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# Design, Synthesis, and Polymerase-Catalyzed Incorporation of Click-Modified Boronic Acid–TTP Analogues

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*On the occasion of the 10th anniversary of click chemistry*

**Abstract:** DNA molecules are known to be important materials in sensing, aptamer selection, nanocomputing, and construction of unique architectures. The incorporation of modified nucleobases affords unique DNA properties for applications in areas that would otherwise be difficult or not possible. Earlier, we demonstrated that the boronic acid moiety can be introduced into DNA through polymerase-catalyzed reactions. In order to study whether such incorporation by polymerase is a general phenomenon, we designed and synthesized four boronic acid-modified thymidine triphosphate (TTP) analogues. The synthesis of certain analogues was through the use of a single dialkyne tether for both the Sonogashira coupling with thymidine and the later Cu-mediated [3+2] cycloaddition for linking the boronic acid moiety. This approach is much more efficient than the previously described method, and paves the way for the preparation of a large number of boronic acid-modified TTPs with a diverse set of structural features. All analogues showed very good stability under polymerase chain reaction (PCR) conditions and were recognized as a substrate by DNA polymerase, and thus incorporated into DNA.

**Keywords:** Boronic acid · click chemistry · DNA · glycoproteins · polymerase chain reaction

## Introduction

The same properties that afford DNA the kind of unique features suitable as genomic materials also allow it to be used in a wide variety of applications such as nanosensing,[1] aptamer selection,[2] nanocomputing,[3] and reaction encoding.[4] Along these lines, modifications of nucleobases often endow DNA with additional properties for enhanced applications.[5] For example, 5-position modified thymidine analogues have been widely used in aptamer selections.[6] On the other hand, it is also well-known that boronic acid is one of the most commonly used building blocks for the design of chemosensors for carbohydrates, owing to its intrinsic affinity with diols, single hydroxy groups, as well as other nucleophiles/Lewis bases.[7] One of our long-standing interests is developing an aptamer selection platform specifically for biologically important carbohydrates and glycoproteins through the incorporation of a boronic acid group into DNA.[7f,8] This is based on the central hypothesis that the incorporation of a boronic acid-modified nucleotide into DNA would allow for enhanced recognition of carbohydrate moieties, which contain many hydroxyl groups.[9] Along this line, we have previously reported the design and synthesis of a thymidine analogue (B-TTP (**1**), Figure 1) modified with 8-quinolinylboronic acid at the 5-position, which can be introduced into DNA through polymerase-catalyzed reactions, as well as the feasibility of boronic acid-modified DNA aptamer selection for biological important glycoproteins.[8–9] In addition, there are boronic acids that change fluorescent properties upon binding.[7f] Incorporation of such boronic acids allows DNA to be used in sensing applications without the need for an additional reporting unit.[8b] As a specific example, we have also demonstrated the synthesis and incorporation of a long wavelength boronic acid-modified TTP (NB-TTP, Figure 1), which shows fluorescence intensity change upon carbohydrate addition. In order to ex-

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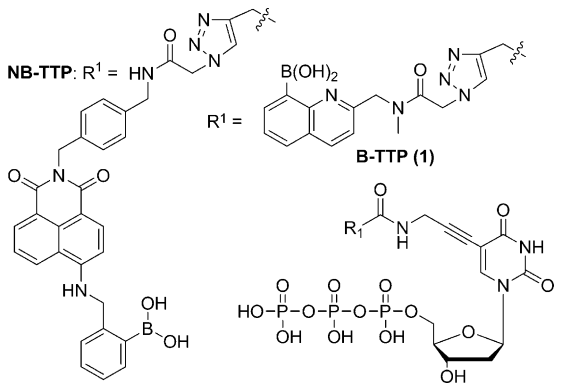



Figure 1. Chemical structures of B-TTP (**1**) and NB-TTP.

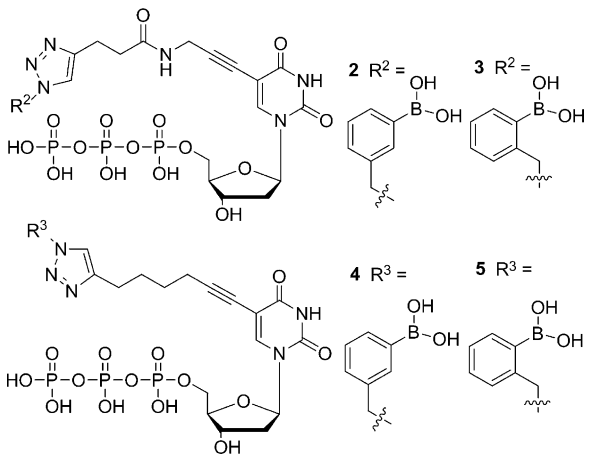



Figure 2. Structures of B-TTP analogues **2**–**5**.

