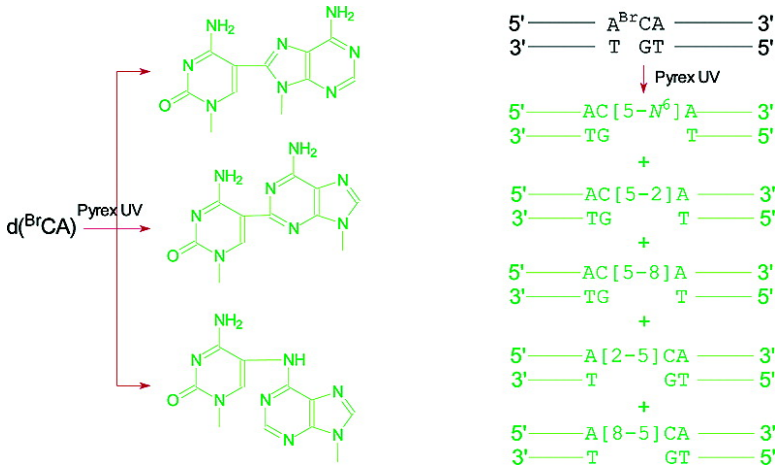
Article

Formation of Intrastrand Cross-Link Products between Cytosine and Adenine from UV Irradiation of d(CA) and Duplex DNA Containing a 5-Bromocytosine

Haizheng Hong, and Yinsheng Wang

J. Am. Chem. Soc., **2005**, 127 (40), 13969-13977• DOI: 10.1021/ja0531677 • Publication Date (Web): 20 September 2005



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 5 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

Journal of the American Chemical Society is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

J|A|C|S
ARTICLES
Published on Web 09/20/2005

Formation of Intrastrand Cross-Link Products between Cytosine and Adenine from UV Irradiation of d(^{Br}CA) and Duplex DNA Containing a 5-Bromocytosine

Haizheng Hong[†] and Yinsheng Wang*[,‡]

Contribution from the Department of Chemistry and Environmental Toxicology Graduate Program, University of California, Riverside, California 92521-0403

Received May 14, 2005; E-mail: yinsheng.wang@ucr.edu

Abstract: Here, we showed that Pyrex-filtered UV light irradiation of d(^{Br}CA) gave rise to three types of intrastrand cross-link products, that is, d(C[5-*N*^{6}]A), d(C[5−2]A), and d(C[5−8]A), where the C5 carbon atom of cytosine is covalently bonded to the *N*^{6} nitrogen atom, C2, and C8 carbon atoms of adenine, respectively. Furthermore, we demonstrated by LC-MS/MS that the UV irradiation of a 5-bromocytosine-containing duplex oligodeoxynucleotide (ODN) led to the formation of five cross-link products, that is, C[5-*N*^{6}]A, C[5−2]A, C[5-8]A, A[2−5]C, and A[8−5]C, under both aerobic and anaerobic conditions. LC-MS/MS quantification results showed that the yields for the formation of these cross-link products are different. The presence of molecular oxygen reduces the yields for the formation of all cross-link products except A[2−5]C. To our knowledge, this is the first report about the formation of intrastrand cross-link products between cytosine and adenine in duplex DNA. The chemistry discovered here may facilitate the future preparations of oxidative cross-link lesion-bearing substrates for biochemical and biophysical studies.

Introduction

Reactive oxygen species (ROS) can be produced by both endogenous and exogenous pathways, and they can damage cellular components including DNA.[1−3] Previous studies by Box, Cadet, their co-workers, and us[4−16] have uncovered several intrastrand cross-link lesions formed between neighboring C and G, T and G, T and A, as well as mC and G (mC is 5-methylcytosine). These products are initiated from γ- or X-ray irradiation of DNA or from the UV irradiation of 5-bromocytosine-containing DNA.

These previous studies also revealed that the intrastrand cross-link lesions are initiated from a single pyrimidine radical.[5−10,12,16] In this regard, hydroxyl radical, which is one of the major ROS produced from γ-radiolysis of water or Fenton reaction,[17] can either be added to the C5 and C6 carbon atoms of cytosine, thymine, and 5-methylcytosine or abstract a hydrogen atom from the 5-methyl carbon atom of the latter two pyrimidine bases. Both pathways result in pyrimidine base-centered secondary radicals,[17] which may attack their neighboring purine bases to yield intrastrand cross-link lesions.[5−10,12,16] In addition, we demonstrated that, under aerobic conditions, the cation radical of cytosine, which is induced from one-electron photooxidation, can deprotonate at the exocyclic amino group, and the resulting nitrogen-centered radical can couple with its neighboring 5′ cytosine to give two types of intrastrand cross-link lesions.[11,13]

Although intrastrand cross-link products have been found to form at adjacent cytosine and guanine, 5-methylcytosine and guanine, thymine and guanine, or thymine and adenine,[5,6,8−10,12,16] no cross-link product formed between cytosine and adenine has been previously reported. We showed recently that the Pyrex-filtered UV light irradiation of duplex DNA containing a G^{Br}CG sequence component results in the facile formation of a type of intrastrand cross-link product in which the C5 carbon atom of cytosine and the C8 carbon atom of its neighboring 5′ guanine are covalently bonded.[14] Moreover, we showed that the same cross-link product can form between guanine and its vicinal 3′ cytosine upon γ irradiation of a double-stranded ODN under anaerobic conditions.[15]

We reason that a similar cross-linking chemistry may occur between cytosine and its adjacent adenine. Here, we examined the corresponding photochemistry of duplex DNA containing

[†] Environmental Toxicology Graduate Program.
[‡] Department of Chemistry.

(1) Lindahl, T. *Nature* **1993**, *362*, 709−715.
(2) Lindahl, T. *NATO ASI Ser., Ser. A* **1999**, *302*, 251−257.
(3) Finkel, T.; Holbrook, N. J. *Nature* **2000**, *408*, 239−247.
(4) Box, H. C.; Budzinski, E. E.; Dawidzik, J. D.; Wallace, J. C.; Evans, M. S.; Gobey, J. S. *Radiat. Res.* **1996**, *145*, 641−643.
(5) Box, H. C.; Budzinski, E. E.; Dawidzik, J. B.; Gobey, J. S.; Freund, H. G. *Free Radical Biol. Med.* **1997**, *23*, 1021−1030.
(6) Budzinski, E. E.; Dawidzik, J. B.; Rajecki, M. J.; Wallace, J. C.; Schroder, E. A.; Box, H. C. *Int. J. Radiat. Biol.* **1997**, *71*, 327−336.
(7) Box, H. C.; Budzinski, E. E.; Dawidzik, J. B.; Wallace, J. C.; Iijima, H. *Radiat. Res.* **1998**, *149*, 433−439.
(8) Romieu, A.; Bellon, S.; Gasparutto, D.; Cadet, J. *Org. Lett.* **2000**, *2*, 1085−1088.
(9) Bellon, S.; Ravanat, J. L.; Gasparutto, D.; Cadet, J. *Chem. Res. Toxicol.* **2002**, *15*, 598−606.
(10) Zhang, Q.; Wang, Y. *J. Am. Chem. Soc.* **2003**, *125*, 12795−12802.
(11) Liu, Z.; Gao, Y.; Wang, Y. *Nucleic Acids Res.* **2003**, *31*, 5413−5424.
(12) Zhang, Q.; Wang, Y. *J. Am. Chem. Soc.* **2004**, *126*, 13287−13297.
(13) Liu, Z.; Gao, Y.; Zeng, Y.; Fang, F.; Chi, D.; Wang, Y. *Photochem. Photobiol.* **2004**, *80*, 209−215.
(14) Zeng, Y.; Wang, Y. *J. Am. Chem. Soc.* **2004**, *126*, 6552−6553.
(15) Gu, C.; Wang, Y. *Biochemistry* **2004**, *43*, 6745−6750.
(16) Zhang, Q.; Wang, Y. *Nucleic Acids Res.* **2005**, *33*, 1593−1603.
(17) von Sonntag, C. *The Chemical Basis of Radiation Biology*; Taylor & Francis: London, 1987.

