

# An Efficient Method for the Construction of Functionalized DNA Bearing Amino Acid Groups through Cross-Coupling Reactions of Nucleoside Triphosphates Followed by Primer Extension or PCR

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**Abstract:** Single step aqueous cross coupling reactions of nucleobase halogenated 2' deoxynucleosides (8 bromo 2' deoxyadenosine, 7 iodo 7 deaza 2' deoxyadenosine, or 5 iodo 2' deoxy uridine) or their 5' triphosphates with 4 boronophenylalanine or 4 ethynylphenylalanine have been developed and used for efficient synthesis of modified 2' deoxynucleoside triphosphates (dNTPs) bearing amino acid groups. These dNTPs were then tested as substrates for DNA polymerases for construction of functionalized DNA through primer extension and PCR.

While 8 substituted adenosine triphosphates were poor substrates for DNA polymerases, the corresponding 7 substituted 7 deazaadenine and 5 substituted uracil nucleotides were efficiently incorporated in place of dATP or dTTP, respectively, by *Pwo* (*Pyrococcus woesei*) DNA polymerase. Nucleotides bearing the amino acid connected through the less bulky acetylene linker

were incorporated more efficiently than those directly linked through a more bulky phenylene group. In addition, combinations of modified dATPs and dTTPs were incorporated by *Pwo* polymerase. Novel functionalized DNA duplexes bearing amino acid moieties were prepared by this two step approach. PCR can be used for amplification of duplexes bearing large number of modifications, while primer extension is suitable for introduction of just one or several modifications in a single DNA strand.

**Keywords:** cross coupling · DNA · nucleosides · nucleotides · polymerase chain reaction

## Introduction

Functional nucleic acids (e.g., DNA aptamers, DNazymes, etc.) are attracting growing interest due to their potential applications in chemical biology, bioanalysis, or nanotechnology.<sup>[1]</sup> To expand the scope of these applications, the introduction of a variety of functional groups into DNA (especially into the nucleobase components) is desirable. Apart from classical oligonucleotide synthesis<sup>[2]</sup> using functionalized nucleoside phosphoramidites or post synthetic oligonu-

cleotide modifications,<sup>[3]</sup> nucleobase functionalized DNA can also be prepared by incorporation of modified nucleoside triphosphates (dNTPs) with the aid of DNA polymerases.<sup>[4-10]</sup> This approach using PCR incorporation of functionalized dNTPs is particularly interesting because of its potential for use in *in vitro* selection. Recently, several types of modified DNA bearing diverse groups, for example, aminoalkynyl or aminoalkyl<sup>[4-6]</sup> and several types of attached functional molecules, for example, biotin,<sup>[6]</sup> acridones,<sup>[7]</sup> ferrocene,<sup>[8]</sup> amino acids,<sup>[9]</sup> carbohydrates,<sup>[10]</sup> or fluorescein labels,<sup>[11]</sup> have been prepared from the corresponding modified dNTPs by this methodology. The dNTP building blocks are usually prepared<sup>[4-9]</sup> by troublesome and laborious triphosphorylation of the corresponding modified nucleosides, in which the functional groups usually have to be protected and deprotected.

We have very recently developed an efficient and rapid single step synthesis<sup>[12]</sup> of modified nucleosides, nucleotides, and nucleoside triphosphates bearing phenylalanine moieties through aqueous phase cross coupling reactions<sup>[13]</sup> between the corresponding unprotected 8 bromoadenine bio molecules and 4 boronophenylalanine. Although an exam

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ple of a Sonogashira reaction of 5 iodo dUTP had been known previously,<sup>[11]</sup> this was the first example of direct cross coupling modification of a purine dNTP. Soon after, other groups reported similar Suzuki Miyaura reactions between 8 bromoGTP and boronic acids<sup>[14]</sup> and coupling of a chloromercury derivative of dUTP with carbohydrate conjugated acrylamide derivatives.<sup>[10]</sup> Here we report on the extension of the aqueous phase Suzuki Miyaura cross coupling methodology to 7 iodo 7 deazaadenine and 5 iodouracil nucleosides and dNTPs, on the development of related aqueous phase Sonogashira reactions with an amino acid linked acetylene, and on the incorporation of these modified dNTPs by DNA polymerases.

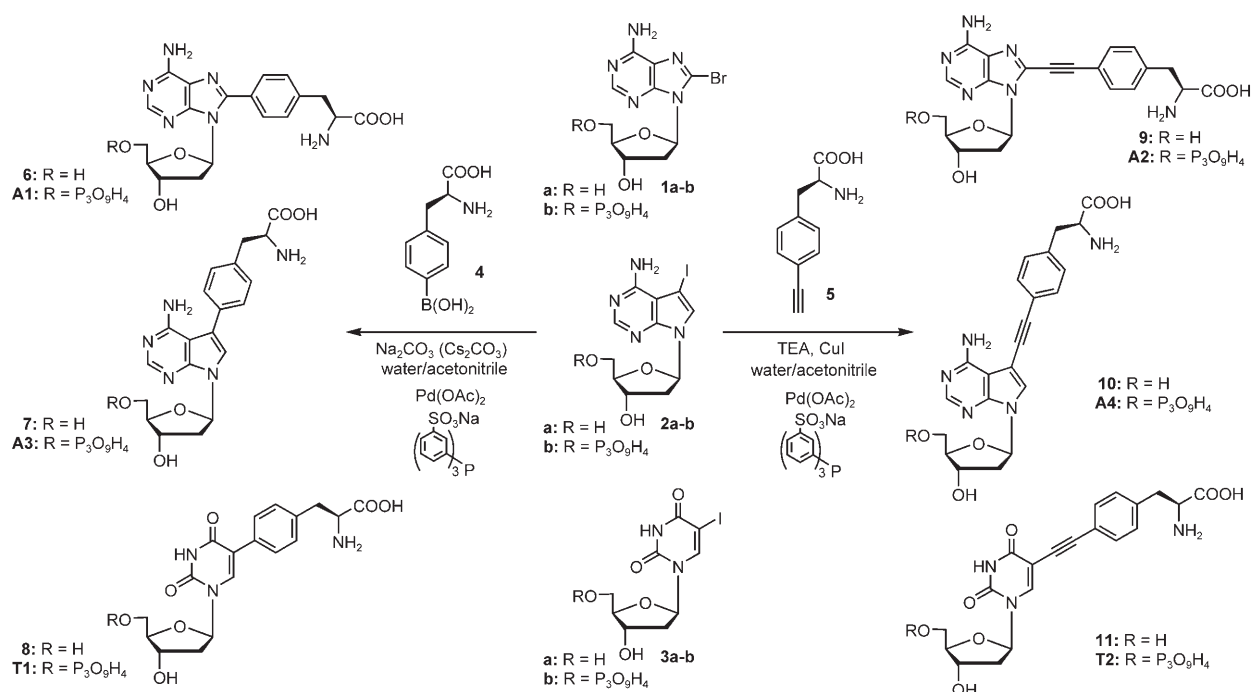
## Results and Discussion

**Synthesis of modified nucleosides and dNTPs:** Cross coupling reactions are efficient tools for the introduction of carbon substituents into nucleobases and nucleosides.<sup>[15]</sup> Until recently, the reactions were usually performed in organic solvents on protected nucleosides. Only the development of Shaughnessy's<sup>[13]</sup> aqueous phase cross coupling reactions using the water soluble  $P(m\text{C}_6\text{H}_4\text{SO}_3\text{Na})_3$  (TPPTS) ligand enabled efficient direct modification of nucleosides. We have applied this aqueous methodology to the synthesis of adenosine phenylalanine conjugates<sup>[16]</sup> and diverse aryl purine bases<sup>[17]</sup> through Suzuki Miyaura cross coupling. Recently, the methodology has been extended to reactions of free adenosine monophosphates and to very labile adenosine triphosphates.<sup>[12]</sup>

Treatment of 8 bromo 2' deoxyadenosine (**1a**) with 4 boronophenylalanine proceeded very smoothly to give conjugate **6** in good yield after RP HPLC isolation (Scheme 1).<sup>[12]</sup> A major problem with the cross coupling reactions of dNTPs was the hydrolysis of triphosphates during the course of the reaction, so the reaction conditions had to be optimized to shorten the reaction times. The reaction with 8 bromo dATP (**1b**) required a higher reaction temperature (125°C) and the use of  $\text{Cs}_2\text{CO}_3$  (Scheme 1) to reach completion within 20 min. The product **A1** was also isolated by RP HPLC in good yield (Table 1, entry 4).<sup>[12]</sup>

In order to extend a scope of this methodology to other nucleosides and nucleoside triphosphates directly applicable to DNA polymerase incorporation, we investigated the aqueous phase Suzuki Miyaura reactions of 7 deaza 2' deoxy 7 iodoadenosine (**2a**) and the corresponding triphosphate **2b**, as well as those of 2' deoxy 5 iodouridine (**3a**) and its triphosphate **3b** with boronophenylalanine **4** (Scheme 1). Both the 7 substituted 7 deazapurine dNTPs<sup>[5]</sup> and the 5 substituted pyrimidine<sup>[4, 10]</sup> dNTPs are known to be tolerated as substrates by some DNA polymerases and to be incorporated into DNA. The reactions of the model halo nucleosides **2a** and **3a** proceeded smoothly at 100°C in the presence of  $\text{Na}_2\text{CO}_3$  base to give the desired products **7** and **8**, respectively, in even higher yields than obtained with 8 substituted adenosine **6** (Table 1, entries 2 and 3).

