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Selective chemical modification of DNA with boronic acids by on-column CuAAC reactions

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Abstract The use of the Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction for the preparation of oligonucleotide conjugates is by now familiar. However, the selective introduction of boronic acids into DNA and RNA sequences by CuAAC reactions has long been considered impossible due to the incompatibility of the boronic acid moiety with copper salts. Here we describe two new methods for the selective on-column functionalization of oligonucleotides with boronic acids via two different CuAAC reactions. The first one allows the introduction of a phenylboronic acid at the 5'-extremity of oligonucleotides, while the selective intrastrand positioning of the modification can be achieved with the second one. Both methods were applied to the DNA and RNA series (up to a 20-mer) with good isolated yields and excellent purities. These results illustrate the potential of the reported methods for selective incorporation of boronic acids into oligonucleotides.

Key words CuAAC, boronic acid, oligonucleotide, bioconjugation, DNA, RNA.

The widespread properties of boronic acid-containing organic compounds has resulted in their use in a large number of applications in various fields, including organic chemistry,¹ medicinal chemistry,² asymmetric synthesis,³ catalysis⁴ and materials.⁵ Due to their trigonal planar sp²-hybridized boron atom bearing two hydroxyl groups and an empty p-orbital, boronic acids can form covalent and reversible complexes with electron-donating centres such as oxygen and nitrogen nucleophiles. In aqueous media, when the pH is higher than the pK_a, this Lewis acid–base reaction results in a rehybridization of the boron atom from sp² to sp³ leading to an anionic tetravalent borate species.⁶ The reversible reaction with 1,2 cis-diols leading to cyclic boronate esters has notably been used to create self-organizing systems, sensors and pH responsive materials.⁷ Given the versatile features of boronic acids, it is therefore not surprising that the incorporation of this moiety into biomolecules is currently attracting a lot of attention to develop new bioconjugates endowing new properties. While the site-selective modification of peptides and proteins with boronic acids has made huge strides in recent years,⁸ their incorporation into DNA remains challenging. One of the reasons is that the cornerstone Cu catalyzed azide-alkyne cycloaddition (CuAAC) reaction is difficult to apply, given the lack of stability of boronic acids in the presence of copper salts.⁹

Modified oligonucleotides (ONs) are usually obtained either by automated chemical synthesis¹⁰ or by enzymatic chain assembly with appropriately modified phosphoramidite building blocks or modified nucleoside triphosphates, respectively.¹¹ Both approaches have the disadvantage of requiring a multi-step synthesis of the modified monomers, which subsequently have to tolerate the

reagents and conditions used for the elongation. An attractive alternative is the site-specific postsynthetic conjugation of a desired functionality to a defined reactive group incorporated into the ON.¹² This latter strategy is highly modular, easier to handle, and usually gives better yields.¹³

Considering the broad variety of applications that can be achieved with borono-based DNA in particular in the field of aptamers,¹⁴ the two strategies described above have been applied to introduce a boronic acid moiety into ONs. In the laboratory, we have extensively studied the phosphoramidite approach that led us to develop enzyme-free DNA- and RNA-templated ligation systems with 5'-boronic acid modified oligonucleotides.¹⁵ We have also demonstrated the ability of borono-based ONs to inhibit nucleases including RNase H.¹⁶ Enzymatic polymerization,¹⁷ amide bond formation¹⁸ and strain-promoted alkyne-azide cycloaddition (SPAAC)¹⁹ have been evaluated as well by other research groups, all of them requiring the prior multi-step synthesis of modified nucleosides or solid-phase synthesis of boronic acid reactive species. In this context, the development of a rapid, efficient

