Published in final edited form as:

J Org Chem. 2014 January 17; 79(2): 501–508. doi:10.1021/jo401901x.

The Leaving Group Strongly Affects H₂O₂-Induced DNA Cross-Linking by Arylboronates

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Abstract

We evaluated the effects of the benzylic leaving group and core structure of arylboronates on H₂O₂-induced formation of bisquinone methides for DNA interstrand cross-linking. The mechanism of DNA cross-linking induced by these arylboronates involves generation of phenol intermediates followed by departure of benzylic leaving group leading to QMs which directly cross-link DNA via alkylation. The QM formation is the rate-determining step for DNA cross-linking. A better leaving group (Br) and stepwise bisquinone methide formation increased interstrand cross-linking efficiency. These findings provide essential guidelines for designing novel anticancer prodrugs.

INTRODUCTION

Interest in the development of cancer therapies with improved selectivity and reduced host toxicity has been growing.^{1–3} One effective approach is to design prodrugs that can be activated under tumor-specific conditions.⁴ Tumor cells produce high levels of reactive oxygen species (ROS), which makes them distinctly different from normal cells.^{5–7} Taking advantage of this difference, our group recently developed a novel prodrug strategy involving H₂O₂-induced DNA cross-linking by a nitrogen mustard cytotoxin for the selective destruction of tumor cells.⁸ Quinone methides (QMs) are the ultimate cytotoxins responsible for the activities of many anticancer drugs.^{9,10} Therefore, the development of pro-drugs that can be activated under tumor-specific conditions to form QMs is a promising approach for the targeted destruction of tumor cells. Various methods have been developed for QM formation, such as photoirradiation, oxidation, thermal extrusion reactions, acid- or base-facilitated reactions, and fluoride- or H₂O₂-induced reactions.^{10–19} However, most of these methods have disadvantages, including the inaccessibility of precursors, undesirably high reaction temperatures, long reaction times, the requirement for additional reagents and acidic or basic conditions, and the occurrence of various side reactions.²⁰ Compared with these other methods, H₂O₂-induced QM generation from arylboronates is more attractive for *in vivo* applications because H₂O₂-induced cleavage of boronate esters is a bioorthogonal reaction and H₂O₂ is bioavailable.

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The authors declare no competing financial interest.

Supporting Information. Copies of ¹H and ¹³C NMR spectra for compounds **1a**, **1b**, **4c**, **10b**, **12–15**, DNA experiments, NMR analysis, and X-ray crystal structures and analysis data of **1a–4a**. This material is available free of charge via the internet at <http://pubs.acs.org>.

Previously, we reported that arylboronic ester quaternary ammonium salt **4b** can be activated by H_2O_2 to generate a bisQM that forms DNA interstrand cross-links (ICLs).¹³ In contrast, QMs are not generated from arylboronic esters **2b** and **3b**, which have similar structures. Preliminary mechanistic studies showed that the structure of the aromatic core, the chemical properties of the benzylic leaving group, and the aromatic substituents affect QM formation.^{13,21} Inspired by these findings, we synthesized three new arylboronic esters (**1a**, **1b**, and **4c**) in this study and used **1–4** to conduct a more-detailed investigation of the effects of the leaving group and the aromatic core structure on the DNA cross-linking ability of these arylboronic esters.

RESULTS AND DISCUSSION

Compound **1a** was synthesized starting from 2-bromo-*m*-xylene (**5**) via palladium-catalyzed borylation followed by bromination (Scheme 1). Quaternization of **1a** with trimethylamine provided **1b** in nearly quantitative yield. Compounds **2–4** were synthesized as previously described.¹³

We investigated the DNA cross-linking abilities of **1–4** by allowing them to react with 49-mer DNA duplex **7** in a phosphate buffer at 37 °C for 24 h for **1a–4a** or 48 h for **1b–4b** (Scheme 2). ICL formation and yields were analyzed via denaturing polyacrylamide gel electrophoresis with phosphor image analysis. Efficient ICL formation was observed with bromide **1a** (cross-linking yield 25%) but not with the corresponding quaternary ammonium salt **1b** (0%; Figure 1, compare lanes 3 and 7). To determine the generality of this leaving-group effect, we investigated the cross-linking abilities of **2a,b** and **3a,b** and found that like **1a**, bromides **2a** (24%) and **3a** (9%) efficiently induced DNA cross-linking, whereas quaternary ammonium salts **2b** and **3b** did not (Figure 1, compare lanes 4 and 5 to lanes 8 and 9, respectively). Bromine, which is a better leaving group than trimethylamine, greatly improved the cross-linking yield. The cross-linking efficiencies of compounds **1a–3a** were affected by their concentrations, the compound/ H_2O_2 ratios, and the pH of the buffer solution (Supporting Information, Figures S1 & S2). The best compound/ H_2O_2 ratios were 2:1 for **1a** and **2a** and 1:1 for **3a**. Cross-linking yields for **1a–3a** were lower under acidic conditions than under basic conditions (SI, Figure S2). These results are consistent with previously reported results.¹³

