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Photochemical Generation of Benzyl Cations That Selectively Cross-Link Guanine and Cytosine in DNA

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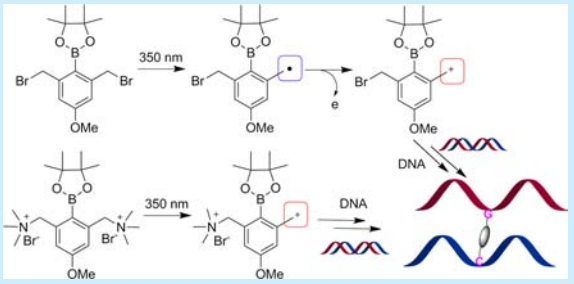
Supporting Information

ABSTRACT: UV irradiation of several aryl boronates efficiently produced bifunctional benzyl cations that selectively form guanine-cytosine cross-links in DNA. Photoinduced homolysis of the C−Br bond took place with the aryl boronate bromides **3a** and **4a**, generating free radicals that were oxidized to benzyl cations via electron transfer. However, photoirradiation of the quaternary ammonium salts **3b** and **4b** led to heterolysis of C−N bond, directly producing benzyl cations. The electron-donating group in the aromatic ring greatly enhanced cross-linking efficiency.



Light-activated molecules that interact with DNA can function by a variety of mechanisms.[1] Some induce DNA photocleavage via free radicals, carbocations, reactive oxygen species, or electron transfer,[2,3] whereas others cross-link DNA via photocycloaddition,[4−7] bifunctional quinone methides,[8−14] or modified nucleotides containing photosensitive functional groups.[15−21] Photochemical DNA cross-linking agents have been used as anticancer drugs,[22,23] for photomanipulation of DNA and therapeutic gene modulation,[24] to probe nucleic acid structures,[25] to study DNA damage and repair,[16,26] and to construct DNA-based reversible photoswitches.[27]

Different from photochemical DNA-cleavage agents that have been extensively studied, photoactivated DNA cross-linking agents are less explored, with the exception of psoralens. So far, psoralen analogues are the only commercial class of drugs known to induce DNA or RNA cross-linking via photocycloaddition. Recently, several modified nucleosides have been reported to induce DNA interstrand cross-link (ICL) formation upon photoirradiation.[4,16−19,28,29] In this vein, Freccero and Zhou reported photochemical generation of bifunctional quinone methides (QMs) capable of cross-linking DNA.[8,9,12,14] In the present study, we describe a novel mechanism for photoinduced DNA ICL formation from several bifunctional aryl boronates (**3a,b** and **4a,b**) (Figure 1), which are activated by 350 nm irradiation to produce bifunctional benzyl cations that efficiently and selectively cross-link DNA at dG and dC sites.

The target compounds investigated in the current study are phenylboronate esters **1**−**4**. Compounds **1**−**3** were synthesized as previously reported.[30,31] Compound **4a** was synthesized via borylation of 2-bromo-5-methoxy-1,3-dimethylbenzene (**5**) using *n*-butyllithium and the isopropoxyboronic acid pinacol ester followed by bromination (Supporting Information (SI), Scheme S1). Reaction of **4a** with trimethylamine provided **4b** in nearly quantitative yield.

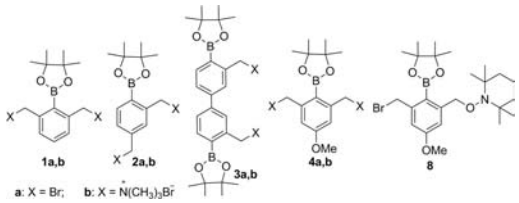


Figure 1. Structures of **1a,b**−**4a,b** and **8**.