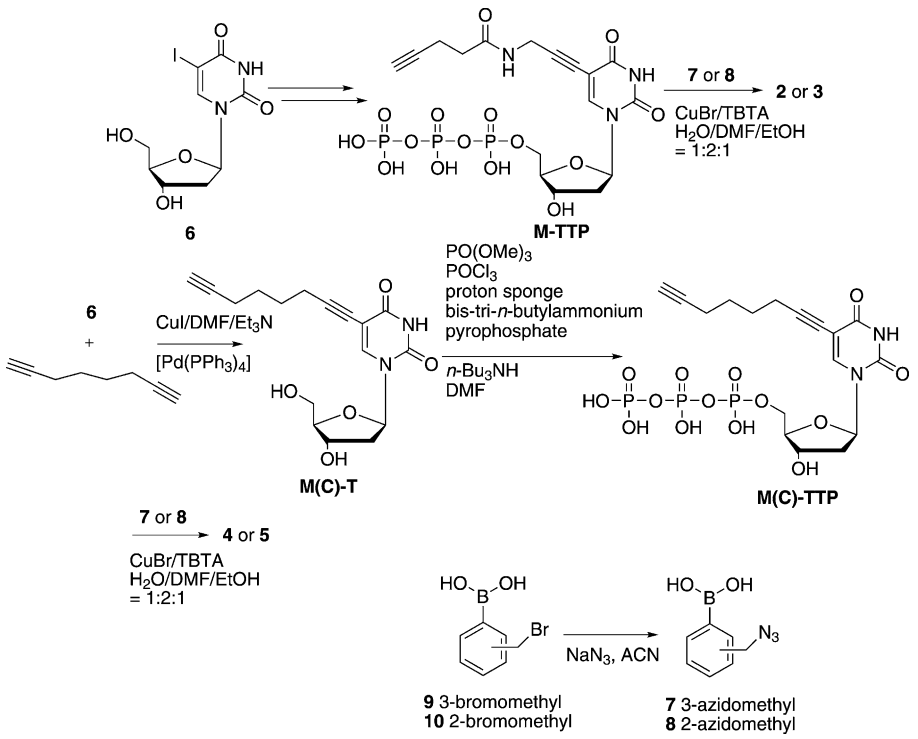
amine the generality of boronic acid incorporation and to broaden the application of boronic acid-modified DNA, we are interested in studying the incorporation of additional boronic acids with different structural features. At the same time, we are also interested in developing a synthetic approach which is more efficient than the previously described method and would allow for easy analogue synthesis.[7f] The resulting increase in structural diversity of boronic acid-modified thymidine analogues will be very important in future applications such as aptamer selection for biologically important carbohydrate biomarkers as well as glycoproteins.

## Results and Discussion

### Design and Synthesis

In our initial design, we were interested in the introduction of *ortho*- and *meta*-substituted phenylboronic acid, which are much easier to synthesize than the previously used boronic acids (B-TTP (**1**) and NB-TTP, Figure 1). In applications where the fluorescent properties are not important, these phenylboronic acid-modified thymidines would be very useful and easy to prepare. Figure 2 shows the structures of the new analogues. In the preparation of these new analogues, we initially followed the general procedures developed for the synthesis of B-TTP (**1**) (Figure 1). Thus, M-TTP (Scheme 1**)** was synthesized following a four-step procedure starting from 5-iodo-2′-deoxyuridine (**6**, Scheme 1) as published previously.[8a] Substituted azidomethylphenylboronic acid (**7** or **8**, Scheme 1) were easily obtained from their bromomethylphenylboronic acid precursors (**9** or **10**, Scheme 1), respectively. Then coupling of the appropriately substituted azidomethylphenylboronic acid (**7** or **8**) through a copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC),[10] gave **2** and **3** in 31 % yield after HPLC purification.

The key design in linking the boronic acid moiety to the 5-position of thymidine is the availability of a terminal alkyne group after the initial coupling. In order to shorten the synthesis, we designed an approach by using a single dialkyne tether for both the Sonogashira coupling with thymi-




Scheme 1. Synthesis of B-TTP analogues **2** to **5**.

dine and the later CuAAC for linking the boronic acid moiety. Such a design shortens the synthesis by two steps and simplifies the structural features of the side chain. Specifically, M(C)-TTP (Scheme 1) was prepared through Sonogashira coupling of 5-iodo-2′-deoxyuridine (**6**, Scheme 1) with 1,7-octadiyne, and the subsequent triphosphorylation was achieved using a modified procedure.[5a,b] Then, CuAAC led to the final products, **4** and **5**, in 29% and 14% yield, respectively, after HPLC purification.

