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Facilitated synthetic access to boronic acid-modified nucleoside triphosphates and compatibility with enzymatic DNA synthesis

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Abstract

Decorating nucleic acids with boronic acids can extend the usefulness of oligonucleotide-based tools to the development of medical imaging agents, the promotion of binding of aptamers to notably more challenging targets, or the detection of (poly)saccharides. However, due to their hygroscopic nature and high intrinsic reactivity, protocols for the introduction of boronic acids into nucleic acids are scarce. Here, we have explored different synthetic routes for the crafting of nucleoside triphosphates equipped with phenyl boronic acids. The strain-promoted azide–alkyne cycloaddition (SPAAC) appears to be the method of choice for such a purpose and enabled us to prepare a modified nucleotide. Enzymatic DNA synthesis permitted the introduction of up to thirteen boronic acid residues in oligonucleotides which bodes well for its extension to SELEX and related methods of *in vitro* selection of functional nucleic acids.

Key words: nucleoside triphosphate • click chemistry • DNA • boron chemistry • SELEX

Introduction

Aptamers^{1, 2} and DNAzymes³ are single-stranded oligonucleotides capable of selective binding to specific targets with high affinity and catalysis of chemical transformations, respectively. These functional nucleic acids are all obtained by application of a Darwinian *in vitro* evolution method coined SELEX (Selection of Ligands by Exponential enrichment).⁴ Aptamers are now well-established tools in numerous applications^{2, 5} and their general usefulness is undisputed since the FDA-approval of Macugen®.^{6, 7} Similarly, even though DNAzymes represent more recent additions to functional nucleic acids, two first clinical trials of DNAzymes against nodular basal-cell carcinoma⁸ and asthma⁹ have recently been reported. Nonetheless, functional nucleic acids suffer from shortcomings that restrict some *in vivo* applications. Consisting of DNA or RNA oligonucleotides, they are prone to rapid nuclease-mediated degradation, display poor cellular penetration capacities, and generally exhibit short *in vivo* residence times due to efficient renal filtration.^{1, 10} These limitations can at least be partially remediated by introducing chemical modifications into the nucleosidic scaffold of aptamers, DNAzymes, and in general in therapeutic oligonucleotides.^{7, 11, 12} These additional groups can be introduced into functional nucleic acids either post-selection by automated solid-phase synthesis (post-SELEX approach) or by supplementing selection experiments with modified nucleoside triphosphates (dN*TPs; mod-SELEX approach).^{12, 13} The latter avoids uncertain and tedious engineering of functional nucleic acids by allowing for direct selection of modified sequences.^{14, 15, 16} The polymerization of dN*TPs broadens the chemical space that can be explored during SELEX, but also allows the introduction of functional groups alien to biology¹⁷ that can be harnessed in applications ranging from electrochemical analysis¹⁸ and surface immobilization¹⁹ to artificial metal base-pair formation.²⁰ In this context, boronic acid moieties specifically interact with *cis*-1,2 or 1,3-diols in aqueous conditions to form the corresponding cyclic boronic esters in a covalent, yet reversible manner.²¹ Due to these intrinsic properties, this functional group has extensively been used for the development of biosensors,²² catalysts,^{23, 24} imaging agents,²⁵ construction of oligonucleotide scaffolds²⁶ therapeutic agents,²⁷ and for surface immobilization.²⁸ In addition, decoration of nucleotides with boronic acids has been suggested to be useful for SELEX to raise aptamers against more demanding targets such as glycosylated proteins.^{23, 29, 30} In addition, appendage of boronic acids on aptameric scaffolds could be harnessed for surface immobilization,³¹ post-SELEX modification strategies,³² or developing potent tools for medical imaging applications.³³ However, despite these intrinsic and alluring properties, synthetic protocols for the facile and robust access to boronic acid-modified DNA nucleobases are scarce.³⁴⁻³⁶

Here, we have explored different synthetic strategies based on copper(I)-catalyzed alkyne–azide cycloaddition (CuAAC or click chemistry)^{16, 35, 37} and strain-promoted copper-free click reactions³⁸

for the reliable introduction of boronic acid modifications on the nucleobase of dN*TPs (Figure 1). We demonstrate that fine tuning of the chemical nature of the substituents on the boronic residues is of critical importance in this approach. Also, we highlight the usefulness of copper-free click reactions for the preparation of such modified nucleotides. Lastly, we highlight the successful enzymatic incorporation of boronic acid-modified dN*TPs into DNA oligonucleotides using polymerase-mediated synthesis.

