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Anthraquinone-Sensitized Photooxidation of 5-Methylcytosine in DNA Leading to Piperidine-Induced Efficient Strand Cleavage

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Abstract: One-electron photooxidations of 5-methyl-2′-deoxycytidine (d^mC) and 5-trideuteriomethyl-2′-deoxycytidine ($[D_3]d^mC$) by sensitization with anthraquinone (AQ) derivatives were investigated. Photoirradiation of an aerated aqueous solution containing d^mC and anthraquinone 2-sulfonate (AQS) afforded 5-formyl-2′-deoxycytidine (d^fC) and 5-hydroxymethyl-2′-deoxycytidine ($d^{hm}C$) in good yield through an initial one-electron oxidation process. The deuterium isotope effect on the AQS-sensitized photooxidation of d^mC suggests that the rate-determining step in the photosensitized oxidation of d^mC involves internal transfer of the C5-hydrogen atom of a d^mC-tetroxide intermediate to produce d^fC and $d^{hm}C$. In the case of a 5-methylcytosine (^mC)-containing duplex DNA with an AQ chromophore that is incorporated into the backbone of the DNA strand so as to be immobilized at a specific position, ^mC underwent efficient direct one-electron oxidation by the photoexcited AQ, which resulted in an exclusive DNA strand cleavage at the target ^mC site upon hot piperidine treatment. In accordance with the suppression of the strand cleavage at 5-trideuterio-methylcytosine observed in a similar AQ photosensitization, it is suggested that deprotonation at the C5-methyl group of an intermediate ^mC radical cation may occur as a key elementary reaction in the photooxidative strand cleavage at the ^mC site. Incorporation of an AQ sensitizer into the interior of a strand of the duplex enhanced the one-electron photooxidation of ^mC, presumably because of an increased intersystem crossing efficiency that may lead to efficient piperidine-induced strand cleavage at an ^mC site in a DNA duplex.

Keywords: isotope effects · DNA cleavage · DNA methylation · nucleic acids · photooxidation

Introduction

Cytosine methylation, recognized to be a physiological modification of DNA in mammalian genomes, is believed to play a key function in the epigenetic regulation of genetic information and has been implicated as an important factor in the control of many cellular processes.[1,2] Consistent with such a crucial function, various types of human diseases are associated with misplaced cytosine methylation.[1,2] The identification and analysis of methylated cytosine sites in human genomes has become important for diagnosis of these diseases, and there is an emerging necessity to develop an effective method to assess the methylation status of specific cytosine residues in genomes. Among the various chemical methods for identifying and analyzing cytosine methylation explored so far,[3–7] the selective chemical modification of methylated cytosines is recognized as a useful reaction that can be applied to identify correctly the methylation site.[6] In particular, with consideration of the evidence that 5-methylcytosine (^mC) has a slightly lower oxidation potential than normal cytosine (C) and thymine, much effort has recently been made to get a discriminating method by which oxidation of a specific target ^mC base in DNA could be selectively induced for sequence-selective DNA methylation analysis.[6–7]

Photosensitized oxidation of DNA has been studied extensively in relation to positive-charge (hole) transfer through a DNA duplex.[8–11] In general, photosensitized one-electron oxidation of a DNA base forms the primary intermediate of a base radical cation, which can migrate through the π stacking between paired DNA bases and thereby produces strand cleavage at the lower-oxidation-potential sites, such as consecutive guanine (G) sites.[10] More recent studies have shown that one-electron oxidation of DNA without G bases also induces selective strand cleavage at thymine sites through transfer of a photosensitizer-injected hole through the DNA duplex.[11] These studies have been performed with a broad variety of organic photosensitizers that are covalently linked to oligonucleotides.[8–11] Our group has also reported that one-electron oxidation and site-selective

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strand cleavage at an ^{m}C base in DNA is caused upon photoirradiation of 2-methyl-1,4-naphthoquinone (NQ)-tethered oligodeoxynucleotides (ODNs).[12] In contrast to oxidative strand cleavage occurring at the target ^{m}C site with an oppositely located NQ photosensitizer, the corresponding normal C site, arranged instead of ^{m}C in the duplex, underwent no such NQ-photosensitized strand cleavage. In addition, competitive strand cleavage was virtually suppressed at a sequence of adjacent G bases, which have a lower oxidation potential than ^{m}C. This striking photooxidative reactivity has been applied to the fluorometric detection of a methylation site in a partial sequence of naturally occurring genomic DNA, by using a combination of ^{m}C-selective strand cleavage and an invasive cleavage reaction.[13] In this case, the photosensitized reaction that directly oxidizes ^{m}C, but not C, has potential for the convenient analysis of cytosine methylation at a specific site in a given DNA. To establish an optimized protocol of photosensitized oxidation and cleavage at the target ^{m}C site in DNA, which can discriminate between C and ^{m}C more efficiently and selectively, the mechanism of ^{m}C photooxidation and the structure–activity relationship of photosensitizers in strand cleavage at the ^{m}C site in DNA are still the subjects for further studies.