Surprisingly, the quaternary ammonium salt **4b**, the core structure of which bears two boronate groups, showed a higher cross-linking yield (23%) than the corresponding bromide **4a** (3.5%; Figure 1, compare lanes 10 and 6), perhaps because **4b** is more water soluble than **4a**. In fact, **4a** precipitated from the reaction mixture. In order to test our hypothesis, we synthesized compound **4c** containing a mixed leaving group - Br and NMe_3 (Scheme 3) and investigated its cross-linking ability. Compound **4c** is completely dissolved in water or a mixture of $\text{H}_2\text{O}/\text{CH}_3\text{CN}$. Precipitation was not observed during the cross-linking reaction.

Next, we determined the rate constants for ICL formation induced by **1a–3a**, **4b**, and **4c** (Table 1). For all compounds, ICL growth followed first-order kinetics (SI, Figure S10). The rate constants for bromides **1a–3a** were 2–3 times the rate constant for quaternary ammonium salt **4b**.¹³ This indicated that better leaving groups facilitate the QM formation.²¹ Surprisingly, the cross-link yields (**4c** > **4b** $\approx$ **1a** $\approx$ **2a** > **3a**) do not correlate well with the kinetics (**2a** $\approx$ **3a** > **1a** > **4b** $\approx$ **4c**). Bromides **1a–3a** showed a larger *k* but a lower ICL yield than the salt **4b** or **4c**. One of the possible reasons is that the better leaving properties of –Br lead to nucleophilic substitution of bromides **1a–3a** by water under reaction condition. The NMR analysis of **1a–3a** in a mixture of DMSO and deuterated phosphate buffer (10:1) without addition of H₂O₂ showed formation of the hydrolysis intermediates (SI, Figure S11B, 12B, and 13B). The degree of the bromide hydrolysis increased with increasing the percentage of water. To ensure all compounds soluble in a mixture of DMSO and phosphate buffer, we could use maximum 9% buffer. Finally, a solvent mixture of 10:1 DMSO/buffer was used for NMR measurement. The hydrolysis of compounds **1a–3a** can be seen from appearance of a variety of new peaks in the region between 4.0 and 6.0 ppm. However, this was not observed with **4b** (SI, Figure S14B). Our recent studies about the substituent effects on QM formation also indicated that both boronate ester and benzylic bromo group were easily hydrolyzed to generate (2-(hydroxymethyl)phenyl)boronic acid derivatives, which underwent intramolecular esterification to form benzo[c][1,2]oxaborol-1(3H)-ol derivatives (**A**).²¹ In addition, bromides could be directly oxidized by excess H₂O₂, leading to 1,2-dihydroxybenzene derivatives (**B**). However, this phenomenon was not observed with the corresponding quaternary ammonium salts.²¹ In summary, both hydrolysis and oxidization reaction of bromide analogues might lead to the lower DNA ICL yields.

Considering compound **4c** also contains one bromo group but led to the highest ICL yield, we fully investigated the effect of other factors, such as the mechanism and the kinetics of the QM generation, on the ICL formation. We propose that the mechanism of ICL formation induced by **1a–4a** and **4c** is similar to that for **4b**:¹³ that is **1a–4a** or **4c** react with H₂O₂ to form phenol intermediates, which directly cross-link DNA via QMs (Scheme 4 and SI, Scheme S1). However, owing to the instability and high reactivity of the phenol intermediates, they were not isolated from the reaction mixtures. NMR analysis of the reaction of **1a** with H₂O₂ showed a variety of new peaks in the region between 4.0 and 6.0 ppm (SI, Figure S15). As described previously, the phenol products generated from the arylboronates having –Br as a leaving group quickly form QMs which react with various nucleophiles or the phenol analogues which could be oxidized by excess H₂O₂ leading to 1,2-dihydroxybenzene derivatives (**B**).²¹ To confirm fast QM formation from **1a**, we performed a QM-trapping experiment with a large excess of ethyl vinyl ether (EVE). When **1a** was incubated at 37 °C for 24 h in the presence of H₂O₂ and EVE, QM-EVE adduct **13** was generated (Scheme 5A). In contrast, the reaction of **1b** with H₂O₂ produced stable phenol product **1e** which was isolated in quantitative yield (Scheme 5C and SI, Figure S16).