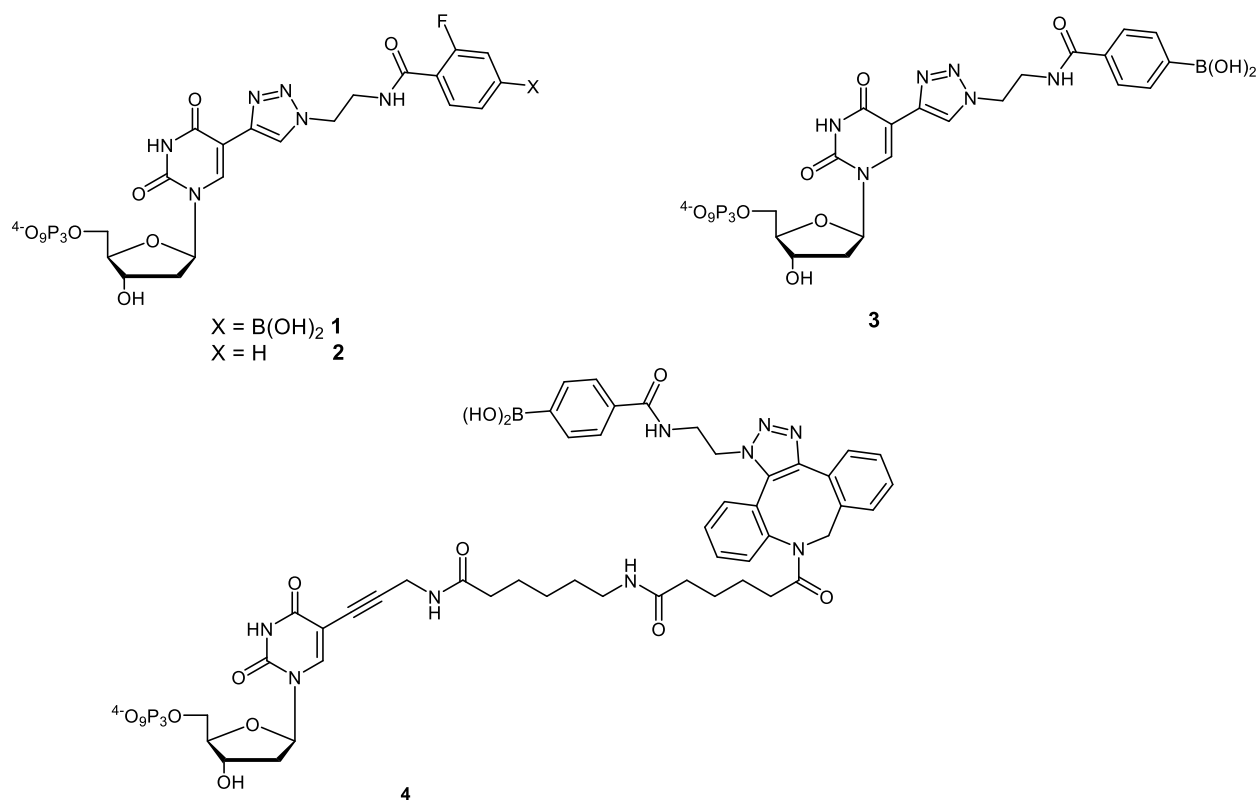
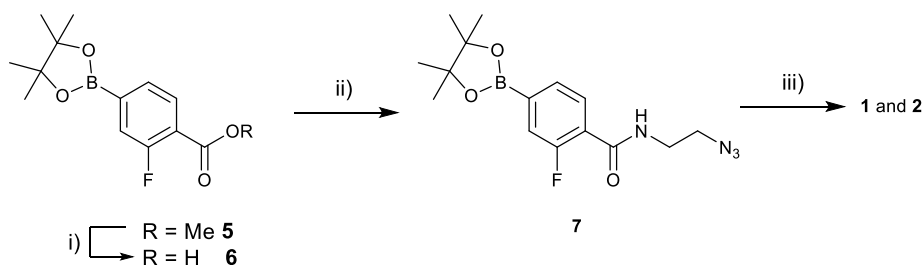


Figure 1. Chemical structures of dU^{BOHF}TP **1**, dU^{PhF}TP **2**, dU^{BOH}TP **3**, and dU^{COBOH}TP **4**.

