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ROS-Inducible DNA Cross-Linking Agent as a New Anticancer Prodrug Building Block

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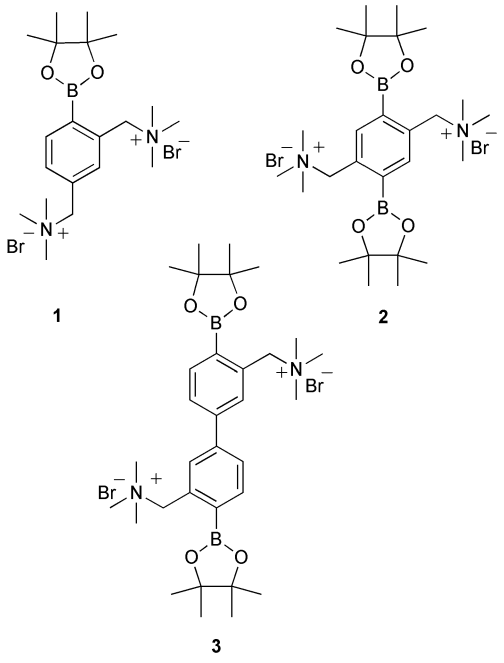
Some antitumor drugs react with DNA by inducing DNA interstrand cross-links (ICLs), which can block DNA transcription and replication.[1] ICL-inducing agents, such as nitrogen mustard, mitomycin C, cisplatin, and psoralens have been used in cancer therapy.[2] However, the major disadvantage of these agents is their poor selectivity for cancer cells. One novel approach to reduce the toxicity of cross-linking agents for normal cells would be the creation of prodrugs that undergo tumor-specific activation. Inducible DNA cross-linking or alkylating agents have been developed by several research groups.[3–6] However, few of them can induce DNA cross-links selectively under tumor-specific conditions. One of the exclusive features of cancer cells is the high level of oxidative stress that is associated with the increased amounts of reactive oxygen species (ROS).[7–10] Therefore, it would be advantageous to develop novel cross-linking agents that can be activated by the high level of ROS in cancer cells.

Among different ROS, such as H_2O_2, hydroxyl radical ($^{\bullet}OH$), and superoxide radical anions ($O_2^{\bullet-}$), H_2O_2 has a pivotal role because it is a stable ROS and generated from nearly all sources of oxygen radicals.[11] Increased levels of H_2O_2 in cancer cells compared to normal cells have been reported.[7,12] These factors make H_2O_2 an ideal candidate as a target to develop new ROS-inducible prodrugs with high selectivity to cancer cells. Arylboronic esters are particularly suitable to this prodrug approach because H_2O_2 can readily cleave the boronic ester to release the quinone methide (QM).[13] QMs are important intermediates in a large number of DNA cross-linking and alkylating processes. With simple modifications suggested by Rokita's group,[4] Freccero and colleagues,[5] Zhou et al.[6] and others,[14] QM can induce DNA ICLs through different strategies for initiation, including UV irradiation, fluoride ions, heating, or oxidation, etc. Meanwhile, Cohen's group reported the activation of matrix metalloproteinase inhibitor in situ by protecting the hydroxyl group of the zinc-binding group (ZBG) with boronic ester as the H_2O_2-sensitive trigger.[15] Boronate-based probes have been developed by Chang and coworkers, Lo's group, and others, for selective detection and imaging of hydrogen peroxide in cells.[16] Recently, our group has shown that a prodrug of nitrogen mustard coupled with an arylboronate can be triggered by H_2O_2 to release active drugs that can kill cancer cells.[17] However, therapeutic utility would require a more efficient trigger that can be coupled with multiple potent effectors to maximize the ROS-inducible cytotoxicity of prodrugs. We expect that the arylboronic ester and biarylboronic ester derivatives **1**–**3** can be activated by H_2O_2 to release biquinone methide and two amine effectors. This work describes the synthesis and biological studies of **1**–**3**.