Initially, the photoreactivity of **1a,b**−**3a,b** toward DNA was investigated by measuring DNA ICL formation upon irradiation with 350 nm light. A 49-mer DNA duplex **7** (**7a**·**7b**) was used for this study. The reaction was carried out in phosphate buffer (8.0) at 37 °C. ICL formation and cross-linking yields were analyzed via denaturing polyacrylamide gel electrophoresis (PAGE) with phosphorimager analysis (Image Quant 5.2). The ICLs were not observed with **1a,b**−**3a,b** without UV irradiation (Figure 2, lanes 2−7), while 350 nm irradiation of **3a** and **1b**−**3b** induced DNA interstrand cross-linking (Figure 2, lanes 10−13). Among **3a** and **1b**−**3b**, the highest ICL yield was observed with **3b** (14.3%) (Figure 2, lane 13). Higher concentration of **3a** or **1b**−**3b** and extended irradiation time further increased ICL yields (**3a**: 6.9% for 8 h vs 24% for 24 h; **3b**: 14.3% for 8 h vs 38.5% for 24 h) (SI, Figures S1−S4). However, DNA cleavages were observed if the irradiation was longer than 24 h. For comparison, 2 mM **3a** or **1b**−**3b** was used for further investigation, and irradiation time was fixed to 8 h.

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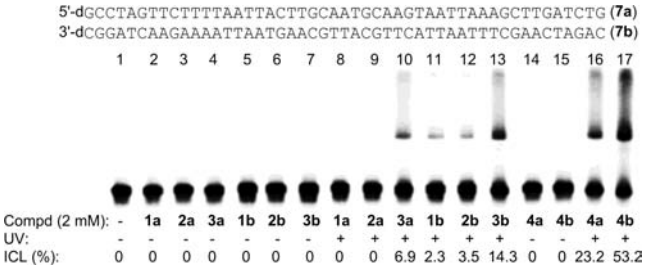

Figure 2. UV-induced DNA cross-link formation by **1a,b**−**4a,b** (ODNs were irradiated at pH 8 phosphate buffer for 8 h).

Previously, we reported that **1a**−**3a** could be activated by H_2O_2 to form bifunctional QMs that cross-link DNA,[30,31] whereas H_2O_2 activation of **1b**−**3b** did not produce ICL products due to the presence of a poor trimethylamine leaving group, thereby prohibiting QM formation. In contrast, UV irradiation of **3a** and **1b**−**3b** induced DNA ICL formation. These results suggested that other reactive species than QMs might be involved in the photoinduced DNA cross-linking process. Trapping experiments provided further evidence that QMs were not generated by UV irradiation of **3a** and **1b**−**3b**. For example, photoirradiation of **3a** and **1b**−**3b** in the presence of excess ethyl vinyl ether (EVE, a QM trapping agent) did not produce QM trapping adducts. We propose that free radicals and/or carbocations may be involved in photoinduced DNA cross-linking by **3a** and **1b**−**3b**.

To test our hypothesis, we performed a free-radical trapping experiment with ICL formation of duplex **7** induced by **3a** and **3b**. Initially, BME was used as a trapping reagent for both carbocations and free radicals. DNA cross-linking induced by **3a** and **3b** was completely inhibited by BME, suggesting that a free radical and/or a carbocation were involved in the cross-linking process (SI, Figures S5 and S6). However, we were unable to distinguish carbocations from free radicals as both react with BME. We then tested the orthogonal trapping reagents, methoxyamine and 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO), separately as competitors for ICL formation upon irradiation of **7** in the presence of **3a** or **3b**. Methoxyamine significantly decreased the DNA cross-linking efficiency of **3a** and **3b**, where 100 mM methoxyamine totally inhibited ICL formation induced by both **3a,b**, indicating that carbocations were responsible for cross-link formation (Figure 3A,B and SI, Figures S7 and S8). Carbocations can be

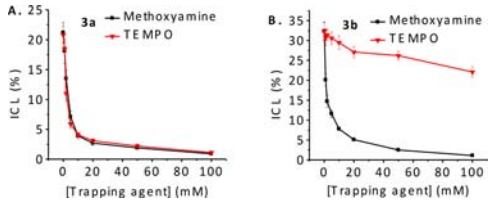

Figure 3. Effect of TEMPO and methoxyamine on DNA ICL formation of duplex **7** induced by **3a** (A) and **3b** (B).