Anthraquinone (AQ) derivatives are among the most potent oxidizing photosensitizers; they undergo efficient intersystem crossing to form the long-lived triplet excited state (^{3}AQ*), which is known to induce hole injection followed by positive-charge transfer through DNA.[11,14] These photochemical properties prompted us to characterize the AQ-photosensitized oxidation of monomeric 5-methyl-2′-deoxycytidine (d^{m}C) and to compare it with the photooxidative strand cleavage reaction at ^{m}C in DNA. Upon photoirradiation of an aqueous solution in the presence of anthraquinone 2-sulfonate (AQS) and d^{m}C, efficient formation of 5-formyl-2′-deoxycytidine (d^{f}C) and 5-hydroxymethyl-2′-deoxycytidine (d^{hm}C) was observed. A similar AQS-sensitized photooxidation of 5-trideuteriomethyl-2′-deoxycytidine instead of d^{m}C suggested that the rate-determining step in the AQ-photosensitized oxidation of d^{m}C involves formation of a tetroxide intermediate and subsequent internal hydrogen transfer at the C5 position to produce the final oxidation products, d^{f}C and d^{hm}C. We also investigated AQ-photosensitized oxidative strand cleavage at an ^{m}C site in DNA with the AQ chromophore incorporated into the DNA backbone so as to be immobilized at a specific position. Thus, we obtained evidence that the AQ chromophore in the triplet excited state formed due to efficient intersystem crossing enhances oxidative DNA strand cleavage at the ^{m}C site. These findings may provide a new guide for the design of highly sensitive photochemical methods to identify and analyze methylated modification of cytosine in DNA.

Results and Discussion

Photooxidation of d^{m}C derivatives sensitized by an AQ chromophore: We initially investigated the photooxidation of d^{m}C sensitized by AQS. Aerobic solutions of d^{m}C (200 μM) and AQS (200 μM) in 2 mM sodium cacodylate buffer containing 20 mM NaCl at pH 7.0 were photoirradiated with 312 nm UV light. Figure 1 shows a representative time course of HPLC profiles observed in the AQS-photosensitized oxidation of d^{m}C. The column eluents were monitored by UV absorbance analysis at 260 nm. A major photooxidation product, d^{f}C, was produced, along with the degradation of d^{m}C, as identified from the characteristic HPLC peaks by reference to the respective authentic samples. In addition to the formation of d^{f}C, a minor product peak assigned to d^{hm}C was also observed. The yields of d^{f}C and d^{hm}C were quantified to be 22% and 14%, respectively, after 1 h of photoirradiation. We further characterized the photooxidation products d^{hm}C and d^{f}C by using mass spectroscopy with reference to the authentic samples. The LC–ESI mass analyses of the characteristic LC peaks indicated the formation of d^{hm}C ($[M-H]^{-}=256.1$) and d^{f}C ($[M-H]^{-}=254.1$), along with several minor products of the d^{m}C degradation (Figure S1 in the Supporting Information). The formation of the final oxidation products, such as d^{f}C and d^{hm}C, has been characterized previously by detailed product analysis of the photosensitized oxidation of d^{m}C with an NQ chromophore.[15–17] The one-electron reduction potential of AQS in the triplet excited state (^{3}AQS*; $E_{rdn}=2.30$ V vs. a normal hydrogen electrode (NHE))[18] has a much higher positive value relative to that of the ^{m}C base ($E_{ox}=1.73$ V vs. NHE).[19] It is therefore most likely that the triplet excited-state chromophore ^{3}AQS* may one-electron oxidize d^{m}C to produce the final products of d^{f}C and d^{hm}C, as in the case of the NQ-photosensitized oxidation of d^{m}C.