Furthermore, no QM-EVE adduct was detected from the reaction of **1e** with EVE. This result is consistent with the results of the cross-linking study.

Similarly, QM-EVE adducts **13–15** were obtained from QM-trapping reactions of bromides **2a–4a** (Scheme 5B). This result indicated that the phenol intermediates generated by the reactions of **2a–4a** with H₂O₂ efficiently formed QMs under physiological conditions. Unlike the reactions of **2a** and **3a**, the reactions of **2b** and **3b** with H₂O₂ yielded stable phenol products that did not undergo QM formation.¹³ In addition, the QM-EVE adduct **15** was formed from **4a**, **4b** and **4c**. The reaction of **4b** and **4c** containing trimethyl leaving group with EVE took more than 48 h, whereas the QM-trapping reaction of **4a** was complete within 3 h. All these results indicated that the bromo leaving group greatly facilitated H₂O₂-induced QM formation from the arylboronates. Different from **1b–3b**, compounds **4b** and **4c** produced QMs even though they contain the poor leaving group (NMe₃). As previously described, the reaction of bisboronates **4b** and **4c** with H₂O₂ induced two electron-donating groups (OH) to the benzene ring (see **4e**) (Scheme 4c).¹³ The addition of the second donating group facilitated QM formation from **4e**.¹³

In order to determine whether formation of the phenol intermediates or QM generation is the rate-determining step for DNA ICL formation, the reaction of these compounds with H₂O₂ was monitored by NMR analysis (SI, Figure S11–14, S17 & S18). Due to the poor solubility of boronate esters in D₂O, all compounds were hydrolyzed to the corresponding boronic acids in DMSO/phosphate buffer (10:1) prior to the addition of H₂O₂. Then, more D₂O was added to reach a condition which is similar to the DNA cross-linking condition. However, if the ratio of phosphate buffer to DMSO is more than 2:3, the boronic acids also precipitated out. Finally, we used a mixture of phosphate buffer/DMSO (2:3) for the kinetic study. From the disappearance of the peaks at about 5.0 ppm (peak **d**), we were able to figure out the relative rate for the phenol intermediate formation. The relative reaction rates of these compounds with H₂O₂ are in the order of **4a** ≈ **4b** ≈ **4c** > **3a** > **1a** > **2a** (Table 2, SI, Figure S19). The reactions of diboronates **4a–4c** were too fast to determine the rate constant by NMR under these conditions (SI, Figure S14D–E, S17D–E, and S18C–D). The relative rates of QM formation were estimated by the formation of the final products (peak **f**, its hydrolyzed compounds or peak **f'**, the formed free NMe₃), which showed the following trend: **4a** > **3a** > **1a** > **2a** > **4b** ≈ **4c** (Table 2, SI, Figure S19). The kinetic data for DNA ICL formation (**2a** ≈ **3a** > **1a** > **4b** ≈ **4c**) (Table 1) showed a similar trend to those for QM formation but different from those for the phenol intermediates formation (Figure 4), which suggested that QM formation is the rate-limiting step for DNA cross-linking. For **4c** with a mixed leaving group –Br and NMe₃, departure of NMe₃ is the rate-determining step which took about 40 hrs while departure of –Br occurred within 3 min (SI, Figure S17).

Among the four bromide analogues, **1a** and **2a** showed ICL yields that were 3 times the ICL yield from **3a** and 9 times that from **4a**. Compound **4c** with a mixed leaving group - Br and NMe₃ proved to be the most efficient for inducing ICL formation. We propose another two possible explanations for the greater cross-linking efficiency of **1a**, **2a**, and **4c**: (1) the better water solubility and (2) stepwise generation of bisQMs from the corresponding phenol derivatives (Scheme 4). Compounds **1a**, **2a**, and **4c** were much more soluble than **3a** and **4a** under the reaction conditions tested. Furthermore, the two quinone methides (**C**, **D** or **E**, **F**) were generated from **1a** or **2a** in a stepwise process (Scheme 4). Once the first QM (**C** or **E**) reacted with one DNA strand, the stepwise formed second QM (**D** or **F**) would have better interaction with the nucleophilic centers in the complementary DNA strand, which resulted in more efficient DNA ICL formation. For **4c**, the fast formation of the first QM (**G**) plays an important role for its higher ICL yield than compound **4b**. In contrast, the reaction of **3a** likely generated two methide groups simultaneously (Scheme S1A), in which case only molecules that were already well-positioned between two nucleophilic centers of two DNA

strands would generate DNA ICLs, and the ICL yield would drop if either of the two methide groups reacted with H₂O prior to addition of a nucleophile from the DNA duplex.²⁴ However, we cannot exclude other explanations for the greater cross-linking efficiency of **1a**, **2a**, and **4c**, such as DNA sequence, molecular structure, or distance between cross-linking sites.