generated by two possible pathways, i.e., through direct heterolysis of C−X bonds or via oxidation of a free radical. Addition of TEMPO up to 100 mM completely quenched DNA ICL formation induced by **3a** (SI, Figure S9) but had little effect on that induced by **3b** (SI, Figure S10), suggesting that free radicals were involved in the cross-linking process of **3a**, but not **3b** (Figure 3A,B). Collectively, these data demonstrated that the photoinduced DNA cross-linking mechanism for **3a** was related to both free radicals and carbocations while that of **3b** was only associated with carbocations. Although TEMPO slightly decreased ICL yields of **3b**, it is unlikely that free radicals were responsible for the ICL formation as complete inhibition of DNA cross-linking was not observed even when the concentrations of TEMPO were increased to 10−100 mM (Figure 3B). The possible reasons for the slight decrease in ICL yields by TEMPO could be the inner filter effect and/or its blockage of the interaction between **3b** and DNA.

To examine the generality of the above observations and to assess the mechanism for DNA cross-linking, we modified these aryl boronates to identify more efficient DNA cross-linking agents. UV spectra suggested that the cross-linking efficiency of these compounds may be correlated with their sensitivity toward 350 nm irradiation. Compounds **1b** and **2b** with a lower cross-linking efficiency have a strong absorption peak at a shorter wavelength (i.e., 225 nm) but a weak absorption peak at 280 nm (SI, Figure S11), while **3b** with a higher cross-linking yield exhibited a strong absorption peak at 270 nm. Further investigation showed that an electron-donating group ($-OCH_3$) on the aromatic ring shifts the absorption maximum to a longer wavelength (SI, Figure S12). DNA cross-linking assay showed that **4a** and **4b** induced efficient ICL formation upon 350 nm irradiation, but ICLs were not observed with **1a** and only 2.3% for **1b** (Figure 2 and SI, Figures S13 and S15). The ICL yields of **4a** and **4b** were even higher than those of **3a** and **3b** as well as traditional DNA alkylating agents, such as nitrogen mustards (Figure 2).[32] In addition, the photochemical activation and transformation of **4a,b** is faster than that of **3a,b**, leading to a faster cross-linking reaction (SI, Figures S2, S4, S14, and S16). The ICL formation was complete within 8 h for **4a** and **4b** but took longer for **3a** (24 h, 25%) and **3b** (24 h, 38.6%).

The high cross-linking efficiency of **4a,b** enabled us to perform detailed mechanistic investigations of their photo-induced DNA cross-linking. Initially, radical and carbocation trapping experiments were carried out with ICL formation from duplex **7**. Similar to **3a**, the DNA ICL formation induced by **4a** was inhibited by TEMPO (SI, Figure S17) and methoxyamine (SI, Figure S18), and complete inhibition was observed with addition of 100 mM TEMPO or methoxyamine (SI, Figure S21A). In contrast, TEMPO only slightly decreased ICL yields of **4b**, and a further increase of concentration of TEMPO (10−100 mM) had no effect on ICL formation (SI, Figures S21B, S19, and S20), which is similar to the results for **3b**. These results suggested that both radicals and cations were involved in the reaction mechanism of ICL formation induced by **4a**, while only cations were involved in **4b**-induced DNA cross-linking. Therefore, we conclude that the leaving groups affect the mechanism for ICL formation. The bromides **3a** and **4a** go through a free-radical mechanism, whereas the trimethylamine salts **3b** and **4b** favor formation of carbocation.

To further explore the mechanism of photoreaction of **4a** and **4b**, we performed monomer reactions using TEMPO as a radical trapping agent. Compounds **4a** and **4b** were irradiated with 350 nm light in the presence of TEMPO in acetonitrile-d_3. The reaction was monitored by NMR spectroscopy, and benzene was used as the internal standard. After 14 h irradiation, the amount of **4a** gradually decreased, and two new peaks appeared in the range of 4.0−6.0 ppm (SI, Figure S22). However, no obvious change was observed with **4b** (SI,