Figure 1 Schematic illustration of on-column CuAAC conjugation of boronic acids. Blue spheres represent the CPG solid-support; Reagents and conditions: Method 1: i. CH₃P(OPh)₃I (500 equiv.), DMF, rt, 15 min; ii. TMGA (300 equiv.), ACN, 60 °C, 1,5 h.; CuAAC: 1 (8 equiv.), CuSO₄•5H₂O (1 equiv.), sodium ascorbate (5 equiv), THPTA (3 equiv.), ACN/H₂O (4/6), 60 °C, 1 h. Method 2: CuAAC: 2 (20 equiv.), CuSO₄•5H₂O (1 equiv.), sodium ascorbate (5 equiv.), THPTA (3 equiv.), 2 (20 equiv.), dioxane/ACN/H₂O (3/1/6), MW 60 °C, 2 h. Cleavage and deprotection: NH₄OH, 55 °C, 5 h for DNA synthesis; DBU 1 M, rt, 3 min, NH₄OH, 37 °C, 3 h for RNA synthesis.

and modular approach for postsynthetic introduction of boronic acid functions into DNA is highly desirable. Herein we report the on column introduction of phenylboronic acid moieties either at the 5'-extremity or at an internal position of the ONs via two different CuAAC reactions (Figure 1): (a) Method 1 involves reaction of a 5'-azido modified ON with 4-ethynylphenylboronic acid pinacol ester 1, and (b) Method 2 implicates an incorporated 2' O propargyluridine and 4-(azidomethyl)phenylboronic acid MIDA ester 2.

The insertion of Cu(I) between carbon-boron bonds is a well-documented side reaction that can hamper the efficacy of the CuAAC reaction.²⁰ This observation has led many research groups to consider that the selective labeling of DNA with boronic acid was incompatible with the CuAAC reaction,^{18b, 19b} although various groups including ours have shown that appropriately protected boronic acids can be successfully used with CuAAC reactions in the context of small molecule synthesis.^{9, 21}

Inspired by these results, we started our study by evaluating the possibility of performing the on-column attachment of commercially available 4-ethynylphenylboronic acid pinacol ester 1 at the 5'-extremity of DNA sequences (Figure 1, Method 1). 5' azido-modified sequences were obtained following the two-steps protocol developed by Kool and co workers.²² All the oligonucleotides were prepared using standard phosphoramidite chemistry on a DNA synthesizer with removal of the terminal DMT group. The 5'-terminal hydroxyl groups were then converted to the corresponding 5' iodide via the on-column reaction with (PhO)₃PCH₃I in DMF for 15 min at r.t.. The conversion of 5'-iodides to 5'-azides proceeded to completion by on-column reaction with a solution of tetramethylguanidinium azide (TMGA) in acetonitrile for 90 min at 60 °C. Excess of TMGA was removed by washing the column with a sodium bisulphite solution. In each step, a syringe with the

reagent was fitted to one end of the DNA synthesis column containing the CPG-bound ODN and an empty syringe was fitted to the other end. 1 mL of reagent was passed through the column from one syringe to the other for the appropriate time before putting the column on a shaker at the appropriate temperature.

The CuAAC reaction conditions were optimized for the tetranucleotide (5'-N3dTCCG-3') until full conversion was observed by HPLC analysis with no noticeable formation of by-products after cleavage from the support. A control experiment was run using 5'-N3dTCCG-3' and 8 equiv. of 4-ethynylphenylboronic acid, the deprotected analogue of 1, CuSO₄•5H₂O (1 equiv.), sodium ascorbate (5 equiv.) and tris(benzyltriazolylmethyl)amine (THPTA, 3 equiv.) for 60 min at 60 °C in a ACN/H₂O mixture. The resulting CPG support was then washed with a saturated solution of EDTA and ODN1 was cleaved off the support by addition of a concentrated aqueous solution of ammonia. MALDI-TOF MS analysis confirmed that the boronic acid was fully oxidized to the corresponding phenol moiety under these conditions (Table 1, entry 1). However, the use of 4 ethynylphenylboronic acid pinacol ester 1 under otherwise identical conditions led to the postsynthetic modification of ODN2 by a phenylboronic acid moiety with 93 % conversion without noticeable degradation of the boronic acid functionality (Table 1, entry 2). RP HPLC spectra of the crude product after three steps and deprotection/cleavage from the solid support showed very little amount of the starting material, indicating complete conversion in all the reactions. This also confirmed the removal of the pinacol protecting group by concentrated aqueous ammonia.^{15c}

Figure 2 HPLC and MALDI-TOF spectra of the A) crude ODN1 and B) pure ODN2.

