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Synthesis of 5-Dihydroxyboryluridine Phosphoramidite and Its Site-Specific Incorporation into Oligonucleotides for Probing Thymine DNA Glycosylase

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Abstract

A concise synthetic strategy to 5-dihydroxyboryldexoyuridine (5boU) phosphoramidite has been developed. 5boU was introduced into short oligonucleotides in a site-specific manner, demonstrating compatibility of the boronic acid moiety with standard solid-phase DNA synthesis chemistry. Electrophilic 5boU DNAs inhibited thymine DNA glycosylase, a cancer-relevant DNA-modifying enzyme. We envisage diverse applications of 5boU in organic synthesis, medicinal chemistry, and chemical biology.

Graphical Abstract

Enzyme-catalyzed chemical modifications on DNA are critical to controlling genome structure and integrity. A notable example includes cytosine methylation at carbon 5 (5mC) by DNA methyltransferases (DNMTs) (Figure 1A).¹ The methyl group undergoes iterative C–H oxidation by the 10–11 translocation (TET) enzymes to furnish 5-carboxycytosine (5caC).^{2–4} In the active DNA demethylation pathway, 5caC is hydrolytically cleaved by thymine DNA glycosylase (TDG) to an abasic site, followed by base-excision repair (BER)

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Supporting Information

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Synthetic procedure and characterization of all relevant compounds, methods for protein expression and purification and biochemical assay, supplementary figures and tables, and NMR spectra (PDF)

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to unmodified cytosine.^{5,6} TDG also cleaves the glycolytic bond in thymine in T:G mismatch, resulting from enzymatic deamination of 5mC, to repair the ensuing abasic site (Figure 1A). Emerging evidence suggests that DNMT, TET, and TDG play fundamental roles during cellular differentiation and organismal development.^{7,8} However, specific small-molecule inhibitors and functional probes targeting these enzymes, particularly TDG, are limited.^{9,10}

Boronic acid containing small molecules have been extensively employed in designing irreversible inhibitors for hydrolytic enzymes.¹¹ The strong electrophilic character of boron due to its vacant p orbital favors transition from a neutral trigonal geometry to a stable anionic tetrahedral structure that effectively mimics the transition state of reactions catalyzed by hydrolytic enzymes.¹² Systematic studies have led to other modes of inhibition where an sp³-hybridized borate traps a substrate in the active site (e.g., tRNA synthetase¹³) or inactivates a nonhydrolytic enzyme (e.g., kinase¹⁴) by reacting with an activated nucleophilic amino acid in the active sites. We reasoned that oligonucleotides carrying a 5-dihydroxyborylpyrimidine nucleobase¹⁵ could constitute covalent binders for DNA-modifying enzymes, particularly those acting on 5-carboxylpyrimidine, such as TDG. The hypothesis is based on the observation that boronic acid mimics carboxylic acid under physiological conditions due to its isosteric and isoelectronic nature (Figure 1B).¹²

Boronic acid containing compounds targeting DNA-modifying enzymes are largely unexplored in comparison to their counterparts developed to inhibit enzymes acting on polypeptide substrates.^{12,16,17} This is likely due to the limited synthetic methods available for accessing nucleotide analogues carrying boronic acid at specific positions. More importantly, there is no precedence for the synthesis of the 5-dihydroxyborylpyrimidine phosphoramidite building block for site-specific introduction of the nucleotide analogue into DNA. Herein, we describe a novel synthesis of 5-dihydroxyboryldexoyuridine (5boU) phosphoramidite **1** via a short strategy (Scheme 1). We then incorporate this uniquely modified base on varied DNA sequences in a site-specific manner to demonstrate its applicability in standard solid-phase DNA synthesis. Finally, we examine the ability of 5boU-containing short oligonucleotides to modulate the hydrolytic activity of TDG. We show that a tetranucleotide bearing 5boU, but not the unmodified nucleobase, could robustly inhibit base-excision potential of TDG, thus providing novel chemical probes to study functions of TDG and likely of other DNA-modifying enzymes.