### Stability Test of B-TTP Analogues under PCR Conditions

After obtaining the B-TTP analogues, we first studied whether these analogues were stable under polymerase chain reaction (PCR) conditions needed for polymerase-mediated incorporation. HPLC was used to monitor the stability of the synthesized B-TTP analogues **2**–**5** (Figure 2). The results showed that all B-TTP analogues are very stable under PCR conditions including three rounds of thermal cycling at 94 °C for 2 min, then 94 °C for 1 min, 46 °C for 1 min, and 72 °C for 1 min, and denaturing treatment (95 °C for 7 min). The HPLC chromatograms of B-TTP analogues **2**–**5** are shown in Figures S1–S4 (Supporting Information). No noticeable degradation was observed.

### Incorporation of B-TTP Analogues by Klenow Fragment-Catalyzed Primer Extension

Next, we studied whether the B-TTP analogues **2**–**5** could be incorporated into DNA in an enzyme-catalyzed reaction, as we have done previously with **1** (Figure 1). Specifically, primer extension, using **2**–**5** and the Klenow fragment, was conducted using a short sequence of 21-nucleotides (nt) oligonucleotide (5′-GGTTCCACCAGCAACCCGCTA-3′) as the template and a 14-nt primer (5′-TAGCGGGTTGCTGG-3′), as shown in Figure 3. The primer and template were designed in such a way that the first incorporated base would be a T, so there are two possible scenarios in the extension, either fully extended product or no extension at all. The latter case could arise from either no incorporation of the modified nucleotide or the inability to extend the sequence with the incorporation of this modified nucleotide. The obtained DNA products were studied using polyacrylamide gel electrophoresis (PAGE). The primer was radio-labeled with $^{32}P$ at the 5′-end using γ-$^{32}P$-ATP and T4 kinase (lane 1, Figure 3). Negative control without the Klenow fragment (lane 2, Figure 3) and without dNTPs (lane 3, Figure 3) showed no full length DNA sequence. The shorter DNA in lane 3 could have resulted from the 3′-5′ exonuclease activity of the Klenow fragment.[8b] The positive control with the Klenow fragment and natural dNTPs showed full length DNA sequence (lane 4, Figure 3). Primer extension using B-TTP analogues **2**–**5** (lanes 5–8, Figure 3) also gave a full length DNA sequence, which clearly indicated that these synthesized B-TTP analogues **2**–**5** were recognized as a substrate by the polymerase, and incorporated into DNA.

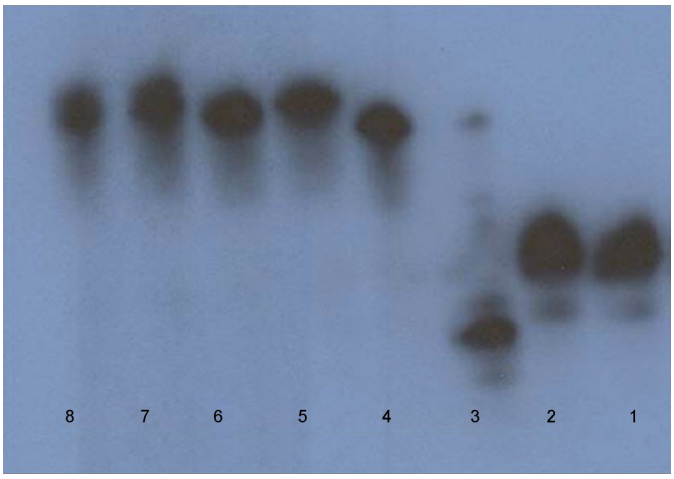



Figure 3. Incorporation of B-TTP analogues by Klenow fragment: 1) Primer only; 2) Primer +dNTPs, no Klenow fragment; 3) Primer+Klenow fragment, no dNTPs; 4) Primer+Klenow fragment+dNTPs; 5) using B-TTP analogue **2** instead of dTTP in 4); 6) using B-TTP analogue **3** instead of dTTP in 4); 7) using B-TTP analogue **4** instead of dTTP in 4); 8) using B-TTP analogue **5** instead of dTTP in 4).