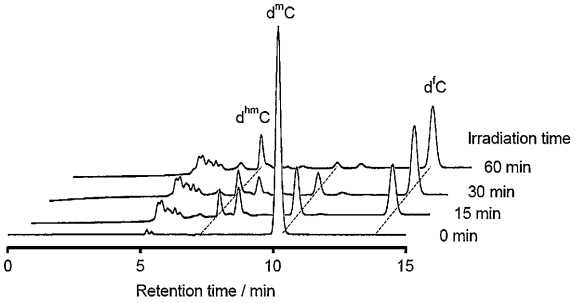


Figure 1. HPLC profiles of the photooxidation of d^{m}C (200 μM) sensitized by AQS (200 μM) in 10 mM sodium cacodylate buffer containing 100 mM NaCl (pH 7.0) upon 312 nm irradiation at 0 °C. The eluents were monitored with UV absorbance at 260 nm.

To obtain mechanistic insight into the AQS-photosensitized one-electron oxidation of d^{m}C, we also performed laser flash photolysis of d^{m}C in aqueous acetonitrile solution (acetonitrile/water, 9:1) containing AQS sensitizer. In accordance with the previous reports,[20] laser flash excitation at 355 nm of sensitizer AQS in deoxygenated aqueous acetonitrile solution without the substrate d^{m}C showed the build-up and subsequent exponential decay of a characteristic transient absorption band at around 380 nm that is assigned

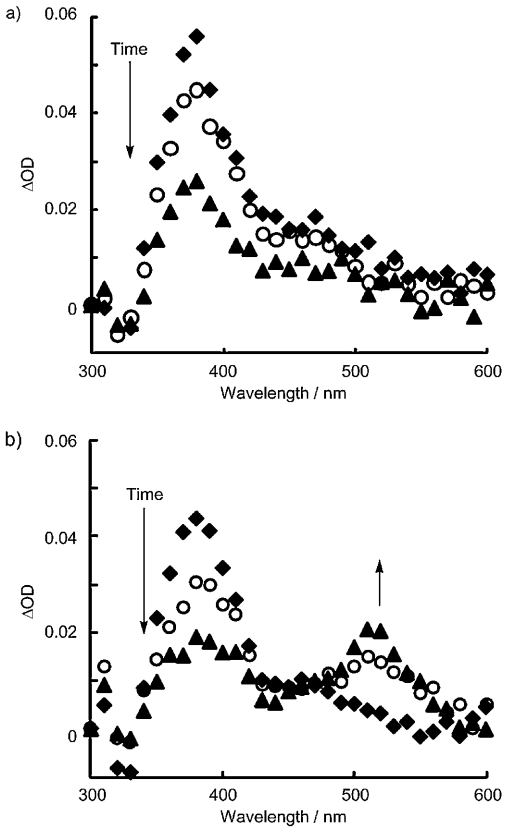


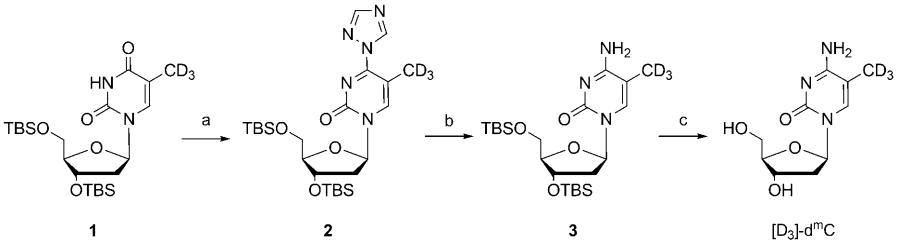
Figure 2. Transient absorption spectra of the intermediates as observed 0.3 (◆), 3 (○), and 20 μs (▲) after 355 nm laser flash photolysis of AQS (50 μM) in the a) absence or b) presence of d^mC (500 μM) in acetonitrile/water (9:1).

