).

In preliminary work we found that 3′-*O*-(2″-nitrobenzyl)-adenosine triphosphate **II** could be incorporated by a DNA polymerase, and that photodeprotection of the 3′-hydroxy was possible facilitating DNA replication.[38] This encouraging result indicated that 3′-modifications could be tolerated by DNA replicating enzymes. Herein we describe syntheses of nucleosides protected with photolabile 3′-O-blocking groups that are also fluorescent, and report preliminary tests for incorporation by DNA polymerase enzymes.

Step 1: add DNA replicating enzyme and analogues of dATP, dTTP, dCTP, and dGTP to the prime DNA to be sequenced

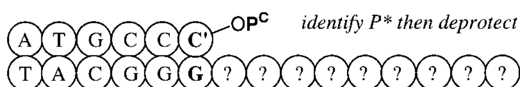


\+ polymerase +

TPO A′ OP^A + TPO T′ OP^T + TPO G′ OP^G + TPO C′ OP^C

enzyme incorporates one base (eg C′) but further replication is blocked by the 3′-group

Step 2: 3′-*O*-blocking group identified spectroscopically, thus indicating the base added and the (previously unknown) next base in the template sequence



Step 3: remove P*, then repeat cycle

the growing strand is now terminated by a natural C

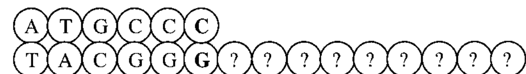


Figure 1. The base addition sequencing scheme (BASS).

Results and Discussion

Syntheses of nucleoside triphosphates with 3′-ether linkages: Initial attempts to prepare 3′-O-protected nucleosides focused on the use of nitrobenzoic acid derivatives as illustrated in Scheme 1. The readily available starting material **1**[44] was coupled with (*N*-allyloxycarbonyl)pentan-5-ol amine, via the acid chloride, to give the ester **2**. A phase transfer catalyst was used to form the critical ether linkage; development of conditions for this step required considerable experimentation. Removal of the 5′-silyl protecting group, triphosphorylation,[45] and removal of the allyloxycarbonyl group then gave the nucleotide amine **6**. A potentially attractive feature of this route was that addition of fluorescent labels at the very end of the synthesis would allow one advanced intermediate to be transformed into several compounds. Unfortunately, labeling of the triphosphate with BODIPY-SE 503/512 was unsuccessful, due to the small

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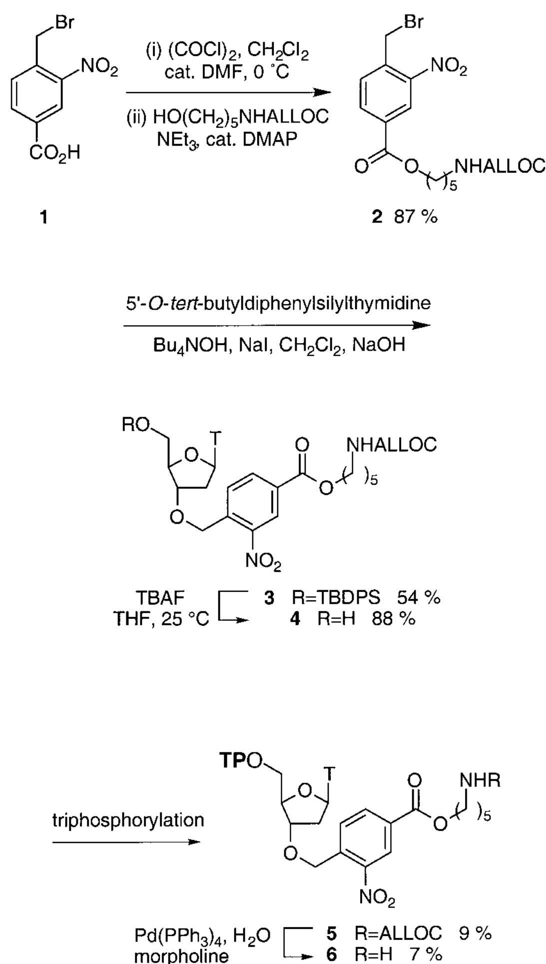
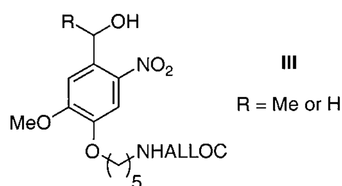


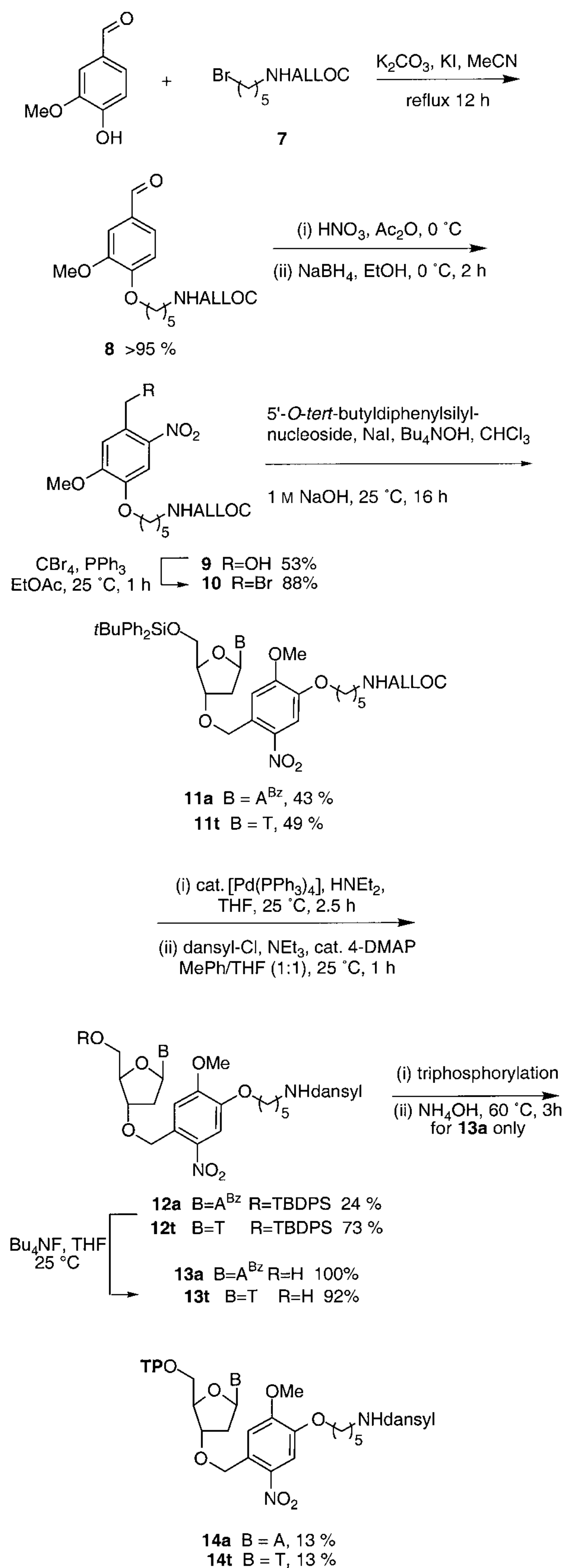
Scheme 1. Synthesis of nucleoside amine **4**.

amounts of material and difficulty in handling triphosphates. A disadvantage of the design **6** is that 2-nitrobenzyl groups substituted with carboxy functionalities cleave less readily under photolytic conditions than comparatively electron-rich systems.[46] This factor, combined with the experimental difficulties associated with the triphosphorylation and labeling steps, led us to investigate alternative routes featuring more photosensitive molecules.

The generic structure **III** represents the photolabile connection sought in the next phase of this work. Photodecomposition of the methyl substituted compounds **III**, R = Me, gives nitroso ketones, whereas the corresponding compounds without this methyl substituent (R = H) give nitroso aldehydes.[47] Nitroso ketones are less reactive by-products hence initial efforts focused on the methyl-substituted compounds. However, the secondary benzylic alcohol **III** where R = Me did not undergo coupling with the 3′-hydroxy of the nucleoside under a variety of conditions.



Coupling of derivatives **III** (R = H) to nucleosides was problematic, but eventually, suitable conditions were developed. Scheme 2 outlines syntheses of derivatives of adenosine



Scheme 2. Preparation of **14a** and **14t**.

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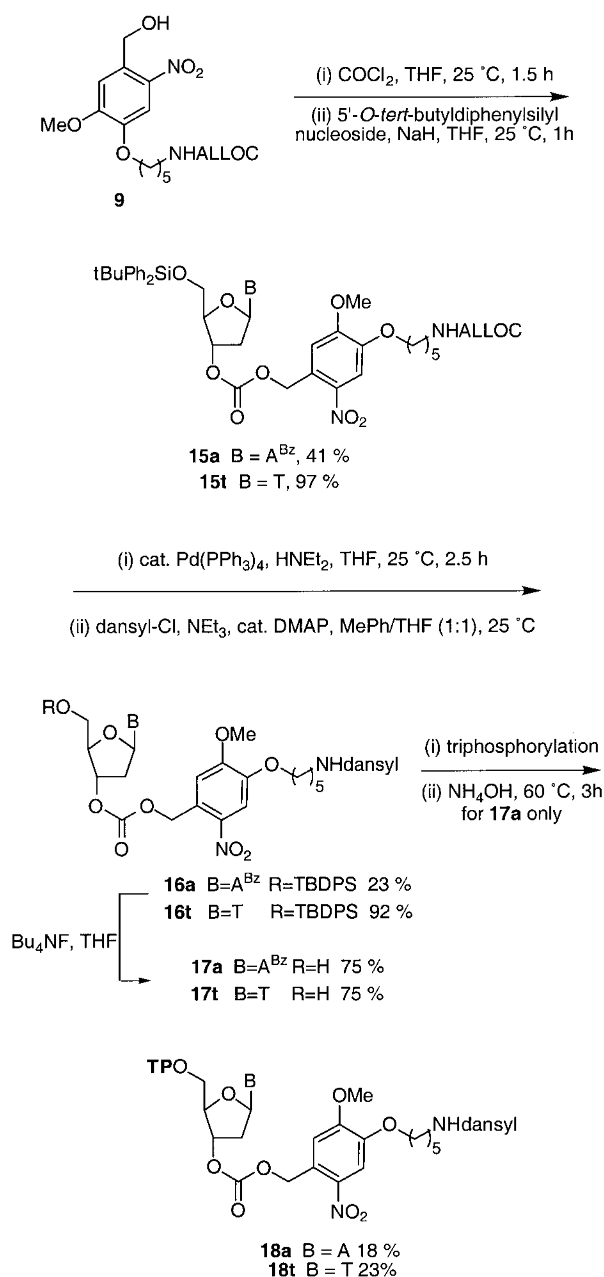
and thymidine. The synthesis began with alkylation of vanillin to install a linker for the fluorescent reporter. Nitration followed by reduction provided the primary benzylic alcohol **9**. The critical coupling step involved conversion of the benzylic alcohol **9** into the corresponding benzyl bromide, then phase transfer catalyzed reaction with a 5′-protected nucleoside under biphasic conditions. Numerous other approaches were attempted, but only phase transfer based methods gave positive results. In preparation for the conversion to the nucleotide, the amine was deprotected then dansylated; 5′-desilation then afforded nucleosides **13a** and **13t**.