1 **2** **3**

Compound **2** was synthesized starting from commercially available 2,5-dibromo-*p*-xylene (**2a**; Scheme 1). Palladium-catalyzed borylation of **2a** provided boronated intermediate **2b**, which reacted with NBS to yield brominated analogue **2c**. Compound **2c** was converted to **2** by using trimethylamine in CH_3CN. In a similar way, **1** and **3** were synthesized (Schemes S1 and S2 in the Supporting Information).

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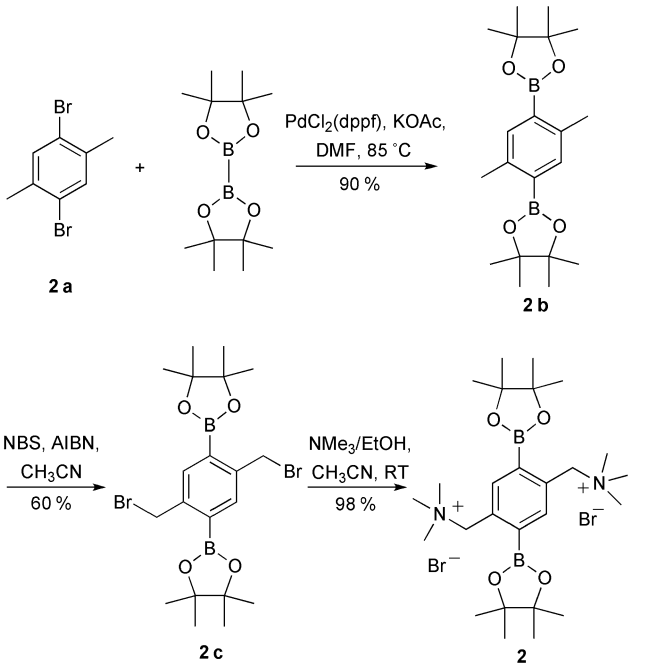
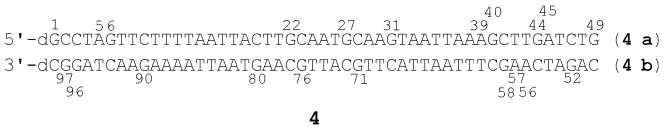


Scheme 1. Synthesis of compound **2**.

Initially, the activity of compounds **1**–**3** towards DNA was investigated by using a 49-mer DNA duplex (**4**) by measuring DNA ICL formation. The treatment of the DNA with **1**–**3** were carried out in phosphate buffer (pH 8.0) at 37 °C. ICL formation and cross-linking yields were analyzed with denaturing polyacrylamide gel electrophoresis (PAGE) with phosphorimager analysis (Image Quant 5.2). In the absence of H_2O_2, ICLs were not observed with **1**–**3** (Figure 1, lanes 2–4), which indicated that QM was not formed. In the presence of H_2O_2, compound **2** efficiently induced ICL formation (Figure 1, lanes 9–11). However, no ICL was observed with **1** and **3** (Figure 1, lanes 6–8 and 12–14). The cross-linking efficiency of **2** was affected by the concentration of the drug, the ratio of drug to H_2O_2, and the pH value of the buffer solution. DNA cross-linking by **2** was observed at a concentration as low as 10 μM (Figure 1, lane 9), and 2 mM of **2** led to 24 % ICLs (Figure 1, lane 11). The best ratio of drug to H_2O_2 was 2:1 (Figure S1 in the Supporting Information). Higher cross-linking yields were observed under basic conditions (pH 8 and 9; Figure S2 in the Supporting Information). This is consistent with the earlier observation that the reaction between arylboronic acid and hydrogen peroxide is pH dependent.[13,17,18] The ICL formation induced by **2** displayed first-order kinetics with a rate constant (k_{obs}) of $4.9(\pm0.3)\times10^{-5}\,s^{-1}$ ($t_{1/2}=4$ h; Figure S3 in the Supporting Information).