Results and Discussion

Our initial design was based on the fluorophenylboronic acid motif which is particularly adapted for efficient, potential interactions between aptamer and sugar-containing targets since it displays very high forward rate constants (k_{on} of around $340 \text{ M}^{-1} \text{ s}^{-1}$) for the formation of boronates and favorable equilibrium constants (K_{eq} in the 10^{-5} - 10^{-6} M^{-1} range) of the resulting boronates in aqueous medium.³⁹ Particularly, while *ortho*-fluoro derivatives display reduced hydrolytic stabilities, 4-carboxy-3-fluorophenylboronic acid-based analogs are stable and have been used in various applications including the immobilization of RGD-peptides on solid supports.²⁸ Despite the presence of copper salts, the CuAAC reaction was recently shown to be compatible with the

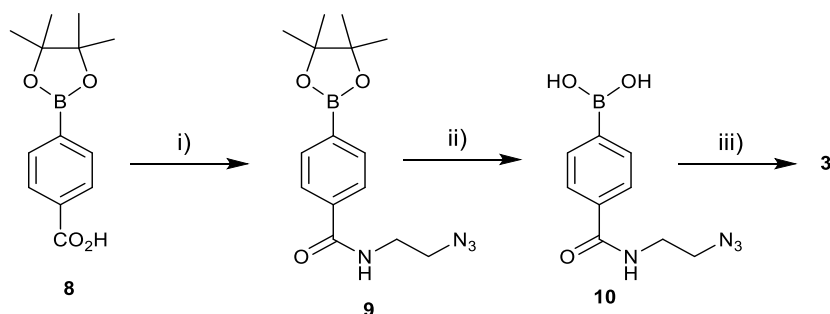
introduction of boronic acids into DNA and RNA oligonucleotides.^{23, 30, 34, 40} Based on these interesting properties, we sought to question whether triphosphates obtained by the modification of the highly useful synthon 5-ethynyl-2'-deoxyuridine 5'-triphosphate (EdUTP)^{41, 42} by the CuAAC reaction could serve as substrates for polymerases. Consequently, we set out to synthesize dUB^{OH}FTP **1** by first converting the pinacol-protected *meta*-fluorophenylboronic acid derivative **5** into the corresponding azide **7** under standard amide-bond forming conditions following hydrolysis of the methyl ester group (Scheme 1). Next, EdUTP was subjected to the CuAAC reaction with azide **7**, which yielded the pinacol-protected triphosphate exclusively (data not shown). The protecting group could be removed by mild treatment of the nucleotide analog with ammonium hydroxide. Surprisingly, besides the expected triphosphate **1** (18% yield), deboronated derivative **2** was formed in rather high yields (29%). The formation of undesired nucleotide **2** was attributed to the electron withdrawing *meta*-fluorine of the arylboronic residue which can induce efficient protodeboronation of arylboronic acids under basic conditions which are required for the deblocking of the pinacol protecting group.⁴³ This analysis of the resulting products was further complicated by the complex behaviour of arylboronic acid derivatives under mass spectrometry conditions.⁴⁴



Scheme 1. Synthesis of modified triphosphates **1** and **2**. Reagents and conditions: i) THF, NaOH, RT, 4h; ii) a) (COCl)₂, DMF (cat), CH₂Cl₂, RT, 1h; b) 2-azidoethylamine, DMAP, NEt₃, CH₂Cl₂, THF, RT, 12h, 31% over 2 steps; iii) a) EdUTP, CuBr, TBTA, H₂O, DMF, rt, 4h, b) NH₄OH, RT, 2h; **1** (18%) and **2** (29%).