The corresponding reactions of boronic acid **4** with dNTPs **2b** and **3b** were also carried out, at a slightly higher temperature (110°C) and in the presence of  $\text{Cs}_2\text{CO}_3$  base, to give the phenylalanine dNTPs conjugates **A3** and **T1** in good preparative yields (Table 1, entries 5 and 6) after HPLC isolation.



Scheme 1. Synthesis of modified nucleosides and dNTPs.

Table 1. Synthesis of modified nucleosides and dNTPs by cross coupling reactions.

| Entry | dN/<br>dNTP | AA <sup>[a]</sup> | Additive                        | T, reaction<br>time | Product   | Yield<br>[%] |
|-------|-------------|-------------------|---------------------------------|---------------------|-----------|--------------|
| 1     | <b>1a</b>   | <b>4</b>          | Na <sub>2</sub> CO <sub>3</sub> | 90°C, 2 h           | <b>6</b>  | 75           |
| 2     | <b>2a</b>   | <b>4</b>          | Na <sub>2</sub> CO <sub>3</sub> | 100°C, 1 h          | <b>7</b>  | 89           |
| 3     | <b>3a</b>   | <b>4</b>          | Na <sub>2</sub> CO <sub>3</sub> | 100°C, 1.5 h        | <b>8</b>  | 78           |
| 4     | <b>1b</b>   | <b>4</b>          | Cs <sub>2</sub> CO <sub>3</sub> | 125°C, 20 min       | <b>A1</b> | 55           |
| 5     | <b>2b</b>   | <b>4</b>          | Cs <sub>2</sub> CO <sub>3</sub> | 110°C, 30 min       | <b>A3</b> | 66           |
| 6     | <b>3b</b>   | <b>4</b>          | Cs <sub>2</sub> CO <sub>3</sub> | 110°C, 30 min       | <b>T1</b> | 56           |
| 7     | <b>1a</b>   | <b>5</b>          | CuI,<br>TEA                     | 60°C, 55 min        | <b>9</b>  | 61           |
| 8     | <b>2a</b>   | <b>5</b>          | CuI,<br>TEA                     | 60°C, 50 min        | <b>10</b> | 94           |
| 9     | <b>3a</b>   | <b>5</b>          | CuI,<br>TEA                     | 60°C, 45 min        | <b>11</b> | 70           |
| 10    | <b>1b</b>   | <b>5</b>          | CuI,<br>TEA                     | 60°C, 1 h           | <b>A2</b> | 61           |
| 11    | <b>2b</b>   | <b>5</b>          | CuI,<br>TEA                     | 60°C, 45 min        | <b>A4</b> | 67           |
| 12    | <b>3b</b>   | <b>5</b>          | CuI,<br>TEA                     | 60°C, 30 min        | <b>T2</b> | 66           |

[a] Amino acid reagent.

As well as nucleoside/dNTP phenylalanine conjugates with the amino acid linked directly through a bulky phenyl group, we also explored the synthesis of a series of conjugates extended by a less bulky acetylene tether (products **9**, **11**, **A2**, **A4**, and **T2**). This type of attachment was designed as an analogy to the modified dNTPs successfully recognized by DNA polymerases with a “nucleobase acetylene linker functionality” structural pattern.<sup>[4–11]</sup> The extended conjugates were prepared by Sonogashira cross coupling reactions of halonucleosides **1a**, **3a** and dNTPs **1b**, **3b** with **4** (ethynyl)phenylalanine (**5**; Scheme 1). Analogously with the Suzuki reactions, the Sonogashira reactions were performed in water/acetonitrile mixtures and with the same water soluble catalytic system (Pd(OAc)<sub>2</sub>/TPPTS), together with triethylamine (TEA) as a base and CuI as an additive. Reactions both with nucleosides and with dNTPs proceeded smoothly at 60°C to give the corresponding products **9**, **11**, **A2**, **A4**, and **T2** in very good yields (Table 1, entries 7–12) within 30 min to 1 h.

**Incorporation of modified dNTPs by DNA polymerases:** All the novel functionalized dATPs **A1**, **A4** and dTTPs **T1** and **T2** were examined as substrates for several types of thermostable DNA polymerases in primer extension experiments and polymerase chain reactions (PCRs). In the initial model PCR experiments, we tested *Thermus aquaticus* (*Taq*), *Thermococcus litoralis* (*Vent (exo<sup>-</sup>)*), and *Pyrococcus woesei* (*Pwo*) DNA polymerases, which had been shown<sup>[5]</sup> to incorporate a broad spectrum of modified dNTPs efficiently. Since *Pwo* DNA polymerase showed the most promising results, further optimization was performed only with this enzyme. The best conditions found involved the addition of 2% DMSO, higher enzyme concentrations, and increasing of the denaturing temperature (98°C) within PCR cycling (see below).

The formation of functionalized DNA in primer extension experiments with *Pwo* DNA polymerase was studied with a 35 mer template in the presence of <sup>32</sup>P labeled 25 mer primer, the modified dATP (**A1**, **A4**) or dTTP (**T1** or **T2**), and three additional natural dNTPs. The reaction products derived from primer extension were tracked by denaturing polyacrylamide gel electrophoresis (PAGE) and phosphorimaging analysis (Figure 1). No incorporation of **A1** and **A2**

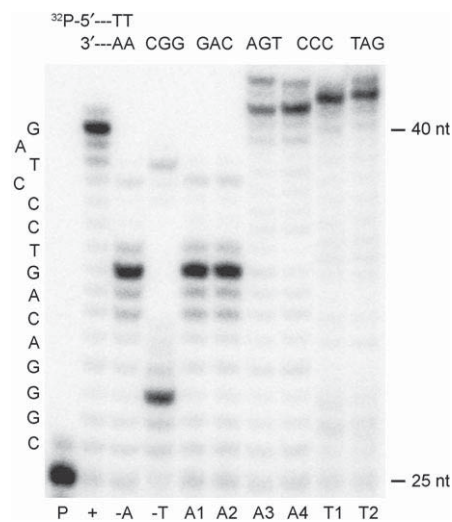


Figure 1. Primer extension with *Pwo* DNA polymerase. 5' <sup>32</sup>P end labeled primer template (sequences as indicated in the figure) was incubated with different combinations of natural and functionalized dNTPs. P: Primer; +: natural dNTPs; -A: dTTP, dCTP, dGTP; -T: dATP, dCTP, dGTP; A1: **A1**, dTTP, dCTP, dGTP; A2: **A2**, dTTP, dCTP, dGTP; A3: **A3**, dTTP, dCTP, dGTP; A4: **A4**, dTTP, dCTP, dGTP; T1: **T1**, dATP, dCTP, dGTP; T2: **T2**, dATP, dCTP, dGTP.

was observed in these experiments, but the other two modified dATPs derived from 7 deazaadenine (**A3** and **A4**), as well as both modified dTTPs (**T1** and **T2**), were incorporated, resulting in full length reaction products (Figure 1). However, the formation of an additional, more slowly migrating band was observed to a varied extent in cases in which the primer was fully extended. 3' 5' exonuclease deficient DNA polymerases are known to add an additional nucleotide in an untemplated fashion under certain circumstances, resulting in an extra band after PAGE analysis.<sup>[18]</sup> Since the generated DNA is highly modified, the 3' 5' exonuclease of *Pwo* DNA polymerase might be functioning less efficiently, which might well be the cause of the observed effect. Alternatively, the effects might arise from secondary structures with different stability or from higher aggregates that cannot be resolved under the conditions applied in standard denaturing PAGE as discussed previously.<sup>[5a]</sup>

These promising results prompted us to attempt simultaneous incorporation of modified adenine and thymine into one DNA strand. The primer extension experiments were performed with combinations of functionalized dATP and dTTP (**A3T1**, **A4T1**, **A3T2**, and **A4T2**) in the presence of





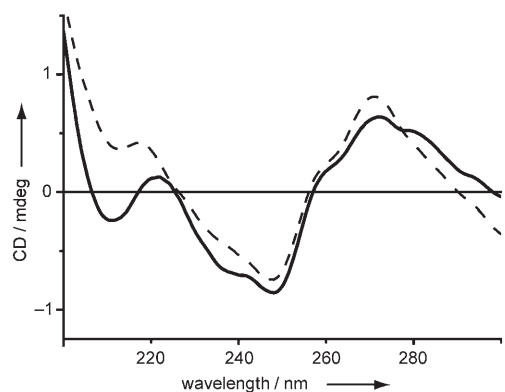


Figure 5. CD spectra of **T2** modified and natural 98 mer duplex. Solid line: unmodified DNA. Dashed line: duplex containing **T2**.