We adopted a modular approach to access **1** from commercially available 5-bromodeoxyuridine **2**. Key to our synthetic strategy toward 5boU phosphoramidite **1** was metal-halogen exchange mediated installation of the boronic acid moiety into suitably protected deoxyuridine (Figures S1–S7, Scheme 1). It has been reported that boronic acid could be introduced into TMS-protected 5-bromodeoxyuridine but only with 12% isolated yield.¹⁸ To circumvent the issues related to labile TMS groups, we generated an organolithium species using hydrolytically stable TBDMS-protected uridine **3** and allowed it to react with tributyl boronate ester for an extended period of time. The desired boronic acid **4** was indeed obtained in high yield (~40% **4**, 20% **3** recovered), a significant improvement on the earlier approach.

We next protected the free boronic acid in **4** as the *N*-methyliminodiacetic acid (MIDA) ester to access **5** in good yield (Scheme 1).¹⁹ The rationale to employ a base-sensitive MIDA protecting group instead of an acid-labile pinacol ester is that the former is expected to remain intact during trichloroacetic acid mediated cleavage of the 5'-dimethoxytrityl (DMT) group during solid-phase DNA synthesis. Furthermore, MIDA can be removed by ammonia while performing global deprotection of the synthesized oligonucleotide and its cleavage from the solid support. Removal of the TBDMS groups by HF-pyridine buffer led to diol **6** in 75% yield, and selective DMT protection on 5'-hydroxyl group proceeded smoothly to furnish **7**. The final 5boU phosphoramidite **1** was obtained in good yield by exposing **7** to 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite and was fully characterized by analytical methods (Figures S1–S7).

After securing the 5boU phosphoramidite, we intended to incorporate it into a short DNA sequence using standard solid-phase procedure. We first introduced 5boU at 5'-end of the palindromic sequence ^{5bo}UCGA (DNA **8**). Ammonia-mediated global deprotection and cleavage from solid support followed by HPLC purification yielded analytically pure DNA **8** carrying the 5boU moiety, as evident from LC–MS data (Figure 2A, Figure S8, Table 1). Given that solid-phase DNA synthesis starts from 3'-end, 5boU was introduced at the last step (5'-end) during assembly of DNA **8**. To examine if the MIDA-protected uridine boronate could be placed at an internal position in a sequence, we designed two more palindromic sequences CA^{5bo}UG (DNA **9**) and CG^{5bo}UCG (DNA **10**). After the solid-phase assembly and postsynthetic ammonia treatment, we obtained the required DNAs bearing intact 5boU unit as confirmed by LC–MS (Figure 2B, Figures S9 and S10, Table 1). These efforts constitute the first synthesis of 5boU phosphoramidite and introduction of this novel nucleotide into DNA strands in a site-specific manner.

We then sought to assess the potential of short DNA sequences carrying the electrophilic 5boU moiety to modulate the hydrolytic activity of TDG. Overexpression of TDG is implicated in multiple cancers, and thus targeting TDG may represent a novel therapeutic approach to human neoplasia.²⁰ As noted above, TDG recognizes a T:G mismatch in double-stranded DNA, flips out the thymine base in its active site, and catalytically cleaves the N-glycosidic bond in thymine to generate an abasic site. Based on this activity, we developed a base-excision assay that involves (i) formation of duplexed DNA carrying T:G mismatch and a fluorescent unit 5'-carboxyfluorescein (FAM), (ii) TDG-mediated base-excision of the duplexed DNA, and (iii) alkaline strand cleavage resulting in a fluorescently labeled short DNA fragment that can be visualized on a denaturing gel (Figure 3A). To examine TDG activity using this assay, we first synthesized a 32-nt substrate DNA **11** bearing a FAM unit and duplexed it with a complementary strand **12** to generate T:G mismatch (Table 1).²¹ Both DNAs were confirmed by LC–HRMS (Figures S11 and S12). We expressed recombinant TDG protein carrying the catalytic domain and examined its activity on the abovementioned duplexed DNA.^{6,22} TDG was indeed able to recognize and excise the T:G mismatch as evident from a shorter DNA segment on the gel upon alkaline strand cleavage (Figure 3B). Consistently, in the negative control lacking TDG, fragmented DNA product was not observed (Figure 3B).