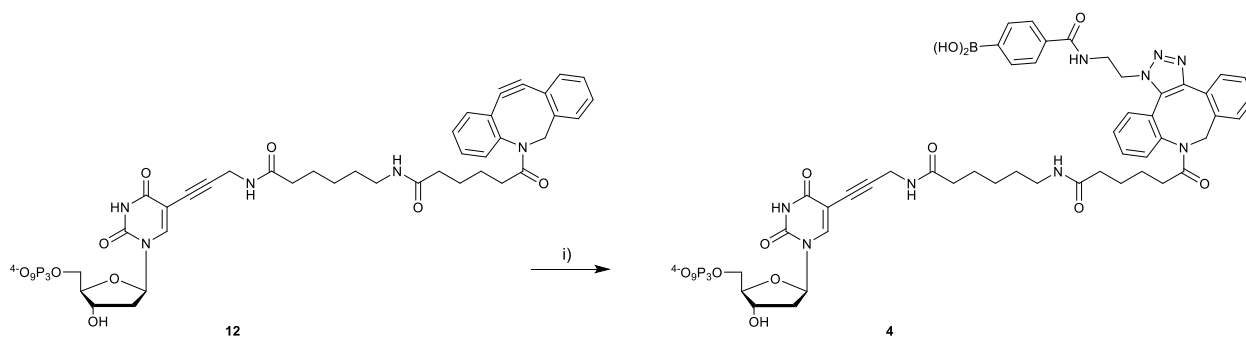
The apparent intrinsic reduced stability of boronic acid **1** under basic reaction conditions required for removal of the protecting group prompted us to synthesize more stable azide building blocks where the *meta*-fluorine substituent was omitted (nucleotide **3** in Figure 1). In addition, we compared synthetic protocols involving copper-dependent to strain promoted, copper-free click reactions. The synthetic route to triphosphate **3** is similar to that of nucleotide **1** with the exception that removal of the pinacol blocking group was carried out at the level of the azide derivative **9**, prior to the CuAAC reaction (Scheme 2). Characterization by NMR, HRMS, and MALDI-ToF of

nucleotide **3**⁴⁵ revealed a predominant compound corresponding to an oxidatively deborylated species previously observed in other related nucleosides and nucleotides.^{34, 35, 44}



Scheme 2. Synthesis of modified triphosphate **3**. Reagents and conditions: i) 2-azidoethylamine, HATU, sym-collidine, DMF, RT, 19h, 64%; ii) NaIO₄, ammonium acetate, acetone, RT, 24h, 98%; iii) EdUTP, CuBr, TBTA, H₂O, DMF, rt, 12h, 29%.

In order to rule out that protodeboration occurred during the copper-catalyzed click reaction, we also carried out the addition of the arylboronic acid moiety on the nucleobase under milder, copper-free conditions. To do so, we first synthesized a nucleotide functionalized with an aza-dibenzocyclooctyne (ADIBO)⁴⁶ moiety (see the Supporting Information). This was achieved by application of standard amide bond formation conditions for the conjugation of dibenzoazacyclooctyne with amino-modified dUTP nucleotide **11** in good yields (64%). The resulting ADIBO-modified nucleotide (dU^{CO}TP) **12** was then subjected to copper-free click reaction conditions⁴⁷ to yield nucleotide analog **4** in 71% yield (Scheme 3).⁴⁸



Scheme 3. Synthesis of modified triphosphate **4**. Reagents and conditions: i) DMF, RT, 12h, 71%.

With the modified nucleotides **1-4** at hand, we sought to question whether the presence of the modifications, particularly the boronic acids, connected to the C5 position of the pyrimidine

nucleobase via rigid triazole linkers would be tolerated by DNA polymerases. To do so, we turned to primer/template systems that have previously been used to evaluate the substrate acceptance of dN*TPs under primer extension reaction (PEX) conditions.^{15, 42, 49} First, we used a 31-mer DNA template **T1** and a 15 nucleotide long primer **P1** carrying a fluorescein dye at the 5'-end (Supporting Information)⁴⁹ and tested family A and B DNA polymerases along with the template-independent polymerase Terminal deoxynucleotidyl Transferase (TdT).⁵⁰

Full extension of primer **P1** was observed when triphosphate dUB^{OHF}TP **1** was used in conjunction with Therminator, Vent (*exo*'), Deep Vent, and the T4 DNA polymerases, despite the propensity of dUB^{OHF}TP **1** to deboronate (Figure S1A). The remaining polymerases (i.e., Taq, *Bst*, and Kf *exo*') were not efficient at accepting nucleotide **1** as a substrate. On the other hand, triphosphate dU^{PhF}TP **2** acted as a moderate substrate for polymerases since formation of expected full length products came at the expense of stalling of the polymerase leading to truncated species (Figure S1B). Like the case of nucleotide **1**, all the polymerases except the T4 DNA polymerase readily accepted triphosphate dU^{BOH}TP **3** as a substrate and yielded fully extended primers (Figure S1C). Of all nucleotides, analog dU^{COBOH}TP **4** displayed the best substrate capacity since full length products could be observed under all conditions with little or no formation of truncated products (Figure S2). As expected, the incorporation of all modified nucleotide analogues into DNA led to important retardation in the gel mobility and thus shifted product bands and can be explained by the presence of the rather bulky modifications combined with the differential interaction of the Lewis acid (boronic acid group) and the gel matrix.^{35, 51} Interestingly, these gel shifts differences were by far the largest when nucleotide **4** was supplemented to the reaction mixtures.