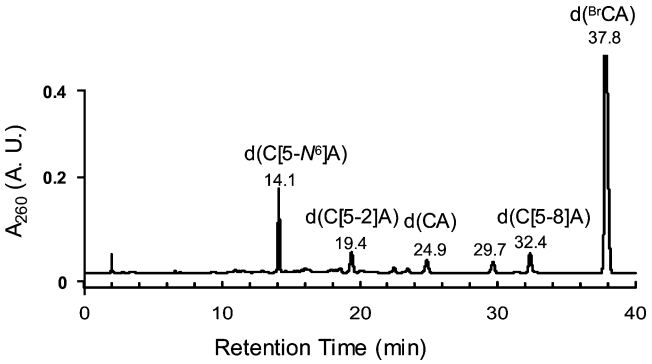


Figure 1. HPLC trace for the separation of 10-min aerobic irradiation mixture of d(BrCA).

an ABrCA sequence motif, and we found that five intrastrand cross-link products, where cytosine and its neighboring 5′ or 3′ adenine are covalently bonded, could be induced in duplex DNA.

Results and Discussion

Isolation and MS Characterization of Novel Cross-Link Products from the Pyrex-Filtered UV Light Irradiation of d(BrCA). For the ease of isolation and characterization of cross-link products, we started with the irradiation of dinucleoside monophosphate d(BrCA), and we were able to isolate from the irradiation mixture three intrastrand cross-link products formed between cytosine and adenine (the retention times for these products were 14.1, 19.4, and 32.4 min, Figure 1). For the brevity of discussion, we designate them as d(C∧A)$_a$, d(C∧A)$_b$, and d(C∧A)$_c$, respectively (Figure 1). Positive-ion ESI-MS of each product gave an ion of *m*/*z* 539, which was 2 Da less than the *m*/*z* value of the $[M + H]^+$ ion of the unmodified d(CA). This result, together with the absence of bromine isotope pattern in the molecular ion peak (data not shown) and the result of accurate mass measurement (Table 1), supports that the products form from the losses of hydrogen and bromine atoms from the starting d(BrCA).

The product−ion spectra of the $[M + H]^+$ ions (*m*/*z* 539) of the three products suggested that the two nucleobases are covalently bonded (Figure 2a−c). In this respect, a common and abundant fragment ion of *m*/*z* 441 was found in the product−ion spectra of all three cross-link products (Figure 2a−c). In addition, we found a product ion of *m*/*z* 245, which is formed in high abundance for d(C∧A)$_a$ and d(C∧A)$_b$ but in low abundance for d(C∧A)$_c$. Exact mass measurements support that the ions of *m*/*z* 441 and 245 are initiated from the neutral losses of a 2-deoxyribose ($C_5H_6O_2$) and the 2-deoxyribose/phosphate backbone, respectively (Table 1). The presence of the ion of *m*/*z* 245 reveals that the cytosine and adenine are covalently bonded. Similar cleavages have been previously observed in the product−ion spectra of several other intrastrand cross-link products.[10,11,13] Likewise, the product−ion spectra of the $[M - H]^-$ ions of the three cross-link products support that the two nucleobases are covalently bonded (Figure S3).

To gain additional information about the structures of the three cross-link products, we acquired the ^{1}H NMR and 2-D NOE spectra of those products. ^{1}H NMR spectrum of d(C∧A)$_a$ shows that it has three singlet aromatic protons (Figure S5a), suggesting that either the H5 or H6 hydrogen atom of cytosine is lost upon the formation of the cross-link product. In addition,

Table 1. Exact Mass Measurements of the $[M + H]^+$ Ions and Product Ions Observed in the MS/MS of the $[M + H]^+$ Ions of the Three d(C∧A) Cross-Link Products

ion identities	calculated mass	measured mass	deviation (ppm)
	d(C[5-*N*^{6}]A)		
$[M + H]^+$	539.1404	539.1401	−0.6
		539.1406	0.4
		539.1406	0.4
-2-deoxyribose	441.1036	441.1031	−1.1
		441.1031	−1.1
		441.1030	−1.4
$[nucleobase + H]^+$	245.0899	245.0902	1.2
		245.0899	0
		245.0899	0
	d(C[5−2]A)		
$[M + H]^+$	539.1404	539.1401	−0.6
		539.1396	−1.5
		539.1399	−0.9
-2-deoxyribose	441.1036	441.1034	−0.5
		441.1035	−0.2
		441.1035	−0.2
$[nucleobase + H]^+$	245.0899	245.0898	−0.4
		245.0899	0.0
		245.0900	0.4
	d(C[5−8]A)		
$[M + H]^+$	539.1404	539.1398	−1.1
		539.1396	−1.5
		539.1398	−1.1
-2-deoxyribose	441.1036	441.1028	−1.8
		441.1029	−1.6
		441.1029	−1.6
$[nucleobase + H]^+$	245.0899	245.0901	0.8
		245.0898	−0.4
		245.0900	0.4

2-D NOE spectrum of this product allows us to assign all proton resonances without ambiguity (Table S1). The result shows that one aromatic proton (δ 8.58 ppm) has strong correlation with the $H_{1'}$, $H_{4'}$, and $H_{5'}$ protons of the 5′ nucleoside, revealing that this aromatic proton is the H6 of cytosine (a portion of the 2-D NOE spectrum is shown in Figure S6a). The remaining two aromatic protons, therefore, can be attributed to the H2 and H8 of adenine. This result supports that neither the C2 nor the C8 carbon atom of adenine participates in coupling with its neighboring nucleobase. Therefore, a nitrogen atom in adenine is involved in the covalent bond formation.

To examine which nitrogen atom of adenine is involved in cross-link bond formation, we treated the product with 10 mM Tris-HCl buffer (pH 7.3) at 70 °C for 1 and 12 h; LC-MS/MS analysis showed that the product is stable under these conditions (data not shown). Therefore, it is unlikely that the *N*1, *N*3, or *N*7 of adenine is involved in cross-linking with the cytosine residue. In this respect, *N*3- and *N*7-substituted adenines are known to undergo depurination under neutral thermal conditions, and the half-lives ($t_{1/2}$) for 3-methyl-2′-deoxyadenosine and 7-methyl-2′-deoxyadenosine in DNA under pH 7.0 at 37 °C are approximately 26 and 3 h, respectively.[18] The *N*1-substituted adenine, on the other hand, can undergo Dimroth rearrangement to give the *N*^{6}-substituted product.[19] We, however, did not observe an apparent change in retention time, MS/MS, or MS^3 (which monitors the fragmentation of the cross-linked nucleobase component) of the product upon incubation at 70 °C under pH 7.3 for up to 12 h, supporting that the *N*^{6} nitrogen atom of

(18) Singer, B.; Grunberger, D. *Molecular Biology of Mutagens and Carcinogens*; Plenum Press: New York and London, 1983.
(19) Fujii, T.; Itaya, T. *Heterocycles* **1998**, *48*, 359−390.

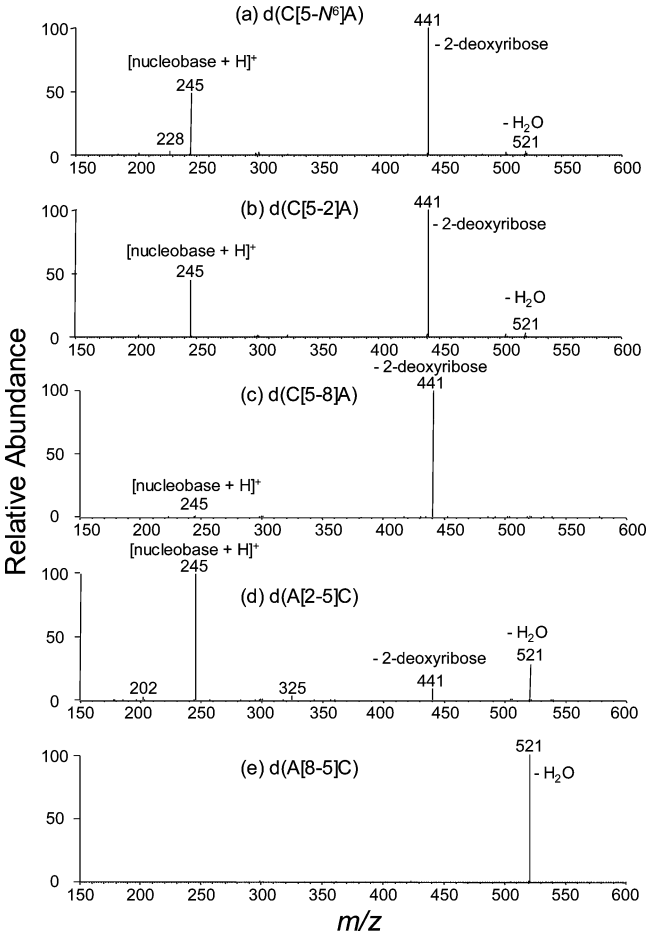


Figure 2. Product−ion spectra of the $[M + H]^+$ ions of d(C[5-*N*^{6}]A) (a), d(C[5−2]A) (b), d(C[5−8]A) (c), d(A[2−5]C) (d), and d(A[8−5]C) (e). The relative collisional energies were 25%.

adenine is involved in the formation of the cross-link product. We, therefore, conclude that the cross-link product is d(C[5-*N*^{6}]A).