To further confirm the results, besides PAGE analysis, we also conducted matrix-assisted laser desorption/ionization (MALDI) analyses of the primer extension product. B-TTP analogue **2** was chosen as an example. From the MALDI spectra (Figures S5–S6, Supporting Information), the following results were obtained. In the control reaction, full extension of the primer using natural dNTPs yielded a DNA with *m*/*z* of 6519 (calculated $[M+H]^+$: 6519) as the extended strand peak in the MALDI analysis. On the other hand, when B-TTP analogue **2** was used instead of TTP, primer extension yielded a DNA with *m*/*z* of 6772 and *m*/*z* of 6787 as new peaks for the corresponding extended strand. Each was assigned as the deborylated (calc. $[M+H-HBO_2]^+$: 6771) and oxidative deborylated sequence (calc. $[M+H-HBO]^+$: 6787). Such behavior in MALDI analysis is common in our past experience with boronic acid compounds.[8] Results obtained further confirmed the full incorporation of the boronic acid-modified TTP analogues.

### PCR Investigation

Encouraged by the successful incorporation of B-TTP analogues **2**–**5** into a short sequence by Klenow fragment-mediated primer extension, we further investigated the PCR amplification of a longer DNA strand with a 90 bases template that we used previously.[9] As can be seen from Figure 4, all four analogues were successfully incorporated. Specifically, very similar bands were found for the PCR products of B-TTP analogues **2**–**5** (lanes 3–6, Figure 4, respectively, group A) compared to those products using dTTP (lane 1, Figure 4 group A) and M-TTP (lane 7, Figure 4 group A). The slightly reduced mobility of DNA with boronic acid incorporation can be explained by the interaction of the boronic acid functional group (a Lewis acid) with the PAGE gel. This can be

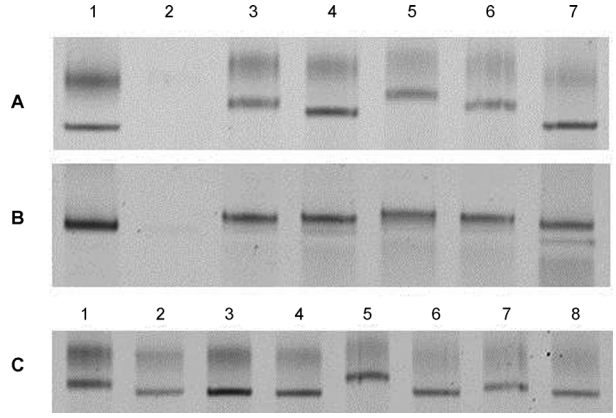



Figure 4. Group A (analyzed with 15% PAGE) and B (15% denaturing PAGE): PCR incorporation using dTTP (lane 1), B-TTP analogues **2**–**5** (lanes 3–6, respectively), or M-TTP (lane 7). Lane 2 is negative control with no dTTP. Group C: (analyzed with 15% PAGE): PCR incorporation using B-TTP analogues **2**–**5** (lanes 1, 3, 5, 7, respectively), DNA being treated with $H_2O_2$ before analyzing (lanes 2, 4, 6, and 8 for B-TTP analogues **2**–**5**, respectively). All reactions were conducted using *Taq* polymerase (See Supporting Information for full gel pictures).

further confirmed by the results of Group C, which represents the gel results of the PCR products after treating with $H_2O_2$ (final concentration of 10 mM, 2 h). All B-TTP analogues **2**–**5** (lanes 2, 4, 6, and 8, respectively, Figure 4 group C) showed almost the same mobility. However, before treatment, a different mobility was observed for B-TTP analogues **2**–**5** (lanes 1, 3, 5, 7, respectively, Figure 4 Group C). As an example, B-TTP analogue **4** shows the biggest difference in mobility (lane 5: before $H_2O_2$ treatment, and lane 6: after $H_2O_2$ treatment, Figure 4 Group C). The most significant evidence of the full incorporation of all the B-TTP analogues came from the denaturing PAGE gel results (15% denaturing PAGE containing 8 M urea). A very clear single band was observed in the case of B-TTP analogues **2**–**5** (lanes 3–6, Figure 4 Group B), compared to that of dTTP (lane 1, Figure 4 group B).

## Conclusions

Four boronic acid-modified TTP analogues were successfully synthesized. Two of them were synthesized using an improved procedure, which uses 1,7-octadiyne as a linker for both the Sonogashira coupling with thymidine and the CuAAC tethering of the boronic acid moiety. All four boronic acid-modified TTP analogues were characterized by $^1$H NMR, $^{31}$P NMR, and MS, and purities were confirmed by HPLC. Moreover, these analogues were successfully incorporated into DNA, suggesting that linker differences and the structural features of the boronic acid part do not have much bearing on polymerase-mediated incorporation. The newly developed synthetic method also paves the way for the preparation of a large number of boronic acid-modified TTP with a diverse set of structural features for future applications such as aptamer selection.