Miyaura or Sonogashira cross coupling reactions with amino acid based boronic acids or acetylenes has been developed. This method does not require protection of any of the reaction components and allows expeditious and simple modifications of dNTPs with functionalized aryl or alkynyl groups. In our view, this approach is more practical than the alternative phosphorylation of functionalized nucleosides used by other groups.<sup>[4-9]</sup> The methodology was used for the synthesis of six types of dNTPs: 8 substituted dATP, 7 substituted 7 deaza dATP, and 5 substituted dUTP bearing phenylalanine moieties linked either directly through a phenyl ring or through an acetylene tether. All the novel dNTPs were tested as substrates for DNA polymerases in the enzymatic construction of functionalized DNA by primer extension and PCR. *Pwo* DNA polymerase was found to be the best suited enzyme, capable of incorporation of 7 substituted 7 deaza dATP (**A3** and **A4**) and 5 substituted dUTP (**T1** and **T2**), while 8 substituted dATP derivatives (**A1** and **A2**) were not suitable as substrates, in accordance with reports by Famulok and colleagues. Other DNA polymerases were not effective, with the sole exception of *Vent* (*exo*<sup>-</sup>) DNA polymerase, which incorporated **T2** in PCR experiment. Simultaneous incorporation of all four combinations of modified A (**A3** or **A4**) and T (**T1** or **T2**) was efficient in primer extension, while in PCR only **A3T2** and **A4T2** combinations were successful. A 98 mer DNA duplex containing **T2** modifications was characterized by  $T_m$  and CD spectroscopy and showed no significant deviation from the stability and B form DNA features of the natural duplex.

The combination of the aqueous phase cross coupling reactions of halogenated dNTPs with enzymatic incorporation by DNA polymerases is a novel and efficient two step approach for the construction of functionalized DNA. Since the modification is introduced in the last chemical step, just prior to enzymatic incorporation, this methodology is well suited for the generation of a series of diverse modified nucleic acids. Primer extension can be efficiently used for incorporation of several modifications to specific positions in a single DNA strand, while PCR is suitable for construction and amplification of DNA duplexes with high degrees of

modification. Further studies are currently focussing on extension of the scope of the cross coupling reactions of dNTPs to other types of reagents and functional groups and on construction of DNA bearing other biorelevant and/or useful functions.

## Experimental Section

NMR spectra were measured on Bruker AMX 3 400 (400 MHz for <sup>1</sup>H and 100.6 MHz for <sup>13</sup>C nuclei) and Bruker DRX 500 (500 MHz for <sup>1</sup>H and 125.8 MHz for <sup>13</sup>C) instruments in D<sub>2</sub>O (referenced to dioxane as internal standard,  $\delta_H = 3.75$  ppm,  $\delta_C = 67.19$  ppm) or in [D<sub>6</sub>]DMSO (referenced to the residual solvent signal). Chemical shifts are given in ppm ( $\delta$  scale), coupling constants ( $J$ ) in Hz. Complete assignment of all NMR signals was achieved by use of a combination of H,H COSY, H,C HSQC, and H,C HMBC experiments. Mass spectra were measured on a ZAB EQ (VG Analytical) spectrometer by FAB (ionization by Xe, accelerating voltage 8 kV, glycerol+thioglycerol matrix) or on an LCQ classic (Thermo Finnigan) spectrometer by ES<sup>-</sup>. Optical rotations were measured at 25°C on an Autopol IV (Rudolph Research Analytical) polarimeter,  $[\alpha]_D^{20}$  values are given in 10<sup>-1</sup> deg cm<sup>2</sup> g<sup>-1</sup>. H<sub>2</sub>O/acetonitrile mixtures were degassed in vacuo and stored under argon. Preparative HPLC separations were performed on a column packed with 10  $\mu$ m C18 reversed phase (Phenomenex, Luna C18(2)). Known starting compounds were either purchased (**3a** from Berry and **4** from Frontier Scientific) or prepared by literature procedures: **1a**,<sup>[19]</sup> **1b**,<sup>[20]</sup> **2a**,<sup>[21]</sup> and **3b**.<sup>[22]</sup> Synthesis and characterization data for compounds **6** and **A1** were reported previously.<sup>[12]</sup>

**(S)-4-Ethynylphenylalanine (5):** A water/acetonitrile mixture 2:1 (30 mL) was added through a septum to an argon purged vial containing (S) 4 iodophenylalanine (730 mg, 2.5 mmol), trimethylsilylacetylene (2.5 mL, 17.7 mmol), Pd(OAc)<sub>2</sub> (22.4 mg, 0.1 mmol), TPPTS (228 mg, 0.4 mmol), CuI (48 mg, 0.25 mmol), and TEA (1.2 mL, 8.6 mmol). The mixture was stirred at ambient temperature for 20 h. Products were isolated from the crude reaction mixture (after filtration) by HPLC on a C18 column with use of a linear gradient of 0.3% AcOH in H<sub>2</sub>O to 0.3% AcOH in MeOH as eluent. Several co distillations with water followed by freeze drying from water gave **5** (118 mg, 25%) as white solid that was directly used in the following step. <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O, ref<sub>dioxane</sub> = 3.75 ppm):  $\delta = 3.13$  (dd,  $J_{gem} = 14.6$ ,  $J_{vic} = 7.9$  Hz, 1H; bCH<sub>2</sub>), 3.28 (dd,  $J_{gem} = 14.6$ ,  $J_{vic} = 5.4$  Hz, 1H; aCH<sub>2</sub>), 3.52 (s, 1H; HC=C), 3.98 (dd,  $J_{vic} = 7.9$ , 5.4 Hz, 1H; CH), 7.30 (m, 2H; H *m* phenylene), 7.54 ppm (m, 2H; H *o* phenylene); <sup>13</sup>C NMR (125.8 MHz, D<sub>2</sub>O, ref<sub>dioxane</sub> = 69.3 ppm):  $\delta = 38.97$  (CH<sub>2</sub>), 58.50 (CH), 81.16 (C=CH), 86.28 (C=CH), 123.36 (C *i* phenylene), 132.23 (CH *m* phenylene), 135.35 (CH *o* phenylene), 139.04 (C *p* phenylene), 178.58 ppm (CO).

**9-(2-Deoxy- $\beta$ -D-erythro-pentofuranosyl)-7-iodo-7-deazapurine 5'-O-tri-phosphate (2b):** Nucleoside **2a** (113 mg, 0.3 mmol) was suspended in trimethyl phosphate (0.75 mL) at 0°C and POCl<sub>3</sub> (35  $\mu$ L, 0.36 mmol) was added. After the mixture had been stirred at 0°C for 45 min, an ice cooled solution of (NHBu<sub>3</sub>)<sub>2</sub>H<sub>2</sub>P<sub>2</sub>O<sub>7</sub> (820 mg, 1.5 mmol) and Bu<sub>3</sub>N (0.3 mL, 1.25 mmol) in dry DMF (3 mL) was added and the mixture was stirred at 0°C for another 30 min. The reaction was then quenched by addition of aqueous TEAB (1M, 2 mL), the solvents were evaporated in vacuo, and the residue was co distilled three times with water. The product was isolated on a DEAE Sephadex column (150 mL) with elution with a gradient of 0 to 1.2M TEAB, evaporated, co distilled with water (3 $\times$ ), and lyophilized to yield a white powder (110 mg, 38%). <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O+Et<sub>3</sub>N, ref<sub>dioxane</sub> = 3.75 ppm):  $\delta = 1.25$  (t,  $J_{vic} = 7.3$  Hz, 27H; CH<sub>3</sub>CH<sub>2</sub>N), 2.51 (ddd,  $J_{gem} = 14.0$ ,  $J_{2,b,1'} = 6.3$ ,  $J_{2,b,3'} = 3.6$  Hz, 1H; H 2'b), 2.69 (ddd,  $J_{gem} = 14.0$ ,  $J_{2,a,1'} = 7.6$ ,  $J_{2,a,3'} = 6.3$  Hz, 1H; H 2'a), 3.15 (q,  $J_{vic} = 7.3$  Hz, 18H; CH<sub>3</sub>CH<sub>2</sub>N), 4.07 4.23 (m, 3H; H 4' and H 5'), 4.76 (dt,  $J_{3,2'} = 6.3$ , 3.6,  $J_{3,4'} = 3.5$  Hz, 1H; H 3'), 6.52 (t,  $J_{1,2'} = 7.6$ , 6.3 Hz, 1H; H 1'), 7.64 (s, 1H; H 8), 8.01 ppm (s, 1H; H 2); <sup>13</sup>C NMR (100.6 MHz, D<sub>2</sub>O+Et<sub>3</sub>N, ref<sub>dioxane</sub> = 69.3 ppm):  $\delta = 10.95$  (CH<sub>3</sub>CH<sub>2</sub>N), 41.05 (CH<sub>2</sub> 2'), 49.26 (CH<sub>3</sub>CH<sub>2</sub>N), 54.49 (C 7), 68.15 (d,  $J_{C,P} = 5$  Hz; CH<sub>2</sub> 5'), 73.56 (CH