Similar NMR analyses enabled us to determine the structures of $d(C\wedge A)_b$ and $d(C\wedge A)_c$. In this respect, we observed two singlet aromatic proton resonances in the ^{1}H NMR spectra of the two products (Figure S5b,d), and we again found a strong correlation peak between H6 proton of cytosine and $H_{1'}$ proton of 2′-deoxycytidine in the 2-D NOE spectra of both cross-links (Figure S6b,d depict a portion of the 2-D NOE spectra of the two products). Therefore, the C5 carbon atom of cytosine is involved in the formation of the covalent bond linking the cytosine and adenine in these two products. In addition, the other aromatic proton of $d(C\wedge A)_b$ strongly correlates with the $H_{1'}$ proton of 2′-deoxyadenosine (Figure S6b). In contrast, no correlation peak between the second aromatic proton and the $H_{1'}$ proton of 3′ nucleoside was present in the 2-D NOE spectrum of $d(C\wedge A)_c$ (Figure S6d). Thus, the H8 proton of adenine remains in $d(C\wedge A)_b$, but this proton is lost in $d(C\wedge A)_c$. The C5 carbon atom of cytosine is, therefore, covalently bonded to the C2 and C8 carbon atoms of adenine in $d(C\wedge A)_b$ and $d(C\wedge A)_c$, respectively, and we designate the two products as d(C[5−2]A) and d(C[5−8]A), respectively. In this regard, we optimized the gas-phase structures of d(C[5−2]A) and d(C[5−8]A) by using the semiempirical PM3 method[20] with Spartan 5.1.3 (Wave function Inc., Irvine, CA). From these structures, we found that the distance between the H8 proton of adenine and $H_{1'}$ proton of 3′ nucleotide is 3.14 Å in d(C[5−2]A), whereas the distance between the H2 proton of adenine and $H_{1'}$ proton of 2′-deoxyadenosine is 4.41 Å in d(C[5−8]A). This result is consistent with the above 2-D NOESY results.

(20) Stewart, J. J. P. *J. Comput. Chem.* **1989**, *10*, 209−220.

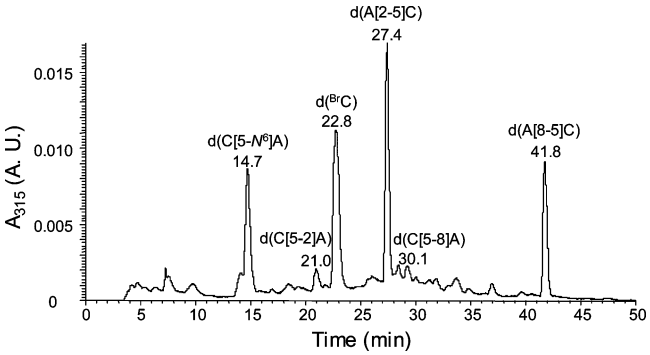


Figure 3. HPLC trace for the separation of the enzymatic digestion mixture (with nuclease P1 and alkaline phosphatase) of d(A^{Br}CA) after 20 min of UV irradiation under aerobic conditions. The PDA detector was set to monitor the absorbance of eluates at 315 nm.

Our above structure assignments are also consistent with the numbers of acidic protons in all three cross-link products as measured by ESI-MS. ESI-MS of d(C[5-*N*^{6}]A) in D_2O/CH_3CN (50/50, v/v) gave *m*/*z* 542 for the $[M - D]^-$ ion (Figure S7a), demonstrating that there are five acidic hydrogen atoms in the $[M - H]^-$ ion of the cross-link product. Similar measurements of d(C[5−2]A), d(C[5−8]A), and the unmodified d(^{Br}CA), however, showed that the deprotonated ion of each of the three compounds has six exchangeable protons (Figure S7b,d). These exchangeable proton measurements are in line with the assigned structures of the three cross-link products.

Isolation and MS Characterization of Novel Cross-Link Products from the Pyrex-Filtered UV Light Irradiation Mixture of d(A^{Br}CA). Next, we examined whether similar cross-linking chemistry occurs between adenine and its neighboring 3′ cytosine. We chose to use d(A^{Br}CA) rather than d(A^{Br}C) because of the ease of preparation of the former substrate by automated solid-phase DNA synthesis. After a similar Pyrex-filtered UV light irradiation, we digested the irradiation mixture with nuclease P1 and alkaline phosphatase. The cross-link product-containing trinucleoside diphosphates can be hydrolyzed, at least in part, to dinucleoside monophosphates containing cross-link products formed at CA or AC site. After HPLC separation, we found, other than the three CA cross-links described above, two other cross-link products eluting at 27.4 and 41.8 min (Figure 3). ESI-MS and UV absorption spectrum of the fraction eluting at 22.8 min showed that it contains d(^{Br}C) (data not shown), which has an absorption maximum at 285 nm and also exhibits some absorbance at 315 nm.

Several lines of evidence support that the two other cross-link products are d(A[2−5]C) and d(A[8−5]C), respectively. First, these two products have the same molecular weight as the three CA cross-link products, though collisional activation of the $[M + H]^+$ ions of the two AC cross-links gives distinctive product−ion spectra (Figure 2d−e) from the corresponding spectra of the three CA cross-link products (Figure 2a−c). In the latter respect, an abundant fragment ion emanating from the loss of a water molecule is observed in both product−ion