To examine if TDG could act on 5boU:G mismatch, we synthesized DNA **13** carrying 5boU in a sequence identical to **11** (Figure 3C, Table 1). Following the protocol similar to that used for DNAs **8–10**, we introduced the 5boU moiety into the 32-mer sequence **13** that also includes a FAM unit at the 5' end (Table S1). The integrity of **13** was established by ESI-HRMS and ¹¹B NMR (Figures S13 and S14), demonstrating the utility of our approach to access long-chain oligonucleotide with 5boU moiety. In the base-excision assay, TDG was indeed able to flip 5boU out into its adaptable active site and cleave the glycosidic bond, reminiscent of its activity toward 5caC (Figure 3D). The hydrolytic activity toward 5boU:G mismatch was, however, significantly lower compared to T:G (3B vs 3D). Given that a stepwise D_N*A_N (departure of the nucleobase leaving group and addition of the nucleophile) pathway predominates in TDG-mediated catalysis,²³ the leaving group aptitude guides the reaction rate significantly. A key marker of leaving group quality is the acidity of the glycosidic nitrogen N1 in uridine; increased acidity ensures greater stability of the conjugate nucleobase and hence better leaving group aptitude. Although an electron-withdrawing dihydroxyboryl moiety is expected to increase N1 acidity of 5boU and enhance TDG activity, the contrary observation that we made in regard to 5boU excision is indicative of a putative tetracoordinated boronate anion in the active site that could potentially destabilize the corresponding conjugate nucleobase. This boronate species could form via nucleophilic addition of an amino acid or an activated water molecule to 5boU in the active site.

We next evaluated the binding of 5boU DNA **13** to TDG. In the electrophoretic mobility shift assay (EMSA), both duplexed DNAs (**11:12** and **13:12**) showed strong binding toward TDG (Figure S15). Encouraged by the results that 5boU DNA could bind to TDG, we proceeded to examine if 5boU could act as an inhibitor of the enzyme. Toward this end, we incubated DNAs **8** and **9**, one at a time, with TDG prior to adding the duplexed substrate **11:12** in the base-excision assay described above. DNAs **8** and **9** are self-complementary due to their palindromic nature and form non T:G duplexes. We observed that TDG was indeed inhibited by both the electrophilic DNAs (Figure 3E). Furthermore, both the DNAs exhibited dose-dependent inhibition of the enzyme activity (Figure 3F, Figure S16). To examine if 5boU is crucial to the inhibition of TDG, we synthesized DNA **14**, which is same as DNA **8** but lacking the 5boU unit (Table 1, Figure S17). This DNA failed to inhibit glycosylase activity of TDG (Figure 3E). To examine if only the monomer 5boU **15** could inhibit TDG, we synthesized the compound from **7** in two steps (Scheme S1, Figure S7). However, the compound failed to inhibit glycosylase activity of TDG (Figure S18), suggesting a DNA fragment is required for optimal binding and inhibition. Although the exact mechanism of inhibition by DNAs **8** and **9** is not defined, a tetrahedral boronate species could form, as discussed above, through nucleophilic addition of an activated amino acid in the catalytic site, likely resulting in covalent inhibition. It would be of future interest to explore the mode of inhibition using mutational and biochemical studies. Taken together, our results provide strong evidence that 5boU moiety could be employed to perturb the enzymatic activity of TDG. To the best of our knowledge, this is the first demonstration that short synthetic DNAs with site-specific boronic acid-containing nucleotide could modulate DNA glycosylases.

In summary, we report the first synthesis of 5boU phosphoramidite building block employing a metal–halogen exchange reaction as key step and its site-specific incorporation