Triphosphorylation of nucleosides **13** and **17**, and of many other unnatural nucleosides prepared in our laboratories, has proved to be experimentally difficult and tends to give poor yields. The protocol developed by Eckstein et al. was the best out of several approaches attempted,[45] although none of those were entirely satisfactory. Fortunately, only small amounts of the product are required for feasibility tests in bioassays. Debenzoylation of the adenosine derivative **13a** and **17a** was performed after the triphosphorylation sequence (NH_4OH, 60 °C, 3 h). Both pairs of final products, **14a**/**14t** and **18a**/**18t**, were purified by chromatography, first on diethylaminoethyl (DEAE) cellulose, then by RP HPLC.

Syntheses of nucleoside triphosphates with 3′-carbonate linkages: Difficulties encountered in the syntheses of the ether linked derivatives, as outlined above, led us to explore preparations of structurally similar, but hopefully more accessible compounds. Syntheses of structural variants would also help probe the tolerance of DNA replicating enzymes to unnatural nucleosides. Consequently, two carbonate-linked compounds were constructed as described in Scheme 3. Alternative routes to the same compounds were attempted, for instance, by forming a chlorocarbonate functionality from the nucleoside 3′-hydroxy, but none worked as well as that shown.

Tests for incorporation of 3′-blocked nucleoside triphosphates: Analogues **14a**, **14t**, **18a**, and **18t** were tested as substrates for a series of commercially available DNA replicating enzymes. The protocol used for these experiments was based on a procedure we have reported previously.[38] Briefly, 5′-fluorescein-labeled universal primer was annealed to a synthetic oligo template, 5′-*TACGGAGGTG*-*G*ACTGGCCGTCGTTTTACA (italic sequence indicates the replication region). The reactions were carried out in the presence of a mixture containing the corresponding enzyme, some dNTPs, and no other added nucleotides (control), a ddNTP (positive control), or a sample of analogue



Scheme 3. Preparation of carbonate-linked compounds.

14a, **14t**, **18a**, or **18t**. After incubation the reactions were stopped and loaded on a 20% acrylamide gel, subjected to gel electrophoresis, the gel was scanned, and the results were visualized with a fragment analysis software.

Table 1 shows the results for the incorporation assays. The enzymes tested were unable to recognize the nucleotide

Table 1. Tests of analogues **14a**, **14t**, **18a**, **18t** as substrates for DNA polymerases.

Analogue	Polymerase				
	Klenow	rTth DNA Pol.	Vent (exo-) DNA Pol.	Ampli Taq DNA Pol.	Ampli Taq FS
14a	inhibition (100 μM)	inhibition (10 μM)	inhibition (100 μM)	nonselective inhibition (100 μM)	nonselective inhibition (100 μM)
14t	no incorporation	nonselective inhibition (6.5 μM)	no incorporation	no incorporation	no incorporation
18a	no incorporation	no incorporation	no incorporation	no incorporation	no incorporation
18t	no incorporation	no incorporation	no incorporation	inhibition (1.8 mM)	no incorporation

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analogues as substrates for the termination of the DNA amplification under the conditions studied. No incorporation was observed in most of the experiments, but in some there was evidence that the analogue being tested inhibited the polymerase under study, that is no incorporation was observed and only a band corresponding to the unreacted primer could be seen. This was the case for Ampli Taq DNA polymerase for which a 1.18 mM final concentration of the thymidine carbonate **18t** caused complete inhibition. Klenow Fragment, rTth, and Vent (exo –) DNA polymerases exhibited the same behavior but at 100 μM of the adenosine ether **14a**. Nonspecific inhibition (termination of the amplification reaction at different positions along the template with no specificity) was observed for Ampli Taq DNA polymerase, FS and Ampli Taq DNA polymerase when a 100 μM of **14a** was used (Figure 2).

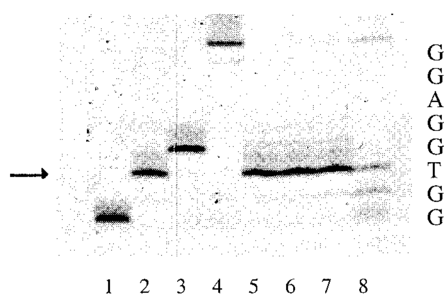

Figure 2. Illustrative data from incorporation assay. Attempted incorporation of **14a** by Ampli Taq DNA polymerase, FS. Arrow marks bands corresponding to no incorporation. Template amplification sequence is shown on the right of the gel. Fluorescein-labeled universal primer was annealed to a complementary oligo template (5′-*TACGGAGGTGG*ACTGGCCGTCGTTTTACA). Lane 1 contained no dNTPs or ddNTPs. Lanes 2–8 contained 0.1 μM dCTP, in addition lanes 3 and 4 contained 0.5 μM ddATP and 0.1 μM dATP, 0.1 μM dTTP, respectively. Lanes 5 to 8 contained 0.1 μM, 2 μM, 10 μM and 100 μM D*ATP, respectively.

Molecular simulations of 3′-blocked nucleoside triphosphates in the active sites of DNA replicating enzymes: Molecular simulations were performed to rationalize the lack of incorporation of the analogues prepared in the course of the work described above. It was perplexing that compound 3′-*O*-(2″-nitrobenzyl)adenosine triphosphate **II** was previously incorporated by a DNA polymerase, whereas similar analogues prepared in the current study were not.

Coordinates for a crystal structure of T7 DNA polymerase encapsulating a primed template and ddGTP were downloaded from the Protein Data Bank and used as a model for this study. This particular set of coordinates was used since they include all the components (enzyme, primed template, and nucleoside triphosphate) and because the data set was recorded at high resolution (2.2 Å). An expansion of the active site of this enzyme complex is shown in Figure 3 top. Removal of the ddGTP entity gave a vacant active site, and several conformers of 3′-*O*-(2″-nitrobenzyl)adenosine triphosphate **II** were fitted in this void by visually docking to form reasonable contacts and avoid unfavorable interactions. A few orientations seemed reasonable, and one illustrative representation is shown in Figure 3 middle. Conversely,

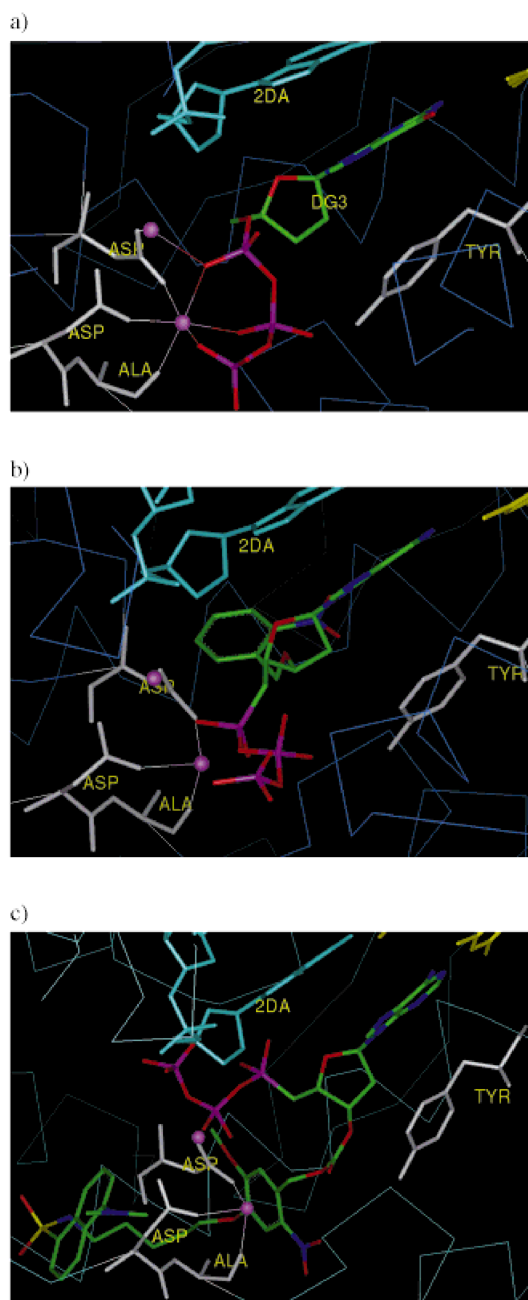

Figure 3. a) Active site of T7 polymerase highlighting two magnesium atoms coordinated to dideoxyguanosine triphosphate with critical side-chains of the protein (grey) and terminus of an encapsulated primer (blue) highlighted; b) as above but with 3′-*O*-(2″-nitrobenzyl)adenosine triphosphate encapsulated; c) as in a) but with the nucleoside triphosphate **18a** encapsulated.

attempts to fit the analogues with fluorescent groups and a methoxy-substituent attached to the aromatic ring were less successful. Figure 3 bottom shows a representation of **18a** in the active site; several interactions were involved that would not be permissible in reality. These docking experiments are too crude to allow detailed conclusions to be formulated, but it does seem clear that 3′-*O*-(2″-nitrobenzyl)adenosine triphosphate **II** is much more easily accommodated in this particular enzyme than any of the analogues prepared in this study.