## Experimental Section

*General*

Chemicals were obtained from Aldrich and Acros, unless indicated otherwise. For all reactions, analytical grade solvents were used. Anhydrous solvents were used for all moisture-sensitive reactions. NMR data were collected on a Bruker 400 MHz spectrophotometer. The chemical shifts are relative to TMS as an internal standard for $^1$H NMR, and 85% $H_3PO_4$ as an external reference for $^{31}$P NMR. Mass spectra were recorded on a Waters Micromass LC-Q-TOF micro spectrometer or an ABI4800 MALDI-TOF/TOF mass spectrometer at Georgia State University, Mass Spectrometry Facilities. HPLC conditions for the purification of B-TTP analogues **2**–**5** were as follows: column: Agilent, semi-preparation column; flow rate: 2.0 mL min$^{-1}$; solvents: A: 0.1 M $NH_4HCO_3$, B: MeOH; program: 0.02–15 min 25% (B%), 35–45 min, 100%, 55–65 min 25%, stop; temperature: 20 °C, detection wavelength: 280 nm.

*Synthesis of M(C)-TTP*

The synthesis of M(C)-TTP followed modified procedure by Carell.[5a,b] Compound M(C)-T[5a,b] (300 mg, 0.9 mmol, 1 eq) and proton sponge (232 mg, 1.08 mmole, 1.2 eq) were dried in vacuo over $P_2O_5$ overnight and then dissolved in anhydrous trimethylphosphate (2.5 mL) under nitrogen in an ice-bath. Then freshly distilled $POCl_3$ (0.10 mL, 1.2 eq) dissolved in anhydrous trimethylphosphate (0.5 mL) was added dropwise using a syringe with stirring. The reaction mixture was further stirred in an ice-bath for 2 h and then a solution of bis-tri-*n*-butylammonium pyrophosphate (2.5 g, 5.27 mmole, 3.5 eq) and tri-*n*-butylamine (2.14 mL) in 3.0 mL of anhydrous DMF was added in one portion. The mixture was stirred at room temperature for 10 min and then triethylammonium bicarbonate solution (0.1 M, pH 8, 70 mL) was added. The reaction mixture was stirred at room temperature for an additional hour, concentrated, and then purified with a DEAE-Sephadex A-25 column using a linear gradient of ammonium bicarbonate (0–0.6 M). The portions eluted out using 0.12–0.15 M ammonium bicarbonate were collected. The process was monitored by the UV absorbance at 290 nm. Lyophilization gave the final product as a white powder (93 mg, 18%). $^1$H NMR ($D_2O$): $\delta$ = 8.00 (s, 1H), 6.27 (t, $J$ = 6.8 Hz, 1H), 4.60 (s, 1H), 4.22 (m, 3H), 2.46 (t, $J$ = 6.8 Hz, 2H), 2.41 (m, 2H), 2.36 (t, $J$ = 2.8 Hz, 1H), 2.27 (m, 2H), 1.68 ppm (m, 2H); $^{31}$P NMR (161 MHz, $D_2O$): $\delta$ = −6.1, −11.1, −21.7 ppm; MS (-ESI) $m/z$: 571 ([$M$−H]$^-$).

*Synthesis of B-TTP analogue **2***

To a solution of M-TTP (5.0 mg, 0.0083 mmol, 1.0 eq) and 3-(azidomethyl)-phenyl-boronic acid (8.0 mg, 0.045 mmol, 5.4 eq) in 120 μL of a mixed solvent ($H_2O$/DMF/EtOH = 1:2:1) was added 50 μL of a solution of TBTA (2.8 mg, 0.0052 mmole, 0.60 eq) and CuBr (0.2 mg, 0.0026 mmole, 0.30 eq) in 100 μL DMF. Then the mixture was stirred vigorously at room temperature for 3 h and centrifuged. Supernatant was removed, and the remaining was washed twice with 100 mM $NH_4HCO_3$ buffer (0.7 mL). The combined washings and supernatant were purified by HPLC to give a white powder after lyophilization (1.9 mg, 31%). $^1$H NMR ($D_2O$): $\delta$ = 7.68 (s, 1H), 7.66 (s, 1H), 7.00 (t, $J$ = 8.4 Hz, 1H), 6.53 (d, $J$ = 8.4 Hz, 1H), 6.34 (s, 1H), 6.05 (t, $J$ = 6.8 Hz, 1H), 5.33 (s, 2H), 4.34 (m, 1H), 4.00 (m, 3H), 3.92 (s, 2H), 2.91 (t, $J$ = 6.4 Hz, 2H), 2.45 (t, $J$ = 6.4 Hz, 2H), 2.12 ppm (m, 2H); $^{31}$P NMR (161 MHz, $D_2O$): $\delta$ = −5.9, −10.8, −19.2 ppm; MS (-ESI) $m/z$: 733 ([$M$−BOH−$H_2O$−H]$^-$).