into synthetic oligonucleotides. In further biochemical experiments, we demonstrate the potential of electrophilic 5boU DNAs in inhibiting developmentally essential and cancer-relevant TDG. We further envisage developing activity-based probes carrying 5boU to capture novel enzymes particularly those implicated in active DNA demethylation pathway. Furthermore, given the importance of boronic acid functionality in metal-catalyzed C–C bond formation, sensor and inhibitor development,^{24–26} we postulate that our efforts delineated here would find widespread applications in synthetic organic chemistry, DNA-based materials, mechanistic enzymology, and pharmacology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- (1). Li E; Zhang Y DNA methylation in mammals. Cold Spring Harbor Perspect. Biol 2014, 6, a019133.
- (2). Tahiliani M; Koh KP; Shen Y; Pastor WA; Bandukwala H; Brudno Y; Agarwal S; Iyer LM; Liu DR; Aravind L; Rao A Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 2009, 324, 930–5. [PubMed: 19372391]
- (3). Ito S; Shen L; Dai Q; Wu SC; Collins LB; Swenberg JA; He C; Zhang Y Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science 2011, 333, 1300–3. [PubMed: 21778364]
- (4). Kriaucionis S; Heintz N The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 2009, 324, 929–30. [PubMed: 19372393]
- (5). He YF; Li BZ; Li Z; Liu P; Wang Y; Tang Q; Ding J; Jia Y; Chen Z; Li L; Sun Y; Li X; Dai Q; Song CX; Zhang K; He C; Xu GL Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. Science 2011, 333, 1303–7. [PubMed: 21817016]
- (6). Maiti A; Drohat AC Thymine DNA glycosylase can rapidly excise 5-formylcytosine and 5-carboxylcytosine: potential implications for active demethylation of CpG sites. J. Biol. Chem 2011, 286, 35334–8. [PubMed: 21862836]
- (7). Song CX; He C Potential functional roles of DNA demethylation intermediates. Trends Biochem. Sci 2013, 38, 480–4. [PubMed: 23932479]
- (8). Pastor WA; Aravind L; Rao A TETonic shift: biological roles of TET proteins in DNA demethylation and transcription. Nat. Rev. Mol. Cell Biol 2013, 14, 341–56. [PubMed: 23698584]
- (9). Dai Q; Lu X; Zhang L; He C Synthesis of DNA oligos containing 2'-deoxy-2'-fluoro-D-arabinofuranosyl-5-carboxylcytosine as hTDG inhibitor. Tetrahedron 2012, 68, 5145–5151. [PubMed: 22711938]
- (10). Bennett MT; Rodgers MT; Hebert AS; Ruslander LE; Eisele L; Drohat AC Specificity of human thymine DNA glycosylase depends on N-glycosidic bond stability. J. Am. Chem. Soc 2006, 128, 12510–9. [PubMed: 16984202]
- (11). Lesnikowski ZJ Recent developments with boron as a platform for novel drug design. Expert Opin. Drug Discovery 2016, 11, 569–78.

- (12). Yang W; Gao X; Wang B Boronic acid compounds as potential pharmaceutical agents. *Med. Res. Rev* 2003, 23, 346–68. [PubMed: 12647314]
- (13). Rock FL; Mao W; Yaremchuk A; Tukalo M; Crepin T; Zhou H; Zhang YK; Hernandez V; Akama T; Baker SJ; Plattner JJ; Shapiro L; Martinis SA; Benkovic SJ; Cusack S; Alley MR An antifungal agent inhibits an aminoacyl-tRNA synthetase by trapping tRNA in the editing site. *Science* 2007, 316, 1759–61. [PubMed: 17588934]
- (14). Ban HS; Usui T; Nabeyama W; Morita H; Fukuzawa K; Nakamura H Discovery of boron-conjugated 4-anilinoquinazoline as a prolonged inhibitor of EGFR tyrosine kinase. *Org. Biomol. Chem* 2009, 7, 4415–27. [PubMed: 19830290]
- (15). Liao TK; Podrebarac EG; Cheng CC Boron-Substituted Pyrimidines. *J. Am. Chem. Soc* 1964, 86, 1869–1870.
- (16). Lin N; Yan J; Huang Z; Altier C; Li M; Carrasco N; Suyemoto M; Johnston L; Wang S; Wang Q; Fang H; Caton-Williams J; Wang B Design and synthesis of boronic-acid-labeled thymidine triphosphate for incorporation into DNA. *Nucleic Acids Res.* 2007, 35, 1222–9. [PubMed: 17267413]
- (17). El Amri C; Martin AR; Vasseur JJ; Smietana M Borononucleotides as substrates/binders for human NMP kinases: enzymatic and spectroscopic evaluation. *ChemBioChem* 2012, 13, 1605–12. [PubMed: 22733592]
- (18). Schinazi RF; Prusoff WH Synthesis of 5-(dihydroxyboryl)-2'-deoxyuridine and related boron-containing pyrimidines. *J. Org. Chem* 1985, 50, 841–847.
- (19). Gillis EP; Burke MD Multistep synthesis of complex boronic acids from simple MIDA boronates. *J. Am. Chem. Soc* 2008, 130, 14084–5. [PubMed: 18837550]
- (20). Mancuso P; Tricarico R; Bhattacharjee V; Cosentino L; Kadariya Y; Jelinek J; Nicolas E; Einarsen M; Beeharry N; Devarajan K; Katz RA; Dorjsuren DG; Sun H; Simeonov A; Giordano A; Testa JR; Davidson G; Davidson I; Larue L; Sobol RW; Yen TJ; Bellacosa A Thymine DNA glycosylase as a novel target for melanoma. *Oncogene* 2019, 38, 3710–3728. [PubMed: 30674989]
- (21). Nabel CS; Jia H; Ye Y; Shen L; Goldschmidt HL; Stivers JT; Zhang Y; Kohli RM AID/APOBEC deaminases disfavor modified cytosines implicated in DNA demethylation. *Nat. Chem. Biol* 2012, 8, 751–8. [PubMed: 22772155]
- (22). Zhang L; Lu X; Lu J; Liang H; Dai Q; Xu GL; Luo C; Jiang H; He C Thymine DNA glycosylase specifically recognizes 5-carboxylcytosine-modified DNA. *Nat. Chem. Biol* 2012, 8, 328–30. [PubMed: 22327402]
- (23). Drohat AC; Maiti A Mechanisms for enzymatic cleavage of the N-glycosidic bond in DNA. *Org. Biomol. Chem* 2014, 12, 8367–78. [PubMed: 25181003]
- (24). Bull SD; Davidson MG; van den Elsen JM; Fossey JS; Jenkins AT; Jiang YB; Kubo Y; Marken F; Sakurai K; Zhao J; James TD Exploiting the reversible covalent bonding of boronic acids: recognition, sensing, and assembly. *Acc. Chem. Res* 2013, 46, 312–26. [PubMed: 23148559]
- (25). Whyte GF; Vilar R; Woscholski R Molecular recognition with boronic acids-applications in chemical biology. *J. Chem. Biol* 2013, 6, 161–74. [PubMed: 24432132]
- (26). Ono T; Wang S; Koo CK; Engstrom L; David SS; Kool ET Direct fluorescence monitoring of DNA base excision repair. *Angew. Chem., Int. Ed* 2012, 51, 1689–92.