A similar trend in substrate acceptance was observed when primer extension reactions were performed with the TdT and 19 nt long, 5'-FAM-labelled primer **P2**. Indeed, longer reaction times combined with Mn²⁺ or Co²⁺ as cofactor and nucleotide **4** led to the formation of larger molecular weight product distributions which are typically observed with canonical nucleotides (Figure S3). On the other hand, tailing reactions with nucleotides **1-3** produced only primers extended with one or two modified nucleotides (Figure S4).

Encouraged by these initial results, we next sought to question whether longer, boronic acid-modified DNA sequences can be obtained since this represents an important prerequisite for their potential use in selection experiments. Consequently, we used a 19-mer 5'-FAM-labelled primer (**P3**) along with a 79-nucleotide long template (**T2**) to determine the substrate acceptance of nucleotide **4**. In addition, we only focused on boronic-acid containing nucleotide dU^{COBOH}TP **4** to synthesize longer DNA sequences given the superior substrate acceptance of this analogue. Analysis of PEX reactions confirmed this since the modified analogue was readily accepted as a

substrate by all polymerases that were evaluated and primers were fully converted to the expected products (Figure 2). As noticed above for PEX reactions with shorter templates and TdT-mediated tailing reactions, the introduction of *thirteen* boronic acid residues induced an important shift in electrophoretic mobility.

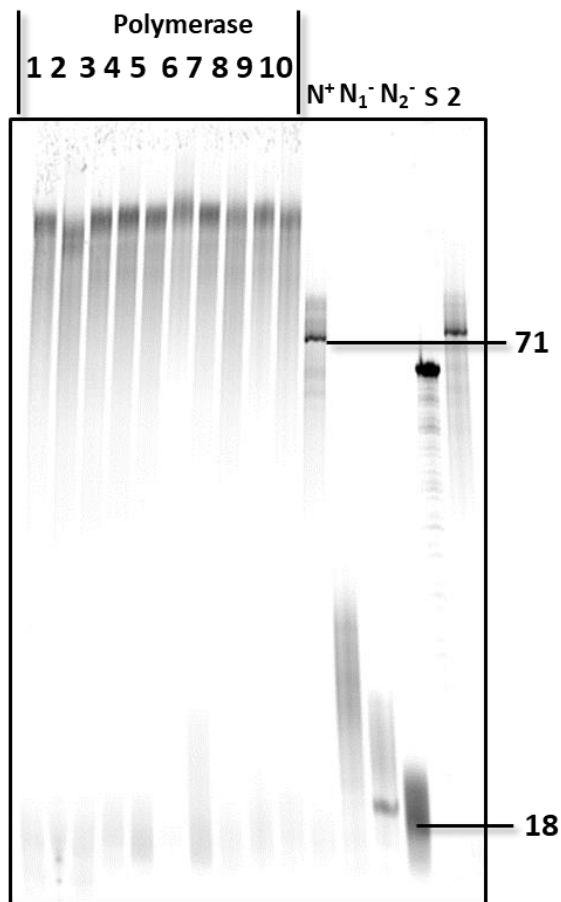


Figure 2. Gel image (PAGE 20%) analysis of primer extension reactions with primer **P3** and template **T2** with dU^{COBOH}TP **4** (200 μ M). Polymerases used: 1: Phusion; 2: HemoKlem Taq; 3: Q5 DNA polymerase; 4: Bst; 5: Taq; 6: Therminator; 7: Vent (*exo*); 8: Dpo4; 9: Deep vent; 10: Klenow *exo*. Control reactions: N⁺: all 4 natural dNTPs; N₂⁻: dATP, dCTP, dGTP; N₁⁻: dATP, dGTP. Lane **S** represents a 5'-FAM-labelled, 71-mer synthetic oligonucleotide reference, lane **2** represents the PEX reaction product obtained with nucleotide **2**, **71** corresponds to expected, 71-nt long full length product, and **18** corresponds to unreacted, 18-nt long primer.