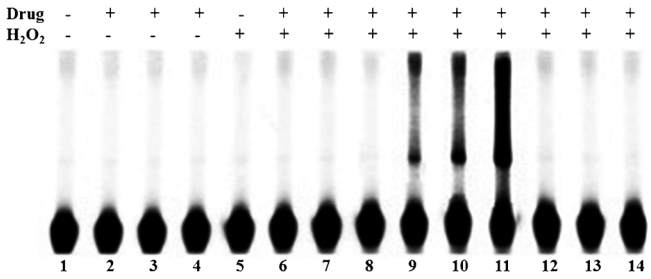


Figure 1. Concentration dependence of compounds **1**–**3** for DNA cross-link formation upon H_2O_2 activation. Lane 1: DNA only (cross-linking yield 0 %); lane 2: DNA with **1** (2 mM) only (cross-linking yield 0 %); lane 3: DNA with **2** (2 mM) only (cross-linking yield 0 %); lane 4: DNA with **3** (2 mM) only (cross-linking yield 0 %); lane 5: DNA with H_2O_2 (10 μM) only (cross-linking yield 0 %); lane 6: 10 μM **1**+5 μM H_2O_2 (0 %); lane 7: 100 μM **1**+50 μM H_2O_2 (0 %); lane 8: 2 mM **1**+1 mM H_2O_2 (0 %); lane 9: 10 μM **2**+5 μM H_2O_2 (2 %); lane 10: 100 μM **2**+50 μM H_2O_2 (8 %); lane 11: 2 mM **2**+1 mM H_2O_2 (24 %); lane 12: 10 μM **3**+5 μM H_2O_2 (0 %); lanes 13: 100 μM **3**+50 μM H_2O_2 (0 %); lane 14: 2 mM **3**+1 mM H_2O_2 (0 %).

The deboronation and activation of **2** was selective for H_2O_2 over other reactive oxygen species (Figure 2). The treatment of **2** with H_2O_2 at 200 μM induced about 8 % ICL formation, whereas the cross-linking yield with *tert*-butylhydroperoxide (TBHP), hydroxyl radical, *tert*-butoxy radical, superoxide, and nitric oxide was less than 1 % and less than 5 % ICL was observed for hypochlorite.

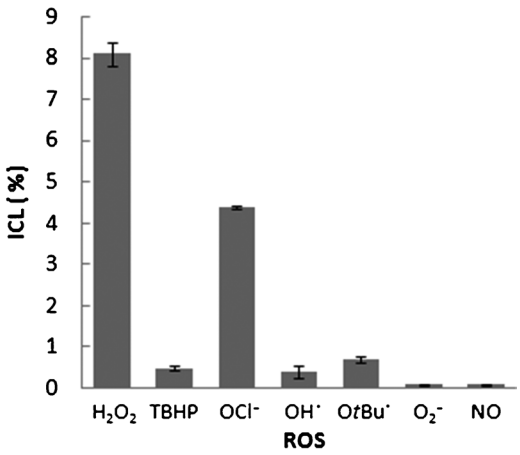


Figure 2. Selectivity of **2** (400 μM) with various reactive oxygen species (ROS) at 200 μM. Data were acquired at 25 °C in phosphate buffer (10 mM, pH 8) after 48 h. H_2O_2: hydrogen peroxide, TBHP: *tert*-butylhydroperoxide, OCl^-: hypochlorite anion, $OH^•$: hydroxyl radical, O*t*Bu$^•$: *tert*-butoxy radical, O_2^-: superoxide, NO: nitric oxide.