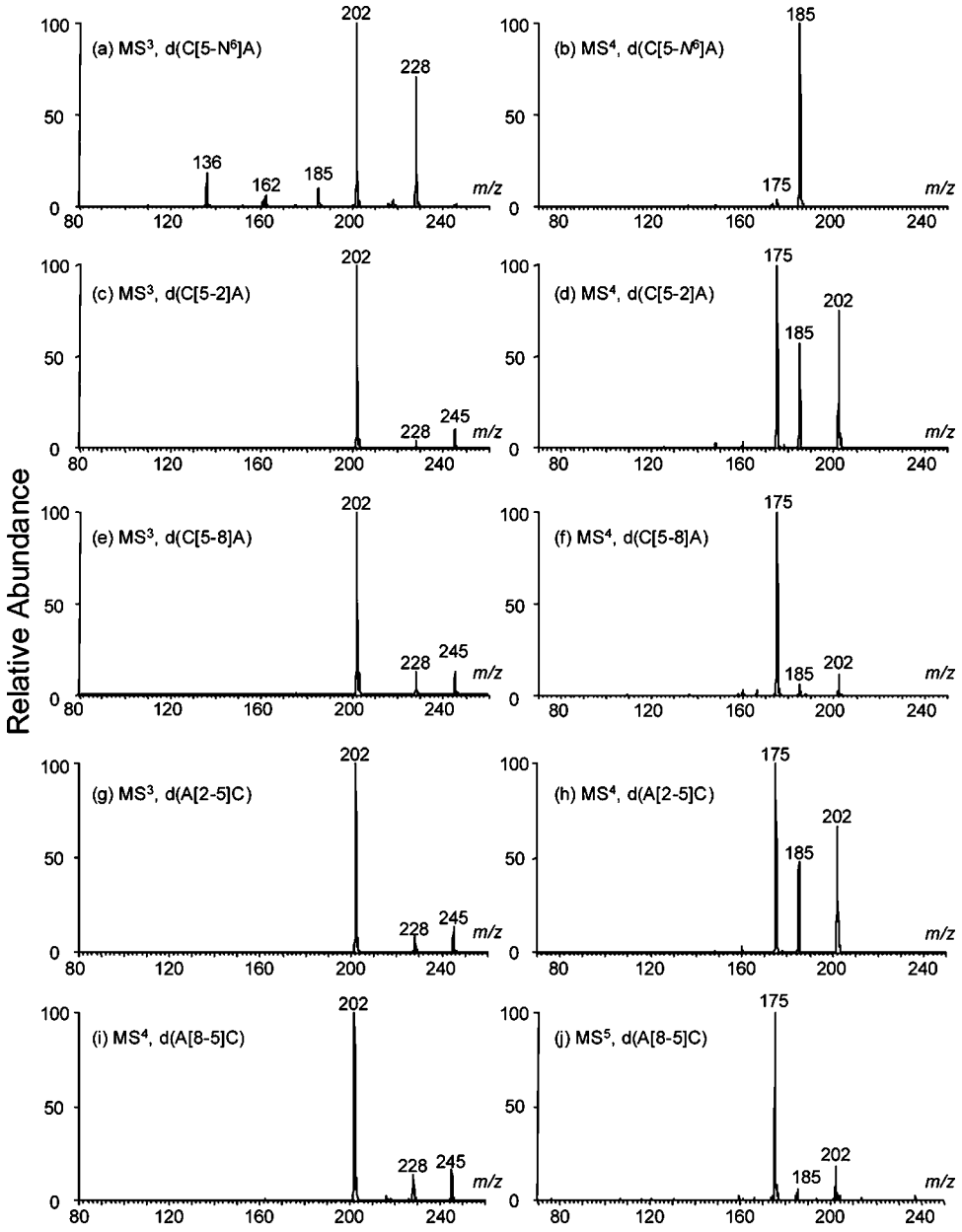


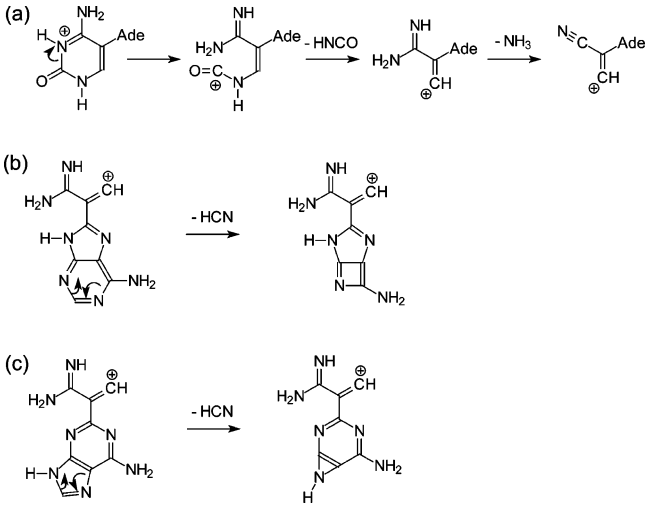
Figure 4. Multistage MS analyses of d(C∧A) and d(A∧C) cross-link products: ESI-MS^3 for monitoring the fragmentation pathway of *m*/*z* 539 → 245 → of authentic d(C[5-N^6]A) (a), d(C[5−2]A) (c), d(C[5−8]A) (e), and the 27.4-min fraction shown in Figure 3 (g); ESI-MS^4 for monitoring the fragmentation pathway of *m*/*z* 539 → 245 → 202 → of authentic d(C[5-N^6]A) (b), d(C[5−2]A) (d), d(C[5−8]A) (f), and the 27.4-min fraction shown in Figure 3 (h). ESI-MS^4 and MS^5 for monitoring the fragmentation pathways of *m*/*z* 539 → 521 → 245 → (i) and *m*/*z* 539 → 521 → 245 → 202 → (j) of the 41.8-min fraction shown in Figure 3. The relative collisional energy was 25% for MS/MS and 35% for MS^3, MS^4, and MS^5.

spectra (Figure 2d−e). In addition, positive-ion ESI-MS/MS of the product eluting at 27.4 min showed the familiar fragment ion of *m*/*z* 245, supporting that the two nucleobases are covalently bonded.

Second, multistage MS experiments support that the nucleobase moieties of the products eluting at 27.4 and 41.8 min have the same structures as those of d(C[5−2]A) and d(C[5−8]A), respectively. In this regard, we produced, in the mass spectrometer, the ion of *m*/*z* 245 from the fragmentation of the $[M + H]^+$ ion of each of the three standard CA cross-link products (Figure 2a−c) and subjected this ion to further collisional activation in an ion-trap mass spectrometer. The resulting product−ion spectra (MS/MS/MS or MS^3) enabled us to differentiate d(C[5-N^6]A) from d(C[5−2]A) and d(C[5−8]A) (Figure 4a, c, e). In this context, the protonated ion of the cross-linked nucleobase portion of d(C[5-N^6]A) can cleave to yield two abundant fragment ions, that is, ions of *m*/*z* 228 and 202, which are attributed to the liberations of NH_3 and HNCO molecules, respectively (Figure 4a). Under identical instrument conditions, the corresponding fragmentation of the *m*/*z*-245 ion from d(C[5−2]A) and d(C[5−8]A), however, leads to the formation of a dominant product ion of *m*/*z* 202 (Figure 4c, e), though the ion of *m*/*z* 228 is also produced in very low abundance.

To explore the possibility of differentiating the d(C[5−2]A) and d(C[5−8]A), we subjected the common and abundant ion

Scheme 1



of *m*/*z* 202 to further collisional activation. The resulting product−ion spectrum, that is, MS^4, of d(C[5−2]A) showed two abundant product ions, that is, ions of *m*/*z* 185 and 175, which are attributed to the neutral losses of NH_3 and HCN, respectively (Figure 4d). The corresponding product−ion spectrum of d(C[5−8]A) showed that the fragment ion of *m*/*z* 175 is still present as the base peak, whereas the ion of *m*/*z* 185 is produced in much lower abundance (<10%, Figure 4f). On the other hand, MS^4 of the ion of *m*/*z* 202 formed from d(C[5-*N*^{6}]A) showed a dominant fragment ion of *m*/*z* 185 (Figure 4b). Therefore, the MS^3 and MS^4 together allowed us to differentiate all three isomeric cross-link products from each other. The proposed structures for the major fragment ions observed in MS^3 and MS^4 are shown in Scheme 1.

We next acquired the corresponding product−ion spectra of the ions of *m*/*z* 245 and 202 for the fractions eluting at 27.4 (Figure 4g, h) and 41.8 min (Figure 4i, j), and it turned out that the spectra for the 27.4- and 41.8-min fractions are nearly identical to those of d(C[5−2]A) (Figure 4c, d) and d(C[5−8]A) (Figure 4e, f), respectively. Therefore, these results support that the two fractions eluting at 27.4 and 41.8 min contain d(A[2−5]C) and d(A[8−5]C), respectively. In this regard, the ion of *m*/*z* 245 is almost undetectable in the product−ion spectrum of the $[M + H]^+$ ion of d(A[8−5]C), and instead, the spectrum is dominated with a fragment ion of *m*/*z* 521 (Figure 2e). Collisional activation of the latter ion (MS^3), however, does lead to the formation of the desired product ion of *m*/*z* 245 (Figure S8). We managed to acquire the product−ion spectrum of the *m*/*z*-245 ion (MS^4, Figure 4i) and that of its product ion of *m*/*z* 202 (MS^5, Figure 4j). For the above multistage MS experiments to be successful, it is important to operate the ion-trap mass spectrometer under slightly different conditions from a typical MS/MS experiment (details shown in the Experimental Section).