to the triplet excited intermediate ^{3}AQS* (Figure 2a and Figure S2 in the Supporting Information). Upon similar 355 nm laser flash excitation of AQS in the presence of d^mC in deoxygenated aqueous acetonitrile solution, an intense transient absorption of ^{3}AQS* was also observed 0.3 μs after the laser flash (Figure 2b). In contrast to the transient spectral behavior in the absence of d^mC (Figure 2a), the decay of ^{3}AQS* in the presence of d^mC accompanied the buildup of a new transient species with an absorption maximum at 520 nm (Figure 2b). By reference to the previous study,[21] this transient species is assigned to an AQS radical anion ($AQS^{\bullet-}$). The $AQS^{\bullet-}$ species formation gave a pseudo-first-order rate constant of $1.38\times10^6\ s^{-1}$, as characterized by the buildup of transient absorption at 520 nm, a value showing good agreement with the decay-rate constant ($1.37\times10^6\ s^{-1}$) of ^{3}AQS* at 380 nm (see Figure S2 in the Supporting Information). This provides strong evidence suggesting that the long-lived triplet excited species ^{3}AQS* could induce the one-electron oxidation of d^mC into a radical cation intermediate ($d^mC^{\bullet+}$), along with formation of the $AQS^{\bullet-}$ species.

An attempt was also made to identify the transient absorption of the possible counterpart radical cation intermediate $d^mC^{\bullet+}$, which may be generated by one-electron transfer from d^mC to ^{3}AQS*. Unfortunately, however, the absorption of the $d^mC^{\bullet+}$ species could not be detected directly in the laser flash photolysis experiment, presumably because of the smaller extinction coefficient of the $d^mC^{\bullet+}$ and because of overlap with the intense absorptions of ^{3}AQS* and $AQS^{\bullet-}$ in the wavelength range of 300–600 nm. In view of the evidence that the growth-time constant of the $AQS^{\bullet-}$ species was well matched with the decay-time constant of ^{3}AQS* in the presence of d^mC, it is reasonable to conclude that the photoinduced one-electron transfer from d^mC to ^{3}AQS* is a key reaction on the pathway leading to the final oxidation products d^fC and $d^{hm}C$ via a $d^mC^{\bullet+}$ intermediate.

A further attempt was made to evaluate the deuterium kinetic isotope effect at the C5-methyl group on the AQS-photosensitized oxidation of d^mC to produce d^fC. The C5-methyl hydrogen atoms of d^mC, as the target site of photosensitized oxidation, were fully deuterated to obtain 5-trideuteriomethyl-2′-deoxycytidine ($[D_3]d^mC$), as prepared from 3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-5-(methyl-d_3)-2′-deoxyuridine (**1**) as outlined in Scheme 1.[22]

An aerobic aqueous solution of $[D_3]d^mC$ (200 μM) containing AQS (200 μM) was irradiated with 312 nm UV light under the conditions described above and analyzed by HPLC. As shown in Figure 3, the yield of 5-deuterioformyl-2′-deoxycytidine ($[D_1]d^fC$) was suppressed in comparison



Scheme 1. Reagents and conditions: a) 1,2,4-Triazole, $POCl_3$, Et_3N, CH_3CN, 72%; b) 25% ammonium deuteroxide, CH_3CN, quant.; c) TBAF, AcOH, THF, 63%. TBAF: tetrabutylammonium fluoride; TBS: *tert*-butyldimethylsilyl; THF: tetrahydrofuran.

with that of normal d^fC, which indicated that deuterium-isotope substitution at the C5-methyl hydrogen atoms of d^mC significantly decreased the formation of d^fC. The kinetic isotope effects on the AQS-photosensitized oxidation of d^mC were quantified by the ratios of the initial rates, to give $k_H/k_D=1.3$ for the d^mC degradation and $k_H/k_D=5.3$ for the d^fC formation. As discussed previously,[15,16,23] the $d^mC^{\bullet+}$ intermediate generated by the one-electron oxidation undergoes deprotonation at the C5-methyl group followed by addition of molecular oxygen to produce the final oxidation products d^fC and $d^{hm}C$ via a stable tetroxide intermediate, according to the Russell mechanism[24] (Scheme 2). Recently, Prado