Figure 1.

DNA-modifying enzymes. (A) Unmodified cytosine is methylated by DNMT to 5mC which undergoes a myriad of enzymatic modifications, such as C–H oxidations and base excision, by TET and TDG enzymes, respectively, as a part of DNA repair and active demethylation. (B) Short DNA strand carrying electrophilic 5boU nucleotide for covalent capture and inhibition of DNA-modifying enzymes.

Figure 2.

Solid-phase synthesis of 5boU-containing DNAs. (A) ESI-HRMS of DNA **8** carrying a terminal 5boU unit in 4-mer palindromic sequence. (B) ESI-HRMS of DNA **9** carrying an internal 5boU unit in 4-mer palindromic sequence.

Figure 3.

Activity of 5boU-containing DNAs toward TDG. (A) Schematic showing assay involving base excision of FAM-labeled DNA by TDG followed by alkaline strand cleavage and visualization using in-gel fluorescence. (B) TDG can excise duplexed DNAs **11** and **12** carrying a T:G mismatch as confirmed by in-gel fluorescence. (C) Duplexed DNAs **13** and **11** carrying 5boU:T mismatch. TDG can excise **13**, albeit to a smaller degree. (D) Inhibition of TDG activity on T:G mismatched DNAs (**11:12**) by 5boU-containing DNAs **8** (^{5bo}UCGA) and **9** (CA^{5bo}UG), but not by nonboron DNA **14** (TCGA), each at 100 μ M concentration. (E) Dose-dependent inhibition of TDG by DNA **8**.

Scheme 1.
Synthesis of 5boU Phosphoramidite 1

Table 1.
Sequences and Expected and Observed Monoisotopic Masses of the Synthetic Oligonucleotides

DNA sequence (5'–3')	expected mass	observed mass
DNA 8: 5boUCGA ^a	600.6107	600.6136
DNA 9: CA5boUG ^a	600.6107	600.6136
DNA 10: CGA5boUCG ^a	909.6601	909.6640
DNA 11: FAM-TCGGATGTTGTGGGTCAGTCATGATAGTGTA	10513.7845	10513.8415
DNA 12: TACACTATCATGGCGCTGACCCACACATCCGA	9677.6630	9677.6863
DNA 13: FAM-TCGGATGTTGTGGGTCAG5boUGCATGATAGTGTA	10543.7758	10543.8080
DNA 14: TCGA ^a	585.6150	585.6134

^aIndicates expected and observed masses are for $z = 2$.