Following RP-HPLC purification, the modified ODN2 was isolated in 34% yield and an excellent purity as shown by RP-HPLC analysis. The RP-HPLC spectrum of ODN1 and ODN2 showed very close retention times (< 1.0 min span). In MALDI-TOF analysis, a single peak at m/z 1291.82 Da (calcd. m/z = 1291.94 Da) was observed for the oxidized sequence ODN1, whereas peaks at m/z = 1318.91 and 1475.12 Da corresponding to [M-H]⁻ (calcd. m/z = 1318.75 Da) and [M+citrate-2H₂O-H]⁻ (calcd. m/z = 1475,85 Da), were observed for ODN2 in accordance with fragmentations and adducts that typically appears with boronic acid-modified oligonucleotides (Figure 2).^{15c, 17b, 18b}

Encouraged by these results, we evaluated the method on sequences varying in length and nature of the residue at the 5' end. As the length of the sequences increased, we observed a decrease in the conversions and isolated yields as exemplified with the 17-mer ODN5, which was obtained with 55 % conversion and 8% isolated yield after purification (Table 1, entry 5). The seemingly low isolated yields are actually not that low considering that they are calculated on 1 μmol scale after automated synthesis (16 cycles), four reaction steps, deprotection and purification by RP-HPLC. In fact, the isolated yields are similar to previously reported syntheses using 5'-azido ONs²³ and far superior to the minimum guaranteed yields provided by major market suppliers for pure custom oligonucleotides (usually less than 50 nmol are guaranteed for modified ONs 10-19mer on a 1 μmol scale purified by RP HPLC). Replacement of 5'-thymidine by 5'-cytidine resulted in similarly good conversions but slightly lower isolated yields (Table 1, entries 6-8). Finally, the method was successfully applied to a hexameric RNA sequence leading to the 5'-boronic acid modified ORN1 with 90 % conversion and 20% isolated yield (Table 1, entry 9).

The successful conjugation of boronic acids at the 5'-extremity of ONs prompted us to evaluate their incorporation inside oligonucleotide strands (Figure 1, Method 2). Commercially available 2'-O-propargyluridine phosphoramidite was used to prepare CPG-bound oligonucleotides (DMT-off). The partner 4 (azidomethyl)phenylboronic acid MIDA ester 2, was synthesized in two steps starting from commercially available 4 (hydroxymethyl)phenylboronic acid 3. The boronic acid moiety was first protected as a N-methyliminodiacetic acid (MIDA) boronate ester under Dean-Stark conditions affording the corresponding derivative 4 in 98% yield. MIDA was selected for its stability and facile deprotection under basic conditions.²⁴ The azidation of 4 was obtained in one step using diphenyl phosphoryl azide (DPPA) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF to afford the desired azide 2 in 89% yield (Scheme 1).

Scheme 1 Synthesis of azide 2

The Cu(I)-catalysed azide–alkyne cycloaddition reaction was then carried out on column by mixing the representative ONs with azide 2 (20 equiv.) in a 2:8 ACN/dioxane mixture in the presence of a freshly prepared aqueous solution of CuSO₄•5H₂O (1 equiv.), sodium ascorbate (5 equiv.) and THPTA (3 equiv.), and by heating the mixture for 120 min at 60 °C under microwave irradiation (Figure 1, Method 2). The resulting CPG supports were then washed with a saturated solution of EDTA, treated with aqueous ammonia and purified by preparative RP-HPLC, leading to the corresponding conjugated oligonucleotides. The boronic acid moiety could be positioned successfully either at the extremity of a sequence or inside the DNA strand although in the latter case we observed a decrease of the overall isolated yield, probably due to steric hindrance (Table 1, entries 10 and 11). The method is equally compatible with RNA sequences although the isolated yields are lower than in the DNA series (Table 1, entry 12). To further demonstrate the applicability of borono-modified sequences in the field of aptamers, the 20-mer ODN11 containing the core sequence of the tobramycin aptamer, used notably for the detection of this aminoglycoside antibiotic in milk and human serum samples,²⁵ was obtained with 48 % conversion and 7% isolated yield (Table 1, entry 13).