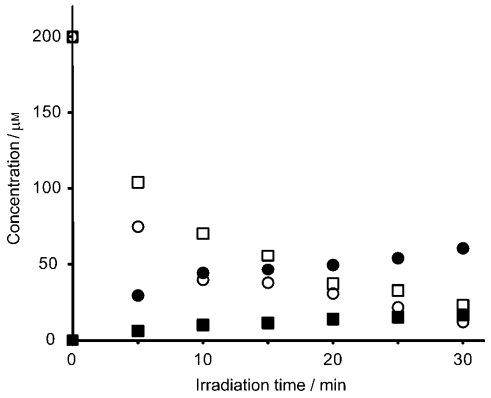
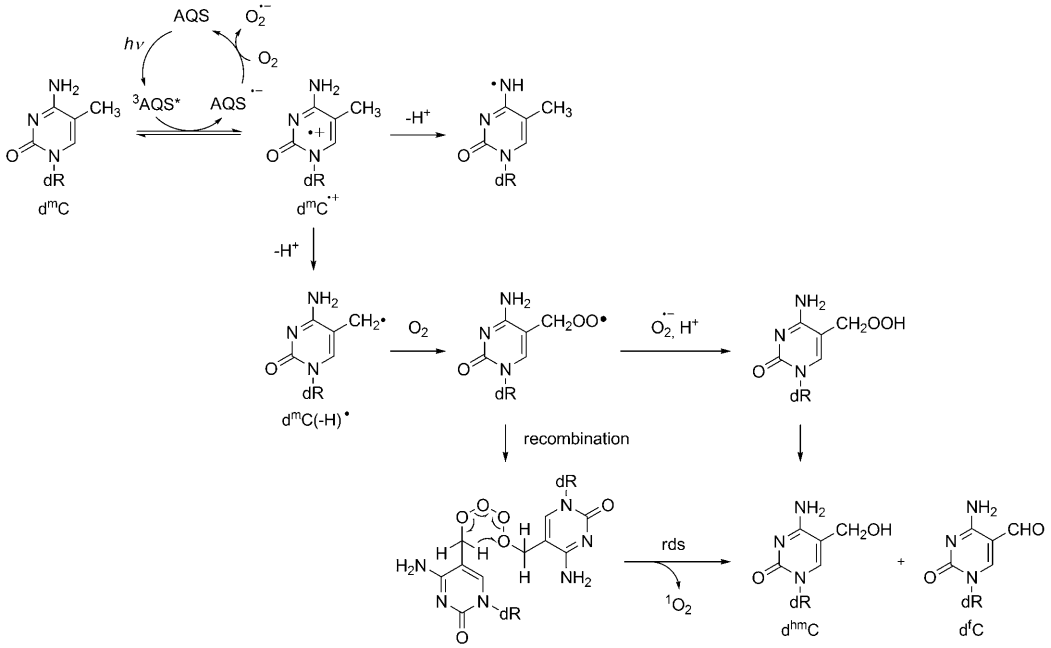


Figure 3. Deuterium isotope effects on the AQS-photosensitized oxidations of d^mC and $[D_3]d^mC$. Aerobic aqueous solutions of d^mC (200 μM) and $[D_3]d^mC$ (200 μM) were photoirradiated at 312 nm at 0 °C in the presence of AQS (200 μM) in 2 mM sodium cacodylate containing 20 mM NaCl (pH 7.0). Degradation of d^mC (○) and $[D_3]d^mC$ (□) and formation of d^fC (●) and deuterated d^fC (■) were monitored by HPLC and quantified by reference to authentic samples. Deuterium isotope effects (k_H/k_D) were estimated from the ratios of the initial rates of the d^mC and $[D_3]d^mC$ degradation and those of the d^fC and deuterated d^fC formation.

and co-workers suggested the formation of a transient tetroxide by recombination of 5-(hydroperoxymethyl)-2′-deoxyuridinyl radicals that underwent decomposition with the release of singlet oxygen ($^1O_2$) by the Russell mechanism.[25] This mechanism was partly supported by spectroscopic measurements indicating the bimolecular and monomolecular decays of $^1O_2$.[26] In this light, the observed kinetic isotope effects may be associated with internal transfer of an α-hydrogen atom in the d^mC-tetroxide intermediate and/or deprotonation of the $d^mC^{\bullet+}$ species into a C5-methyl carbon-centered radical. It has also been proposed that the primary intermediate, $d^mC^{\bullet+}$, may undergo rapid competitive deprotonation from the methyl group and the N4-exocyclic amino group, in what is called a proton-coupled electron-transfer process.[16] Although the d^mC-tetroxide intermediate has not yet been identified, the rate-determining step in the photosensitizied oxidation of d^mC is presumed to involve the formation of a tetroxide intermediate followed by C5-hydrogen transfer to produce d^fC and $d^{hm}C$. In addition, the yields of d^fC and $d^{hm}C$ as indicated in Figure 1 may be partly accounted for by the occurrence of further oxidation from $d^{hm}C$ to d^fC during AQS-photosensitization.[23]