Conclusions

3′-O-Blocked nucleoside triphosphates, wherein the 3′-protecting group is both photolabile and fluorescent, are synthetically accessible. However, they tend to be too big to fit into the active site of DNA polymerases as evidenced by the data from the activity screens and the molecular-simulation experiments. It seems clear that modifications to the polymerase enzyme as well as to the nucleoside triphosphate are required if BASS is to be developed into a viable sequencing scheme. There are indications that modern methods for combinatorial mutagenesis of proteins, gene shuffling,[48] have the potential to overcome this obstacle, but those methods must be refined further before this goal can be realized.

Experimental Section

General procedures: High-field NMR spectra were recorded on a Unity+ 300 spectrometer (^{1}H at 300 MHz, ^{13}C at 75 MHz), ^{1}H chemical shifts are reported in δ relative to $CHCl_3$ (7.23 ppm) as internal standard, and ^{13}C chemical shifts are reported in ppm relative to $CDCl_3$ (77.0 ppm) unless otherwise specified. Multiplicities in ^{1}H NMR are reported as (br) broad, (s) singlet, (d) doublet, (t) triplet, (q) quartet, (p) quintet and (m) multiplet. Thin-layer chromatography was performed on silica gel 60 F_{254} plates from Whatman. Flash chromatography was performed on SP silica gel 60 (230–600-mesh ASTM). BODIPY 503/512 was purchased from Molecular Probes. Other chemicals were purchased from commercial suppliers and used as received.

(*N*-Allyloxycarbonyl)-5-aminopent-1-yl 4-bromomethyl-3-nitrobenzoate (2): 4-Bromomethyl-3-nitrobenzoic acid (2.0 g, 7.7 mmol), CH_2Cl_2 (38 mL), and DMF (0.1 mL) were cooled to 0 °C under a nitrogen atmosphere. Oxalyl chloride (1.95 g, 15.4 mmol, 1.3 mL) was added, resulting in a vigorous evolution of gas. The reaction mixture was stirred for 3 h, concentrated, and taken up in CH_2Cl_2 (38 mL). A solution of triethylamine (1.56 g, 15.4 mmol, 2.15 mL), catalytic DMAP, and (*N*-allyloxycarbonyl)-5-aminopentanol (1.44 g, 7.7 mmol) in CH_2Cl_2 (10 mL) was added to the above acid chloride, and the mixture was stirred for 3 h. The reaction mixture was then diluted with CH_2Cl_2 (200 mL), washed with HCl (0.5 M 2 × 250 mL), and the aqueous layer was back extracted with CH_2Cl_2 (50 mL). Purification of the crude product by flash chromatography with a gradient of 25 % to 30 % EtOAc/hexanes as the eluant gave **2** as a thick liquid with an orange tinge (2.9 g, 87 % yield). R_f = 0.66 (50 % EtOAc/hexanes); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.57 (d, J = 1.5 Hz, 1 H), 8.21 (dd, J = 8.1, 1.5 Hz, 1 H), 7.74 (d, J = 8.1 Hz, 1 H), 5.90–5.77 (m, 1 H), 5.20 (d, J = 17.1 Hz, 1 H), 5.12 (d, J = 10.2 Hz, 1 H), 4.94 (s, 2 H), 4.88 (brs, 1 H), 4.47 (d, J = 5.1 Hz, 2 H), 4.31 (t, J = 6.6 Hz, 2 H), 3.14 (q, J = 6.6 Hz, 2 H), 1.76 (p, J = 7.5 Hz, 2 H), 1.58–1.36 (m, 4 H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 163.9, 156.2, 147.8, 136.5, 134.0, 132.7, 131.7, 126.0, 117.4, 65.7, 65.3, 42.2, 40.6, 29.5, 28.1, 27.9, 23.0.

3′-*O*-[(4″-(*N*-Allyloxycarbonyl)-5‴-aminopent-1-yl-oxycarbonyl)-2″-nitrophenylmethyl]-5′-*O*-(*tert*-butyldiphenylsilyl)thymidine (3): A solution of **2** (676 mg, 1.57 mmol) in CH_2Cl_2 (4.0 mL, 0.39 M) was added dropwise over 10 min to a stirred mixture of 5′-*O*-(*tert*-butyldiphenylsilyl)thymidine (771 mg, 1.60 mmol), aqueous Bu_4NOH (70 %, 0.5 mL), NaI (cat), CH_2Cl_2 (5 mL), H_2O (5 mL), NaOH (1 M, 5 mL), and stirred for 17 h at 25 °C. The reaction mixture was diluted with CH_2Cl_2 (100 mL) and extracted with HCl (0.5 M, 2 × 75 mL). The combined aqueous layers were then back extracted with CH_2Cl_2 (50 mL). Purification of the crude product was by flash chromatography with a gradient of 4:1:5 EtOAc/hexanes/CH_2Cl_2 increased to 100 % EtOAc as the eluting solvent. Appropriate fractions were combined and concentrated to yield **3** as a yellow foam (717 mg, 54 % yield). R_f = 0.28 (50 % EtOAc/hexanes); IR (neat): $\tilde{\nu}$ = 3448, 2934, 2859, 1715, 1668 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.59 (d, J = 1.8 Hz, 1 H), 8.10 (dd, J = 8.1, 1.5 Hz, 1 H), 7.64–7.61 (m, 4 H), 7.54 (d, J = 1.2 Hz, 1 H), 7.45–7.37 (m, 6 H), 7.25 (d, J = 8.1 Hz, 1 H), 6.34 (dd, J = 7.8, 5.4 Hz, 1 H), 5.93–5.80 (m, 1 H), 5.49 (s, 2 H), 5.24 (dd, J = 17.1, 1.5 Hz, 1 H), 5.16 (dd, J = 10.5, 1.5 Hz, 1 H), 4.78 (brs, 1 H), 4.55–4.50 (m, 3 H), 4.30 (t, J = 6.3 Hz, 2 H), 4.00–3.80 (m, 3 H), 3.16 (q, J = 6.6 Hz, 2 H), 2.35 (ddd, J = 13.2, 5.7, 2.4 Hz, 1 H), 2.22–2.12 (m, 1 H), 1.75 (p, J = 7.2 Hz, 2 H), 1.63 (s, 3 H), 1.59–1.38 (m, 4 H), 1.06 (s, 9 H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 164.3, 163.2, 156.3, 150.7, 148.8, 136.9, 135.5, 135.2, 134.1, 133.8, 132.8, 132.2, 130.5, 130.2, 130.1, 128.4, 128.0, 127.9, 125.9, 117.6, 110.3, 87.0, 85.4, 72.0, 65.5, 64.0, 41.6, 41.1, 40.8, 29.6, 28.2, 27.0, 23.1, 19.3, 12.8; HRMS (positive-ion FAB, nitrobenzyl alcohol (NBA)): calcd for $C_{43}H_{52}N_4O_{11}SiNa$ 851.3299, found 851.3327.

3′-*O*-[(4″-(*N*-Allyloxycarbonyl)-5‴-aminopent-1-yl-oxycarbonyl)-2″-nitrophenylmethyl]thymidine (4): Compound **3** (700 mg, 0.84 mmol), THF (8 mL), and terabutylammonium fluoride (TBAF) (1.26 mL, 1.26 mmol) were stirred at 25 °C for 15 min. The reaction was concentrated and purified directly by flash chromatography with a gradient of 100 % EtOAc, then 1 to 5 % MeOH in EtOAc as the eluant to give **4** as a foam (589 mg, 88 % yield). R_f = 0.11 (75 % EtOAc/hexanes); IR (neat): $\tilde{\nu}$ = 3479, 2947, 1720, 1642 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.54 (d, J = 1.8 Hz, 1 H), 8.06 (dd, J = 8.1, 1.5 Hz, 1 H), 7.60 (s, 1 H), 7.24 (d, J = 8.1 Hz, 1 H), 6.17 (t, J = 6.6 Hz, 1 H), 5.89–5.76 (m, 1 H), 5.44 (s, 2 H), 5.21 (d, J = 17.4 Hz, 1 H), 5.12 (d, J = 10.5 Hz, 1 H), 4.97 (br, 1 H), 4.47–4.41 (m, 3 H), 4.28 (t, J = 6.3 Hz, 2 H), 4.00 (t, J = 6.6 Hz, 2 H), 3.89 (br, 1 H), 3.81–3.69 (m, 2 H), 3.12 (q, J = 6.6 Hz, 2 H), 3.95 (br, 1 H), 2.25–2.19 (m, 2 H), 1.99 (s, 3 H), 1.74 (p, J = 7.5 Hz, 2 H), 1.58–1.35 (m, 4 H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 164.3, 163.2, 156.4, 150.7, 148.7, 136.8, 135.5, 133.9, 132.8, 130.5, 128.4, 125.8, 117.5, 110.1, 87.0, 86.5, 71.1, 65.6, 65.4, 62.1, 41.5, 40.7, 40.3, 29.5, 28.2, 23.0, 13.1; HRMS (positive-ion FAB, NBA): calcd for $C_{27}H_{34}N_4O_{11}Na$ 613.2122, found 613.2122.

3′-*O*-[(4″-(*N*-Allyloxycarbonyl)-5‴-aminopent-1-yl-oxycarbonyl)-2″-nitrophenylmethyl]thymidine-5′-*O*-triphosphate (5): Compound **4** was azeotroped with PhH (3 × 5 mL) and placed in a high vacuum dessicator overnight. Compound **4** (369 mg, 0.46 mmol), 1,8-bis(dimethylamino)naphthalene (199 mg, 0.93 mmol), and trimethyl phosphate (4.6 mL) were cooled to 0 °C under a nitrogen atmosphere, and phosphorus oxychloride (142 mg, 0.93 mmol) was added in one portion. This mixture was stirred at 0 °C for 140 min. A premixed solution of tributylammonium pyrophosphate (894 mg, 1.6 mmol) and tributylamine (296 mg, 1.60 mmol) in DMF (2.0 mL) was then added. The reaction was stirred for 5 min and then quenched at 0 °C with triethylammonium bicarbonate (1 M, pH = 5.7). The reaction mixture was allowed to warm to 25 °C, stirred for an additional 1 h, diluted with deionized H_2O (10 mL), lyophilized, taken up in EtOH (3 × 15 mL), and concentrated at 25 °C on a high vacuum rotary evaporator. Purification by ion exchange chromatography with DEAE-sephadex A-25 resin, eluting with a 0 to 1.0 M triethylammonium bicarbonate (500 mL each) gradient gave the product **5**. This was detected by monitoring the absorbance at 302 nm. Appropriate fractions were then combined, concentrated, redissolved in deionized water (5 mL), and concentrated again to yield compound **5** (34 mg, 8.9 % yield). ^{31}P NMR (D_2O, 121 MHz): δ = 3.82 (d, J = 24.3 Hz), −5.40 (d, J = 21.4 Hz), −19.0 (t, J = 20.8 Hz); MS (MALDI-TOF): calcd for $C_{27}H_{35}N_4O_{20}P_3$ 828, found 828.