*Synthesis of B-TTP Analogue **3***

To a solution of M-TTP (5.0 mg, 0.0083 mmol, 1.0 eq) and 2-(azidomethyl)-phenyl-boronic acid (5.1 mg, 0.029 mmol, 3.4 eq) in 120 μL of a mixed solvent ($H_2O$/DMF/EtOH = 1:2:1) was added 45 μL of a solution of TBTA (2.8 mg, 0.0052 mmole, 0.62 eq) and CuBr (0.4 mg, 0.0028 mmole, 0.34 eq) in 90 μL DMF. Then the mixture was stirred vigorously at room temperature for 3 h and centrifuged. Supernatant was removed, and the remaining was washed twice with 100 mM $NH_4HCO_3$ buffer (0.7 mL). The combined washings and supernatant were purified by HPLC to give a white powder after lyophilization (2.0 mg, 31%). $^1$H NMR ($D_2O$): $\delta$ = 7.69 (s, 1H), 7.65 (s, 1H), 7.01 (m, 1H), 6.57 (m, 1H), 6.37 (m, 2H), 6.04

(t, $J$ = 6.8 Hz, 1H), 5.61 (s, 2H), 4.35 (m, 1H), 4.04 (m, 3H), 3.92 (s, 2H), 2.92 (t, $J$ = 6.4 Hz, 2H), 2.46 (t, $J$ = 6.4 Hz, 2H), 2.11 ppm (m, 2H); $^{31}$P NMR (161 MHz, $D_2O$): $\delta$ = −5.1, −10.0, −18.5 ppm; MS (-ESI) $m/z$: 669 ([$M$−BOH−$PO_3$H−H]$^-$).

*Synthesis of B-TTP Analogue **4***

To a solution of M(C)-TTP (5.0 mg, 0.0087 mmol, 1.0 eq) and 3-(azidomethyl)-phenyl-boronic acid (8.0 mg, 0.045 mmol, 5.2 eq) in 120 μL of a mixed solvent ($H_2O$/DMF/EtOH = 1:2:1) was added 50 μL of a solution of TBTA (2.8 mg, 0.0052 mmol, 0.60 eq) and CuBr (0.4 mg, 0.0026 mmol, 0.30 eq) in 100 μL DMF. The mixture was stirred vigorously at room temperature for 3 h and centrifuged. Supernatant was removed, and the remaining was washed twice with 100 mM $NH_4HCO_3$ buffer (0.7 mL). The combined washings and supernatant were purified by HPLC to give a white powder after lyophilization (1.9 mg, 29%). $^1$H NMR ($D_2O$): $\delta$ = 7.86 (s, 2H), 7.63 (d, $J$ = 7.2 Hz, 1H), 7.55 (s, 1H), 7.36 (t, $J$ = 7.2 Hz, 1H), 7.24 (d, $J$ = 7.6 Hz, 1H), 6.25 (t, 1H), 5.59 (s, 1H), 4.58 (m, 2H), 4.22 (m, 3H), 2.76 (t, $J$ = 6.8 Hz, 2H), 2.41 (m, 4H), 1.79 (t, $J$ = 6.4 Hz, 2H), 1.57 ppm (t, $J$ = 6.4 Hz, 2H); $^{31}$P NMR (161 MHz, $D_2O$): $\delta$ = −5.4, −10.3, −18.7 ppm; MS (-ESI) $m/z$: 355.6 ([$M$−2$H_2O$−2H]$^{2-}$).

*Synthesis of B-TTP Analogue **5***

To a solution of M(C)-TTP (5.0 mg, 0.0087 mmole, 1.0 eq) and 2-(azidomethyl)-phenyl-boronic acid (8.0 mg, 0.045 mmol, 5.2 eq) in 120 μL of a mixed solvent ($H_2O$/DMF/EtOH = 1:2:1) was added 50 μL of a solution of TBTA (2.8 mg, 0.0052 mmole, 0.60 eq) and CuBr (0.4 mg, 0.0026 mmole, 0.30 eq) in 100 μL DMF. The mixture was stirred vigorously at room temperature for 3 h and centrifuged. Supernatant was removed, and then the remaining was washed twice with 100 mM $NH_4HCO_3$ buffer (0.7 mL). The combined washings and supernatant were purified by HPLC to give a white powder after lyophilization (0.9 mg, 14%). $^1$H NMR ($D_2O$): $\delta$ = 7.70 (s, 1H), 7.60 (s, 1H), 7.41 (d, $J$ = 6.4 Hz, 1H), 7.24 (m, 1H), 7.09 (d, $J$ = 6.4 Hz, 1H), 6.74 (m, 1H), 6.07 (t, $J$ = 6.4 Hz, 1H), 5.53 (s, 2H), 4.41 (m, 1H), 4.04 (m, 3H), 2.56 (t, $J$ = 6.8 Hz, 2H), 2.20 (m, 4H), 1.59 (t, $J$ = 7.6 Hz, 2H), 1.37 ppm (t, $J$ = 7.6 Hz, 2H); $^{31}$P NMR (161 MHz, $D_2O$): $\delta$ = −5.1, −10.0, −18.5 ppm; MS (-ESI) $m/z$: 355.6 ([$M$−2$H_2O$−2H]$^{2-}$).