3'), 85.35 (CH 1'), 87.94 (d,  $J_{C,P}=9$  Hz; CH 4'), 106.37 (C 5), 129.74 (CH 8), 151.67 (C 4), 154.21 (CH 2), 159.56 ppm (C 6);  $^{31}\text{P}$  ( $^1\text{H}$  dec.) NMR (162 MHz,  $\text{D}_2\text{O}+\text{Et}_3\text{N}$ ,  $\text{ref}_{\text{H}_3\text{PO}_4}=0$  ppm):  $\delta=-22.65$  (t,  $J=20.9$ , 19.8 Hz;  $\text{P}_\beta$ ),  $-11.36$  (d,  $J=19.8$  Hz;  $\text{P}_\alpha$ ),  $-6.54$  ppm (d,  $J=20.9$  Hz,  $\text{P}_\gamma$ ); MS (ES $^-$ ):  $m/z$ : 615 (100)  $[M-1]^-$ , 517 (75)  $[M-\text{PO}_3\text{H}_2-1]^-$ ; HRMS (ES $^-$ ):  $m/z$ : calcd for  $\text{C}_{11}\text{H}_{15}\text{N}_4\text{O}_{12}\text{P}_3$ : 614.8944; found: 614.8952.

**Synthesis of modified nucleosides—Suzuki–Miyaura cross-coupling—General Procedure A:** A water/acetonitrile mixture 2:1 (1.2 mL) was added through a septum to an argon purged vial containing halogenated nucleosides **1a** **3a** (0.1 mmol), boronic acid **4** (27 mg, 0.13 mmol), Pd(OAc) $_2$  (1.12 mg, 0.005 mmol), TPPTS (14.2 mg, 0.025 mmol), and  $\text{Na}_2\text{CO}_3$  (32 mg, 0.3 mmol). The mixture was stirred with heating (for temperature and reaction time see Table 1). The products were isolated from the crude reaction mixture by HPLC on a C18 column with use of a linear gradient of 0.3% AcOH in  $\text{H}_2\text{O}$  to 0.3% AcOH in MeOH as eluent. Several co distillations with water, followed by freeze drying from water, gave the products as white solids.

**Synthesis of modified nucleosides—Sonogashira cross-coupling—General Procedure B:** A water/acetonitrile mixture (2:1, 1.2 mL) was added through a septum to an argon purged vial containing halogenated nucleosides **1a** **3a** (0.1 mmol), acetylene **5** (28 mg, 0.15 mmol), Pd(OAc) $_2$  (1.12 mg, 0.005 mmol), TPPTS (11.4 mg, 0.02 mmol), CuI (2 mg, 0.1 mmol), and TEA (80  $\mu\text{L}$ , 0.57 mmol). The mixture was stirred at 60°C (for reaction time see Table 1). Products were isolated from the crude reaction mixture by HPLC on a C18 column with use of a linear gradient of 0.3% AcOH in  $\text{H}_2\text{O}$  to 0.3% AcOH in MeOH as eluent. Several co distillations with water, followed by freeze drying from water, gave the products as white solids.

**Synthesis of modified dNTPs—Suzuki–Miyaura cross-coupling—General Procedure C:** A water/acetonitrile mixture (2:1, 0.5 mL) was added through a septum to an argon purged vial containing the halogenated dNTP **1b** **3b** (0.05 mmol), boronic acid **4** (20 mg, 0.1 mmol), and  $\text{Cs}_2\text{CO}_3$  (81 mg, 0.25 mmol). After the solids had dissolved, a solution of Pd(OAc) $_2$  (1.12 mg, 0.005 mmol) and TPPTS (14.2 mg, 0.025 mmol) in water/acetonitrile (2:1, 0.3 mL) was added and the mixture was stirred with heating (for temperature and reaction time see Table 1). The products were isolated from the crude reaction mixture by HPLC on a C18 column with use of a linear gradient of 0.1 M TEAB (triethylammonium bicarbonate) in  $\text{H}_2\text{O}$  to 0.1 M TEAB in  $\text{H}_2\text{O}/\text{MeOH}$  1:1 as eluent. Several co distillations with water, followed by freeze drying from water, gave the products as white solids.

**Synthesis of modified dNTPs—Sonogashira cross-coupling—General Procedure D:** A water/acetonitrile mixture (2:1, 0.5 mL) was added through a septum to an argon purged vial containing the halo dNTP **1b** **3b** (0.05 mmol), acetylene **5** (19 mg, 0.1 mmol), CuI (2 mg, 0.01 mmol), and TEA (50  $\mu\text{L}$ , 0.36 mmol). After the solids had dissolved, a solution of Pd(OAc) $_2$  (1.12 mg, 0.005 mmol) and TPPTS (14.2 mg, 0.025 mmol) in water/acetonitrile (2:1, 0.3 mL) was added and the mixture was stirred with heating at 60°C (for reaction time see Table 1). The products were isolated from the crude reaction mixture by HPLC on a C18 column with use of a linear gradient of 0.1 M TEAB in  $\text{H}_2\text{O}$  to 0.1 M TEAB in  $\text{H}_2\text{O}/\text{MeOH}$  (1:1) as eluent. Several co distillations with water, followed by freeze drying from water, gave the products as white solids.

**(S)-2-Amino-3-[4-[[6-amino-9-(2-deoxy- $\beta$ -D-erythro-pentofuranosyl)-purin-8-yl]ethynyl]phenyl]propanoic acid (9):** This compound was prepared by Method B from **1a**, yield 61%. [ $\alpha$ ] $_{\text{D}}^{20}=-37.7$  ( $c=1.60$ , DMSO);  $^1\text{H}$  NMR (400 MHz,  $[\text{D}_6]\text{DMSO}$ ):  $\delta=2.26$  (ddd,  $J_{\text{gem}}=13.2$ ,  $J_{2b,1'}=6.5$ ,  $J_{2b,3'}=2.6$  Hz, 1H; H 2'b), 2.94 (dd,  $J_{\text{gem}}=14.0$ ,  $J_{\text{vic}}=8.1$  Hz, 1H; bCH $_2$ ), 3.15 (ddd,  $J_{\text{gem}}=13.2$ ,  $J_{2a,1'}=7.9$ ,  $J_{2a,3'}=6.2$  Hz, 1H; H 2'a), 3.19 (dd,  $J_{\text{gem}}=14.0$ ,  $J_{\text{vic}}=4.8$  Hz, 1H; aCH $_2$ ), 3.45 (dd,  $J_{\text{vic}}=8.1$ , 4.8 Hz, 1H; CH), 3.51 (dd,  $J_{\text{gem}}=11.8$ ,  $J_{5b,4'}=4.9$  Hz, 1H; H 5'b), 3.68 (dd,  $J_{\text{gem}}=11.8$ ,  $J_{5a,4'}=4.4$  Hz, 1H; H 5'a), 3.91 (td,  $J_{4,5'}=4.9$ , 4.4,  $J_{4,3'}=2.9$  Hz, 1H; H 4'), 4.51 (dt,  $J_{3,2'}=6.2$ , 2.6,  $J_{3,4'}=2.9$  Hz, 1H; H 3'), 5.30 and 5.50 (br, 2H; OH 3',5'), 6.52 (dd,  $J_{1,2'}=7.9$ , 6.5 Hz, 1H; H 1'), 7.40 (m, 2H; H *m* phenylene), 7.58 (m, 4H; NH $_2$ , H *o* phenylene), 8.17 ppm (s, 1H; H 2);  $^{13}\text{C}$  NMR (100.6 MHz,  $[\text{D}_6]\text{DMSO}$ ):  $\delta=37.22$  (CH $_2$ ), 37.96 (CH $_2$  2'), 55.48 (CH), 62.39 (CH $_2$  5'), 71.42 (CH 3'), 82.94 (C=C phenylene), 85.18 (CH 1'), 88.45 (CH 4'), 94.84 (C=C phenylene), 118.14 (C *i* phenylene),

119.75 (C 5), 130.34 (CH *m* phenylene), 132.04 (CH *o* phenylene), 133.12 (C 8), 140.79 (C *p* phenylene), 148.80 (C 4), 153.57 (CH 2), 156.24 (C 6), 169.29 ppm (CO); IR (KBr):  $\tilde{\nu}=3392$ , 3172, 2212, 1643, 1602, 1401, 1338, 1095, 1058, 796  $\text{cm}^{-1}$ ; MS (FAB):  $m/z$ : 439 (100)  $[M+1]^+$ ; HRMS (FAB):  $m/z$ : calcd for  $\text{C}_{21}\text{H}_{23}\text{N}_6\text{O}_5$ : 439.1730; found: 439.1721.