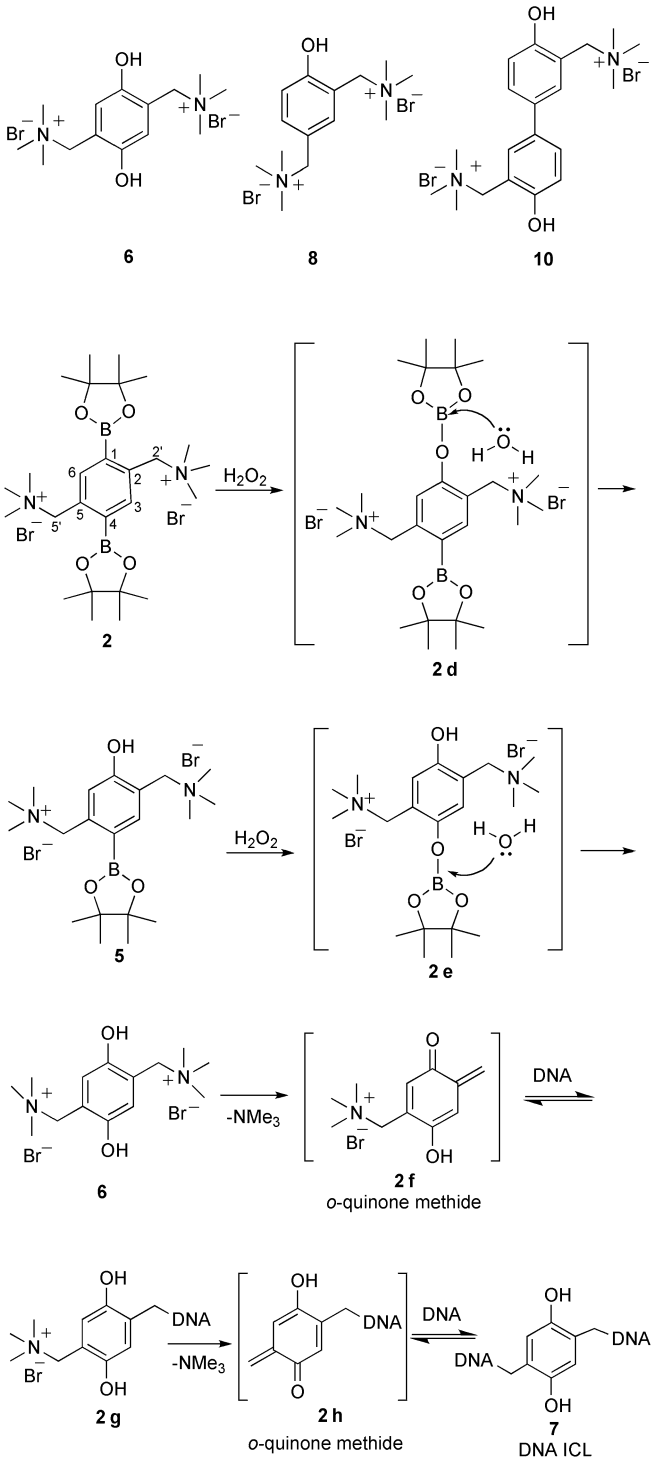
In order to acquire further insight into the reactivity of **2**, we determined DNA monoalkylations by examining the stability and reactivity of the purified ICL products and the treated single stranded DNA. The stability and alkaline lability of DNA alkylation products depend upon the reaction site. The N7-alkylated products of purines are labile to piperidine treatment, which can result in DNA cleavage.[3c,19]

The ICLs formed from **2** are stable at high temperatures in phosphate buffer (90 °C, pH 7.2, 30 min) and partially stable in piperidine (1 M; 90 °C, 30 min; Figure S4, lanes 3 and 4, in the Supporting Information). DNA cleavage bands were observed with all dGs and dAs when the purified ICLs were treated with piperidine (1 M; Figure S4, lanes 4, 11, 18, and 25, in the Supporting Information). Weak cleavage bands were observed with dCs flanked by dAs and/or dGs. These results indicated that the alkylations occurred at N7 of dG and dA, and partially with dC. The stable ICLs observed after piperidine treatment suggested that N7 is not the only alkylation site (Figure S4, lanes 4, 11, 18, and 25, in the Supporting Information). It is highly likely that the alkylation took place at exocyclic amines. Monoalkylation of all guanine and adenine units was also observed with single-stranded DNA **4a** or **4b** (Figure S4, lanes 2, 9, 16, and 23, in the Supporting Information). In a control experiment, oligonucleotide **4** was treated with 10 μM, 100 μM, and 1.0 mM H_2O_2 alone; weak cleavage bands were observed at the purine and pyrimidine sites (Figure S5 in the Supporting Information). This indicated that H_2O_2 alone induced trace amounts of hydroxyl radical upon heating, which led to the equal cleavages with purine and pyrimidine nucleotides.[20]