Third, ^{1}H NMR and 2-D NOE spectra of the two products support our structure assignments. We found two singlet aromatic proton resonances in the ^{1}H NMR spectra of both products (Figure S5c,e). In addition, there is a strong correlation peak between the H6 proton of cytosine and the $H_{1'}$ proton of 2′-deoxycytidine in the 2-D NOE spectra of both cross-links (Figure 6c,e depict a portion of the 2-D NOE spectra),

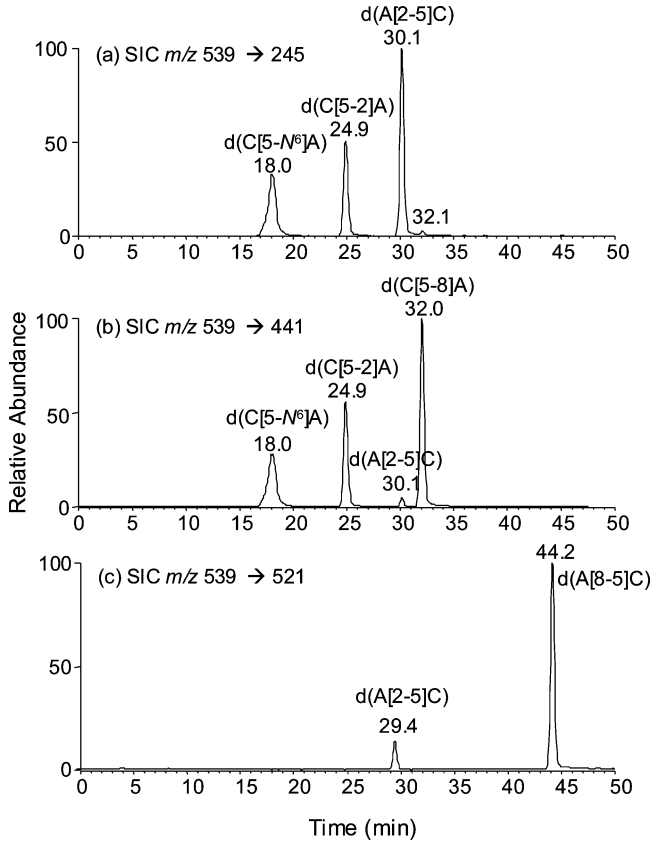


Figure 5. LC-MS/MS trace for the analysis of a solution containing all five C∧A and A∧C cross-link products (2 pmol each). The selected-ion chromatograms (SICs) for monitoring the transitions of *m*/*z* 539 → 245 (a), *m*/*z* 539 → 441 (b), and *m*/*z* 539 → 521 (c) are shown.

supporting that the C5 carbon atom of cytosine is involved in the cross-link bond formation.

Last, these two products have the same number of acidic hydrogens and very similar UV absorption spectra as the two corresponding CA cross-link products. Negative-ion ESI-MS of d(A[2−5]C) and d(A[8−5]C) gave *m*/*z* 543 for the $[M - D]^-$ ions (Figure S7c,e), which are consistent with the presence of six acidic hydrogens in the deprotonated ions of the two cross-link products. The $[M - D]^-$ ions were formed from the corresponding compounds after hydrogen/deuterium exchange. In addition, d(C[5−2]A) and d(A[2−5]C) showed almost identical absorption spectra in the wavelength range of 200−400 nm (Figure S9b). Likewise, the UV absorption spectra of d(C[5−8]A) and d(A[8−5]C) are very similar to each other (Figure S9c), but they are different from those of d(C[5−2]A) and d(A[2−5]C). d(C[5-*N*^{6}]A) has a distinct UV absorption spectrum from the other four cross-link products (Figure S9a). In addition, all five cross-link products exhibit significant absorbance at ∼315 nm. Taken together, the above results support that the fractions eluting at 27.4 and 41.8 min contain the d(A[2−5]C) and d(A[8−5]C), respectively.

We observed marked downfield displacement of the $H_{2'}$ proton of the 2′-deoxycytidine in d(C[5−2]A), d(C[5−8]A), and d(A[8−5]C) (Table S1 and Figure S5). Pyrimidine nucleosides with *N*-glycosidic bond in the syn conformation have been shown to result in the placement of C2 carbonyl moiety on top of the 2-deoxyribose ring and to lead to downfield displacement of the $H_{2'}$ proton of the 2-deoxyribose.[21,22] The downfield shift of the $H_{2'}$ proton may, therefore, indicate that the *N*-glycosidic

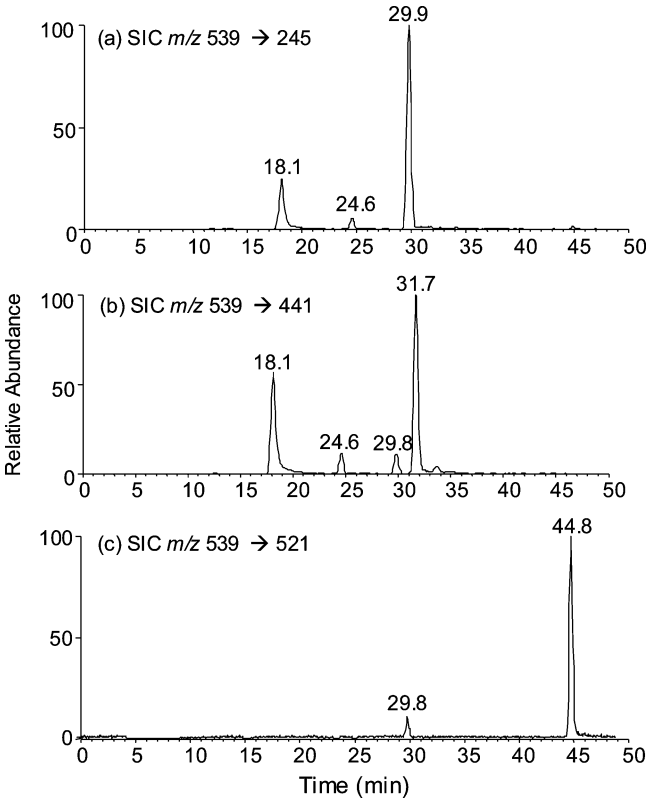


Figure 6. LC-MS/MS analysis of the enzymatic digestion products of 1 nmol duplex ODN d(ATGGCA^{Br}CACTAT)/d(ATAGTGTGCCAT) that was irradiated with UV under saturated O_2 conditions. The selected-ion chromatograms (SICs) for monitoring the transitions of *m*/*z* 539 → 245 (a), *m*/*z* 539 → 441 (b), and *m*/*z* 539 → 521 (c) are shown.

bond of the 2′-deoxycytidine moiety in these three products adopts a syn conformation. No downfield shift, however, was observed for the corresponding $H_{2'}$ proton of d(C[5-*N*^{6}]A) and d(A[2−5]C) (Table S1 and Figure S5).

Identification and Quantification of Cross-Link Products Formed from the Pyrex-Filtered UV Light Irradiation of 5-Bromocytosine-Containing Duplex DNA. With those five cross-link standards in hand, we next asked whether the UV irradiation of 5-bromocytosine-bearing duplex DNA can also result in the formation of any of the above cross-link products. To this end, we irradiated a double-stranded dodecamer harboring an A^{Br}CA sequence motif, d(ATGGCA^{Br}CACTAT)/d(ATAGTGTGCCAT); unfortunately, we were not able to separate the photoproducts from each other and from the complementary strand by HPLC. To determine which cross-link products are induced in duplex DNA, we degraded the irradiation mixture with four enzymes (i.e., nuclease P1, alkaline phosphatase, snake venom phosphodiesterase, and calf intestinal phosphodiesterase) and analyzed the digestion mixture by LC-MS/MS. These four enzymes were chosen because they have been successfully employed for the release from duplex DNA, dipyrimidine photoproducts,[23] and intrastrand cross-link lesions formed between G and T[9] or mC.[16]

It turned out that all five cross-link products can be induced under both aerobic and anaerobic conditions. We monitored the LC eluates by MS/MS where the product−ion spectra of the $[M + H]^+$ ions of the cross-link products were recorded. The SICs for the *m*/*z* 539 → 245, *m*/*z* 539 → 441, and *m*/*z* 539 → 521 transitions for the analysis of the five standard cross-link products are shown in Figure 5. In the SIC of the *m*/*z* 539 → 245 transition for the analysis of the digestion products of duplex DNA, which was irradiated with UV under saturated oxygen conditions, we found three major peaks at 18.1, 24.6, and 29.9 min (Figures 5a, 6a), supporting that d(C[5-*N*^{6}]A), d(C[5−2]A), and d(A[2−5]C) are formed. While the LC-MS/MS data were plotted as the SIC for the *m*/*z* 539 → 441 transition, we found peaks at similar retention times as the corresponding peaks for the standard d(C[5-*N*^{6}]A), d(C[5−2]A), d(A[2−5]C), and d(C[5−8]A) (18.1, 24.6, 29.8, and 31.7 min in Figure 6b). In addition, the SIC for the *m*/*z* 539 → 521 transition clearly supports the formation of d(A[2−5]C) and d(A[8−5]C) (Figures 5c, 6c). The product−ion spectra of the ion of *m*/*z* 539 for those fractions from the UV irradiation mixture are very similar to the corresponding spectra for the standards, which further support the formation of the cross-link products in duplex DNA (Figure S10). Likewise, LC-MS/MS results showed that UV irradiation under anaerobic conditions can also lead to the formation of the five cross-link products (Figures S11−12).