**(S)-2-Amino-3-[4-[[6-amino-9-(2-deoxy- $\beta$ -D-erythro-pentofuranosyl)-7-deazapurin-7-yl]phenyl]propanoic acid (7):** This compound was prepared by Method A from **2a**, yield 89%. [ $\alpha$ ] $_{\text{D}}^{20}=-19.5$  ( $c=2.12$ ,  $\text{H}_2\text{O}$ );  $^1\text{H}$  NMR (500 MHz,  $[\text{D}_6]\text{DMSO}$ ):  $\delta=2.20$  (ddd,  $J_{\text{gem}}=13.2$ ,  $J_{2b,1'}=6.0$ ,  $J_{2b,3'}=2.7$  Hz, 1H; H 2'b), 2.56 (ddd,  $J_{\text{gem}}=13.2$ ,  $J_{2a,1'}=8.3$ ,  $J_{2a,3'}=5.8$  Hz, 1H; H 2'a), 2.90 (dd,  $J_{\text{gem}}=14.1$ ,  $J_{\text{vic}}=8.8$ , 1H; bCH $_2$ ), 3.22 (dd,  $J_{\text{gem}}=14.1$ ,  $J_{\text{vic}}=4.3$  Hz, 1H; aCH $_2$ ), 3.46 (dd,  $J_{\text{vic}}=8.8$ , 4.3 Hz, 1H; CH), 3.51 (dd,  $J_{\text{gem}}=11.8$ ,  $J_{5b,4'}=4.3$  Hz, 1H; H 5'b), 3.58 (dd,  $J_{\text{gem}}=11.8$ ,  $J_{5a,4'}=4.6$  Hz, 1H; H 5'a), 3.84 (td,  $J_{4,5'}=4.6$ , 4.3,  $J_{4,3'}=2.4$  Hz, 1H; H 4'), 4.37 (dt,  $J_{3,2'}=5.8$ , 2.7,  $J_{3,4'}=2.4$  Hz, 1H; H 3'), 5.12 and 5.32 (2 $\times$  brs, 2 $\times$  1H; OH 3',5'), 6.20 (brs, 2H; NH $_2$ ), 6.58 (dd,  $J_{1,2'}=8.3$ , 6.0 Hz, 1H; H 1'), 7.35 (s, 4H; H *o,m* phenylene), 7.47 (s, 1H; H 8), 8.13 ppm (s, 1H; H 2);  $^{13}\text{C}$  NMR (125.8 MHz,  $[\text{D}_6]\text{DMSO}$ ):  $\delta=36.73$  (CH $_2$ ), 39.92 (CH $_2$  2'), 55.93 (CH), 62.19 (CH $_2$  5'), 71.28 (CH 3'), 83.18 (CH 1'), 87.56 (CH 4'), 100.55 (C 5), 116.56 (C 7), 120.30 (CH 8), 128.82 and 130.10 (CH *o,m* phenylene), 132.79 (C *i* phenylene), 136.28 (C *p* phenylene), 150.57 (C 4), 151.88 (CH 2), 157.44 (C 6), 169.48 ppm (CO); IR (KBr):  $\tilde{\nu}=3391$ , 2926, 1626, 1587, 1539, 1506, 1468, 1398, 1219, 1094, 1053, 951, 922, 769  $\text{cm}^{-1}$ ; MS (FAB):  $m/z$ : 414 (25)  $[M+1]^+$ , 255 (100); HRMS (FAB):  $m/z$ : calcd for  $\text{C}_{20}\text{H}_{24}\text{N}_5\text{O}_5$ : 414.1777; found: 414.1789.

**(S)-2-Amino-3-[4-[[6-amino-9-(2-deoxy- $\beta$ -D-erythro-pentofuranosyl)-7-deazapurin-7-yl]ethynyl]phenyl]propanoic acid (10):** This compound was prepared by Method B from **2a**, yield 94%. [ $\alpha$ ] $_{\text{D}}^{20}=-23.3$  ( $c=2.40$ , DMSO);  $^1\text{H}$  NMR (500 MHz,  $[\text{D}_6]\text{DMSO}$ ):  $\delta=2.20$  (ddd,  $J_{\text{gem}}=13.1$ ,  $J_{2b,1'}=6.0$ ,  $J_{2b,3'}=2.9$  Hz, 1H; H 2'b), 2.49 (ddd,  $J_{\text{gem}}=13.1$ ,  $J_{2a,1'}=8.1$ ,  $J_{2a,3'}=5.7$  Hz, 1H; H 2'a), 2.91 (dd,  $J_{\text{gem}}=14.4$ ,  $J_{\text{vic}}=8.0$  Hz, 1H; bCH $_2$ ), 3.16 (dd,  $J_{\text{gem}}=14.4$ ,  $J_{\text{vic}}=4.7$  Hz, 1H; aCH $_2$ ), 3.50 (dd,  $J_{\text{vic}}=8.0$ , 4.7 Hz, 1H; CH), 3.52 (dd,  $J_{\text{gem}}=11.7$ ,  $J_{5b,4'}=4.4$  Hz, 1H; H 5'b), 3.60 (dd,  $J_{\text{gem}}=11.7$ ,  $J_{5a,4'}=4.6$  Hz, 1H; H 5'a), 3.84 (td,  $J_{4,5'}=4.6$ , 4.4,  $J_{4,3'}=2.6$  Hz, 1H; H 4'), 4.36 (dt,  $J_{3,2'}=5.7$ , 2.9,  $J_{3,4'}=2.6$  Hz, 1H; H 3'), 5.10 and 5.32 (2 $\times$  brs, 2 $\times$  1H; OH 3',5'), 6.51 (dd,  $J_{1,2'}=8.1$ , 6.0 Hz, 1H; H 1'), 6.70 (brs, 2H; NH $_2$ ), 7.32 (m, 2H; H *m* phenylene), 7.51 (m, 2H; H *o* phenylene), 7.86 (s, 1H; H 8), 8.15 ppm (s, 1H; H 2);  $^{13}\text{C}$  NMR (125.8 MHz,  $[\text{D}_6]\text{DMSO}$ ):  $\delta=36.95$  (CH $_2$ ), 39.86 (CH $_2$  2'), 55.32 (CH), 62.06 (CH $_2$  5'), 71.14 (CH 3'), 82.94 (C=C phenylene), 83.40 (CH 1'), 87.76 (CH 4'), 91.38 (C=C phenylene), 94.96 (C 7), 102.31 (C 5), 120.79 (C *i* phenylene), 126.78 (CH 8), 129.98 (CH *m* phenylene), 131.25 (CH *o* phenylene), 138.29 (C *p* phenylene), 149.58 (C 4), 153.01 (CH 2), 157.80 (C 6), 169.42 ppm (CO); IR (KBr):  $\tilde{\nu}=3435$ , 2211, 1629, 1593, 1573, 1505, 1456, 1400, 1236, 1088, 1057, 922, 794  $\text{cm}^{-1}$ ; MS (FAB):  $m/z$ : 438 (20)  $[M+1]^+$ , 419 (100), 375 (40), 322 (10)  $[M-\text{dRF}+2]^+$ ; HRMS (FAB):  $m/z$ : calcd for  $\text{C}_{22}\text{H}_{24}\text{N}_5\text{O}_5$ : 438.1777; found: 438.1797.