In an effort to identify the interstrand cross-linking site of compound **2**, we used hydroxyl radical cleavage analysis of the purified cross-linking product.[20] However, a completely cleaved ladder similar to the hydroxyl radical control experiment was observed with stronger cleavage bands at the positions of dGs and dAs (Figure S4, lanes 5, 12, 19, and 26, in the Supporting Information). This further confirmed that the interstrand cross-linking should occur at these positions. However, the exact interstrand cross-linking site and pattern are still unknown. This is caused by three factors: 1) high reactivity of the quinone methide generated from **2** upon H_2O_2 activation; 2) the complexity of the reaction products, which include the interstrand cross-linking product, intrastrand cross-linking and monoalkylated products, and adducts formed with dG, dA, and dC; 3) the cross-linking and alkylated products are labile in piperidine.

To understand the difference for ICL formation between compounds **2** and **1** or **3**, their reaction mechanism was investigated by NMR spectroscopy analysis of the monomer reactions. The treatment of **1**–**3** with H_2O_2 was performed in deuterated potassium phosphate buffer (10 mM; pH 8.0) as well as in DMSO/D_2O. When phosphate buffer was used as a solvent, the reaction was too fast to observe any intermediates. Compounds **1**–**3** were completely consumed within 5 min and converted to the phenol derivatives **6**, **8**, and **10** (Figures S6B, S7B, and S9B in the Supporting Information). The formation of **6**, **8**, and **10** was confirmed by ^1H,^{13}C NMR, and HRMS-ESI analysis of the isolated materials (Figures S21–S27 in the Supporting Information).

When the treatment of **2** with H_2O_2 was carried out in a mixture of DMSO/D_2O, we were able to observe all intermediates formed (Scheme 2 and Figure 3A–D). The integral change of protons C3−H, C6−H, C2′−H and C5′−H characterized the kinetic transformation of compound **2** into the phenol derivative **6**. Analysis 3 h after addition of H_2O_2 revealed that compound **2** was completely consumed (Figure 3C), as evidenced by the absence of diagnostic resonances at $\delta = 7.99$ ppm (singlet) and $\delta = 4.86$ ppm (singlet) corresponding to the vinyl and methylene protons of **2**, respectively. The resonances corresponding to **2** were replaced by



Scheme 2. Tandem QM generation and ICL formation induced by **2** upon H_2O_2 activation.

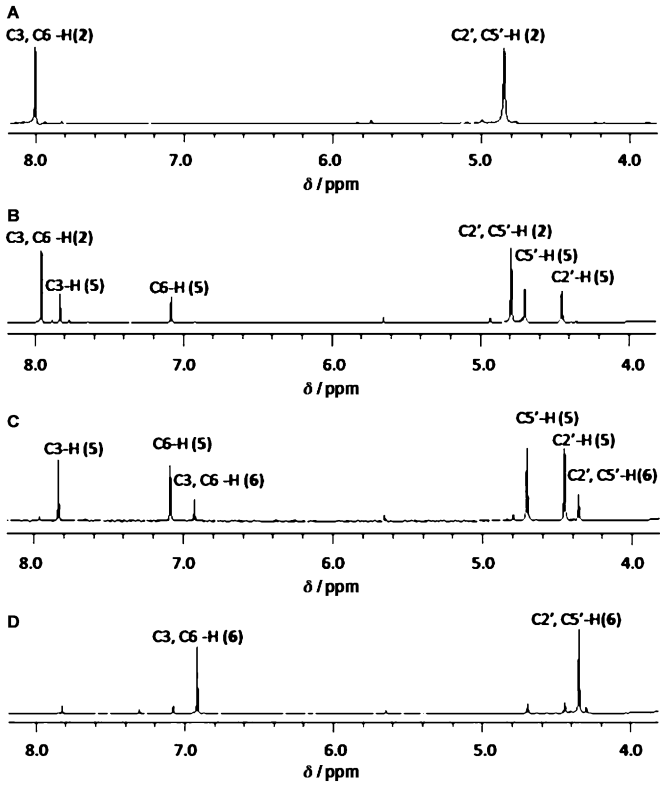
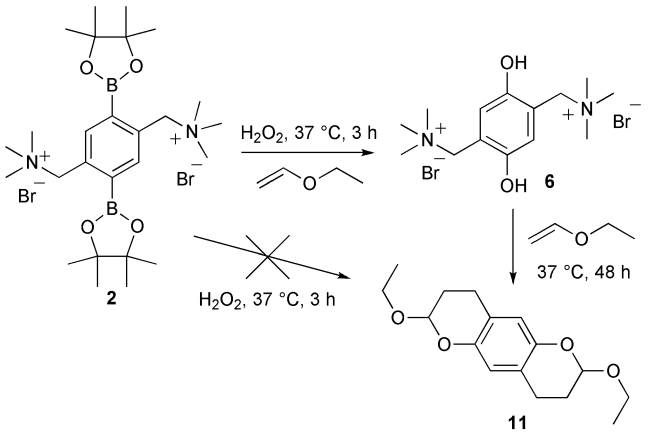