Table 2. The ϵ_{260} and ϵ_{315} Values of the Five A∧C or C∧A Cross-Link Products

	d(C[5-*N*^{6}]A)	d(C[5−2]A)	d(C[5−8]A)	d(A[2−5]C)	d(A[8−5]C)
ϵ_{260}	16 100	14 500	25 600	16 000	24 200
ϵ_{315}	6150	12 000	8720	11 500	9240

To quantify the cross-link products formed in duplex DNA by LC-MS/MS, we need to determine the concentrations of standard cross-link products. UV absorption measurement offers a convenient and sensitive method for such determination. For these measurements, we, however, need to first establish the extinction coefficients of the cross-link products. For this and other kinds of modified nucleosides or nucleotides, it is often difficult to obtain enough material for determining accurately their weight by a balance. To circumvent this, we came up with a novel method for determining the extinction coefficient of a substrate when its amount is enough for acquiring a ^{1}H NMR spectrum. In this context, the application of ^{1}H NMR for quantitative measurements has been recently reviewed.[24]

In this method, we mixed a certain amount of a cross-link product (for a diluted portion of which a UV absorption spectrum had been taken) with a known amount of thymidine, acquired a ^{1}H NMR spectrum for the mixture, and determined the amount/concentration of the cross-link product from the relative peak areas of an aromatic proton of the cross-link product and the H6 proton of thymidine (Figure S13). Afterward, the extinction coefficients at 260 and 315 nm for the cross-link product can be calculated by using Beer's Law with the measured absorbances at these two wavelengths and the concentration determined by the above NMR measurement. The extinction coefficients at 260 and 315 nm for these five cross-link products determined by this method are listed in Table 2. The ^{1}H NMR spectra were recorded on a 500-MHz instrument with a regular NMR probe and a Shigemi NMR tube (Shigemi

(21) George, A. L.; Hruska, F. E.; Ogilvie, K. K.; Holy, A. *Can. J. Chem.* **1978**, *56*, 1170−1176.
(22) Cadet, J.; Ducolomb, R.; Hruska, F. E. *Biochim. Biophys. Acta* **1979**, *563*, 206−215.
(23) Douki, T.; Court, M.; Sauvaigo, S.; Odin, F.; Cadet, J. *J. Biol. Chem.* **2000**, *275*, 11678−11685.
(24) Pauli, G. F.; Jaki, B. U.; Lankin, D. C. *J. Nat. Prod.* **2005**, *68*, 133−149.

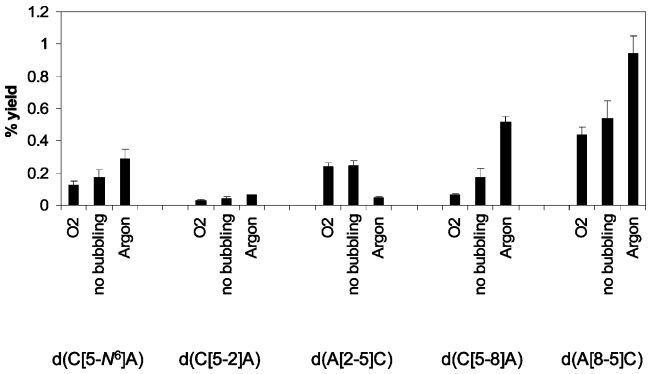


Figure 7. The yields for the formation of A∧C and C∧A cross-link products in 5-bromo-2′-deoxycytidine-containing duplex DNA upon Pyrex-filtered UV irradiation. (The yields refer to percent damage of the parent duplex and the values were averaged from three independent measurements. Error bars represent the standard deviations.)

Inc., Allison Park, PA), and we were able to determine the extinction coefficients if 50−100 nmol of a pure cross-link product is available. With an NMR microprobe, we anticipate to be able to determine the extinction coefficient for a substrate while a much smaller quantity of material is available.

After having established a robust method for determining the concentrations of the five standard cross-link products, we subjected a series of standard solutions, which contain all five authentic cross-link products, to LC-MS/MS experiment and analyzed, under the same experimental conditions, the enzymatic digestion products of duplex DNA. The calibration curves were linear (Figure S14). To quantify the amounts of cross-link products formed in duplex DNA by LC-MS/MS with standard d(C[5-N^6]A), d(C[5−2]A), d(C[5−8]A), d(A[2−5]C), and d(A[8−5]C), we also need to evaluate whether the enzymatic digestion procedure allows for the quantitative release of the cross-link products as dinucleoside monophosphates. To this end, we examined by LC-MS/MS whether some of the C∧A or A∧C cross-link products are released as trinucleoside diphosphates, that is, d(C∧AC) and d(A∧CA), or tetranucleoside triphosphates, that is, d(AC∧AC) and d(CA∧CA). It turned out that, under our digestion conditions, the C∧A and A∧C cross-link product can be liberated as dinucleoside monophosphates. Incomplete digestion products may constitute at most a total of 5% of the dinucleoside monophosphate products (data not shown).

The quantification results showed that the yields for different cross-link products are quite different and molecular oxygen exerts some effects on the formation of cross-link products (Figure 7). In this respect, we found that d(A[8−5]C) is more abundant than other types of cross-link products under all three irradiation conditions. Other than this product, significant amounts of other cross-link products are also produced. We measured the distances between atoms in cytosine and adenine that are involved in the formation of C∧A or A∧C in standard B-DNA geometry (Table 3). The relative yields for the formation of different cross-link products under aerobic and saturated O_2 conditions appear to correlate reasonably well with the distances between the relevant atoms for the formation of the cross-link bonds: the shorter the distance, the higher the yield. In this regard, the distance between the C5 carbon atom of cytosine and the C8 carbon atom of its vicinal 5′ adenine is the shortest, which is in accord with the highest yield for the formation of d(A[8−5]C). Similarly, the relevant distances for the formation of d(A[N^6-5]C) and d(C[5−2]A) are longer than other cross-link products, which is consistent with that these two products are either in low abundance, for d(C[5−2]A), or undetectable, for d(A[N^6-5]C). Similar effects of distance on the formation of other types of intrastrand cross-link products have been observed previously.[9,10,14]

In addition, the yields for the formation of d(C[5-N^6]A), d(C[5−2]A), d(C[5−8]A), and d(A[8−5]C) under anaerobic conditions were higher than those under aerobic conditions (Figure 7). This might be due to the consumption of some reactive intermediates by oxygen. Surprisingly, the yield for d(A[2−5]C) under anaerobic conditions was approximately 5 times lower than that under aerobic conditions. The exact reason for the decreased formation of d(A[2−5]C) under anaerobic conditions is unclear, though we speculate that different conformations for the *N*-glycosidic bond of the 2′-deoxycytidine moiety (vide supra) in the five cross-link products might make partial contribution to different effects of molecular oxygen on their formation.

Mechanisms for the Formation of Cross-Link Products from UV Irradiation. The foregoing discussion illustrated that the Pyrex-filtered UV light irradiation of duplex DNA containing an $A^{Br}CA$ sequence motif yields five isomeric intrastrand cross-link products formed between cytosine and its neighboring adenine. On the basis of previous investigations on the formation of protein−nucleic acid conjugate[25] and intrastrand cross-link product between cytosine and guanine from the photoactivation of 5-halopyrimidine,[14] we proposed tentative mechanisms for the formation of the cross-link products. In this context, the Pyrex-filtered UV irradiation can result in the transferring of an electron from adenine to its neighboring 5-bromocytosine. The resulting anion radical of 5-bromocytosine can then eliminate a bromide ion to give the cytosin-5-yl radical (Scheme 2). The latter radical can couple with the C2 or C8 carbon atom of the vicinal adenine, and the resulting products can deprotonate to offer cross-link products where the C5 carbon atom of cytosine is covalently linked to the C2 or C8 carbon atom of adenine (Scheme 2). Conceivably, the C−Br bond of 5-bromocytosine may also undergo homolytic cleavage[26] and the resulting C5-centered radical may attack the C2 or C8 carbon atom of its neighboring adenine to give the corresponding cross-link products.