**(S)-2-Amino-3-[4-[1-(2-deoxy- $\beta$ -D-erythro-pentofuranosyl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl]phenyl]propanoic acid (8):** This compound was prepared by Method A from **3a**, yield 78%. [ $\alpha$ ] $_{\text{D}}^{20}=-17.8$  ( $c=2.72$ , DMSO);  $^1\text{H}$  NMR (400 MHz,  $[\text{D}_6]\text{DMSO}$ ):  $\delta=2.16$  (ddd,  $J_{\text{gem}}=13.3$ ,  $J_{2b,1'}=6.2$ ,  $J_{2b,3'}=3.7$  Hz, 1H; H 2'b), 2.22 (ddd,  $J_{\text{gem}}=13.3$ ,  $J_{2a,1'}=7.0$ ,  $J_{2a,3'}=5.9$  Hz, 1H; H 2'a), 2.87 (dd,  $J_{\text{gem}}=14.2$ ,  $J_{\text{vic}}=7.9$ , 1H; bCH $_2$ ), 3.14 (dd,  $J_{\text{gem}}=14.2$ ,  $J_{\text{vic}}=4.3$  Hz, 1H; aCH $_2$ ), 3.45 (dd,  $J_{\text{vic}}=7.9$ , 4.3 Hz, 1H; CH), 3.57 (dd,  $J_{\text{gem}}=11.8$ ,  $J_{5b,4'}=3.3$  Hz, 1H; H 5'b), 3.62 (dd,  $J_{\text{gem}}=11.8$ ,  $J_{5a,4'}=3.1$  Hz, 1H; H 5'a), 3.82 (q,  $J_{4,5'}=3.3$ , 3.1,  $J_{4,3'}=3.1$  Hz, 1H; H 4'), 4.29 (dt,  $J_{3,2'}=5.9$ , 3.7,  $J_{3,4'}=3.1$  Hz, 1H; H 3'), 5.10 5.40 (br, 2H; OH 3',5'), 6.23 (t,  $J_{1,2'}=7.0$ , 6.2 Hz, 1H; H 1'), 7.26 (m, 2H; H *m* phenylene), 7.48 (m, 2H; H *o* phenylene), 8.16 (s, 1H; H 6), 11.49 ppm (brs, 1H; NH);  $^{13}\text{C}$  NMR (100.6 MHz,  $[\text{D}_6]\text{DMSO}$ ):  $\delta=36.78$  (CH $_2$ ), 40.29 (CH 2'), 55.55 (CH), 61.19 (CH $_2$  5'), 70.47 (CH 3'), 84.67 (CH 1'), 87.73 (CH 4'), 113.54 (C 5), 127.93 (CH *o* phenylene), 129.36 (CH *m* phenylene), 131.54 (C *i* phenylene), 136.70 (C *p* phenylene), 137.80 (CH 6), 150.11 (C 2), 162.30 (C 4), 169.62 ppm (CO); IR (KBr):  $\tilde{\nu}=3586$ , 3404, 1695, 1665, 1649, 1599, 1471, 1415, 1350, 1293, 1099, 1039, 785  $\text{cm}^{-1}$ ; MS (FAB):  $m/z$ : 414 (100)  $[M+\text{Na}]^+$ ; HRMS (FAB):  $m/z$ : calcd for  $\text{C}_{18}\text{H}_{21}\text{N}_5\text{O}_7\text{Na}$ : 414.1277; found: 414.1267.

**(S)-2-Amino-3-(4-[[1-(2-deoxy-β-D-erythro-pentofuranosyl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl]ethynyl]phenyl)propanoic acid (I1):** This compound was prepared by Method B from **3a**, yield 70%. [ $\alpha$ ]<sub>D</sub><sup>20</sup> = -20.9 (c = 2.06, DMSO); <sup>1</sup>H NMR (500 MHz, [D<sub>6</sub>]DMSO): δ = 2.15 (ddd,  $J_{\text{gem}} = 13.5$ ,  $J_{2b,1} = 6.3$ ,  $J_{2b,3} = 4.2$  Hz, 1H; H 2'b), 2.17 (ddd,  $J_{\text{gem}} = 13.5$ ,  $J_{2a,1} = 6.8$ ,  $J_{2a,3} = 5.8$  Hz, 1H; H 2'a), 2.88 (br, 1H; bCH<sub>2</sub>), 3.17 (br, 1H; aCH<sub>2</sub>), 3.42 (br, 1H; CH), 3.59 and 3.65 (2 × dd,  $J_{\text{gem}} = 11.9$ ,  $J_{5',4'} = 3.4$  Hz, 2H; H 5'), 3.81 (q,  $J_{4',5'} = 3.4$ ,  $J_{4',3'} = 3.2$  Hz, 1H; H 4'), 4.26 (dt,  $J_{3',2'} = 5.8$ , 4.2,  $J_{3',4'} = 3.2$ , 1H; H 3'), 5.23 and 5.33 (2 × brs, 2 × 1H; OH 3',5'), 6.12 (t,  $J_{1,2} = 6.8$ , 6.3 Hz, 1H; H 1'), 7.30 (brm, 2H; H *m* phenylene), 7.37 (brm, 2H; H *o* phenylene), 8.37 ppm (s, 1H; H 6); <sup>13</sup>C NMR (100.6 MHz, [D<sub>6</sub>]DMSO): δ = 40.43 (CH<sub>2</sub> 2'), 55.21 (CH), 61.00 (CH<sub>2</sub> 5'), 70.11 (CH 3'), 82.31 (C=C phenylene), 85.06 (CH 1'), 87.80 (CH 4'), 92.14 (C=C phenylene), 98.51 (C 5), 120.68 (C *i* phenylene), 129.99 (CH *m* phenylene), 131.36 (CH *o* phenylene), 143.97 (CH 6), 149.66 (C 2), 161.69 (C 4), 167.66 ppm (CO) (CH<sub>2</sub> and C *p* phenylene not observed due to signal broadening); IR (KBr):  $\tilde{\nu} = 3423$ , 2815, 1695, 1642, 1618, 1533, 1511, 1464, 1412, 1314, 1274, 1199, 1154, 1098, 1052, 966 cm<sup>-1</sup>; MS (FAB):  $m/z$ : 438 (20) [M+Na]<sup>+</sup>, 416 (89); HRMS (FAB):  $m/z$ : calcd for C<sub>20</sub>H<sub>22</sub>N<sub>3</sub>O<sub>7</sub>: 416.1458; found: 416.1438; elemental analysis (%) calcd for C<sub>20</sub>H<sub>21</sub>N<sub>3</sub>O<sub>7</sub>·2H<sub>2</sub>O (451.4): C 53.21, H 5.58, N 9.31; found: C 52.91, H 5.31, N 8.99.

**(S)-2-Amino-3-(4-[[6-amino-9-(2-deoxy-β-D-erythro-pentofuranosyl)-purin-8-yl]ethynyl]phenyl)propanoic acid 5'-O-triphosphate (A2):** This compound was prepared by Method D from **1b**, yield 61%. <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O+Et<sub>3</sub>N, ref<sub>dioxane</sub> = 3.75 ppm): δ = 1.12 (t,  $J_{\text{vic}} = 7.2$  Hz, 27H; CH<sub>3</sub>CH<sub>2</sub>N), 2.56 (brm, 1H; H 2'b), 2.83 (q,  $J_{\text{vic}} = 7.2$  Hz, 18H; CH<sub>3</sub>CH<sub>2</sub>N), 2.87 (dd,  $J_{\text{gem}} = 13.5$ ,  $J_{\text{vic}} = 7.2$  Hz, 1H; bCH<sub>2</sub>), 3.03 (dd,  $J_{\text{gem}} = 13.5$ ,  $J_{\text{vic}} = 4.9$  Hz, 1H; aCH<sub>2</sub>), 3.45 3.55 (m, 2H; H 2'a and CH), 4.13 (brm, 1H; H 5'b), 4.28 4.32 (m, 2H; H 4' and H 5'a), 4.85 (q,  $J_{3',2'} = J_{3',4'} = 4.1$  Hz, 1H; H 3'), 6.67 (t,  $J_{1,2} = 7.2$  Hz, 1H; H 1'), 7.27 (m, 2H; H *m* phenylene), 7.57 (m, 2H; H *o* phenylene), 8.22 ppm (s, 1H; H 2); <sup>13</sup>C NMR (125.8 MHz, D<sub>2</sub>O+Et<sub>3</sub>N, ref<sub>dioxane</sub> = 69.3 ppm): δ = 11.85 (CH<sub>3</sub>CH<sub>2</sub>N), 38.73 (CH<sub>2</sub> 2'), 43.55 (CH<sub>2</sub>), 48.69 (CH<sub>3</sub>CH<sub>2</sub>N), 59.85 (CH), 68.34 (CH<sub>2</sub> 5'), 73.73 (CH 3'), 79.61 (C=C pur), 87.42 (CH 1'), 88.29 (d,  $J_{\text{C,P}} = 9$  Hz; CH 4'), 99.95 (C=C pur), 120.19 (C *i* phenylene), 121.25 (C 5), 132.50 (CH *m* phenylene), 134.86 (CH *o* phenylene), 137.94 (C 8), 144.12 (C *p* phenylene), 151.32 (C 4), 156.32 (CH 2), 157.78 (C 6), 184.34 ppm (CO); <sup>31</sup>P (1H dec.) NMR (162 MHz, D<sub>2</sub>O+Et<sub>3</sub>N, ref<sub>H<sub>3</sub>PO<sub>4</sub></sub> = 0 ppm): δ = -22.49 (brt,  $J = 20.8$ , 19.5 Hz; P<sub>β</sub>), -11.19 (d,  $J = 19.5$  Hz; P<sub>α</sub>), -6.40 ppm (d,  $J = 20.8$  Hz; P<sub>γ</sub>); MS (ES<sup>-</sup>):  $m/z$ : 677 (100) [M-1]<sup>-</sup>, 579 (25); HRMS (ES<sup>-</sup>):  $m/z$ : calcd for C<sub>21</sub>H<sub>24</sub>N<sub>6</sub>O<sub>14</sub>P<sub>3</sub>: 677.0563; found: 677.0552.