Figure 3. ^{1}H NMR spectroscopic kinetic analysis of **2** in a mixture of deuterated DMSO (550 μL) and D_2O (96 μL) with H_2O_2 (57.7 mM) and **2** (23.1 mM). A) Sample **2** in deuterated DMSO only, as reference; B) 30 min after addition of H_2O_2; C) 3 h after addition of H_2O_2; D) 24 h after addition of H_2O_2.

those that were indicative of molecule **5**. Evidence for these intermediates was obtained from the appearance of two distinct aromatic protons ($\delta \approx 7.85$, 7.11 ppm; Figure 3B and C) and two different methylene protons ($\delta \approx 4.72$, 4.46 ppm). After incubating the sample at room temperature for 24 h (Figure 3D), the resonances corresponding to **5** were replaced by those of **6** ($\delta \approx 6.95$, 4.37 ppm). Similarly, the activation of **1** and **3** with H_2O_2 followed by the formation of phenol derivatives were observed by NMR spectroscopy analysis (Figures S8 and S10, and Schemes S4 and S5 in the Supporting Information).

The NMR spectroscopic analysis of the monomer reaction indicated that compound **2** can be efficiently activated by H_2O_2 to give the intermediate **6**. The formation of **6** was triggered by the stepwise oxidation of the carbon–boron bond initiated by nucleophilic attack by H_2O_2 (**2**→**2d** and **5**→**2e**) followed by deboronation (**2d**→**5** and **2e**→**6**; Scheme 2). We believed that compound **6** directly generated *o*-QMs (**2f** and **2h**) under physiological conditions that alkylate and cross-link DNA. By comparison with compounds **8** and **10**, an additional hydroxyl group in **6** leads to an electron-rich aromatic ring, which is an important factor for efficient ICL formation induced by **2**. Rokita et al. have shown that the electron-donating group favors QM generation and regeneration, while the electron-withdrawing group hinders the formation of QM.[4d,e,5b] This could explain the obvious difference between compounds **1**/**3** and **2** regarding the cross-linking properties.

In order to determine whether QM was generated from the oxidation process or from the intermediate **6**, QM trapping experiments with a large excess of ethyl vinyl ether (EVE) were performed. When **2** was incubated at 37 °C for 3 h in the presence of H_2O_2 and EVE, no detectable QM–EVE adduct (**11**) was observed and **2** was completely converted to **6** (Scheme 3). However, **11** was detected if **6** was incubated at the same temperature for 48 h. Compound **11** was confirmed by ^{1}H and ^{13}C NMR spectroscopy analysis. The generation of QM via **6** was further supported by the cross-linking capability of **6** (Figure S11 in the Supporting Information).



Scheme 3. Trapping reactions in the presence of EVE.