*PAGE Analysis of Klenow-Fragment-Catalyzed Primer Extension*

The mixture of 14-nt primer DNA (50 μM), T4 polynucleotide kinase (0.5 units μL$^{-1}$, Biolabs, Inc.) and γ-$^{32}$P-ATP (0.6 μL, from Perkin–Elmer Corp.) in 12 μL T4 kinase buffer solution was incubated at 37 °C for 1 h. The harvested $^{32}$P-labeled DNA was then purified using Microcon YM-3 centrifugal filter (Millipore Corp.) to remove low molecular weight molecules. Using a similar primer extension protocol as previously described,[8] the $^{32}$P-labeled primer alone, reaction mixture without enzyme, reaction mixture without dNTPs, reaction mixture with both enzyme and natural dNTPs, and reaction mixture with B-TTP analogues **2**–**5** together with the other 3 dNTPs as well as enzyme were incubated at 37 °C for 1 h. After reaction, the mixtures were quenched with 2 × DNA loading dye. 3 μL of samples from each reaction was taken and run on 15% PAGE at 300 V for 3 h. After being isolated, fixed, and dried, the gel was developed using autoradiography (overnight) to obtain the film.

*Primer Extension using the Klenow Fragment for MALDI-TOF-MS Studies*

10 μL of 21-nt template (100 μM), 15 μL of 14-nt primer (100 μM), 1 μL of Klenow (5 units μL$^{-1}$), 4 μL of dNTPs (0.2 mM each), 5 μL of 10 × NEB buffer 2, 15 μL of deionized water in a total volume of 50 μL of solution was prepared for the control experiment; 10 μL of 21-nt template (100 μM), 15 μL of 14-nt primer (100 μM), 1 μL of Klenow (5 units μL$^{-1}$), 4 μL of B-TTP analogue 2 (2.5 mM), 4 μL of three other dNTPs (0.2 mM each), 5 μL of 10 × NEB buffer 2, and 11 μL of de-ionized water in a total volume of 50 μL of solution was prepared for the reaction by using B-TTP analogue **2**. The prepared solutions were then incubated at 37 °C for 1 h. After further purification using Microcon YM-3 centrifugal filter (Millipore Corp.) to remove dNTPs and other low molecular weight molecules, the harvested DNA was directly sent for MALDI analysis.

*PCR Incorporation using B-TTP Analogues*

PCR was performed on an Eppendorf Mastercycler thermal cycler with ethidium bromide fluorescent imaging. *Taq* DNA polymerase was purchased from New England Biolabs. Reaction buffer was used as provided by the vendor. 50 μL reaction mixture contains template (90-mer single-strand DNA pool 5′-CCTTCGTTGTCTGCCTTCGT-50N-ACCCTTCA-GAATTCGCACCA-3′, with a final concentration 10 nM, where 50N stands for 50 randomized positions), primer 1 (100 μM, 5′-TGGTGCGAATTCTGAAGGGT-3′), primer 2 (1 μM, 5′-CCTTCGTTGTCTGCCTTCGT-3′), dATP, dCTP, dGTP, dTTP, or one of the B-TTP analogues or MTTP (each with a final concentration of 200 mM), 1 × reaction buffer, and *Taq* polymerase (0.5 U). Reaction thermocycling is composed of initial denaturation (95 °C 2 min), 30 cycles of 95 °C 20 s, 48 °C 20 s, and 72 °C 30 s, and final extension (72 °C 10 min) then held at 4 °C. Reaction product was purified by washing with $H_2O$ in a Millipore Amicon Ultra 10 kDa spin column. The product was then analyzed with 15% PAGE by loading with 1 × gel-loading dye (2.5% Ficoll 400, 11 mM EDTA, 3.3 mM Tris-HCl, 0.017% SDS, 0.015% bromophenol blue at pH 8.0). Denaturing gel analysis was performed by denaturing the purified PCR product at 95 °C for 2 min, followed by addition of 1 × loading dye with 8 M urea at 70 °C and kept for 2 min. The sample was then loaded onto 15% denaturing PAGE containing 8 M urea. One portion of purified PCR product was treated with $H_2O_2$ (final concentration 10 mM) at RT for 2 h before loading onto 15% PAGE.