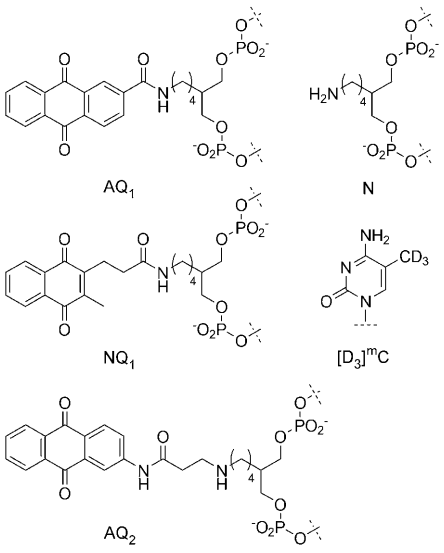
In a separate experiment, we also examined similar AQS-photosensitized degradation of d^mC and $[D_3]d^mC$ in D_2O solution, under which conditions the N4-hydrogen atoms of d^mC and $[D_3]d^mC$ are fully deuterated (Figure S3 in the Supporting Information). If the competitive deprotonation from the N4-exocyclic amino group of the $d^mC^{\bullet+}$ species is involved in this reaction mechanism,[16] the rate of d^mC degradation as a result of the deprotonation from the C5-methyl group of the $d^mC^{\bullet+}$ species is expected to be enhanced upon replacement of the solvent H_2O by D_2O. The inverse solvent isotope effects on the initial rates of the d^mC and $[D_3]d^mC$ degradations were evaluated as $k_{H_2O}/k_{D_2O}=0.90$ and 0.54, respectively. Although the difference in the inverse isotope effect between d^mC and $[D_3]d^mC$ is not fully understood at present, it is presumable that the enhanced degradation in D_2O solution is attributable to the occurrence of competitive deprotonation from the exocyclic amino group of the $d^mC^{\bullet+}$ or $[D_3]d^mC^{\bullet+}$ species.

Photooxidation and strand cleavage at ^{m}C derivatives in DNA sensitized by AQ-tethered ODNs: The photosensitized one-electron oxidation and strand cleavage at ^{m}C in



Scheme 2. A plausible reaction mechanism for the AQ-photosensitized oxidation of d^mC.

DNA was characterized by using a modified ODN with an AQ sensitizer in the interior of a DNA strand (ODN 1 (AQ_1)). To incorporate an AQ sensitizer into the DNA backbone, ODN 1(AQ_1) was prepared by coupling AQ succinimidyl ester[27] with ODN (N), which possessed an amino linker in the middle of sequential bases.[28] In this study, we used a partial sequence of the human p53 gene corresponding to codons 280–285 of exon 8 and targeted the ^{m}C at codon 282.[29] The sequences and structures of ODNs used in this study are summarized in Scheme 3.



Scheme 3. Sequences and structures of ODNs used in this study.

Photoirradiation at $\lambda_{ex}=312$ nm in air of a duplex of ODN 1(AQ_1) with ^{32}P-5′-end-labeled ODN 2(C) or ODN 2(^{m}C) was performed in 10 mM sodium cacodylate buffer (pH 7.0) containing 100 mM NaCl at 20 °C.[30] The photoirradiated solution was treated with hot piperidine and then analyzed by polyacrylamide gel electrophoresis (PAGE). As shown in Figure 4, specific strand cleavage occurred at the target ^{m}C site in the ODN 1(AQ_1)/ODN 2(^{m}C) duplex after photoirradiation, whereas only a background level of strand cleavage was observed at the corresponding unmethylated C site for the ODN 1(AQ_1)/ODN 2(C) duplex. When the hot piperidine treatment was not employed after AQ-sensitized photooxidation, the strand cleavage bands were substantially suppressed. In a control photoreaction of a similar duplex comprising ODN 1(G) and ^{32}P-5′-end-labeled ODN 2(^{m}C) with a complementary G base instead of the AQ sensitizer, no strand cleavage band was observed at the target ^{m}C site. This suggests that the present photoirradiation conditions could not produce possible alkali-labile dimeric photoproducts, such as pyrimidine (6-4)