The crystal structures of bromides **1a–4a** suggest that they could easily enter the minor or major groove of DNA (Figure 5, SI, and Figures S38 & S39). For example, the distance between B1A and B2A for the largest molecule (**3a**) is 10.2 Å; comparatively, the widths of the DNA minor groove and the major groove are 12 Å and 22 Å respectively. Thus, these molecules can interact with DNA either through minor groove or major groove. The bromomethylene groups extended almost perpendicularly from the central benzene ring like two arms and were in a *syn* orientation for **2a** and **3a** and an *anti* orientation for **1a** and **4a**. This result indicates that the C–Br bonds could freely rotate. The distances between the two methide groups were 5.1 Å for **1a**, 5.0 Å for **2a**, 8.6 Å for **3a** and 5.8 Å for **4a**. In addition, the two central benzene rings in **3a** were rotated relative to each other at a torsion angle C2–C1–C14–C19 of 44.9(7)°. This angle results in a more flexible spatial conformation. Overall, the medium size, the structural flexibility, and the medium distance between the two formed methide groups make all these molecules suitable for interaction with DNA duplexes to form ICLs.

CONCLUSIONS

In summary, our investigation of H₂O₂-induced reactions of nine arylboronate esters with DNA revealed that the benzylic leaving group and the aromatic core structure significantly affected the DNA cross-linking ability of the arylboronates. The mechanism of ICL formation induced by these arylboronates involves generation of phenol intermediates which directly produce QMs capable of cross-linking DNA. The QM formation is the rate-determining step for DNA cross-linking. Bromine, the better of the two leaving groups, facilitate the efficient generation of QMs. Meanwhile, the core structure determined whether bisQM formation was stepwise or simultaneous, which greatly altered the cross-linking ability of the bisQMs. More importantly, incorporation of mixed leaving groups such as Br and NMe₃ greatly enhances the DNA cross-linking efficiency. Overall, our results suggest an effective strategy for designing promising anticancer drugs that release QMs upon activation by ROS *in vivo*. Our findings may also be useful for designing novel arylboronic esters that can serve as ROS bio-sensors and other applications in medicine and chemical biology.

EXPERIMENTAL SECTION

General Methods

All chemicals were commercially purchased and used without further purification. Thin layer chromatography (TLC) was carried out on precoated silica gel plates and visualized under UV light. Oligonucleotides were synthesized via standard automated DNA synthesis techniques. Deprotection of the synthesized DNA was carried out under mild deprotection conditions (28% aq. NH₃, room temperature, overnight). Oligonucleotides were purified by 20% denaturing polyacrylamide gel electrophoresis. Radiolabeling was carried out according to the standard protocols. Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics Phosphorimager equipped with ImageQuant Version 5.1 software. Enzymatic digestion products were purified with a HPLC, and mass spectra were available on an electron spray injection mass Spectrometer (ESI). ¹H, ¹³C NMR spectra were collected on a 300 MHz and 500 MHz FT-NMR spectrometer. High resolution mass spectrometry was carried out on an atmospheric-pressure chemical

ionization (APCI) TOF mass spectrometer. X-ray crystallography was performed on a diffractometer and crystal structures were solved with the Olex2 software.

2-(2,6-Bismethylphenyl)-4,4,5,5-tetramethyl-[1,3,2]dioxaborolane (**6**)

2-Bromo-1,3-dimethyl-benzene (**5**: 0.74 g, 4 mmol), bis(pinacolato)diboron (1.53 g, 6 mmol), KOAc (1.18 g, 12 mmol), and PdCl₂(dppf) (98 mg, 0.12 mmol) were dissolved in DMF (40 mL) under argon atmosphere. The mixture was heated at 85 °C for 48 h and cooled to room temperature. Then, water (100 mL) was added and the mixture was extracted with CH₂Cl₂ (3 x 50 mL). The combined organic layer was washed with water and brine dried over anhydrous Na₂SO₄, filtrated, and the solvent was evaporated. The crude product was purified through column chromatography with 0–50% EtOAc in hexane to provide **6** as colorless oil (0.74 g, 80%). ¹H NMR (300 MHz, CDCl₃) δ 7.13 (t, *J* = 7.0 Hz, 1H), 6.95 (d, *J* = 7.0 Hz, 2H), 2.42 (s, 6H), 1.41 (s, 12H). ¹³C NMR (500 MHz, CDCl₃) δ 141.6, 129.1, 126.4, 83.4, 24.9, 22.2. The NMR spectra were consistent with literature value.²⁵