3′-*O*-[4″-(5‴-Aminopent-1-yl-oxycarbonyl)-2″-nitrophenylmethyl]thymidine-5′-*O*-triphosphate (6): Compound **5** (81 mg, 0.10 mmol), degassed HPLC grade H_2O (1.0 mL, 0.1 M), [$Pd(PPh_3)_4$] (11 mg, 0.01 mmol), and degassed morpholine (85 mg, 0.98 mmol) were stirred for 19 h at 25 °C. The reaction was then filtered through a 45 µm HPLC filter. Purification was performed by C18 RP-HPLC (analytical) with a gradient of 0 % B (time t = 0 min); 50 % B (t = 20 min); 70 % B (t = 30 min) where A is 0.1 M triethylammonium acetate, and B is 70 % MeCN/A. This procedure was repeated 15 times for small portions of the reaction mixture on an analytical instrument. Fractions containing the product **6** were assayed by UV absorption at 302 nm, combined, and lyophilized to yield a white hygroscopic powder (5.1 mg, 7 % yield). MS (MALDI-TOF): calcd for $C_{23}H_{32}N_4O_{18}P_3$ 745, found 745.

4-[(*N*-Allyloxycarbonyl)-5-aminopent-1-yl-oxy]-3-methoxybenzaldehyde (8): Compound **7** (3.35 g, 13 mmol), MeCN (40 mL), vanillin (1.98 g, 0.01 mmol), KI (cat), and K_2CO_3 (5.6 g, 0.04 mol) were refluxed for 12 h. The crude reaction mixture was filtered through a plug of silica gel on Celite and diluted with EtOAc (100 mL). The organic layer was extracted with water (2 × 50 mL), HCl (0.5 M, 2 × 50 mL), and brine (25 mL), dried over Na_2SO_4, filtered, and concentrated to yield **8** as a thick orange liquid (4.2 g, 100 % yield). R_f = 0.38 (50 % EtOAc/hexanes); IR (neat): $\tilde{\nu}$ = 3454, 2945, 2866, 1700, 1266 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 9.75 (s, 1 H),

7.35 (dd, J = 8.1, 1.8 Hz, 1H), 7.32 (s, 1H), 6.87 (d, J = 1.8 Hz, 1H), 5.89–5.76 (m, 1H), 5.20 (dd, J = 17.1, 1.2 Hz, 1H), 5.11 (dd, J = 10.2, 0.9 Hz, 1H), 4.94 (brs, 1H), 4,46 (d, J = 5.4 Hz, 2H), 4.01 (t, J = 6.6 Hz, 2H), 3.83 (s, 3H), 3.13 (q, J = 6.3 Hz, 2H), 1.82 (p, J = 6.9, 2H), 1.57–1.41 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 190.8, 156.2, 153.9, 149.6, 132.8, 129.7, 126.6, 117.3, 111.2, 109.0, 68.6, 65.2, 55.8, 40.6, 29.5, 28.3, 23.0; HRMS (positive-ion FAB, NBA): calcd for $C_{17}H_{24}N_1O_5$ 322.1654, found 322.1646.

1-[(*N*-Allyloxycarbonyl)-5′-aminopent-1-yl-oxy]-4-(hydroxymethyl)-2-methoxy-5-nitrobenzene (9): Compound **8** (4.23 g, 13.2 mmol) in Ac_2O (17 mL) was slowly added to a stirred mixture of HNO_3 (66 mL) and Ac_2O (17 mL) at 0 °C. After 2 h the reaction was allowed to warm to 25 °C and stirred for an additional 2 h. The resulting solution was diluted with EtOAc (200 mL), washed with brine (4 × 100 mL), $NaHCO_3$ (6 × 100 mL), brine (100 mL), dried over Na_2SO_4, filtered through a plug of silica gel on Celite, and concentrated yielding a thick yellow oil. This crude material was dissolved in absolute EtOH (66 mL) and cooled to 0 °C. Solid $NaBH_4$ (1.0 g, 26 mmol) was added in about 50 mg portions; the reduction was monitored by TLC until starting material was completely consumed (approximately 4 h). The reaction was then quenched with saturated aqueous NH_4Cl, allowed to warm to room temperature, and diluted with EtOAc (200 mL). The crude reaction mixture was extracted with water (2 × 100 mL), brine (100 mL), dried over Na_2SO_4, filtered through a plug of silica gel on Celite, and concentrated. The crude product was purified by flash chromatography with a gradient of 50 to 60 % EtOAc/hexanes as the eluting solvent yielding **9** (0.73 g, 53 % yield over two steps) as a yellow oil which crystallized upon standing. R_f = 0.36 (3 × 30 % EtOAc/hexanes); IR (neat): $\tilde{\nu}$ = 3439, 2943, 2862, 1711, 1576, 1323, 1064 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.65 (s, 1H), 7.15 (s, 1H), 5.93–5.80 (m, 1H), 5.24 (dd, J = 17.4, 1.5 Hz, 1H), 5.14 (dd, J = 10.2, 1.2 Hz, 1H), 4.91 (s, 2H), 4.85 (br, 1H), 4.49 (d, J = 5.4 Hz, 2H), 4.02 (t, J = 6.6 Hz, 2H), 3.93 (s, 3H), 3.16 (q, J = 6.3 Hz, 2H), 3.10–2.85 (br, 1H), 1.84 (p, J = 6.9 Hz, 2H), 1.60–1.42 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 156.3, 154.1, 147.2, 139.4, 132.8, 132.0, 117.6, 110.8, 109.1, 69.1, 65.4, 62.6, 56.3, 40.7, 29.6, 28.4, 23.0; HRMS (positive-ion FAB, NBA): calcd for $C_{17}H_{25}N_2O_7$ 369.1661, found 369.1666.

1-[(*N*-Allyloxycarbonyl)-5′-aminopent-1-yl-oxy]-4-bromomethyl-2-methoxy-5-nitrobenzene (10): Compound **9** (114 mg, 0.31 mmol), in an oven-dried flask, was azeotroped with PhH (2 mL), charged with distilled EtOAc (1.5 mL), triphenylphosphine (122 mg, 0.46 mmol), and stirred until homogeneous at 25 °C. Carbon tetrabromide (154 mg, 0.46 mmol) was added and the resulting orange-red solution was stirred for 1 h. The reaction was exothermic and a gummy material formed. The crude reaction mixture was filtered through a plug of silica gel on Celite and washed with EtOAc (50 mL). Purification of the crude product by flash chromatography with a gradient of 30 to 40 % EtOAc/hexanes as the eluting solvent gave compound **10** (117 mg, 88 % yield), as an off-white solid. M.p. 107–108 °C; R_f = 0.36 (40 % EtOAc/hexanes); IR ($CDCl_3$): $\tilde{\nu}$ = 3452, 2942, 1718, 1526, 1282 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.60 (s, 1H), 6.88 (s, 1H), 5.93–5.81 (m, 1H), 5.25 (ddd, J = 17.4, 3.0, 1.2 Hz, 1H), 5.16 (ddd, J = 10.2, 2.7, 1.5 Hz, 1H), 4.82 (s, 2H), 4.83–4.76 (brs, 1H), 4.50 (d, 5.7 Hz, 2H), 4.03 (t, J = 6.3 Hz, 2H), 3.93 (s, 3H), 3.17 (q, J = 6.3 Hz, 2H), 1.85 (dq, J = 6.6 Hz, 2H), 1.61–1.41 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 156.2, 153.4, 148.3, 140.0, 132.9, 127.2, 117.5, 113.7, 109.4, 69.1, 65.4, 56.4, 40.7, 30.2, 29.6, 28.3, 23.0; HRMS (positive-ion FAB, NBA): calcd for $C_{17}H_{24}N_2O_6Br$ 431.0817, found 431.0830.

3′-*O*-[4″-((*N*-Allyloxycarbonyl)-5‴-aminopent-1-yl-oxy)-5″-methoxy-2″-nitrophenylmethyl]-5′-*O*-(*tert*-butyldiphenylsilyl)thymidine (11t): A solution of **10** (800 mg, 1.85 mmol) in $CHCl_3$ (5.0 mL, 0.37 M) was added over 10 min to a vigorously stirred solution of 5′-*O*-(*tert*-butyldiphenylsilyl)-thymidine (891 mg, 1.85 mmol), Bu_4NOH (0.5 mL), NaI (cat), NaOH (1 M, 5.0 mL), and $CHCl_3$ (5.0 mL). After 16 h the reaction was extracted with H_2O (20 mL), and brine (20 mL). Material in the combined aqueous fractions was back extracted into $CHCl_3$ (2 × 10 mL), dried (Na_2SO_4), filtered, and concentrated. The crude product was purified by flash chromatography with 50 to 60 % EtOAc/hexanes as eluant giving **11t** as an off-white foam (1.56 g, 49 % yield). R_f = 0.30 (60 % EtOAc/hexanes); IR (neat): $\tilde{\nu}$ = 3425, 3271, 2937, 1713, 1646, 1520 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.64–7.61 (m, 4H), 7.51 (s, 2H), 7.45–7.33 (m, 6H), 6.64 (s, 1H), 6.34 (dd, J = 8.1, 6.0 Hz, 1H), 5.94–5.81 (m, 1H), 5.46 (d, J = 2.7 Hz, 2H), 5.24 (dd, J = 17.1, 1.5 Hz, 1H), 5.15 (dd, J = 10.5, 1.2 Hz, 1H), 4.84–4.76 (brm, 1H), 4.56–4.48 (brm, 3H), 4.01–3.80 (m, 6H), 3.77 (s, 3H), 3.16 (q, J = 6.3 Hz, 2H), 2.39–2.32 (m, 1H), 2.19–2.10 (m, 1H), 1.82 (p, J = 6.9 Hz, 2H), 1.64 (d, J = 0.9 Hz, 3H), 1.56–1.42 (m, 4H), 1.05 (s, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 163.4, 156.3, 153.5, 150.8, 147.3, 141.9, 135.5, 135.3, 133.9, 133.0, 132.9, 132.4, 130.2, 130.1, 128.0, 128.0, 126.7, 117.5, 114.0, 110.9, 109.5, 86.9, 85.5, 72.0, 69.2, 65.4, 64.0, 56.2, 41.4, 41.0, 29.6, 28.5, 27.0, 23.1, 19.3, 12.8; HRMS (positive-ion FAB, NBA): calcd for $C_{43}H_{54}N_4O_{11}SiNa$ 853.3456, found 853.3479.