Given the excellent substrate capacity of nucleotide **4**, we next evaluated the possibility of producing boronic acid-modified DNA using PCR. To do so, we performed PCR with the 79-mer template **T3** and primers **P4** and **P5** using five different polymerases to see whether amplicons

could be produced with nucleotide **4** (Figure 3). This analysis revealed that Vent (*exo*) and HemoKlem Taq accepted modified nucleotide **4** and produced full length amplicons, while all other polymerases failed to yield any significant amount of expected product. Also here, boronic acid modified DNA presented an important shift in electrophoretic mobility, presumably due to a substantial interaction between the boronic acid moieties and the cis-diols of the galactose sugar of agarose.

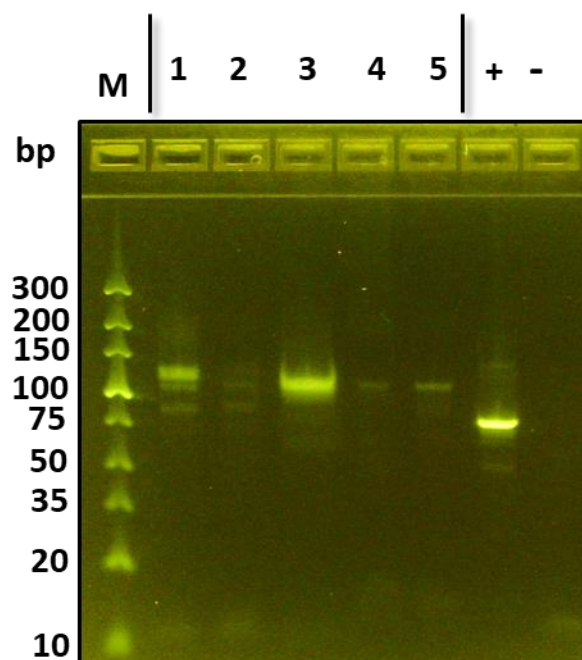


Figure 3. Agarose gel (4%) analysis of PCR products obtained with template **T3**, primers **P4** and **P5**, and a mixture of dATP, dCTP, dGTP, and nucleotide **4** (all 200 μ M). Polymerases used: 1: Vent (*exo*); 2: Taq; 3: HemoKlem Taq; 4: Q5 DNA polymerase; 5: Phusion. Control reactions: +: PCR with all four natural nucleotides and -: PCR without any polymerase and with all four natural dNTPs. Full experimental details can be found in the Supporting Information.

In order to confirm the identity of the product stemming from the PEX reactions with nucleotide analogue **4**, we carried out a digestion-LC-MS analysis protocol.⁵² To do so, we first converted triphosphate **4** to the corresponding nucleoside with Shrimp Alkaline Phosphatase (rSAP). LC-MS analysis clearly revealed the presence of the expected nucleoside analogue equipped with a boronic acid moiety, further confirming the chemical nature of dU^{COBOH}TP **4**. Next, we performed a PEX reaction with the 20-nucleotide long template **T4** and the 5'-FAM-labelled, 19-mer primer **P6** with Vent (*exo*) and dU^{COBOH}TP **4**. The resulting modified DNA sequence containing a single dU^{COBOH}MP residue was then nuclease-digested, dephosphorylated and the resulting deoxynucleosides were then analyzed by LC-MS (Figure 4). This analysis clearly demonstrates

the successful incorporation of the modified nucleotide without inducing the loss of the boronic acid moiety.