2-(2,6-Bisbromomethylphenyl)-4,4,5,5-tetramethyl-[1,3,2]dioxaborolane (**1a**)

Compound **6** (0.83 g, 3.6 mmol) was dissolved in CH₃CN (55 mL), and NBS (1.6 g, 9 mmol) and AIBN (62.9 mg) were added. The mixture was refluxed at 90 °C for 3 h. Then the mixture was concentrated and dissolved in DCM (100 mL). The organic phase was washed with H₂O (3 x 50 mL) and dried with anhydrous Na₂SO₄. The solution was evaporated and the residue was subjected to column chromatography on silica gel with 0–50% DCM in hexane to give **1a** as a white solid (0.7 g, 50 %): mp 159–163 °C; ¹H NMR (300 MHz, CDCl₃) δ 7.31 (m, 3H), 4.84 (s, 4H), 1.49 (s, 12H). ¹³C NMR (500 MHz, CDCl₃) δ 144.4, 130.8, 130.0, 84.4, 34.0, 25.2; HRMS (EI) *m/z* Calcd for C₁₄H₁₉BBBr₂O₂ [M]⁺ 387.9845, found 387.9829. The NMR spectra were consistent with literature value.²⁵

1,1'-(2-(4,4,5,5-Tetramethyl-[1,3,2]dioxaborolan-2-yl)-1,3-phenylene)bis(*N,N,N*-trimethylmethanaminium) bromide (**1b**)

Compound **1a** (0.182 g, 0.47 mmol) was suspended in CH₃CN (10 mL), and 4.2 M trimethylamine (0.34 mL, 1.41 mmol) in ethanol was added dropwise with stirring. The reaction mixture was stirred at rt for 12 h and concentrated resulting in **1b** as a white solid (0.22 g, 95%): mp 250–256 °C; ¹H NMR (300 MHz, D₂O): δ 7.73–7.70 (m, 3H), 4.79 (s, 4H), 3.05 (s, 18H), 1.41 (s, 12H). ¹³C NMR (500 MHz, DMSO): δ 136.3, 135.0, 131.3, 85.9, 68.1, 52.9, 25.3; HRMS (ESI): *m/z* Calcd for C₂₀H₃₇BBBr₂N₂O₂ [(M-2Br)/2]⁺ 174.1474, found 174.1460.

1-(4-(bromomethyl)-2,5-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)-*N,N,N*-trimethylmethanaminium bromide (**4c**)

Bromide **4a** (50 mg, 0.1 mmol) was dissolved in CH₃CN (2 mL), and 4.2 M trimethylamine (24 μL, 0.1 mmol) in ethanol was added dropwise while stirring. The reaction mixture was concentrated after 24 h at room temperature. The residue was purified by column chromatography with 0–15% methanol in DCM to afford the compound **4c** as white solid (7 mg, 12%): mp 216–220 °C; ¹H NMR (300 MHz, CDCl₃) δ 8.02 (s, 1H), 7.87 (s, 1H), 4.99 (s, 2H), 4.91 (s, 2H), 3.46 (s, 9H), 1.39 (s, 24H); ¹³C NMR (500 MHz, CDCl₃) δ 146.3, 140.6, 139.2, 131.8, 85.1, 84.7, 68.3, 53.1, 32.3, 25.0, 24.9; HRMS (ESI) *m/z* Calcd for C₂₃H₃₉B₂Br₂NO₄ [M-Br]⁺ 494.2243, found 494.2247.

1,1'-(2-Hydroxy-1,3-phenylene)bis(*N,N,N*-trimethylmethanaminium) bromide (**10b**)

A solution of **1b** (50 mg) in a mixture of H₂O (3 mL), 1 M potassium phosphate buffer (52 μL, pH 8), and H₂O₂ (1.9 equivalent of **1b**) was incubated at 37 °C for 3 h, then rinsed with ethyl acetate (3 x 5 mL) and DCM (3 x 5 mL). The aqueous phase was dried under vacuum

yielding **10b** as white solid quantitatively: mp 214–218 °C; ^1H NMR (500 MHz, D_2O): δ 7.32 (d, J = 7.5 Hz, 2H), 6.56 (t, J = 7.5 Hz, 1H), 4.36 (s, 4H), 2.96 (s, 18H). ^{13}C NMR (500 MHz, DMSO): δ 137.3, 118.2, 112.8, 65.6, 52.2; HRMS (ESI): m/z Calcd for $\text{C}_{14}\text{H}_{26}\text{Br}_2\text{N}_2\text{O}$ [(M-2Br)/2] $^+$ 119.1017, found 119.1022.