In conclusion, we have developed three H_2O_2-inducible DNA bisalkylating and/or cross-linking agents (**1**–**3**), which contain boronic ester. Among these compounds, the nontoxic compound **2** can be selectively activated by H_2O_2 to generate a powerful and reversible DNA alkylating agent **6**, which directly produces QMs under physiological conditions and releases the leaving group trimethyl amine. The mechanism of H_2O_2 activation, ICL formation, and DNA alkylation was determined by NMR spectroscopy analysis as well as by a QM trapping experiment. Although the ICL yield by **2** is moderate, the alkylating and cross-linking potency can be improved by introducing an alkylating, DNA binding, or intercalating agent in the position of trimethyl amine. Therefore, an effective strategy has been developed to design and synthesize novel potent anticancer prodrugs that can be activated under tumor-specific conditions (high level of ROS) to release multiple active species by using compound **2** as a building block. Such a model will also be equally applicable to the development of prodrugs for the treatment of other diseases that are associated with H_2O_2. The synthesis of boronate-based prodrugs with different functional leaving groups is underway in our laboratory.

Experimental Section

1,4-Dimethyl-2,5-di(4,4,5,5-tetramethyl[1,3,2]dioxaborolan-2-yl)phenyl (2b): 2,5-Dibromo-*p*-xylene (**2a**; 1.06 g, 4 mmol), bis(pinacolato)diboron (3.05 g, 12 mmol), KOAc (2.36 g, 24 mmol), and $PdCl_2$(dppf) (196 mg, 0.24 mmol) were dissolved in DMF (40 mL) under argon atmosphere. The mixture was heated at 85 °C for 48 h, cooled and then water (100 mL) was added, the mixture was extracted with CH_2Cl_2 (3×50 mL). The combined organic layer was washed with water and brine, and then dried over Na_2SO_4, and the solvent was evaporated. The crude product was purified through column chromatography with 0–50% EtOAc in hexane to provide compound **2b** as white solid (1.28 g, 90%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.39 (s, 24H), 2.51 (s, 6H), 7.57 ppm (s, 2H); ^{13}C NMR (500 MHz, $CDCl_3$): δ = 140.58, 136.94, 83.43, 24.91, 21.52 ppm.

1,4-Dibromomethyl-2,5-di(4,4,5,5-tetramethyl[1,3,2]dioxaborolan-2-yl)-phenyl (2c): Compound **2b** (1.08 g, 3 mmol) was dissolved in CH_3CN (45 mL), and NBS (1.34 g, 7.5 mmol) and AIBN (52.4 mg) were added. The mixture was refluxed at 90 °C for 6 h. Then the mixture was concentrated and dissolved in DCM (100 mL). The organic phase was washed with H_2O (3×50 mL) and dried with anhydrous Na_2SO_4. The solution was evaporated and the residue was subjected to column chromatography on silica gel with 0–50% DCM in hexane to give the desired product **2c** as a white solid (0.71 g, 60%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.38 (s, 24H), 4.88 (s, 4H), 7.79 ppm (s, 2H); ^{13}C NMR (500 MHz, CDCl3): δ = 143.36, 137.85, 84.13, 33.43, 24.90 ppm.

1,4-Di(trimethylammonium bromide-methyl)-2,5-di(4,4,5,5-tetramethyl-[1,3,2]dioxaborolan-2′-yl)phenyl (2): Compound **2c** (0.1 g, 0.19 mmol) was suspended in CH_3CN (5 mL) and trimethylamine (4.2 M; 0.14 mL, 0.57 mmol) in ethanol was added dropwise with stirring. The reaction mixture was concentrated after 12 h at room temperature and gave **2** as a white solid (0.12 g, 95%). ^{1}H NMR (300 MHz, DMSO): δ = 1.35 (s, 24H), 3.05 (s, 18H), 4.86 (s, 4H), 8.02 ppm (s, 2H); ^{13}C NMR (500 MHz, DMSO): δ = 141.57, 134.66, 84.76, 65.84, 52.10, 24.49 ppm; HRMS (ESI) *m/z* calcd (%) for $C_{26}H_{48}B_2Br_2N_2O_4$ $[(M-2Br)/2]^+$: 237.1900; found: 237.1862.

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Keywords: antitumor agents · arylboronates · DNA alkylation · DNA cross-linking · prodrugs

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