The formation of C[5-N^6]A may follow a different mechanism. The cation radical of 2′-deoxyadenosine has a low pK_a (<1), and it can deprotonate to give an exocyclic nitrogen-centered radical,[27−30] which can couple with the C5 carbon atom of the neighboring 5′ cytosine. The resulting product can then eliminate a bromide ion to give C[5-N^6]A. Alternatively, it might be feasible for the anion radical of 5-bromocytosine to eliminate a bromide ion to give the C5-centered radical of cytosine, which can couple with the N^6 radical of adenine to give the C[5-N^6]A (Scheme 2).

(25) Meisenheimer, K. M.; Koch, T. H. *Crit. Rev. Biochem. Mol. Biol.* **1997**, *32*, 101−140.
(26) Chen, T.; Cook, G. P.; Koppisch, A. T.; Greenberg, M. M. *J. Am. Chem. Soc.* **2000**, *122*, 3861−3866.
(27) Steenken, S. *Chem. Rev.* **1989**, *89*, 503−520.
(28) Melvin, T.; Bothe, E.; Schulte-Frohlinde, D. *Photochem. Photobiol.* **1996**, *64*, 769−776.
(29) Wang, Y.; Liu, Z. *J. Org. Chem.* **2002**, *67*, 8507−8512.
(30) Wang, Y.; Liu, Z.; Dixon, C. *Biochem. Biophys. Res. Commun.* **2002**, *291*, 1252−1257.

Scheme 2

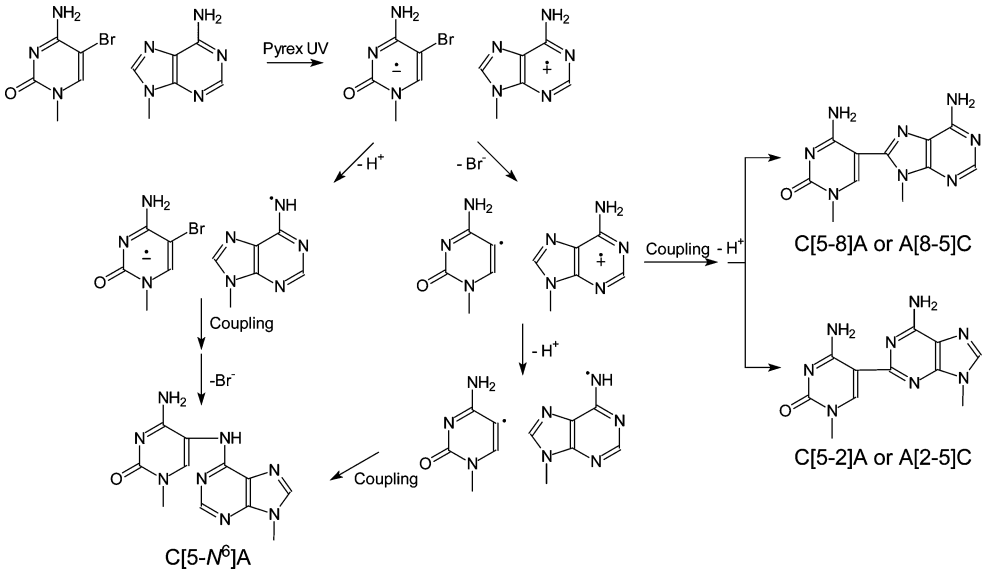


Table 3. Distances (in Å) between Relevant Atoms in Cytosine and Its Adjacent Adenine That Are Involved in the Formation of Intrastrand Cross-Link Products[a]

	5-CA-3	5-AC-3
C5 (Cyt)-N^6 (Ade)	4.52	5.44
C5 (Cyt)-C2 (Ade)	6.73	4.24
C5 (Cyt)-C8 (Ade)	5.15	3.86

[a] Distances were determined from a model duplex DNA built in Insight II with standard B-DNA geometry (axial rise = 3.38 Å, tilt angle = 0°, roll angle = 0°, twist angle = −36°).

Comparing to the results that we obtained previously for the duplex DNA containing a G^{Br}CG sequence motif, the total yield for the formation of all five cross-link products from the UV irradiation of the A^{Br}CA-bearing duplex DNA reported here is much lower (>10-fold) than the yield for the d(G[8−5]C) formed from the G^{Br}CG-containing duplex DNA.[14] In line with this, we found that approximately 65% of 5-bromo-2′-deoxycytidine remained undamaged in the irradiation mixture of A^{Br}CA-containing duplex DNA. These results are consistent with the fact that guanine has a much lower ionization potential than adenine[31,32] and, to some extent, support our proposed electron-transfer mechanism. Along this line, Saito and coworkers[33] observed a contrathermodynamic sequence selectivity (5′-deoxyadenosine > 5′-deoxyguanosine) for UV-irradiation-induced strand break in duplex DNA bearing a 5-bromo-2′-deoxyuridine. This might be due to preferential formation of intrastrand cross-link product while the flanking 5′ nucleobase is a guanine rather than an adenine. In this respect, our preliminary results showed that cross-link products can form between uracil and its neighboring 5′ guanine upon UV irradiation of 5-bromouracil-containing duplex DNA (data not shown).

Experimental Section

All chemicals and enzymes were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Calf intestinal alkaline phosphatase and nuclease P1 were obtained from New England Biolabs (Beverly, MA) and US Biological (Swampscott, MA), respectively. Reagents used for solid-phase DNA synthesis were purchased from Glen Research Inc. (Sterling, VA). Substrates containing a 5-bromocytosine were synthesized on a Beckman Oligo 1000S DNA synthesizer (Fullerton, CA) by using the commercially available 5-bromo-2′-deoxycytidine phosphoramidite building block (Glen Research Inc.) The nucleobase deprotection was carried out in 29% ammonia at room temperature for 48 h, and the deprotection at room temperature is necessary for minimizing the decomposition of the halogenated nucleoside. The identity and purity of d(^{Br}CA), d(A^{Br}CA), and d(ATGGCA^{Br}CACTAT) were confirmed by ESI-MS and MS/MS (Figure S1).

Photoirradiation and Enzymatic Digestion. Dinucleoside monophosphate d(^{Br}CA) or trinucleoside diphosphate d(A^{Br}CA) (150 nmol) was dissolved in 5 mL doubly distilled water and was transferred to a 13 × 100 mm (O. D. × length) Pyrex tube (Catalog No. 99447-13, Corning Inc., Corning, NY). The absorption spectrum of the Pyrex tube is shown in Figure S2. Photoirradiation was carried out for 20 min unless otherwise specified with a Hanovia 450-W medium-pressure mercury lamp. During irradiation, the Pyrex tube and lamp were immersed in an ice−water bath. The solution was exposed to air during irradiation and, after irradiation, the mixture was dried by using a Savant Speed-vac (Savant Instrument Inc., Holbrook, NY).

The dried residue of the d(^{Br}CA) irradiation mixture was redissolved in water and was employed for HPLC analysis. The irradiation products of d(A^{Br}CA) were, however, digested with nuclease P1 and alkaline phosphatase prior to HPLC separation. In this respect, 2 units of nuclease P1 was added to the irradiation products of 150 nmol d(A^{Br}CA), and the digestion was carried out at 37 °C for 3 h. The resulting solution was dried and redissolved in a 200-μL solution of 50-mM Tris-HCl (pH 8.9), to which solution was then added 50 units of alkaline phosphatase and the digestion was carried out at 37 °C for 3 h. The digestion mixture was extracted with an equal volume of chloroform to remove the enzymes, dried, redissolved in water, and subjected to HPLC separation.