QM Trapping Assay. General Procedure

A solution of bromides **1a–4a** (50 mg) in a mixture of CH_3CN (3 mL) and 1 M potassium phosphate buffer (52 μL , pH 8) was incubated at 37 °C for 30 min with excess ethyl vinyl ether (EVE). Then H_2O_2 (1.9 equivalent of bromides) was added to initiate the reaction. The reaction mixture was stirred at 37 °C for 24 h, then evaporated. Water (2 mL) was added to the residue, and extracted with ethyl acetate (3×5 mL). The organic phase was combined, dried over anhydrous Na_2SO_4 , and evaporated. The crude product was purified through column chromatography with 0–50% EtOAc in hexane to provide QM-EVE adducts **12–15**.

(2-Ethoxychroman-8-yl)methanol (**12**)

Colorless oil, 16% yield (4.9 mg). ^1H NMR (300 MHz, CDCl_3): δ 7.20–6.82 (m, 3H), 5.35 (s, 1H), 4.78–4.58 (m, 2H), 3.98–3.84 (m, 1H), 3.72–3.58 (m, 1H), 3.05–2.88 (m, 1H), 2.68–2.56 (m, 1H), 2.28 (s, 1H), 2.12–1.92 (m, 2H), 1.21 (t, J = 7.2 Hz, 3H). ^{13}C NMR (500 MHz, CDCl_3): δ 150.1, 128.9, 128.5, 126.5, 122.6, 120.4, 97.2, 63.9, 62.0, 26.5, 20.5, 15.1; HRMS (APCI): m/z Calcd for $\text{C}_{12}\text{H}_{16}\text{O}_3$ [M-H] $^+$ 207.1021, found 207.1025.

(2-Ethoxychroman-6-yl)methanol (**13**)

Colorless oil, 11% yield (3.4 mg). ^1H NMR (300 MHz, CDCl_3): δ 7.16–7.08 (m, 2H), 6.82 (d, J = 8.1 Hz, 1H), 5.27 (s, 1H), 4.61 (s, 2H), 3.98–3.84 (m, 1H), 3.72–3.58 (m, 1H), 3.08–2.92 (m, 1H), 2.72–2.58 (m, 1H), 2.12–1.92 (m, 2H), 1.61 (br, 1H), 1.21 (t, J = 7.2 Hz, 3H). ^{13}C NMR (500 MHz, CDCl_3): δ 151.9, 133.0, 128.5, 126.5, 122.7, 117.1, 97.0, 65.3, 63.7, 26.5, 20.5, 15.1; HRMS (APCI): m/z Calcd for $\text{C}_{12}\text{H}_{16}\text{O}_3$ [M-H] $^+$ 207.1021, found 207.1023.

2,7-Diethoxy-2,3,4,7,8,9-hexahydropyrano[2,3-g]chromene (**14**)

Colorless oil, 21% yield (8.8 mg). ^1H NMR (300 MHz, CDCl_3): δ 6.54 (s, 2H) 5.21 (s, 2H), 3.96–3.86 (m, 2H), 3.72–3.58 (m, 2H), 3.04–2.90 (m, 2H), 2.64–2.54 (m, 2H), 2.08–1.90 (m, 4H), 1.21 (t, J = 7.2 Hz, 6H). ^{13}C NMR (500 MHz, CDCl_3): δ 145.8, 121.5, 116.5, 96.8, 63.5, 26.7, 20.5, 15.1; HRMS (APCI): m/z Calcd for $\text{C}_{16}\text{H}_{22}\text{O}_4$ [M+NH $_4$] $^+$ 296.1862, found 296.1861.

2,2'-Diethoxy-6,6'-bichroman (**15**)

Colorless oil, 26% yield (13.8 mg). ^1H NMR (300 MHz, CDCl_3): δ 7.34–7.24 (m, 4H), 6.87 (d, 2H, J = 8.1 Hz), 5.30 (s, 2H), 3.98–3.88 (m, 2H), 3.72–3.62 (m, 2H), 3.08–2.96 (m, 2H), 2.74–2.62 (m, 2H), 2.08–1.92 (m, 4H), 1.21 (t, J = 7.2 Hz, 6H). ^{13}C NMR (500 MHz, CDCl_3): δ 151.3, 133.7, 127.6, 125.7, 122.7, 117.2, 97.0, 63.7, 26.6, 20.7, 15.2; HRMS (APCI): m/z Calcd for $\text{C}_{22}\text{H}_{26}\text{O}_4$ [M+NH $_4$] $^+$ 372.2175, found 372.2178.