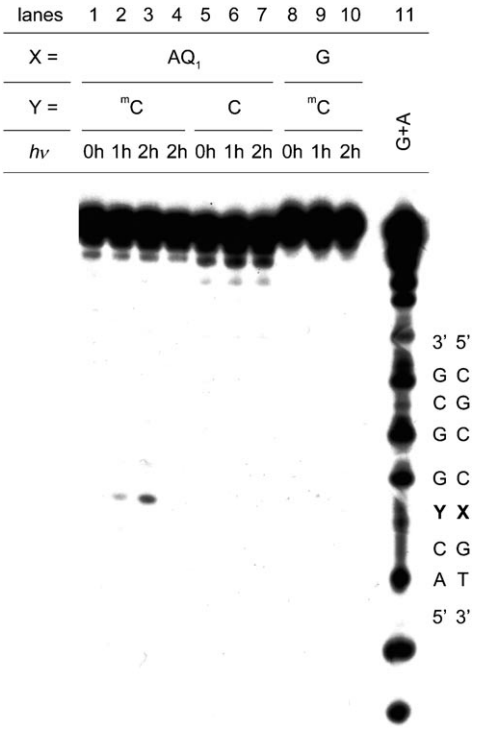


Figure 4. Photosensitized oxidation of AQ-tethered ODNs. A representative autoradiogram of denaturing gel electrophoresis for ODN 1(X) and ^{32}P-5′-end-labeled ODN 2(Y) upon 312 nm photoirradiation for 0 h (lanes 1, 5, and 8), 1 h (lanes 2, 6, and 9), and 2 h (lanes 3, 4, 7, and 10) in 10 mM sodium cacodylate buffer containing 100 mM NaCl (pH 7.0) at 20 °C. After treatment with hot piperidine (90 °C, 20 min), the samples were electrophoresed through denaturing 20% polyacrylamide/7 M urea: Lanes 1–4: ODN 1(AQ_1)/ODN 2(^{m}C) duplex; lanes 5–7: ODN 1(AQ_1)/ODN 2(C) duplex; lanes 8–10: ODN 1(G)/ODN 2(^{m}C) duplex; lane 11: Maxam–Gilbert G+A sequencing lanes. The sample in lane 4 was not treated with hot piperidine.

photoproducts and their Dewar valence isomers.[31] The previous ESI mass experiments showed that ^{m}C residue in DNA is converted into 5-formylcytosine by photosensitization with ODNs possessing an NQ chromophore at the 5′-end.[12a] In addition, 5-formyluracil, one of the thymidine oxidation products, has been shown to form alkali-labile lesions in DNA.[11,32] In this context, the present results clearly indicate that photoexcitation of the AQ sensitizer in the interior of the strand can one-electron oxidize ^{m}C to produce potentially alkali-labile oxidation products such as 5-formylcytosine and can thus result in strand cleavage at the ^{m}C site. In contrast, substantially less strand cleavage at the unmethylated C site is attributable to the less efficient AQ-photosensitized one-electron oxidation of C into the corresponding radical cation intermediate than that of ^{m}C, probably because of the considerably smaller free-energy change of charge separation ($-\Delta G_{CS}=0.02$ eV) between C and AQ in the triplet excited state (^{3}AQ*).[33,34]

In conjunction with the intense cleavage at the target ^{m}C site, possible strand cleavages at the adjacent G sites, which are one-electron oxidized the most readily in the DNA duplex,[10] were negligible in the present AQ-sensitized pho-

tooxidation. Such negligible strand cleavage at the adjacent guanine bases is consistent with the NQ-sensitized photooxidation of ^{m}C in DNA.[12] Thus, the secondary intermediate, the guanine radical cation ($G^{\bullet+}$), formed through hole transfer from the primary intermediate $^{m}C^{\bullet+}$ mostly undergoes rapid charge recombination with the neighboring $AQ^{\bullet-}$ to suppress net strand cleavage at adjacent G sites.[14c] In contrast to the behavior of the more stable radical cation $G^{\bullet+}$, the charge recombination of $^{m}C^{\bullet+}$ with $AQ^{\bullet-}$ may be inefficient, probably because the $^{m}C^{\bullet+}$ species undergoes kinetically more favorable deprotonation at the C5-methyl group.[35] As a consequence, selective strand cleavage proceeds almost exclusively at the target ^{m}C site without unfavorable cleavage at the adjacent G sites upon AQ-sensitized photooxidation. In view of the evidence that ^{m}C is usually generated in CpG-rich regions of DNA,[1,2,29] the suppression of a competitive strand cleavage at adjacent G doublets should be a strong advantage for distinct detection of the ^{m}C site on a sequencing gel.