Finally, to further prove the presence of the boronic acid moiety in the modified sequences, we performed a colorimetric displacement assay with Alizarin red S (ARS). Indeed, the combination of this dye with boronic acids is well known for the detection of various analytes.²⁶ The binding of the boronic

Table 1 On-column CuAAC reaction with DNA and RNA sequences

Entry	Name	Sequence 5'→ 3'a	Conversion (%) ^b	Yield (nmol/ (%) ^c
1	ODN1	PhOH-d(TCCG) -	-	-
2	ODN2	PhBn-d(TCCG) 93	348,6 (34)	
3	ODN3	PhBn-d(TCTCCG)	95	145,8 (15)
4	ODN4	PhBn-d(TCTCTCCG)	93	148,7 (15)
5	ODN5	PhBn d(TACAACGACTCTCTCCG) 55		77,1 (8)
6	ODN6	PhBn-d(CCG) 95	98,9 (10)	
7	ODN7	PhBn-d(CTCCG) 95	141,5 (14)	

8	ODN8	PhBn-d(CTCTCCG)	97	61,5 (6)
9	ORN1	PhBn-r(UCUCCA)d	90	200,7 (20)
10	ODN9	UPhBnd(CCAGCTGACCG)	95	236.6 (24%)
11	ODN10	d(GCCAGCUPhBnGACCG)	66	111.6 (11%)
12	ORN2	r(UPhBnCCAGCUGACCA)d	69	86.0 (9%)
13	ODN11	d(ACTTGGTTUPhBnAGGTAATGAGT)	48	70.47 (7%)

a PhOH and PhBn refer respectively to a phenol or a phenylboronic acid moiety linked to the 5'-extremity. UPhBn refers to a phenylboronic acid moiety linked to the 2'-position of a uridine residue. b Determined by RP-HPLC. c Isolated yields based on 1 μ mol scale. d RNA synthesis performed with 2'-OPivOM residues.^{23, 27}

Figure 3 Naked-eye detection of the formation of DNA-ARS and DNA-fructose complexes formed in situ. A) ODN3; B) ODN10; (ARS, 0,1 mM; DNA, 0,6 mM, in 40% DMSO/PBS buffer (pH 7.7) at 25 °C).

acid to ARS induces a significant colour change from purple to yellow within a few seconds, which can be readily visualised by the naked-eye (Figure 3). The addition of 100 equiv. of fructose results, in less than 1 min, in the displacement of the ARS dye and the formation of a DNA-fructose complex, which ultimately restores the purple colour.

In conclusion, we have described the first successful use of CuAAC reactions as a convenient strategy for postsynthetic on column conjugation of oligonucleotides with boronic acids. The phenylboronic acid moiety could be selectively positioned either at the 5'-extremity or at an internal position of ONs by the use of the appropriately protected 4-ethynyl- or 4-(azidomethyl)-phenylboronic acid. The functionalized oligonucleotide products were obtained after preparative HPLC in good isolated yields and excellent purities in both the DNA and the RNA series. The demonstrated practicability of this new procedure for postsynthetic modification of oligonucleotides with boronic acids increases significantly the repertoire of possible DNA and RNA conjugation chemistries and will undoubtedly find use for the design of functional ONs.

The experimental section has no title; please leave this line here.