3′-*O*-[4″-((*N*-Allyloxycarbonyl)-5‴-aminopent-1-yl-oxy)-5″-methoxy-2″-nitrophenylmethyl]-2′-deoxy-5′-*O*-(*tert*-butyldiphenylsilyl)-*N*-6-benzoyladenosine (11a): Compound **11a** was prepared by a similar procedure to that used for **11t** but with **10** (409 mg, 0.95 mmol) in $CHCl_3$ (3.2 mL), 6-*N*-benzoyl-5′-*O*-*tert*-butyldiphenylsilyl-2′-deoxy-*N*-6-benzoyladenosine (564 mg, 0.95 mmol), Bu_4NOH (0.5 mL), NaI (cat), NaOH (1 M, 3.0 mL), and $CHCl_3$ (3.2 mL) for a reaction time of 23 h. Chromatography with 60 to 70 % EtOAc/hexanes as the eluant gave **11a** (384 mg, 43 % yield) as a yellow foam. R_f = 0.34 (70 % EtOAc/hexanes); IR ($CDCl_3$): $\tilde{\nu}$ = 3451, 2943, 2863, 1713, 1578, 1520 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.49 (s, 1H), 8.02 (s, 1H), 7.62–7.54 (m, 5H), 7.44–7.20 (m, 11H), 7.13–7.08 (m, 2H), 6.34 (t, J = 6.6 Hz, 1H), 5.96 (s, 2H), 5.93–5.80 (m, 1H), 5.23 (dq, J = 17.1, 1.5 Hz, 1H), 5.14 (dq, J = 10.2, 1.5 Hz, 1H), 4.77 (brs, 1H), 4.66–4.59 (brm, 1H), 4.50 (d, J = 5.1 Hz, 2H), 4.03–3.93 (m, 3H), 3.86–3.77 (m, 2H), 3.75 (s, 3H), 3.15 (t, J = 6.3 Hz, 2H), 2.65–2.56 (m, 1H), 2.48–2.40 (m, 1H), 2.35 (brs, 1H), 1.79 (p, J = 7.5 Hz, 2H), 1.57–1.38 (m, 4H), 1.00 (s, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 181.7, 172, 156.3, 154.1, 152.3, 146.8, 142.1, 140.2, 135.5, 135.4, 133.0, 132.7, 130.9, 130.0, 130.0, 128.7, 127.9, 127.2, 117.5, 110.5, 109.6, 87.0, 84.4, 71.8, 69.2, 65.4, 63.8, 56.4, 49.7, 40.8, 40.3, 29.6, 28.5, 26.9, 23.1, 19.2; HRMS (positive-iom FAB, NBA): calcd for $C_{50}H_{57}N_7O_{10}SiNa$ 966.3834, found 966.3892.

3′-*O*-[4″-((*N*-Dansyl)-5‴-aminopent-1-yl-oxy)-5″-methoxy-2″-nitrophenylmethyl]-5′-*O*-(*tert*-butyldiphenylsilyl)thymidine (12t): Compound **11t** (681 mg, 0.82 mmol) was dissolved in freshly distilled THF (4.0 mL) at 25 °C under N_2 atmosphere. Degassed distilled diethylamine (1.2 g, 16.4 mmol) and $[Pd(PPh_3)_4]$ (95 mg, 0.82 mmol) were added and the solution was stirred at 25 °C for 75 min. The reaction was concentrated, dissolved in $CHCl_3$ (5 mL), and concentrated again. This cycle was repeated two more times to remove residual diethylamine. The reaction mixture was charged with THF (4 mL), TEA (triethylamine; 331 mg, 3.3 mmol), dimethylaminopyridine (DMAP) (cat), and stirred at 25 °C. A slurry of dansyl chloride (221 mg, 0.82 mmol) in THF/DMF (2:1 mL) was added to the above reaction mixture, and this was then stirred for 6 h at 25 °C. The crude product was purified by flash chromatography with 2.5:2.5:1 to 3:2:1 EtOAc/hexanes/CH_2Cl_2 as eluant and gave **12t** as a off-white foam (582 mg, 73 % yield). R_f = 0.57 (70 % EtOAc/hexanes); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.46 (d, J = 8.7 Hz, 1H), 8.24 (d, J = 8.7 Hz, 1H), 8.18 (dd, J = 7.2, 1.2 Hz, 1H), 7.94 (s, 2H), 7.62–7.30 (m, 12H), 7.10 (d, J = 7.8 Hz, 1H), 6.61 (s, 1H), 6.36 (dd, J = 8.1, 5.7 Hz, 1H), 5.46 (s, 2H), 5.21 (t, J = 6 Hz, 1H), 4.56–4.50 (brm, 1H), 4.00–3.75 (m, 5H), 3.74 (s, 3H), 3.54 (d, J = 3.6 Hz, 1H) 2.86 (brm, 2H), 2.82 (s, 3H), 2.81 (s, 3H), 2.35 (ddd, J = 13.2, 5.7, 2.1 Hz, 1H), 2.16–2.07 (m, 1H), 1.61 (s, 3H), 1.55 (p, J = 7.5 Hz, 2H), 1.37 (p, J = 6.9 Hz, 2H), 1.28–1.20 (m, 2H), 1.03 (s, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 163.3, 153.4, 151.9, 150.7, 147.1, 141.7, 135.4, 135.2, 134.0, 132.9, 132.4, 132.0, 131.9, 130.2, 130.0, 129.9, 129.8, 129.6, 129.3, 128.5, 128.3, 128.2, 127.8, 127.8, 126.8, 123.0, 118.7, 115.1, 110.2, 110.0, 109.3, 87.0, 85.4, 71.7, 68.9, 64.0, 56.1, 45.2, 42.9, 41.3, 40.9, 28.9, 28.0, 26.9, 22.7, 19.2, 12.7; HRMS (positive-ion FAB, NBA): calcd for $C_{51}H_{61}N_5O_{11}SiSNa$ 1002.3755, found 1002.3791.

3′-*O*-[4″-((*N*-Dansyl)-5‴-aminopent-1-yl-oxy)-5″-methoxy-2″-nitrophenylmethyl]-2′-deoxy-5′-*O*-(*tert*-butyldiphenylsilyl)-*N*-6-benzoyladenosine (12a): Compound **12a** was prepared by a similar procedure to that used for **12t** but with compound **11a** (174 mg, 0.18 mmol), diethylamine (0.27 g, 3.6 mmol, 381 µL), $[Pd(PPh_3)_4]$ (21 mg, 0.82 mmol), THF (1.0 mL) for a reaction time of 75 min. After the reaction mixture was concentrated to dryness, it was then dissolved in THF (1.0 mL), triethylamine (75 mg, 0.74 mmol), (cat) DMAP, dansyl chloride (50 mg, 0.18 mmol) in 2:1 THF/DMF (1.0 mL) for a reaction time of 6 h. Flash chromatography with 3:2:1 to 3.5:1.5:1 EtOAc/hexanes/CH_2Cl_2 as eluant gave **12a** (47.8 mg, 24 % yield) as a yellow foam. R_f = 0.56 (70 % EtOAc/hexanes); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.50 (s, 1H), 8.50–8.46 (m, 1H), 8.19 (dd, J = 1.2 Hz, 1H), 8.01 (d, J = 7.2 Hz, 1H), 7.62–7.08 (m, 21H), 6.31 (dd, J = 8.7, 6.0 Hz, 1H), 5.98 (s, 2H), 5.62–5.58 (m, 1H), 4.72 (t, J = 6.3 Hz, 1H), 4.20–4.15 (m, 1H), 3.85 (t, J = 3.3 Hz, 2H), 3.79–3.71 (m, 2H), 3.75 (s, 3H), 2.92–2.86 (m, 2H), 2.83 (s, 3H), 2.80–2.70 (m, 1H), 2.54–2.46 (m,

1H), 1.60–1.50 (m, 2H), 1.40–1.32 (m, 2H), 1.29–1.20 (m, 2H), 0.99 (s, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 172.2, 159.9, 153.9, 153.8, 152.4, 152.1, 146.6, 141.8, 139.9, 135.5, 135.4, 134.6, 132.5, 132.3, 131.1, 130.4, 130.0, 129.8, 129.6, 129.5, 128.9, 128.7, 128.3, 127.9, 127.9, 127.0, 123.1, 118.5, 115.1, 110.1, 109.2, 85.0, 84.2, 74.0, 68.8, 63.4, 56.4, 49.8, 45.3, 42.9, 37.9, 29.0, 28.4, 27.9, 26.8, 22.7, 19.1.