**(S)-2-Amino-3-(4-[[6-amino-9-(2-deoxy-β-D-erythro-pentofuranosyl)-7-deazapurin-7-yl]phenyl]propanoic acid 5'-O-triphosphate (A3):** This compound was prepared by Method C from **2b**, yield 66%. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O, ref<sub>dioxane</sub> = 3.75 ppm): δ = 1.26 (t,  $J_{\text{vic}} = 7.4$  Hz, 27H; CH<sub>3</sub>CH<sub>2</sub>N), 2.45 (ddd,  $J_{\text{gem}} = 14.2$ ,  $J_{2b,1} = 6.4$ ,  $J_{2b,3} = 3.3$  Hz, 1H; H 2'b), 2.68 (ddd,  $J_{\text{gem}} = 14.2$ ,  $J_{2a,1} = 7.9$ ,  $J_{2a,3} = 6.2$  Hz, 1H; H 2'a), 3.16 (dd,  $J_{\text{gem}} = 14.3$ ,  $J_{\text{vic}} = 8.5$  Hz, 1H; bCH<sub>2</sub>), 3.17 (q,  $J_{\text{vic}} = 7.4$  Hz, 18H; CH<sub>3</sub>CH<sub>2</sub>N), 3.36 (dd,  $J_{\text{gem}} = 14.3$ ,  $J_{\text{vic}} = 4.7$  Hz, 1H; aCH<sub>2</sub>), 4.02 (dd,  $J_{\text{vic}} = 8.5$ , 4.7 Hz, 1H; CH), 4.10 4.25 (m, 3H; H 4' and H 5'), 4.74 (brm, 1H; H 3'), 6.64 (t,  $J_{1,2} = 7.9$ , 6.4 Hz, 1H; H 1'), 7.39 (m, 2H; H *m* phenylene), 7.44 (m, 2H; H *o* phenylene), 7.58 (s, 1H; H 8), 8.17 ppm (s, 1H; H 2); <sup>13</sup>C NMR (100.6 MHz, D<sub>2</sub>O, ref<sub>dioxane</sub> = 69.3 ppm): δ = 10.94 (CH<sub>3</sub>CH<sub>2</sub>N), 38.86 (CH<sub>2</sub>), 41.53 (CH<sub>2</sub> 2'), 49.37 (CH<sub>3</sub>CH<sub>2</sub>N), 58.71 (CH), 68.35 (d,  $J_{\text{C,P}} = 5$  Hz; CH<sub>2</sub> 5'), 74.06 (CH 3'), 85.82 (CH 1'), 87.98 (d,  $J_{\text{C,P}} = 9$  Hz; CH 4'), 103.11 (C 5), 121.07 (C 7), 123.76 (CH 8), 131.96 (CH *o* phenylene), 132.83 (CH *m* phenylene), 134.98 (C *i* phenylene), 137.28 (C *p* phenylene), 151.63 (CH 2), 151.85 (C 4), 158.06 (C 6), 176.67 ppm (CO); <sup>31</sup>P (1H dec.) NMR (162 MHz, D<sub>2</sub>O, ref<sub>H<sub>3</sub>PO<sub>4</sub></sub> = 0 ppm): δ = -23.17 (t,  $J = 19.7$ , 19.0 Hz; P<sub>β</sub>), -11.40 (d,  $J = 19.7$  Hz; P<sub>α</sub>), -10.47 ppm (d,  $J = 19.0$  Hz; P<sub>γ</sub>); MS (ES<sup>-</sup>):  $m/z$ : 652 (100) [M-1]<sup>-</sup>, 572 (20), 554 (20); HRMS (ES<sup>-</sup>):  $m/z$ : calcd for C<sub>20</sub>H<sub>25</sub>N<sub>5</sub>O<sub>14</sub>P<sub>3</sub>: 652.0611; found: 652.0579.

**(S)-2-Amino-3-(4-[[6-amino-9-(2-deoxy-β-D-erythro-pentofuranosyl)-7-deazapurin-7-yl]ethynyl]phenyl)propanoic acid 5'-O-triphosphate (A4):** This compound was prepared by Method D from **2b**, yield 67%. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O, ref<sub>dioxane</sub> = 3.75 ppm): δ = 1.25 (t,  $J_{\text{vic}} = 7.4$  Hz,

27H; CH<sub>3</sub>CH<sub>2</sub>N), 2.46 (brddd,  $J_{\text{gem}} = 14.2$ ,  $J_{2b,1} = 6.0$ ,  $J_{2b,3} = 3.0$  Hz, 1H; H 2'b), 2.64 (brdt,  $J_{\text{gem}} = 14.2$ ,  $J_{2a,1} = J_{2a,3} = 6.5$  Hz, 1H; H 2'a), 3.07 (dd,  $J_{\text{gem}} = 14.6$ ,  $J_{\text{vic}} = 8.5$  Hz, 1H; bCH<sub>2</sub>), 3.16 (q,  $J_{\text{vic}} = 7.4$  Hz, 18H; CH<sub>3</sub>CH<sub>2</sub>N), 3.30 (dd,  $J_{\text{gem}} = 14.6$ ,  $J_{\text{vic}} = 4.9$  Hz, 1H; aCH<sub>2</sub>), 3.98 (dd,  $J_{\text{vic}} = 8.5$ , 4.9 Hz, 1H; CH), 4.11 4.26 (m, 3H; H 4' and H 5'), 4.73 (brm, 1H; H 3'), 6.42 (t,  $J_{1,2} = 6.5$ , 6.0 Hz, 1H; H 1'), 7.22 (m, 2H; H *m* phenylene), 7.38 (m, 2H; H *o* phenylene), 7.77 (s, 1H; H 8), 8.13 ppm (s, 1H; H 2); <sup>13</sup>C NMR (100.6 MHz, D<sub>2</sub>O+Et<sub>3</sub>N, ref<sub>dioxane</sub> = 69.3 ppm): δ = 11.42 (CH<sub>3</sub>CH<sub>2</sub>N), 41.14 (CH<sub>2</sub>), 44.89 (CH<sub>2</sub> 2'), 48.98 (CH<sub>3</sub>CH<sub>2</sub>N), 59.98 (CH), 68.18 (d,  $J_{\text{C,P}} = 6$  Hz; CH<sub>2</sub> 5'), 73.56 (CH 3'), 84.29 (C=C phenylene), 85.56 (CH 1'), 88.12 (d,  $J_{\text{C,P}} = 9$  Hz; CH 4'), 95.01 (C=C phenylene), 99.49 (C 7), 105.63 (C 5), 122.93 (C *i* phenylene), 128.80 (CH 8), 132.34 (CH *m* phenylene), 133.98 (CH *o* phenylene), 141.82 (C *p* phenylene), 151.56 (C 4), 155.18 (CH 2), 160.36 (C 6), 184.29 ppm (CO); <sup>31</sup>P (1H dec.) NMR (162 MHz, D<sub>2</sub>O, ref<sub>H<sub>3</sub>PO<sub>4</sub></sub> = 0 ppm): δ = -23.05 (t,  $J = 20.0$ , 19.3 Hz; P<sub>β</sub>), -11.28 (d,  $J = 20.0$  Hz; P<sub>α</sub>), -10.35 (d,  $J = 19.3$  Hz; P<sub>γ</sub>); MS (ES<sup>-</sup>):  $m/z$ : 676 (100) [M-1]<sup>-</sup>; HRMS (ES<sup>-</sup>):  $m/z$ : calcd for C<sub>22</sub>H<sub>25</sub>N<sub>6</sub>O<sub>14</sub>P<sub>3</sub>: 676.0611; found: 676.0629.

**(S)-2-Amino-3-(4-[[1-(2-deoxy-β-D-erythro-pentofuranosyl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl]phenyl]propanoic acid 5'-O-triphosphate (T1):** This compound was prepared by Method C from **3b**, yield 56%. <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O, ref<sub>dioxane</sub> = 3.75 ppm): δ = 1.27 (t,  $J_{\text{vic}} = 7.3$  Hz, 27H; CH<sub>3</sub>CH<sub>2</sub>N), 2.39 2.48 (m, 2H; H 2'), 3.05 (dd,  $J_{\text{gem}} = 14.6$ ,  $J_{\text{vic}} = 9.5$  Hz, 1H; bCH<sub>2</sub>), 3.21 (q,  $J_{\text{vic}} = 7.3$  Hz, 18H; CH<sub>3</sub>CH<sub>2</sub>N), 3.41 (dd,  $J_{\text{gem}} = 14.6$ ,  $J_{\text{vic}} = 4.3$  Hz, 1H; aCH<sub>2</sub>), 3.98 (dd,  $J_{\text{vic}} = 9.5$ , 4.3 Hz, 1H; CH), 4.13 4.24 (m, 3H; H 4' and H 5'), 4.65 (td,  $J_{3',2'} = 4.4$ ,  $J_{3',4'} = 2.7$  Hz, 1H; H 3'), 6.38 (t,  $J_{1,2} = 7.0$  Hz, 1H; H 1'), 7.40 (m, 2H; H *m* phenylene), 7.51 (m, 2H; H *o* phenylene), 7.99 ppm (s, 1H; H 6); <sup>13</sup>C NMR (125.8 MHz, D<sub>2</sub>O, ref<sub>dioxane</sub> = 69.3 ppm): δ = 10.96 (CH<sub>3</sub>CH<sub>2</sub>N), 39.00 (CH<sub>2</sub>), 41.71 (CH<sub>2</sub> 2'), 49.39 (CH<sub>3</sub>CH<sub>2</sub>N), 58.85 (CH), 68.10 (d,  $J_{\text{C,P}} = 5$  Hz; CH<sub>2</sub> 5'), 73.41 (CH 3'), 88.22 (CH 1'), 88.48 (d,  $J_{\text{C,P}} = 9$ , CH 4'), 118.41 (C 5), 132.17 (CH *o* phenylene), 132.33 (CH *m* phenylene), 133.99 (C *i* phenylene), 138.07 (C *p* phenylene), 141.66 (CH 6), 154.05 (C 2), 167.54 (C 4), 176.88 ppm (CO); <sup>31</sup>P (1H dec.) NMR (162 MHz, D<sub>2</sub>O, ref<sub>H<sub>3</sub>PO<sub>4</sub></sub> = 0 ppm): δ = -23.33 (t,  $J = 20.1$ , 19.9 Hz; P<sub>β</sub>), -11.87 (d,  $J = 19.9$  Hz; P<sub>α</sub>), -10.86 ppm (d,  $J = 20.1$  Hz; P<sub>γ</sub>); MS (ES<sup>-</sup>):  $m/z$ : 630 (100) [M-1]<sup>-</sup>, 532 (30); HRMS (ES<sup>-</sup>): calcd for C<sub>18</sub>H<sub>23</sub>N<sub>5</sub>O<sub>16</sub>P<sub>3</sub>: 630.0291; found: 630.0294.