For the irradiation of duplex oligodeoxynucleotide (ODN), d(ATGGCA^{Br}CACTAT) was annealed with its complementary strand d(ATAGTGTGCCAT) in a buffer containing 50 mM NaCl and 50 mM phosphate (pH 6.9). The resulting duplex mixture (50 nmol) was diluted to 2.5 mL with an aqueous solution bearing 1 mM NaCl and 1 mM phosphate buffer (pH 6.9), was transferred to the Pyrex tube, and was irradiated in an ice−water bath for 80 min. For irradiation under anaerobic and saturated oxygen conditions, the solution was bubbled with argon and O_2, respectively, for 30 min prior to irradiation, and the bubbling was continued during irradiation, whereas the solution

(31) Seidel, C. A. M.; Schulz, A.; Sauer, M. H. M. *J. Phys. Chem.* **1996**, *100*, 5541−5553.
(32) Steenken, S.; Jovanovic, S. V. *J. Am. Chem. Soc.* **1997**, *119*, 617−618.
(33) Sugiyama, H.; Tsutsumi, Y.; Saito, I. *J. Am. Chem. Soc.* **1990**, *112*, 6720−6721.

was exposed to air during irradiation for experiments under aerobic conditions. The irradiation mixture was concentrated to 0.2 mL by Speed-vac prior to enzymatic digestion.

Five units of nuclease P1, 0.005 unit of calf spleen phosphodiesterase, and a 10-μL buffer containing 300 mM sodium acetate (pH 5.0) and 10 mM zinc acetate were added to the sample, and the digestion was carried out at 37 °C for 6 h. To the digestion mixture were then added 200 units of calf intestinal phosphatase, 0.05 unit of snake venom phosphodiesterase, and 20 uL of 0.5 M Tris-HCl buffer (pH 8.9). The digestion was continued at 37 °C for 6 h. The digestion mixture was extracted with an equal volume of chloroform to remove the enzymes, and the aqueous layer was dried by using the Speed-vac and was redissolved in water for LC-MS/MS analysis. The amount of double-stranded 12-mer DNA injected was quantified by UV absorbance measurement.

HPLC. The HPLC separation was performed on a Surveyor system (ThermoFinnigan, San Jose, CA) with a photodiode array (PDA) detector, which was set at 260 nm for monitoring the effluents in most separations. The PDA detector was set at 315 nm to monitor the separation of the digestion mixture of d(A^{Br}CA). A 4.6 × 250 mm Apollo reverse-phase C18 column (5 μm in particle size and 300 Å in pore size, Alltech Associates Inc., Deerfield, IL) was used. A gradient of 5 min 0−3% acetonitrile followed by a 35 min 3−9% acetonitrile in 10 mM ammonium formate (pH 6.3) was employed to separate the irradiation mixture of d(^{Br}CA), and the flow rate was 0.8 mL/min. The HPLC fractions were then dried in the Speed-vac. For NMR analysis, the products were further desalted by using a reverse-phase C18 column (10 × 250 mm, 5 μm in particle size, and 300 Å in pore size, Varian, Walnut Creek, CA). After the sample was loaded, the column was washed with water for 10 min, and the sample was eluted from the column by 50% methanol in water.

NMR. All NMR spectra were acquired on a Varian Unity Inova 500 MHz instrument (Palo Alto, CA). The residual proton signal of the solvent serves as internal reference. Two-dimensional (2-D) NOESY (nuclear Overhauser effect spectroscopy) experiments were performed at 298 K with 600-ms mixing time, and the NOE spectra were obtained from 2 × 256 × 2048 data matrixes with 16−48 scans per t1 value.

Mass Spectrometry. Electrospray ionization-mass spectrometry (ESI-MS) and tandem MS (MS/MS) experiments were conducted on an LCQ Deca XP ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). An equal-volume solvent mixture of acetonitrile and water was used as the carrier and electrospray solvent, and a 2-μL aliquot of 5 μM sample solution was injected in each run. The spray voltages were 4.5 and 3.0 kV for experiments in the positive- and negative-ion modes, respectively. The mass width for precursor ion selection in MS/MS mode was 3 *m*/*z* units. Each spectrum was obtained by averaging approximately 50 scans, and the time for each scan was 0.1 s.

Multistage MS (MS^3, MS^4, and MS^5) experiments were carried out by infusing directly 10−20 μM sample solution to the LCQ Deca XP ion-trap mass spectrometer. The time for each scan was 1−3 s, and the signal from 5 to 10 min acquisition was averaged to give a spectrum. In addition, the "automated gain control" (AGC) feature was employed and the maximum number of ions was set to be 1×10^8 in the multistage MS experiments rather than 2×10^7 ions for a typical MS or MS/MS measurement.

An IonSpec HiResESI external ion source FTICR mass spectrometer (IonSpec Co., Lake Forest, CA) equipped with a 4.7 T unshielded superconducting magnet was used for high-resolution MS and MS/MS measurements. Dinucleoside monophosphate d(^{Br}CA) and 2′-deoxyguanosine were used as internal standards for mass calibration.

Exchangeable Proton Measurement. To measure the number of exchangeable protons, purified d(C∧A) or d(A∧C) cross-link products were incubated in D_2O for 24 h, dried by Speedvac, and redissolved in D_2O. The resulting solution was injected directly for ESI-MS and MS/MS analysis, and acetonitrile in D_2O (50/50, v/v) was used as electrospray solvent.

LC-MS/MS. A 0.5 × 150 mm Zorbax SB−C18 column (particle size, 5 μm, Agilent Technologies, Palo Alto, CA) was used for the separation of the enzymic digestion products from the UV irradiation of the 5-bromocytosine-containing duplex ODN, and a 100-min gradient of 0−35% acetonitrile in 20 mM ammonium acetate was employed. The flow rate was 6.0 μL/min and the effluent was coupled to the LCQ Deca XP ion-trap mass spectrometer. The mass spectrometer was set up to monitor the fragmentation of the $[M + H]^+$ ions of the cross-link products (*m*/*z* 539). Approximately 1 nmol irradiation mixture was injected in each run. To construct the calibration curves for quantification, we injected five standards, that is, d(C[5-*N*^{6}]A), d(C[5−2]A), d(C[5−8]A), d(A[2−5]C), and d(A[8−5]C) (0.010, 0.040, 0.20, 0.50, 1.0, 2.0, and 4.0 pmol each), to the column in separate LC-MS/MS runs with identical experimental setup as for the digestion mixture.

Determination of the Extinction Coefficients of d(C∧A) and d(A∧C). A certain amount of cross-link product, for a diluted portion of which a UV absorption spectrum had been taken, was mixed with 100 nmol of thymidine. A ^{1}H NMR spectrum of the mixture was then acquired, and the amount of the cross-link product was calculated from the ratio of the peak area of the H6 proton of dT over that of an aromatic proton of the cross-link product. The extinction coefficients at 260 and 315 nm were then calculated by using Beer's Law.

Conclusions

We showed for the first time that the Pyrex-filtered UV light irradiation of d(^{Br}CA) results in the formation of three isomeric intrastrand cross-link products, d(C[5-*N*^{6}]A), d(C[5−2]A), and d(C[5−8]A). Similar irradiation of d(A^{Br}CA) gave, other than the three cross-links formed at the CA site, A[2−5]C and A[8−5]C formed at the AC site. The structures of these five products in dinucleoside monophosphates were established by rigorous mass spectrometric and NMR spectroscopic characterizations. In this respect, multistage MS plays a very important role in the structure elucidation of d(A[2−5]C) and d(A[8−5]C). Similar irradiation of a duplex ODN containing an A^{Br}CA sequence motif, however, showed that all five cross-link products, that is, C[5-*N*^{6}]A, C[5−2]A, C[5−8]A, A[2−5]C, and A[8−5]C, can be induced. The multistage MS approach for the structure elucidation and the method that we reported here for the determination of the extinction coefficients should be applicable to other modified nucleosides and nucleotides. Moreover, the photochemistry reported here offers a synthetic route for the preparations of oxidative intrastrand cross-link lesion-containing substrates. In this respect, our preliminary studies showed that some of the cross-link products reported here can also be induced in duplex DNA by γ irradiation.

Acknowledgment. The authors thank the National Institute of Health for supporting this research (R01 CA96906).

Supporting Information Available: Spectroscopic characterizations of products and LC-MS/MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

JA0531677