3′-*O*-[4″-((*N*-Dansyl)-5‴-aminopent-1-yl-oxy)-5″-methoxy-2″-nitrophenylmethyl]thymidine (13t): Compound **12t** (570 mg, 0.58 mmol), THF (2.9 mL), and TBAF in THF (1M, 870 μL) were stirred for 45 min at 25 °C. The reaction mixture was concentrated, diluted with CH_2Cl_2 (50 mL), then washed with HCl (0.5M, 20 mL), H_2O (20 mL), and brine (20 mL). The organic layer was dried (Na_2SO_4) filtered, and concentrated. The crude product was purified by flash chromatography with EtOAc eluant giving **13t** (397 mg, 92% yield) as a yellow foam. R_f = 0.32 (100% EtOAc); IR ($CDCl_3$): $\tilde{\nu}$ = 3502, 2937, 1703, 1527, 1247 cm^{-1}; ^{1}H NMR ([D_6]acetone, 300 MHz): δ = 8.50 (dt, J = 5.1, 1.2 Hz, 1H), 8.38 (dt, J = 8.7, 0.9 Hz, 1H), 8.20 (dd, J = 7.2, 1.2 Hz, 1H), 7.99 (s, 1H), 7.95 (d, J = 1.5 Hz, 1H), 7.72–7.49 (m, 3H), 7.19 (dd, J = 7.8, 0.9 Hz, 1H), 6.76 (t, J = 6.0 Hz, 1H), 6.72 (s, 1H), 6.32 (t, J = 6.9 Hz, 1H), 5.41 (s, 2H), 4.52–4.46 (brs, 1H), 4.38–4.32 (brs, 1H), 3.97–3.93 (brm, 1H), 3.83 (t, J = 6.6 Hz, 2H), 3.82–3.76 (m, 2H), 3.74 (s, 3H), 2.86–2.94 (m, 2H), 2.81 (s, 6H), 2.29–2.23 (m, 2H), 1.85 (d, J = 0.9 Hz, 3H), 1.55 (p, J = 6.9 Hz, 2H), 1.47–1.25 (m, 4H); ^{13}C NMR ([D_6]acetone, 75 MHz): δ = 163.8, 154.3, 152.6, 151.7, 148.0, 142.3, 137.1, 136.1, 132.6, 130.5, 129.6, 128.5, 127.2, 124.0, 120.0, 115.8, 110.6, 109.7, 109.7, 88.6, 86.6, 71.8, 69.4, 62.5, 56.4, 45.5, 43.4, 41.6, 41.2, 28.9, 23.4, 13.2; HRHRMS (positive-ion FAB, NBA): calcd for $C_{35}H_{43}N_5O_{11}SNa$ 764.2577, found 764.2605.

3′-*O*-[4″-((*N*-Dansyl)-5‴-aminopent-1-yl-oxy)-5″-methoxy-2″-nitrophenylmethyl]-2′-deoxy-*N*-6-benzoyladenosine (13a): Compound **13a** was prepared by a similar procedure to that used for **13t** but with compound **12a** (40 mg, 0.04 mmol), THF (0.5 mL), and TBAF (1M) in THF (55 μL) for a reaction time of 45 min. Chromatography with 100% EtOAc to 10% MeOH/EtOAc as eluant gave **13a** (32 mg, 100% yield) as a yellow foam. R_f = 0.41 (100% EtOAc); IR ($CDCl_3$): $\tilde{\nu}$ = 3513, 2942, 1701, 1579, 1252 cm^{-1}; ^{1}H NMR ([D_6]acetone, 300 MHz): δ = 8.58 (s, 1H), 8.51 (s, 1H), 8.49 (dt, J = 8.4, 1.2 Hz, 1H), 8.36 (dt, J = 8.7, 0.9 Hz, 1H), 8.18 (dd, J = 7.5, 1.5 Hz, 1H), 8.00 (s, 1H), 7.64–7.47 (m, 4H), 7.39–7.30 (m, 2H), 7.25–7.18 (m, 3H), 6.70 (t, J = 6.0 Hz, 1H), 6.44 (dd, J = 7.8, 6.0 Hz, 1H), 5.93 (s, 2H), 4.61–4.56 (m, 1H), 4.50–4.45 (m, 1H), 4.04–4.00 (m, 2H), 3.82–3.74 (m, 2H), 3.76 (s, 3H), 2.94–2.87 (m, 3H), 2.80 (s, 6H), 2.78–2.65 (m, 1H), 2.38–2.31 (m, 1H), 1.53 (p, J = 6.9 Hz, 2H), 1.40 (p, J = 7.5 Hz, 2H), 1.32–1.25 (m, 2H); ^{13}C NMR ([D_6]acetone, 75 MHz): δ = 172.5, 154.9, 154.8, 154.6, 153.2, 152.7, 152.3, 147.9, 141.0, 137.2, 136.8, 131.7, 130.0, 130.5, 129.7, 129.5, 129.3, 128.7, 128.6, 128.3, 124.1, 120.3, 115.9, 111.1, 109.9, 89.6, 86.4, 79.1, 72.6, 69.5, 63.2, 56.7, 53.7, 49.9, 45.5, 43.5, 41.2, 26.2, 23.4, 20.4, 13.8; HRMS (positive-ion FAB, NBA): calcd for $C_{42}H_{46}N_8O_{10}SNa$ 877.2955, found 877.2955.

3′-*O*-[4″-((*N*-Dansyl)-5‴-aminopent-1-yl-oxy)-5″-methoxy-2″-nitrophenylmethyl]thymidine 5′-*O*-triphosphate (14t): Compound **13t** was azeotroped with anhydrous pyridine (3 × 1.0 mL) and stored for 20 h in a vacuum dessicator over P_2O_5. A solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (1.0M 4.3 mg, 21.2 μmol) in dioxane (21 μL) was added to **13t** (14.3 mg, 19.3 μmol) in pyridine (38 μL) and dioxane (116 μL), and the reaction was then stirred at 25 °C under a nitrogen atmosphere for 10 min. A solution of tributylammonium pyrophosphate (0.5M, 13.2 mg, 29 μmol) and tributylamine (14.8 mg, 79 μmol, 19 μL) in DMF (158 μL, 0.5M) was prepared in a glove box. This was added to the **13t** solution and stirred for 15 min. A solution of 1% I_2 (7.8 mg, 31 μmol) in pyridine (764 μL) and H_2O (16 μL) was added and the resulting solution was stirred for 15 min, then quenched with saturated aqueous $NaHSO_3$ (added until the red color dissipated, approximately four drops). The reaction was concentrated to dryness, taken up in H_2O (2 mL) and concentrated to dryness again. The residue was purified by ion exchange chromatography with DEAE-sephadex A-25 and a gradient of H_2O (300 mL) to triethylammonium acetate pH = 7.5 (1.0M, 300 mL) giving **14t** (2.1 mg, 13% yield). ^{31}P NMR (D_2O, 121 MHz): δ = −3.70 (brs), −8.1 (brs), −19.00 (brs); MS (MALDI-TOF): calcd for $C_{35}H_{44}N_5O_{20}P_3S$ 978, found 978.

3′-*O*-[4″-((*N*-Dansyl)-5‴-aminopent-yl-oxy)-5″-methoxy-2″-nitrophenylmethyl]-2′-deoxy-*N*-6-benzoyladenosine 5′-*O*-triphosphate (14a): Compound **13a** was prepared by a similar procedure to that used for **14t**, except that the solution was heated to 60 °C with NH_4OH (5.0 mL) for 5.5 h before ion exchange purification. The product was isolated as an off-white solid (21 mg, 13% yield). ^{31}P NMR (D_2O, 121 MHz): δ = −7.02 (brs), −8.09 (brs), −19.60 (brs); MS (MALDI-TOF): calcd for $C_{35}H_{43}N_8O_{18}P_3S$ 988 , found 988.

3′-*O*-[4″-((*N*-Allyloxycarbonyl)-5‴-aminopent-1-yl-oxy)-5″-methoxy-2″-nitrophenylmethoxycarbonyl]-5′-*O*-(*tert*-butyldiphenylsilyl)thymidine (15t): Compound **9** (144 mg, 0.39 mmol), THF (2 mL), and a solution of phosgene (2.1 mL, 1.9M) in toluene (3.91 mmol) were stirred at 25 °C for 1.5 h. The mixture was concentrated using a rotary evaporator in a hood (CAUTION: phosgene is a toxic gas), and azeotroped with benzene (2 × 2 mL). The residual chlorocarbonate formed was dissolved in THF (4 mL) and cooled to 0 °C. In a separate flame-dried flask, 5′-*O*-(*tert*-butyldiphenylsilyl)thymidine (169 mg, 0.35 mmol), was dissolved in THF (2 mL), and NaH (17 mg, 0.70 mmol, 60% in oil) was added (without removal of oil), and this slurry was stirred at 25 °C for 1 h. The anion was transferred slowly to the chlorocarbonate solution via a cannula, over a 4 min period, then washed in with THF (2 × 1 mL). The reaction mixture was stirred at 0 °C for 30 min then at 25 °C for an additional 1.5 h. The product was purified by flash chromatography with 4:1 CH_2Cl_2/EtOAc eluant which gave **15t** (336 mg, 97% yield) as a white foam. R_f = 0.35 (3 × 50% EtOAc/hexanes); IR (neat): $\tilde{\nu}$ = 3574, 3499, 3297, 2966, 2858, 1961, 1717, 1456 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 9.61 (d, J = 5.7 Hz, 1H), 7.67 (s, 1H), 7.64–7.59 (m, 4H), 7.44 (d, J = 1.2 Hz, 1H), 7.40–7.32 (m, 6H), 7.03 (s, 1H), 6.41 (dd, J = 9.3, 5.1 Hz, 1H), 5.93–5.80 (m, 1H), 5.54 (s, 2H), 5.36 (d, J = 5.7 Hz, 1H), 5.23 (d, J = 17.1 Hz, 1H), 5.13 (d, J = 10.5 Hz, 1H), 4.96 (brs, 1H), 4.55 (brs, 1H), 4.50 (d, J = 5.4 Hz, 1H), 4.16 (s, 1H), 4.03 (t, J = 6.0 Hz, 2H), 4.00–3.90 (m, 2H), 3.95 (s, 3H), 3.17 (q, J = 6.3 Hz, 2H), 2.52 (dd, J = 14.1, 5.1 Hz, 1H), 2.31–2.21 (m, 1H), 1.84 (p, J = 7.2 Hz, 2H), 1.58–1.45 (m, 4H), 1.51 (s, 3H), 1.06 (s, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 163.8, 156.2, 154.0, 153.9, 150.5, 147.7, 139.3, 135.4, 135.0, 134.7, 132.9, 132.7, 131.8, 130.1, 130.0, 128.0, 127.9, 125.9, 117.4, 111.5, 109.7, 109.1, 84.7, 84.1, 78.7, 69.1, 66.6, 65.3, 64.2, 56.5, 40.6, 37.8, 29.5, 28.3, 26.9, 23.0, 19.3, 11.8; HRMS (positive-ion FAB, NBA): calcd for $C_{44}H_{54}N_4O_{13}SiNa$ 897.3354, found 897.3369.