Commercially available reagents were used as received. Pyridine was distilled on calcium hydride. The reaction mixtures were monitored by TLC using silica gel plates (Merck) with fluorescent indicator ($\lambda = 254$ nm). The purification of the products was performed by flash column chromatography using silica gel 60 (43–63 μ m) from Merck-Millipore. NMR analyses were made on a Bruker spectrometer 400 MHz at 20 °C. Chemical shifts are reported in parts per million (ppm) and are referenced to the residual solvent resonance as the internal standard (CD₃CN $\delta = 1.94$ for ¹H NMR and CD₃CN $\delta = 1.32$ for ¹³C NMR). Data are reported as follows: chemical shift, multiplicity (standard abbreviations), coupling constant (Hz), and integration. HRMS were recorded on a Micromass Q-TOF spectrometer with an electrospray ionisation (ESI) in negative or positive mode. All the oligonucleotide syntheses were conducted on an ABI 394 DNA/RNA synthesizer by classical phosphoramidite chemistry with commercial phosphoramidites and CPG (Control Pore Glass) solid support using standard conditions.

Crude sequences were purified by semi-preparative HPLC Dionex 600 in reverse phase with a gradient of buffer B (TEAAc 0.05M + 80% ACN) in buffer A (TEAAc 0.05M + 1% ACN) at 30 °C at a flow rate of 4 mL/min. Purified DNA samples were analysed by analytic HPLC with a gradient of buffer B (TEAAc 0.05M + 80% ACN) in buffer A (TEAAc 0.05M + 1% ACN) at a flow of 1 mL/min. MALDI-TOF mass spectra were recorded on an Axima assurance spectrometer (Shimadzu Biotech) using 1 µL of purified DNA sample mixed with 5 µL of a 6-aza-2-thiothimine (ATT) saturated matrix and ammonium citrate (0.1 M) as co-matrix. Samples were then spotted on a stainless steel plate and air dried before analysis. Reactions under micro-wave irradiation were realized on an monowave 400 (Anton Paar) in G10 reaction flask with a power of 850 W and a speed of 400t/min.

4-(Hydroxymethyl)phenylboronic acid MIDA ester (3)

An oven-dried flask topped with a Dean–Stark apparatus and condenser was charged sequentially with 4-(hydroxymethyl)phenylboronic acid 2 (200 mg, 1.32 mmol), MIDA (387 mg, 2.63 mmol) anhydrous benzene (2.7 mL) and DMSO (0.3 mL). The Dean-Stark trap was filled with benzene and the resulting mixture was heated at reflux for 20 h (reaction monitored by TLC). The solution was cooled to r.t., concentrated under reduced pressure and the resulting residue was purified by flash column chromatography over silica gel (8:2, EtOAc/Acetone) to provide compound 3 (340 mg, 98%). Rf : 0.35 (8:2, EtOAc/Acetone)

¹H NMR (400 MHz, CD₃CN): δ = 2.49 (s, 3H), 3.91 (d, J = 17.2 Hz, 2H), 4.11 (d, J = 17.2 Hz, 2H), 4.57 (s, 2H), 7.34 (d, J = 8.0 Hz, 2H), 7.45 (d, J = 8.0 Hz, 2H)

¹³C NMR (100 MHz, CD₃CN): δ = 41.4, 62.8, 64.6, 127.2, 133.5, 144.2, 169.6

¹¹B NMR (128 MHz, CD₃CN): δ = 11.59

HRMS (ESI+): m/z calcd for C₁₂H₁₄NO₅B [M+H]⁺: 264.1043; found: 264.1039.

4-(Azidomethyl)phenylboronic acid MIDA ester (4)

Compound 3 (200.0 mg, 0.76 mmol) was dissolved in anhydrous DMF (4 mL) and the resulting solution was cooled to 0 °C and treated by dropwise addition of DPPA (212 µL, 0.91 mmol) and DBU (136 µL, 0.91 mmol). The reaction mixture was stirred at r.t. for 15 h (reaction monitored by TLC). After addition of EtOAc, the organic layer was extracted with water (3 × 5 mL), dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The crude product was purified by flash column chromatography over silica gel (8:2, EtOAc/Acetone) to afford compound 4 (195.0 mg, 89%) as a white solid; Rf: 0.50 (8:2, EtOAc/Acetone).