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[1] R. Metzler, T. Ambjornsson, A. Hanke, Y. L. Zhang, S. Levene, *J. Comput. Theor. Nanosci.* **2007**, *4*, 1–49.

[2] a) L. C. Bock, L. C. Griffin, J. A. Latham, E. H. Vermaas, J. J. Toole, *Nature* **1992**, *355*, 564–566; b) D. Proske, M. Blank, R. Buhmann, A. Resch, *Appl. Microbiol. Biotechnol.* **2005**, *69*, 367–374; c) M. A. Syed, S. Pervaiz, *Oligonucleotides* **2010**, *20*, 215–224.

[3] V. Maojo, F. Martin-Sanchez, C. Kulikowski, A. Rodriguez-Paton, M. Fritts, *Pediatr. Res.* **2010**, *67*, 481–489.

[4] a) D. C. Pregibon, M. Toner, P. S. Doyle, *Science* **2007**, *315*, 1393–1396; b) J. Scheuermann, D. Neri, *ChemBioChem* **2010**, *11*, 931–937.

[5] a) J. Gierlich, G. A. Burley, P. M. E. Gramlich, D. M. Hammond, T. Carell, *Org. Lett.* **2006**, *8*, 3639–3642; b) J. Gierlich, K. Gutsmiedl, P. M. E. Gramlich, A. Schmidt, G. A. Burley, T. Carell, *Chem. Eur. J.* **2007**, *13*, 9486–9494; c) P. M. E. Gramlich, C. T. Wirges, A. Manetto, T. Carell, *Angew. Chem.* **2008**, *120*, 8478–8487; *Angew. Chem. Int. Ed.* **2008**, *47*, 8350–8358; d) C. F. Dai, L. F. Wang, J. Sheng, H. J. Peng, A. B. Draganov, Z. Huang, B. H. Wang, *Chem. Commun.* **2011**, *47*, 3598–3600.

[6] a) K. Sakthivel, C. F. Barbas, *Angew. Chem.* **1998**, *110*, 2998–3002; *Angew. Chem. Int. Ed.* **1998**, *37*, 2872–2875; b) J. D. Vaught, C. Bock, J. Carter, T. Fitzwater, M. Otis, D. Schneider, J. Rolando, S. Waugh, S. K. Wilcox, B. E. Eaton, *J. Am. Chem. Soc.* **2010**, *132*, 4141–4151.

[7] a) J. Yoon, A. W. Czarnik, *J. Am. Chem. Soc.* **1992**, *114*, 5874–5875; b) T. D. James, S. Shinkai, *Top. Curr. Chem.* **2002**, *218*, 159–200; c) W. Wang, X. Gao, B. Wang, *Curr. Org. Chem.* **2002**, *6*, 1285–1317; d) W. Yang, X. Gao, B. Wang, *Med. Res. Rev.* **2003**, *23*, 346–368; e) J. Yan, H. Fang, B. Wang, *Med. Res. Rev.* **2005**, *25*, 490–520; f) S. Jin, Y. F. Cheng, S. Reid, M. Li, B. Wang, *Med. Res. Rev.* **2010**, *30*, 171–257.

[8] a) N. Lin, J. Yan, Z. Huang, C. Altier, M. Li, N. Carrasco, M. Suyemoto, L. Johnston, S. Wang, Q. Wang, H. Fang, J. Caton-Williams, B. Wang, *Nucleic Acids Res.* **2007**, *35*, 1222–1229; b) X. C. Yang, C. F.

  

Dai, A. Dayan, C. Molina, B. H. Wang, *Chem. Commun.* **2010**, *46*, 1073–1075.
[9] M. Y. Li, N. Lin, Z. Huang, L. P. Du, C. Altier, H. Fang, B. H. Wang, *J. Am. Chem. Soc.* **2008**, *130*, 12636–12638.
[10] a) R. Huisgen in *1,3–Dipolar Cycloaddition Chemistry*, *Vol.* (Ed.: A. Padwa), Wiley, New York, **1984**, pp. 1–176; b) H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem.* **2001**, *113*, 2056–2075; *Angew. Chem. Int. Ed.* **2001**, *40*, 2004–2021; c) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 2708–2711; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596–2599; d) C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057–3064.

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