3′-*O*-[4″-((*N*-Allyloxycarbonyl)-5‴-aminopent-1-yl-oxy)-5″-methoxy-2″-nitrophenylmethoxycarbonyl]-2′-deoxy-5′-*O*-(*tert*-butyldiphenylsilyl)-*N*-6-benzoyladenosine (15a): Compound **15a** was prepared by a similar procedure to that used for **15t** but with compound **9** (150 mg, 0.41 mmol), THF (2 mL), phosgene (2.2 mL, 4.1 mmol) for a reaction time of 1.5 h. 6-*N*-Benzoyl-5′-*O*-*tert*-butyldiphenylsilyl-2′-deoxyadenosine (75.5 mg, 0.13 mmol), NaH (15 mg, 0.25 mmol, 60% in oil), and THF (2.0 mL) were mixed in another flask and stirred for 30 min. The nucleoside anion was added slowly with a cannula to the chlorocarbonate solution for a reaction time of 17 h. Chromatography with a 3:1 to 1:2 CH_2Cl_2/EtOAc eluant gave **15a** (57 mg, 41% yield) as a yellow foam. R_f = 0.20 (20% EtOAc/hexanes); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 9.12 (brs, 1H), 8.69 (s, 1H), 8.18 (s, 1H), 7.98 (d, J = 7.2 Hz, 2H), 7.69 (s, 1H), 7.63–7.29 (m, 13H), 7.03 (s, 1H), 6.52 (dd, J = 8.7, 5.4 Hz, 1H), 5.94–5.81 (m, 1H), 5.57 (s, 2H), 5.50 (d, J = 6.0 Hz, 1H), 5.24 (dd, J = 17.4, 1.5 Hz, 1H), 5.15 (dd, J = 10.5, 1.2 Hz, 1H), 4.83 (br, 1H), 4.51 (d, J = 5.4 Hz, 2H), 4.30 (br, 1H), 4.05 (t, J = 6.3 Hz, 2H), 3.99–3.87 (m, 2H), 3.96 (s, 3H), 3.18 (q, J = 6.3 Hz, 2H), 2.94–2.84 (m, 1H), 2.73 (dd, J = 13.2, 5.4 Hz, 1H), 1.86 (p, J = 7.2, 2H), 1.59-1.42 (m, 4H), 1.03 (s, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 165.5, 155.9, 153.6, 153.5, 152.1, 151.1, 149.3, 147.5, 140.6, 139.1, 135.2, 135.1, 135.0, 133.1, 132.5, 132.2, 132.1, 131.9, 129.6, 128.2, 127.5, 127.5, 127.4, 125.3, 123.0, 117.0, 109.8, 108.8, 84.7, 83.9, 78.3, 68.7, 64.9, 63.4, 56.1, 40.3, 37.6, 29.2, 28.0, 26.5, 22.7, 18.8; HRMS (positive-ion FAB, NBA): calcd for $C_{51}H_{57}N_7O_{12}SiNa$ 1010.3732, found 1010.3711.

3′-*O*-[4″-((*N*-Dansyl)-5‴-aminopent-1-yl-oxy)-5″-methoxy-2″-nitrophenylmethoxycarbonyl]-5′-*O*-(*tert*-butyldiphenylsilyl)thymidine (16t): Compound **15t** (821 mg, 0.94 mmol), THF (4.7 mL, 0.26M), $HNEt_2$ (686 mg, 9.4 mmol, 970 μL), and [$Pd(PPh_3)_4$] (108 mg, 0.09 mmol, 10 mol%) was stirred at 25 °C for 2.5 h and concentrated giving the deprotected amine. A sample of the crude amine prepared as above (16 mg, 0.02 mmol) was dissolved in 1:1 toluene/THF (100 μL); NEt_3 (16 μL, 0.12 mmol) and DMAP (cat.) was added, and the solution was stirred at 25 °C for 15 min. A solution of dansyl chloride (5.5 mg, 0.02 mmol) in 1:1 toluene/THF (0.5 mL) was added, and the solution was stirred at 25 °C for 1 h, then concentrated to dryness. The residue was purified by flash chromatography with 1:1:2 to 6:4:5 EtOAc/hexanes/CH_2Cl_2 eluant which gave **16t** (19 mg, 92% yield) as a yellow foam. R_f = 0.36 (60% EtOAc/hexanes); IR (neat):

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$\tilde{\nu}$ = 3387, 2939, 1751, 1682, 1519, 1274 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 9.13–9.04 (brm, 1H), 8.48 (d, J = 8.7 Hz, 1H), 8.25 (d, J = 8.7 Hz, 1H), 8.21 (dd, J = 7.5, 1.2 Hz, 1H), 7.65–7.60 (m, 5H), 7.52–7.34 (m, 9H), 7.13 (d, J = 7.5 Hz, 1H), 7.02 (s,1H), 6.42 (dd, J = 6.9, 5.1 Hz, 1H), 5.52 (s, 2H), 5.38 (d, J = 6.0 Hz, 1H), 4.99–4.92 (brm, 1H), 4.17 (d, J = 1.2 Hz, 1H), 3.97 (qd, J = 11.4, 1.8 Hz, 2H), 3.95 (s, 3H), 3.85 (t, J = 6.3 Hz, 2H), 2.90 (q, J = 6.6 Hz, 2H), 2.84 (s, 6H), 2.53 (dd, J = 13.8, 5.1 Hz, 1H), 2.32-2.22 (m, 1H), 1.64 (p, J = 6.9 Hz, 2H), 1.52 (s, 3H), 1.43 (p, J = 6.9 Hz, 2H), 1.36–1.29 (m, 2H), 1.07 (s, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 163.6, 154.0, 152.0, 150.4, 147.7, 139.3, 135.4, 135.1, 134.8, 134.7, 132.7, 131.8, 130.3, 130.2, 130.1, 129.8, 129.6, 128.3, 128.1, 128.0, 125.9, 123.1, 118.6, 115.1, 111.6, 109.8, 109.1, 84.7, 84.2, 78.8, 68.9, 66.7, 64.3, 56.6, 45.3, 42.9, 37.9, 29.1, 28.0, 26.9, 22.7, 19.3, 11.9; HRMS (positive-ion FAB, NBA): calcd for $C_{52}H_{61}N_5O_{13}SiSNa$ 1046.3650, found 1046.3662.

3′-*O*-[4″-((*N*-Dansyl)-5‴-aminopent-1-yl-oxy)-5″-methoxy-2″-nitrophenyl-methoxycarbonyl]-2′-deoxy-5′-*O*-(*tert*-butyldiphenylsilyl)-*N*-6-benzoyl-adenosine (16a): Compound **16a** was prepared by a similar procedure to that used for **16t** but with compound **15a** (260 mg, 0.26 mmol), $HNEt_2$ (192 mg, 2.6 mmol, 272 µL), $Pd(PPh_3)_4$ (30 mg, 0.03 mmol) in THF (1.3 mL) for a reaction time of 3 h. A portion of the amine prepared above (134 mg, 0.15 mmol) was combined with NEt_3 (90 mg, 0.89 mmol, 124 µL), DMAP (cat.), and dansyl chloride (40 mg, 0.15 mmol) in THF (1.3 mL) and stirred at 25 °C for 17 h. Chromatography with 60 to 90% EtOAc/hexanes eluent, gave **16a** (38 mg, 23% yield) as an off-white foam. R_f = 0.34 (70% EtOAc/hexanes); IR (neat): $\tilde{\nu}$ = 3162, 2934, 2863, 1754, 1612, 1527 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 9.12 (s, 1H), 8.71 (s, 1H), 8.49 (d, J = 8.7 Hz, 1H), 8.25 (d, J = 9.0 Hz, 1H), 8.21 (dd, J = 7.2, 1.2 Hz, 1H), 8.19 (s, 1H), 7.99 (d, J = 7.2 Hz, 2H), 7.67–7.30 (m, 16H), 7.12 (d, J = 6.9 Hz, 1H), 5.52 (dd, J = 9.0, 5.4 Hz, 1H), 5.58 (s, 2H), 5.50 (d, J = 5.7 Hz, 1H), 4.92 (t, J = 6.0 Hz, 1H), 4.34–4.28 (m, 1H), 3.97 (s, 3H), 4.00–3.85 (m, 4H), 2.90 (q, J = 6.6 Hz, 2H), 2.84 (s, 6H), 2.76 (d, J = 4.5 Hz, 1H), 2.71 (d, J = 5.7 Hz, 1H), 1.95 (brs, 1H), 1.65 (p, J = 7.2 Hz, 2H), 1.95 (brs, 1H), 1.65 (p, J = 7.2 Hz, 2H), 1.48–1.28 (m, 4H), 1.04 (s, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 164.6, 153.9, 152.7, 152.0, 151.5, 149.6, 147.8, 140.8, 139.5, 135.5, 135.4, 134.7, 133.1, 132.8, 132.5, 132.3, 132.1, 132.0, 132.0, 131.9, 130.4, 130.0, 129.8, 129.9, 128.8, 128.5, 128.4, 128.3, 127.9, 125.8, 123.2, 118.6, 115.1, 110.2, 109.1, 85.2, 84.2, 78.8, 69.0, 66.8, 63.8, 56.6, 45.3, 42.9, 38.3, 29.1, 28.0, 26.9, 22.8, 19.2; HRMS (positive-ion FAB, NBA): calcd for $C_{59}H_{64}N_8O_{12}SiSNa$ 1159.4030, found 1159.4058.