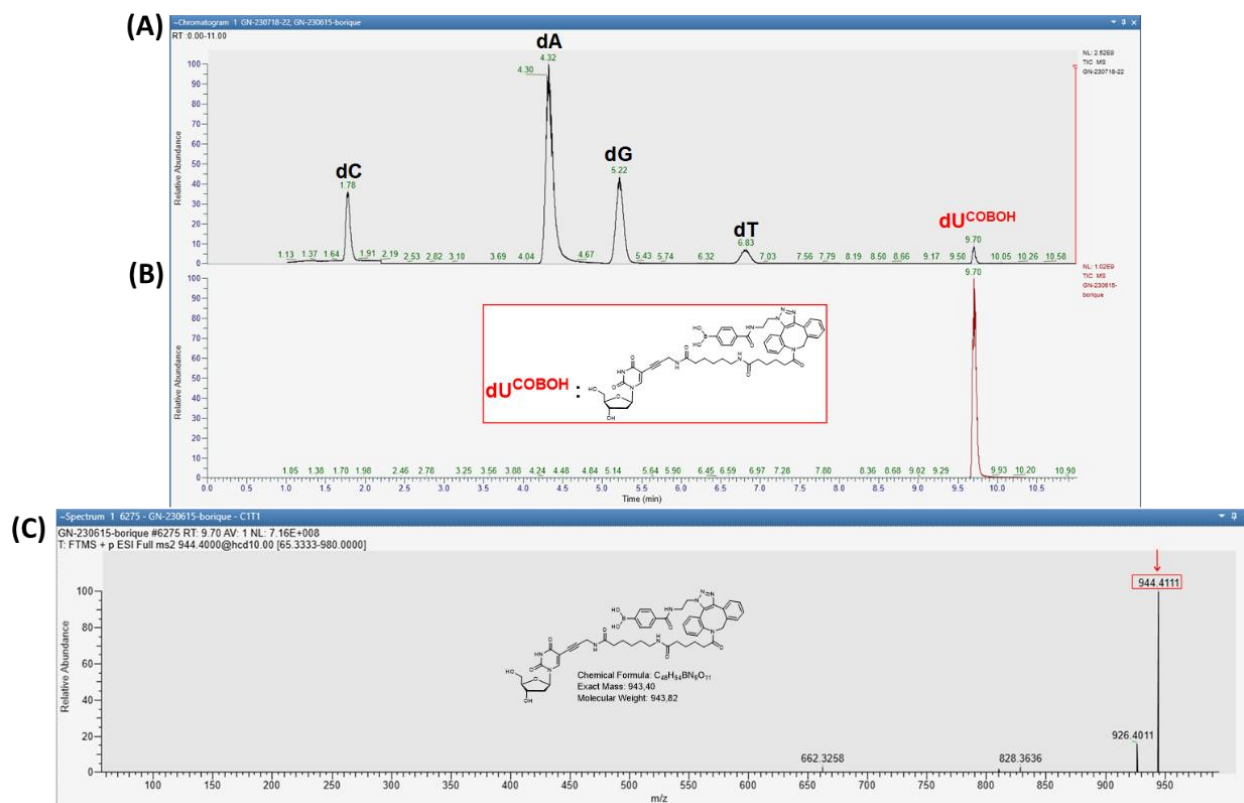


Figure 4. Analysis of digested, modified dsDNA. (A) LC-MS chromatogram showing the nucleoside digestion profile of double-stranded DNA after PEX reaction with template **T4** and primer **P6**. The chromatogram reveals five distinct peaks, four of which correspond to the canonical nucleosides of the template and primer: deoxycytidine (dC), deoxyadenine (dA), deoxyguanine (dG) and deoxythymidine (dT). The additional peak represents the modified deoxyuridine (dU^{COBOH}), indicating the successful insertion of dU^{COBOH}TP during PEX reactions and its subsequent digestion into dU^{COBOH}. (B) LC-MS chromatogram serves as the (dU^{COBOH}) standard. (C) MS spectrum of dU^{COBOH} after digestion.

Conclusion

Decorating nucleic acids with boronic acids is an alluring aim since these functional groups can be harnessed for post synthetic and orthogonal modification strategies including conversion of oligonucleotides into medical imaging devices. These functional groups might also endow aptamers with the possibility of interacting with highly glycosylated targets. However, due to their

inherent reactivity, synthetic methods for the introduction of boronic acids, especially in longer oligonucleotides are scarce. Here, we explored the effect of substituents of aryl boronic acid residues on the stability of these synthons in copper-catalyzed click reactions and investigated the efficiency of the coupling strategy. While the presence of *ortho*-fluoro substituents on phenylboronic acids might be useful for numerous applications, such synthons do not seem to be suitable for CuAAC reactions on nucleotides. In addition, due to the milder conditions and the absence of copper, strain-promoted azide–alkyne cycloaddition (SPAAC) appears as the method of choice for the crafting of nucleotides decorated with boronic acids. The resulting nucleotide dU^{COBOH}TP **4** proved to be an excellent substrate for a large number of DNA polymerases under primer extension reactions and could also be used under PCR conditions. The combination of this modified nucleotide with polymerase-mediated synthesis allowed the generation of DNA sequences containing up to thirteen boronic acid residues. Such an approach will be useful for the preparation of nucleotides modified with boronic acids in order to raise aptamers with enhanced properties and target scope.