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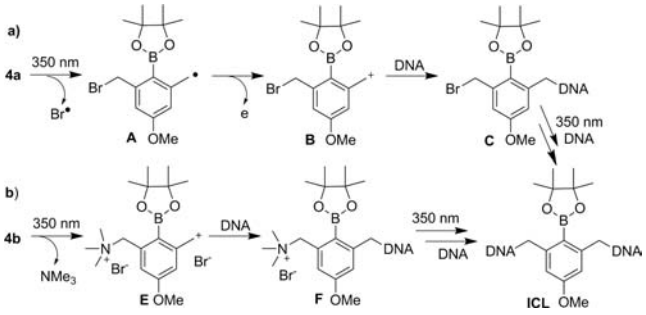
Figure S23). Additionally, a new product **8** was isolated from reaction of **4a** and TEMPO (10 equiv) after 5 days of irradiation (36% yield) (Figure 1 and SI, Scheme S2), while no adduct was formed between **4b** and TEMPO. At this point, we conclude that a radical mechanism is involved in the photoreaction of **4a** but not **4b**, which is consistent with DNA cross-linking reaction. Thus, we propose that photoirradiation of the bromides (**3a** and **4a**) generates free radicals (**A**) first, which undergo one-electron transfer to produce carbocations (**B**) that alkylate DNA directly (Scheme 1a and SI, Scheme S3). We propose that the bromo radical could be the electron acceptor. To test our hypothesis, a $AgNO_3$ solution was added to a 20 mM **4a** solution in CH_3CN after 8 h UV irradiation. The immediate formation of yellow precipitate AgBr suggested the presence of bromide ions (SI, Scheme S4). Similar photoinduced C−X bond cleavage and electron-transfer processes were proposed to be involved in photochemical activation of several modified thymidines,[15,20] 5-bromocytosine and 5-bromouracil,[21,33,34] as well as binitroimidazole in DNA.[35] In contrast, the trimethylamine salts (**3b** and **4b**) undergo heterolysis of the C−X bond upon 350 nm irradiation, which directly forms carbocations (**E**) that alkylate DNA complementary strands (Scheme 1b and SI, Scheme S3). Cross-linking from carbocations **B** or **E** provides a simple explanation for why efficient ICL formation was observed under aerobic conditions since the ionic intermediate (**B** or **E**) does not react with O_2.

Scheme 1. Proposed Mechanism for ICL Formation Induced by 4a and 4b



Having established the chemistry for DNA cross-linking induced by these aryl boronates, we determined which nucleotides were covalently bonded with one another. Initially, we tested the heat stability of purified cross-linked products and monoalkylated single-stranded DNA (**7a′**) formed with **4a** and **4b**. The ICLs were stable toward heating in phosphate buffer, while DNA cleavage was observed with piperidine treatment that is known to induce cleavage of N7-alkylated purines according to the Maxam and Gilbert reaction mechanism.[36−38] Cleavage bands were observed mainly at dG sites and also dC sites upon heating in 1.0 M piperidine (SI, Figure S24, lanes 3 and 7). Similar cleavage patterns were observed with ICL products formed by **3a**,**b** upon heating in 1.0 M piperidine (SI, Figure S25, lanes 2 and 5). This indicated that the cross-linking reactions of these benzyl cations mainly occurred with dGs and dCs via alkylation. To confirm this, we synthesized two DNA duplexes, one containing self-complementary dAT sequences (**9**) and the other having mixed dA, dT, dG, and dC sequences (**10**) (Figure 4). Compounds **3b** and **4a**,**b** induced DNA ICL formation with duplex **10** but not with **9** upon 350 nm irradiation (SI, Figure S26), which provided further evidence to support that cross-linking reactions took place with dG and dC

Figure 4. DNA sequences of duplex **9**, **10**, **15**, and **16**.

but not with dA and dT. Although there are plenty of nucleophilic centers in four nucleobases, N7 of guanine is the most active nucleophilic site that is most likely to bond to the carbocation,[39,40] subsequently, the second carbocation reacts with the opposing C via a more favorable intramolecular reaction leading to ICL formation.