Interstrand cross-link formation and kinetics study with duplex DNA

The ^{32}P -labelled oligonucleotide (0.5 μM) was annealed with 1.5 equiv of the complementary strand by heating to 65 °C for 3 min in a buffer of 10 mM potassium phosphate (pH 7) and 100 mM NaCl, followed by slow-cooling to room temperature overnight. The ^{32}P -labeled oligonucleotide duplex (0.5 μM , 2 μL) was mixed with 1 M NaCl (2 μL), 100 mM potassium phosphate (2 μL , pH 8.0), 10 mM H_2O_2 (2 μL), and compounds **1b–4b** (concentration range: 10 μM to 7 mM) and appropriate amount of

autoclaved distilled water to give a final volume of 20 μL . For Bromides **1a–4a**, 6 μL CH_3CN was added in reaction mixture to facilitate their dissolution. The reaction was incubated at 37 $^\circ\text{C}$ for 24 h and quenched by an equal volume of 90% formamide loading buffer, then subjected to 20% denaturing polyacrylamide gel analysis. For kinetics study, aliquots (final concentration: 50 nM ^{32}P -labeled oligonucleotide duplex, 100 mM NaCl, 10 mM potassium phosphate, 1 mM H_2O_2 , 2 mM of **1–4**) were taken at the prescribed times and immediately quenched by 90% formamide loading buffer, and stored at $-20\text{ }^\circ\text{C}$ until subjected to 20% denaturing PAGE analysis.

Enzyme digestion of cross-linked oligonucleotides

Inter-strand cross-linked oligonucleotide (38 nmol) was dissolved in 0.1 M Tris-HCl buffer, pH 8.0 (300 μL) and snake-venom phosphodiesterase (8.0 μL , 0.34 U) in a buffer of 110 mM Tris-HCl, pH 8.9, 110 mM NaCl, 15 mM MgCl_2 , and 50% glycerol was added. The mixture was incubated at 37 $^\circ\text{C}$ for 1 h. Then, alkaline phosphatase (8.0 μL , 80 U) in 16 μL alkaline phosphatase buffer (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl_2 and 1 mM dithiothreitol) was added. The reaction mixture was incubated at 37 $^\circ\text{C}$ for another hour. The digested products were passed through a Microcon cellulose filter (10,000 molecular cutoff, Amicon Inc.) by centrifugation at 15,000 RPM. The filtrate was collected, lyophilized, redissolved in H_2O (500 μL), and analyzed by reversed-phase HPLC (RP-18, at 260 nm) using the following gradient: 0–30 min 2–20% MeOH in water, 30–35 min 20–50% MeOH in water, 35–42 min 50–0% MeOH in water, 42–50 min 0% MeOH in water, at a flow rate 1.0 mL/min.

Stability study of ICL product formed with DNA Duplex²

After the cross-linking reaction, the reaction mixtures (0.35 μM DNA duplex, 20 μL) were co-precipitated with calf thymus DNA (2.5 mg/mL, 5 μL) and NaOAc (3 M, 5 μL) in the presence of EtOH (90 μL) at $-80\text{ }^\circ\text{C}$ for 30 min, followed by centrifuging for 5 min at 15000 rpm. The supernatant was removed, and the pellet was washed with cold 75% EtOH and lyophilized for 30 min in a Centrivap Concentrator of LABCONCO at 37 $^\circ\text{C}$. The dried DNA fragments were dissolved in H_2O (30 μL) and divided into three portions. One portion (10 μL) was incubated with piperidine (2 M, 10 μL) at 90 $^\circ\text{C}$ for 30 min, and the second portion (10 μL) was incubated with 0.1 M NaCl and 10 mM potassium phosphate buffer (pH 8, 10 μL) under the same condition, and the third portion was used as control sample. The samples were subjected to electrophoresis on a 20% denaturing polyacrylamide gel.

Hydroxyl radical reaction (Fe-EDTA reaction)³

Fe(II)-EDTA cleavage reactions of ^{32}P -labelled oligonucleotide (0.1 μM) were performed in a buffer containing 50 μM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, 100 μM EDTA, 5 mM sodium ascorbate, 0.5 M NaCl, 50 mM sodium phosphate (pH 7.2) and 1 mM H_2O_2 for 3 min at room temperature (total substrate volume 20 μL), then quenched with 100 mM thiourea (10 μL). Samples were lyophilized, and incubated with 1 M piperidine (20 μL) at 90 $^\circ\text{C}$ for 30 min. The mixture was lyophilized again, dissolved in 20 μL H_2O : 90% formamide loading buffer (1:1) and subjected to 20% denaturing PAGE analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Cancer Institute (1R15CA152914-01), Great Milwaukee Foundation (Shaw Scientist Award), and UWM Research Growth Initiative (RGI101X234).