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Supporting Information

YES (this text will be updated with links prior to publication)

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45. Synthesis of modified nucleotide **3**:
 5-EdUTP (4.7 mg, 9.5 μ mol) was dissolved in water (500 μ L). To this solution the azide **9** (5 mg, 19 μ mol, 2 eq.) and EtOH (500 μ L) was added. Finally, a freshly prepared solution of TBTA (3 mg, 5.7 μ mol, 0.6 eq.) Cul (1 mg, 2.8 μ mol, 0.3 eq.) in DMF (1 mL) was added and the reaction mixture was stirred at RT. After 3.5h additional catalyst TBTA (3 mg, 5.7 μ mol, 0.6 eq.) Cul (1 mg, 2.8 μ mol, 0.3 eq.) in DMF (100 μ L) was added and the reaction mixture was stirred for O.N. in the fridge. The product was precipitated in NaClO₄ 2% in acetone (10 mL), centrifuged, the pellet redissolved in ammonium hydroxide 30% (2 mL) and stirred for 2h. The reaction mixture was then concentrated, pre-purified by precipitation in NaClO₄ 2% in acetone (10 mL) and purified by RP-HPLC (30% B in 20min; Buffer A, 20 mM TEAA in H₂O; Buffer B, 30% 20 mM TEAA in H₂O / 70% MeCN) chromatography to yield (2 mg, 29%) of the desired triphosphate **3**.
 HR ESI MS of **3** [M-B(OH)]⁻ C₂₀H₂₄N₆O₁₆P₃⁻ *m/z* calc. 697.0467; found [M-B(OH)]⁻ 697.0253.
 MALDI of **3** [M-B(OH)]⁻ C₂₀H₂₄N₆O₁₆P₃⁻ *m/z* calc. 697.0467; found [M-B(OH)]⁻ 696.546.
¹H-NMR (D₂O) of **3** δ = 2.43 (2H, s), 3.04 (2H, m), 3.87 (2H, s), 4.21 (4H, s), 6.31 (1H, s), 6.92 (1.5H, s), 7.59 (2H, s), 7.80 (0.5H, s), 8.22 (1H, s), 8.39 (1H, b).
³¹P-NMR (D₂O) of **3** δ = -4.93 (1P, b), -9.89 (1P, b), -21.03 (1P, b).
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48. Synthesis of modified nucleotide **4**:
 In a 1.5 ml Eppendorf tube, **10** (3.95 mg, 0.017 mmol, 5eq.) is dissolved in 100 μ L DMF and 98,5 μ L of 34.2 mM stock solution of dU^{CO}TP **12** (3.2 mg, 0.003 mmol, 1eq.) is added in the Eppendorf. The reaction mixture is shaken at 1000 rpm for 12 hours. DMF was then evaporated *in vacuo* and the crude resuspended in 200 μ L H₂O. The mixture was poured in 12 mL NaClO₄ 2% in acetone to precipitate the crude product, centrifuged at 4000 rpm for 15 minutes, and the supernatant was discarded. The crude was dissolved in 1 mL H₂O and the product was purified by semi-prep reverse phase HPLC (10-50% B in 15 minutes, hold at 50% for 10 minutes, 50-90% B in 5 minutes, 50-90% B in 5 minutes; Buffer A, 20 mM TEAA in H₂O; Buffer B, 30% 20 mM TEAA in H₂O / 70% MeCN) with a Kinetex 5 μ m C18 100 Å LC column to yield 2.82 mg of triphosphate (71%).
¹H NMR (500 MHz, D₂O) δ 7.97 (d, *J* = 17.7 Hz, 1H), 7.89-7.86 (m, 2H), 7.72-7.62 (m, 4H), 7.58-7.38 (m, 7H), 6.83 (d, *J* = 7.8 Hz, 1H), 6.10 (q, *J* = 6.4 Hz, 1H), 5.34 (d, *J* = 17.3 Hz, 1H), 5.07 (dd, *J* = 21.1, 11.0 Hz, 1H), 4.31-4.10 (m, 9H), 3.98 (d, *J* = 14.7 Hz, 2H), 3.47-3.37 (m, 1H), 2.41-2.04 (m, 8H), 1.88 (t, *J* = 7.3 Hz, 1H), 1.67 (dp, *J* = 15.5, 7.5 Hz, 3H), 1.59-1.50 (m, 3H).

^{31}P NMR (202 MHz, D_2O) δ -10.85 (d, J = 19.8 Hz), -11.54 (d, J = 20.6 Hz), -23.27 (t, J = 20.3 Hz).

HRMS (ESI) calcd for $\text{C}_{48}\text{H}_{57}\text{BN}_9\text{O}_{20}\text{P}_3$, $[\text{M-BOH}]^-$ = 1155.29, found $[\text{M-BOH}]^-$ = 1155.2892.

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