¹H NMR (400 MHz, CD₃CN): δ = 2.50 (s, 3H), 3.90 (d, J = 17.2 Hz, 2H), 4.08 (d, J = 17.2 Hz, 2H), 4.41 (s, 2H), 7.37 (d, J = 8.0 Hz, 2H), 7.31 (d, J = 8.0 Hz, 2H).

¹³C NMR (100 MHz, CD₃CN) δ = 48.5, 55.1, 62.9, 128.9, 133.9, 138.9, 169.6.

¹¹B NMR (128 MHz, CD₃CN) δ = 11.44.

HRMS (ESI+): m/z calcd for C₁₂H₁₃N₄O₄B [M-N₂+H]⁺: 261.1043; found: 261.1071.

On-column Iodination

Upon completion of the syntheses of the desired sequences (DMT-off), the columns were removed from the synthesizer and 1 mL syringes were fitted to each end. 1 mL of a solution of (PhO)₃PCH₃I (0.5 M in anhydrous DMF, 500 µmol) was then passed through the column at r.t. for 15 min. The

column was then washed with 10 mL of DCM, 10 mL of ACN and 10 mL of DCM, and eventually dried under argon.

On column Azidation

The column containing the 5'-iodo oligodeoxyribonucleotide bound to a CPG resin was fitted in each end by 1 mL syringes. 1 mL of a solution of TMGA (0.3 M in anhydrous ACN, 300 μ mol) was then passed through the column 10 to 15 times before placing the column in an oven at 60 °C for 60 min. The column was then washed with 10 mL of ACN, 5 mL of sodium bisulphite, 5 mL of DCM and 5 mL of ACN and dried under argon.

On-column CuAAc reactions

Incorporation of alkyne-boronic acid 1

All solutions were freshly prepared and degassed with argon bubbling. The column containing the 5'-azido oligodeoxyribonucleotide bound to a CPG resin was fitted in each end by 1 mL syringes. 1 mL of a solution containing boronic acid 1 (80 μ L, 0.1 M in ACN, 8 μ mol), THPTA (30 μ L, 0.1 M in water, 3 μ mol), copper sulphate (25 μ L, 0.04 M in water, 1 μ mol) and sodium ascorbate (50 μ L, 0.1 M in water, 5 μ mol) was then passed through the column 10 to 15 times before putting the column in a shaker at 60 °C for 60 min. The column was then washed with 5 mL of aqueous EDTA disodium salt, 5 mL of ACN and dried under argon.

Incorporation of azide-boronic acid 2

All solutions were freshly prepared and degassed with argon bubbling. Protected oligodeoxyribonucleotide attached to CPG beads was placed into a 10 mL reaction flask (G10). THPTA (30 μ L, 0.1 M in water, 3 μ mol) and boronic acid 2 (200 μ L, 0.1 M in a 8:2 dioxane/ACN mixture, 20 μ mol), copper sulphate (25 μ L, 0.04 M in water, 1 μ mol) and sodium ascorbate (50 μ L, 0.1 M in water, 5 μ mol) were added and the resulting mixture was then stirred for 120 min at 60 °C under microwave irradiation (power: 850W, speed: 400t/min).²⁸ The beads were then successively washed with 5 mL of aqueous EDTA disodium salt and 5 mL of ACN, and eventually dried under argon. ODN11 was obtained using 100 μ mol of boronic acid 2 after 180 min under microwave irradiation at 60 °C.

Deprotection, analysis and purification of oligonucleotides

Deprotection and cleavage of the DNA sequences from the solid support was achieved upon mixing with concentrated NH₄OH at 55 °C for 5 h.

Deprotection and cleavage of the RNA sequences from the solid support, on the other hand, was possible after passing a solution of DBU 1 M in ACN through the column for 3 min before treating the beads with concentrated NH₄OH at 37 °C for 3 h.

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Supporting Information

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

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