3′-*O*-[4″-((*N*-Dansyl)-5‴-aminopent-1-yl-oxy)-5″-methoxy-2″-nitrophenyl-methoxycarbonyl]thymidine (17t): Compound **16t** (55.9 mg, 0.06 mmol), THF (275 µL), and TBAF (61 µL) were stirred for 1 h at 0 °C then allowed to warm to room temperature. The reaction was concentrated to dryness, dissolved in CH_2Cl_2 (30 mL), then extracted with aqueous HCl (0.5 M, 2 × 30 mL), and brine (30 mL), dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography with 30:4:1 to 45:5:1 EtOAc/hexanes/CH_2Cl_2 eluant which gave **17t** as a yellow foam (32 mg, 75% yield). R_f = 0.53 (90% EtOAc/hexanes), 0.28 (80% EtOAc/hexanes); IR (neat): $\tilde{\nu}$ = 2925, 2860, 1760, 1695, 1515, 1285 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 9.04 (br, 1H), 8.49 (d, J = 8.4 Hz, 1H), 8.23 (d, J = 9 Hz, 1H), 8.20 (d, J = 7.2 Hz, 1H), 7.60 (s, 1H), 7.49 (m, 3H), 7.12 (d, J = 7.8 Hz, 1H), 7.00 (s, 1H), 6.23 (t, J = 7.2 Hz, 1H), 5.53 (s, 2H), 5.31 (brm, 1H), 4.96 (t, J = 6.0 Hz, 1H), 4.17 (brm, 1H), 3.49 (s, 3H), 3.90 (br, 2H), 3.87 (m, 2H), 2.89 (q, J = 6.3 Hz, 2H), 2.80 (br, 1H), 2.83 (s, 6H), 2.44 (m, 2H), 1.86 (s, 3H), 1.64 (p, J = 7.2 Hz, 2H), 1.41 (m, 2H), 1.32 (m, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 163.7, 154.1, 152.0, 150.4, 147.8, 139.5, 136.5, 134.6, 130.4, 129.8, 129.6, 128.3, 125.7, 123.2, 118.6, 115.0, 111.4, 110.2, 109.2, 86.2, 84.9, 78.9, 69.0, 66.8, 62.6, 56.6, 45.3, 42.9, 37.1, 29.1, 28.0, 22.8, 12.5; HRMS (positive-ion FAB, NBA): calcd for $C_{36}H_{43}N_5O_{13}SNa$ 808.2476, found 808.2510.

3′-*O*-[4″-((*N*-Dansyl)-5‴-aminopent-1-yl-oxy)-5″-methoxy-2″-nitrophenyl-methoxycarbonyl]-2′-deoxy-*N*-6-benzoyladenosine (17a): Compound **17a** was prepared by a similar procedure to that used for **17t** but with compound **16a** (37 mg, 0.03 mmol), THF (330 µL), TBAF (33 µL) for a reaction time of 18 min. Chromatography with 90% EtOAc/hexanes then EtOAc then 5 to 10% MeOH/EtOAc as eluant gave **17a** (22 mg, 75% yield) as an off-white foam. R_f = 0.13 (70% EtOAc/hexanes), 0.52 (100% EtOAc); IR (neat): $\tilde{\nu}$ = 3322, 2989, 2961, 2887, 1766 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 9.09 (br, 1H), 8.74 (s, 1H), 8.50 (d, J = 8.4 Hz, 1H), 8.22 (t, J = 9.0 Hz, 2H), 8.06 (s, 1H), 7.99 (d, J = 7.2 Hz, 2H), 7.62 (s, 1H), 7.62–7.56 (m, 1H), 7.52–7.46 (m, 4H), 7.14 (d, J = 7.5 Hz, 1H), 7.00 (s, 1H), 6.34 (dd, J = 9.6, 5.4 Hz, 1H), 5.92 (brd, J = 9.3 Hz, 1H), 5.56 (s, 2H), 5.50 (d, J = 5.4 Hz, 1H), 4.82 (t, J = 5.7 Hz, 1H), 4.37 (s, 1H), 3.96 (s, 3H), 4.00–3.87 (m, 2H), 3.88 (t, J = 6.6 Hz, 2H), 3.25–3.50 (m, 1H), 2.90 (q, J = 6.3 Hz, 2H), 2.85 (s, 6H), 2.54 (dd, J = 13.8, 5.1 Hz, 1H), 1.90-1.70 (brs, 1H), 1.66 (p, J = 6.9 Hz, 2H), 1.49–1.30 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 153.9, 153.8, 152.1, 150.3, 148.0, 142.5, 139.9, 134.6, 133.3, 133.0, 130.4, 129.8, 129.6, 128.9, 128.3, 127.9, 125.4, 123.2, 118.6, 115.1, 111.0, 109.2, 87.5, 87.1, 80.1, 69.0, 67.0, 63.2, 56.5, 45.4, 42.9, 37.7, 29.1, 28.0, 22.8; HRMS (positive-ion FAB, NBA); calcd for $C_{43}H_{46}N_8O_{12}SNa$ 921.2853, found 921.2858.

3′-*O*-[4″-((*N*-Dansyl)-5‴-aminopent-1-yl-oxy)-5″-methoxy-2″-nitrophenyl-methoxycarbonyl]thymidine 5′-*O*-triphosphate (18t): Compound **17t** (35 mg, 0.04 mmol) was azeotroped with benzene (3 × 2 mL) and stored overnight in a desiccator over P_2O_5. 2-Chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (10 mg, 0.05 mmol), DMF (160 µL) then pyridine (64 µL) were added and the solution was stirred at 25 °C for 15 min. A solution of tributylammonium pyrophosphate (0.5 M, 30 mg, 0.07 mmol) in DMF (132 µL) and tributylamine (44 µL, 0.19 mmol) were added simultaneously, and the mixture was stirred at 25 °C for 11 min. A solution of 1% iodine (18 mg, 0.07 mmol) in pyridine/water (98/2) (1760 µL/36 µL) was added, and the mixture was stirred at 25 °C for 11 min. The reaction was quenched by dropwise addition of 5% aqueous $NaHSO_3$, until the red color disappeared. The reaction mixture was stirred for 1 h at 25 °C after the quench, then concentrated under a high vacuum. The residue was purified by semi-prep C18 RP-HPLC with a gradient of 0 to 100% B (over 70 min) at 10 mL min^{-1} pump rate. The product (R_t = 47 min) **18t** (10.3 mg, 23% yield) was isolated as a pale orange fluffy powder. MS (positive-ion FAB, NBA): calcd for $C_{36}H_{44}N_5O_{22}P_3S$ 1023, found 1023; ^{31}P NMR (D_2O, 121 MHz) δ = −1.46 (d, J = 20.8 Hz), −6.83 (d, J = 18.9 Hz), −17.62 (t, J = 20.2 Hz).

3′-*O*-[4″-((*N*-Dansyl)-5‴-aminopent-1-yl-oxy)-5″-methoxy-2″-nitrophenyl-methoxycarbonyl]-2′-deoxyadenosine 5′-*O*-triphosphate (18a): Compound **18a** was prepared by a similar procedure to that used for **18t** but with compound **17a** (22 mg, 25 µmol), 2-Chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (5.5 mg, 27 µmol), DMF (100 µL), and pyridine (40 µL) for a reaction time of 11 min. A solution of tributylammonium pyrophosphate (0.5 M, 17 mg, 37 µmol,) in DMF (80 µL) and tributylamine (30 µL, 126 µmol) were added simultaneously and the resulting solution was stirred for 14 min. A solution of 1% iodine (11 mg, 39 µmol) in 98:2 pyridine/water was added, and the solution was stirred for 15 min. The reaction was quenched by dropwise addition of 10% aqueous $NaHSO_3$, until the red color disappeared. The solution was stirred at 25 °C for 10 min after the quench, then concentrated under a high vacuum. The residue was dissolved in 29% NH_4OH (10 mL) and heated to 60 °C for 2 h then concentrated to dryness. Purification of the residue was by semi-prep C18 RP-HPLC with 0 to 100% B over 70 min at 10 mL min^{-1}. The product **18a** was collected as an off-white solid (4 mg, 18% yield). MS (MALDI-TOF): calcd for $C_{36}H_{43}N_8O_{20}P_3S$ 1032, found 1032.

Incorporation assays: Klenow fragment of DNA polymerase I was purchased from Boehringer Mannheim. *Bst* DNA polymerase, Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase, and $Vent_R$ (exo −) DNA polymerase were purchased from New England Bio-Labs. r*Tth* DNA polymerase was purchased from Pharmacia. Ampli Taq DNA polymerase and Ampli Taq DNA polymerase, FS were purchased from Perkin Elmer. Fluorescein-labeled universal primer (5′-TGTAAAAC-GACGGCCAGT) and oligonucleotide template (5′-*TACGGAGGTG-G*ACTGGCCGTCGTTTTACA) were purchased from Life Technologies (italic sequence represents that part of the oligonucleotide that remains for amplification).

The single-stranded template (1 pmol) was mixed with 5′-fluorescein tagged universal primer (1 pmol) in the corresponding buffer solution. This mixture was warmed to 80 °C for 7 min and then allowed to reach room temperature slowly. Extension of the primer-template complex was performed in the absence of the natural nucleotide when either a ddNTP or a 3′-modified nucleotide was tested. Each reaction mixture contained 5 µL of the annealed DNA duplex and 5 µL of the corresponding mixtures containing the enzyme and the nucleotides in the specific buffer. Conditions for all the enzymatic reactions can be found in reference 38 except for those of Ampli Taq DNA polymerase, FS. In this case, the buffer was 80 mM Tris, 2 mM $MgCl_2$, pH 9.0; dNTPs were used at a final concentration of 0.1 µM, and ddNTPs at a final concentration of 0.5 µM.

The incubation temperature was 65 °C in the presence of 1 unit of the enzyme.

The reactions were incubated for 10 min and then stopped by the addition of 5 µL of loading solution containing 98% deionized formamide, EDTA (10mM, pH 8.0) bromophenol blue (0.025%) and xylene cyanol (0.025%). All samples were heated up to 85 °C for 7 min, chilled on ice, and 5 µL of each reaction mixture loaded on a 20% acrylamide gel. After electrophoresis at 35 W the gel was transferred to a special 10″ × 8.25″ glass plate and scanned with a Molecular Dynamics Fluoroimager 595. The scanned gel was then analyzed with fragment analysis software provided with the instrument.

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