To determine whether ICLs occurred with opposing GCs or staggered GCs, we designed and synthesized two new DNA duplexes, **15** (**15a**·**15b**) with only opposing GCs and **16** (**16a**·**16b**) with mixed opposing GCs and staggered GCs (Figure 4). Duplex **15** allows us to examine whether ICL takes place with opposing GCs. If the ICL would occur with staggered GCs, there would be more cross-linking sites in **7** and **16** than that in **15**. Thus, the order of cross-linking efficiency would be **16** > **7** > **15**. We then performed a cross-linking assay with these three DNA duplexes in parallel and compared their ICL yields. Efficient cross-link formation was observed with **15** in the presence of **4b**, suggesting that ICLs occurred with opposing GCs (SI, Figure S27). The ICL yield of **15** (47.6 ± 1.7%) was comparable to those with **7** (46.7 ± 1.9%) and **16** (47.5 ± 0.8%), indicating staggered GCs did not increase ICL yields (SI, Figure S27). The same phenomenon was observed with **4a** (SI, Figure S28). These results suggested that opposing GCs are more favorable for ICL formation than staggered GCs. This is different from ICL formation induced by psoralen, which selectively occurred with staggered TTs via [2 + 2] cycloaddition.[41] The cross-linking efficiency of psoralen depends on the position and number of T−A sites.[42]

To exploit this further, we used liquid chromatography-tandem mass spectrometry (LC−MS/MS) to determine the identities of the nucleobases in two complementary strands that are cross-linked by these compounds. As the highest cross-linking yield was observed with **4b**, we isolated the DNA cross-linking products formed with **4b**. We then digested the isolated ICL products obtained from duplex **7** with a cocktail of four enzymes to release the ICL as a dinucleoside remnant, following previously published procedures,[43,44] and subjected the resulting mixture to LC−MS/MS analyses. Our LC−MS/MS results revealed the presence of dinucleosides in the digestion mixture, where **4b** is conjugated with a dG and a dC (Scheme 2 and SI, Figure S29 and Scheme S5) (note: the boronate ester **4b** was hydrolyzed to the boronic acid derivative **4b*** during cross-linking and/or purification process) (SI, Figure S30).[45] The MS/MS for the $[M + H]^+$ ion of dG-**4b***-dC revealed a peak eluting at 26.4 min in the selected-ion chromatogram (SIC) for the m/z 671.3 (**11**) → 555.2 (**12**) transition, which monitors the neutral loss of a 2-deoxyribose. Fragment ion of m/z 439.0 (**13**) was also found in the MS/MS due to the neutral loss of the second 2-deoxyribose. Loss of cytosine from **13** resulted in **14** (m/z 328.1).

In summary, we discovered a novel mechanism for photoinduced DNA interstrand cross-link formation induced by bifunctional aromatic compounds. Photoirradiation of two series of aryl boronates generated bifunctional benzyl cations

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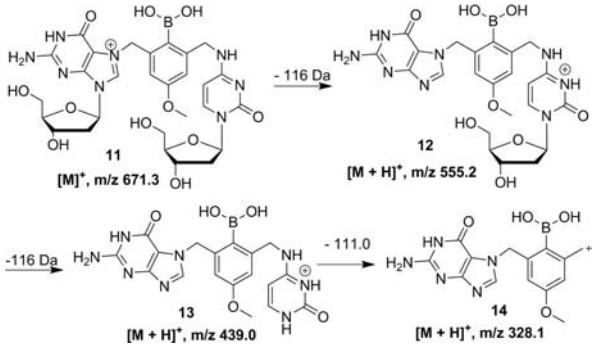
Scheme 2. Proposed Structures for the ICL Product and the Proposed Major Fragmentation Pathway for the $[M]^+$ Ion of dG-4b*-dC Cross-Link Observed in LC−MS/MS



that selectively alkylate dG and dC. The leaving groups in C−X bonds affected the pathway of cation formation. Photoirradiation of bromides **3a** and **4a** yielded free radicals that were further transformed to carbocations via one-electron transfer. In contrast, photoinduced heterolysis of C−N bonds took place with the ammonium salts **3b** and **4b** directly forming carbocations. The mechanism for photoactivation was determined using the orthogonal traps, TEMPO and methoxyamine that react with either free radicals or carbocations but not both.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b00755.

Experimental procedures for all reactions and analyses, preparation and characterization of **4a,b**, and **8**, effects of TEMPO and methoxyamine on ICL formation, autoradiograms of Fe·EDTA, and piperidine treatment of the cross-linked products and reacted single-stranded DNA (PDF)

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Notes

The authors declare no competing financial interest.

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