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Figure 1.

H₂O₂-induced DNA ICL formation by compounds **1–4**. Lane 1: DNA only (cross-linking yield 0%); lane 2: DNA with 100 μ M H₂O₂ (0%); lane 3: 2 mM **1a** (25%); lane 4: 2 mM **2a** (24%); lane 5: 2 mM **3a** (9%); lane 6: 2 mM **4a** (3.5%); lane 7: 2 mM **1b** (0%); lane 8: 2 mM **2b** (0%); lane 9: 2 mM **3b** (0%); lane 10: 2 mM **4b** (23%); lane 11: 2 mM **4c** (33.7%); lane 12: DNA marker; [H₂O₂] = 1 mM for **1**, **2**, and **4**, and 2 mM for **3** (Reaction mixture was incubated at 37°C for 48 h).

Figure 2. Determination of the reaction sites of 1a

Phosphorimage autoradiogram of 20% denaturing PAGE analysis of the isolated ICL products and monoalkylated single stranded DNA (**7a'**) upon heating in piperidine or phosphate buffer. The ICL product and **7a'** were produced by incubation of duplex **7** with 1 mM **1a** and 1 mM H₂O₂. **7a** was radiolabeled at 5'-terminus. lane 1: isolated monoalkylated single stranded DNA (**7a'**); lane 2: **7a'** was heated in phosphate buffer at 90 °C for 30 min; lane 3: **7a'** was heated in 1.0 M piperidine at 90 °C for 30 min; lane 4: the ICL was heated in phosphate buffer at 90 °C for 30 min; lane 5: the ICL product was heated in 1.0 M piperidine at 90 °C for 30 min; lane 6: Fe-EDTA treatment of ICL; lane 7: G+A sequencing; lane 8: Fe-EDTA treatment of **7**.

Figure 3.

HPLC profiles of the enzymatic analysis. (A) DNA duplex **7** only as control; (B) **1a** treated single stranded **7a** and **7b**; (C) ICL products induced by **1a**, obtained by digestion with snake venom phosphodiesterase followed by alkaline phosphatase (analyzed by reverse-phase HPLC (RP-18, at 260 nm) using gradient: 0–30 min 2–20% MeOH in water, 30–35 min 20–50% MeOH in water, 35–42 min 50–100% MeOH in water, 42–50 min 100% MeOH in water, at a flow rate 1.0 mL/min).

Figure 4.

The relationship between the rate constant (k) of ICL formation (y-axis) vs. that of QM formation (x-axis).

Figure 5.
X-ray crystal structures of **1a** and **2a**.

Scheme 1.
Synthesis of **1a** and **1b**

Scheme 3.
Synthesis of **4c** that contains mixed leaving groups

Scheme 4.

Mechanism of H_2O_2 -induced QM generation and ICL formation by 1a, 2a, and 4c.

Scheme 5.

Effect of the leaving group on QM formation. (A) QM-trapping product 12 was generated by reaction of 1a, H₂O₂, and EVE; (B) QM-trapping products 13–15 were generated from 2a–4a; (C) no QM-EVE adduct was generated from quaternary ammonium salt 1b; phenol product 1e was obtained instead.

Table 1

Rate of ICL growth from 7 upon treatment with bromides and salts

<i>compound</i>	$k_{obs}, 10^{-5} \text{ s}^{-1}$	$t_{1/2}, \text{ min}$
<i>1a</i>	8.8 ± 1.3	130 ± 13
<i>2a</i>	14.1 ± 1.5	82 ± 8
<i>3a</i>	13.8 ± 1.2	84 ± 9
<i>4b</i>	4.9 ± 0.5	234 ± 9
<i>4c</i>	3.7 ± 0.3	312 ± 10

Table 2

Rate of starting material disappearance and QM formation

Compounds	Disappearance of starting materials		QM formation	
	Time of completion (min)	k_{obs} (10^{-5} s^{-1})	Time of completion (min)	k_{obs} (10^{-5} s^{-1})
1a	60	39.0 ± 1.5	60	9.5 ± 0.2
2a	90	36.7 ± 3.8	90	12.2 ± 1.1
3a	30	77.0 ± 2.1	30	20.0 ± 4.0
4a	< 3	n.d.	< 3 min	n.d.
4b	< 3	n.d.	~3000	4.6 ± 0.3
4c	< 3	n.d.	~2400	4.8 ± 0.5

^a
n.d.: not determined