**(S)-2-Amino-3-(4-[[1-(2-deoxy-β-D-erythro-pentofuranosyl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl]ethynyl]phenyl)propanoic acid 5'-O-triphosphate (T2):** This compound was prepared by Method D from **3b**, yield 66%. <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O+Et<sub>3</sub>N, ref<sub>dioxane</sub> = 3.75 ppm): δ = 1.22 (t,  $J_{\text{vic}} = 7.3$  Hz, 27H; CH<sub>3</sub>CH<sub>2</sub>N), 2.36 (ddd,  $J_{\text{gem}} = 14.0$ ,  $J_{2b,1} = 7.3$ ,  $J_{2b,3} = 6.6$  Hz, 1H; H 2'b), 2.38 (ddd,  $J_{\text{gem}} = 14.0$ ,  $J_{2a,1} = 6.5$ ,  $J_{2a,3} = 4.3$  Hz, 1H; H 2'a), 2.34 (dd,  $J_{\text{gem}} = 13.9$ ,  $J_{\text{vic}} = 8.3$  Hz, 1H; bCH<sub>2</sub>), 3.08 (dd,  $J_{\text{gem}} = 13.9$ ,  $J_{\text{vic}} = 5.4$  Hz, 1H; aCH<sub>2</sub>), 3.08 (q,  $J_{\text{vic}} = 7.3$  Hz, 18H; CH<sub>3</sub>CH<sub>2</sub>N), 3.57 (dd,  $J_{\text{vic}} = 8.3$ , 5.4 Hz, 1H; CH), 4.16 4.27 (m, 3H; H 4' and H 5'), 4.46 (dt,  $J_{3',2'} = 6.6$ , 4.3,  $J_{3',4'} = 3.6$  Hz, 1H; H 3'), 6.35 (t,  $J_{1,2} = 7.3$ , 6.5 Hz, 1H; H 1'), 7.29 (m, 2H; H *m* phenylene), 7.56 (m, 2H; H *o* phenylene), 8.06 ppm (s, 1H; H 6); <sup>13</sup>C NMR (125.8 MHz, D<sub>2</sub>O+Et<sub>3</sub>N, ref<sub>dioxane</sub> = 69.3 ppm): δ = 11.19 (CH<sub>3</sub>CH<sub>2</sub>N), 41.28 (CH<sub>2</sub> 2'), 42.82 (CH<sub>2</sub>), 49.16 (CH<sub>3</sub>CH<sub>2</sub>N), 59.85 (CH), 68.02 (d,  $J_{\text{C,P}} = 5$  Hz; CH<sub>2</sub> 5'), 73.03 (CH 3'), 85.58 (C=C pyr), 87.87 (d,  $J_{\text{C,P}} = 9$  Hz; CH 4'), 87.99 (CH 1'), 95.46 (C=C pyr), 102.71 (C 5), 123.62 (C *i* phenylene), 132.28 (CH *m* phenylene), 134.29 (CH *o* phenylene), 141.20 (C *p* phenylene), 145.82 (CH 6), 160.76 (C 2), 176.71 (C 4), 183.52 ppm (CO); <sup>31</sup>P (1H dec.) NMR (162 MHz, D<sub>2</sub>O+Et<sub>3</sub>N, ref<sub>H<sub>3</sub>PO<sub>4</sub></sub> = 0 ppm): δ = -22.57 (t,  $J = 21.0$ , 19.8 Hz; P<sub>β</sub>), -11.20 (d,  $J = 19.8$  Hz; P<sub>α</sub>), -6.41 ppm (d,  $J = 21.0$  Hz; P<sub>γ</sub>); MS (ES<sup>-</sup>):  $m/z$ : 654 (100) [M-1]<sup>-</sup>, 448 (70); HRMS (ES<sup>-</sup>):  $m/z$ : calcd for C<sub>24</sub>H<sub>20</sub>N<sub>2</sub>O<sub>16</sub>P<sub>2</sub>: 654.0288; found: 654.0287.

**Primer extension experiments:** The reaction mixture (20 μL) contained *Pwo* DNA polymerase (PeqLab, 1 unit), DMSO (2%), dNTPs (either natural or functionalized, 200 μM), primer (150 nM, LT25TH: 5' CAA GGA CAA AAT ACC TGT ATT CCT T 3'), and template (225 nM, FVL35 A: 5' GAT CCC TGA CAG GGC AAG GAA TAC AGG TAT TTT GT 3') in *Pwo* reaction buffer supplied by the manufacturer. LT25TH was labeled by use of [<sup>32</sup>P]ATP according to standard techniques. Reaction mixtures were incubated for 30 min at 55°C in a thermal cycler and were stopped by addition of stop solution (40 μL, 80%



[v/v] formamide, 20 mM EDTA, 0.025 % [w/v] bromophenol blue, 0.025 % [w/v] xylene cyanol). Reaction mixtures were separated by use of a 12 % denaturing PAGE. Visualization was performed by phosphoimaging.

**Polymerase chain reactions:** The PCR reaction mixture (20  $\mu$ L) contained *Pwo* DNA polymerase (PeqLab, 2 units), DMSO (2%), dNTPs (either natural or functionalized, 200  $\mu$ M), primers LT25TH and L20 (5' GACATCATGAGAGACATCGC 3'), and a 98 mer template (5' GAC ATC ATG AGA GAC ATC GCC TCT GGG CTA ATA GGA CTA CTT CTA ATC TGT AAG AGC AGA TCC CTG GAC AGG CAA GGA ATA CAG GTA TTT TGT CCT TG 3') in *Pwo* reaction buffer supplied by the manufacturer. 30 PCR cycles were run under the following conditions: denaturation for 1 min at 98 °C, annealing for 1 min at 55 °C, extension for 1.5 min at 72 °C, followed by a final extension step of 5 min at 72 °C. PCR products were analyzed on a 2.5 % agarose gel in 0.5  $\times$  TBE buffer, followed by staining with ethidium bromide.

**CD spectroscopy and thermal denaturation studies:** CD spectra and melting temperatures were determined for a functionalized DNA duplex in which all natural dT had been replaced with the modified **T2**. A DNA duplex containing all natural nucleotides served as control. For preparative purposes a total volume of 500  $\mu$ L PCR was run as mentioned above and purification was carried out with a MinElute PCR Purification Kit (Qiagen). Samples were eluted with Tris HCl (10 mM, pH 8.5) in 160  $\mu$ L. Absorbance at 260 nm was determined with the aid of an ND 1000 spectrophotometer (NanoDrop) and was as follows: Abs<sub>260</sub>(control)=0.948; Abs<sub>260</sub>(sample **T2**)=0.533. 10  $\times$  PBS (0.1 M phosphate buffer with 27 mM KCl and 1.37 M NaCl, pH 7.4) was added to a final concentration of 1  $\times$ . CD spectra were recorded with a Jasco 720 instrument. The duplex DNA samples were heated to 94 °C for 5 min and allowed to cool slowly to room temperature prior to measurements. A spectrum of the buffer was measured separately and subtracted from the spectra resulting from the samples. An average of 10 spectra was recorded in each experiment.

Melting curves were recorded on a Cary 100 bio UV/Vis instrument with temperature controller. Data were obtained from three individual cooling/heating cycles. Melting temperatures ( $T_m$  values in °C) were obtained by plotting temperature versus absorbance and by applying a sigmoidal curve fit. The samples were the same as for CD spectra.

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