To gain further insight into the suppression of photooxidative strand cleavage at G sites, the photoreactivity of a ODN 1(G)/^{32}P-labeled ODN 2(^{m}C) duplex was examined in the presence of exogenous AQS sensitizer instead of ODN 1 (AQ_1). Efficient strand cleavage at the 5′-G site of a G doublet was observed after 2 h photoirradiation followed by treatment with hot piperidine (Figure S4 in the Supporting Information), which indicated that a positive charge injected on a DNA base from the exogenous ^{3}AQS* migrated through the DNA duplex until it was trapped by a G doublet. In this case, longer separation of the positive and negative charges occurs between the exogenous AQS chromophore and the G doublet: the charge recombination process between the $G^{\bullet+}$ and exogenous $AQS^{\bullet-}$ species may become more difficult and, therefore, preferential strand cleavage at the G site can proceed. Thus, arrangement of the AQ sensitizer in the vicinity of the target ^{m}C residue would be essential for selective strand cleavage at ^{m}C residues without unfavorable cleavage at adjacent G residues.

The thermal stability of duplexes was evaluated by monitoring the melting temperatures (T_m) in 10 mM sodium cacodylate buffer (pH 7.0) containing 100 mM NaCl (Figure S5 in the Supporting Information). The lower T_m value of the ODN 1(N)/ODN 2(^{m}C) duplex ($T_m = 55.1$ °C) relative to a reference duplex of ODN 1(G)/ODN 2(^{m}C) ($T_m = 59.6$ °C) suggests that the introduction of an amino linker moiety into the DNA backbone reduces the thermal stability of the duplex, probably because of the partial absence of hydrogen bonding. In contrast, slight stabilization was observed for the AQ-modified duplex of ODN 1(AQ_1)/ODN 2(^{m}C) ($T_m =$ 62.7 °C). These results suggest that the observed thermal stabilization is responsible for the intercalation of the AQ chromophore between flanking base pairs.

One-electron transfer from the intercalated AQ derivatives with nπ* configuration can also occur to form a base radical cation and the $AQ^{\bullet-}$ species, due to orbital overlap between nucleobases and the AQ chromophore; this leads to oxidative strand cleavage upon treatment with piperidine.[36] To confirm whether the one-electron transfer process may be involved in the selective strand cleavage at the ^{m}C site, we conducted photoreactions of duplexes with a different structure of AQ analogue. Breslin and Schuster reported that a 2-aminoanthraquinone chromophore (AQ_2) has the lowest ππ* triplet excited state and can react only by electron transfer.[37] A duplex of ODN 1(AQ_2), bearing AQ_2 instead of AQ_1, with its complementary ODN 2(^{m}C) was prepared and photoirradiated at 312 nm in a similar manner. As illustrated in Figure 5, we could detect specific

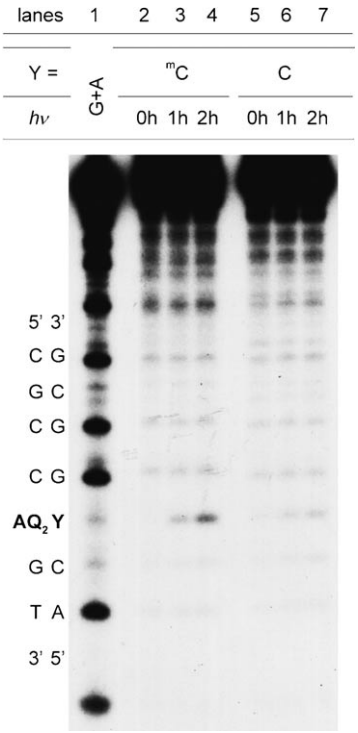


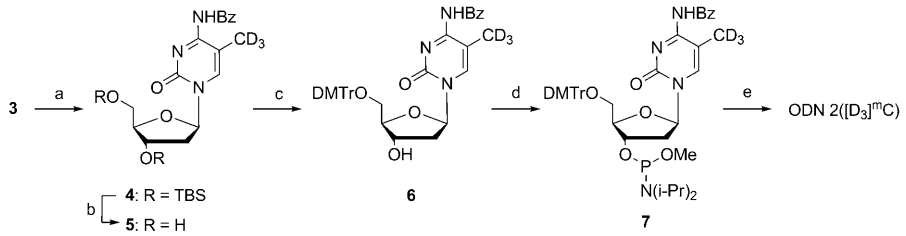
Figure 5. A representative autoradiogram of denaturing gel electrophoresis for ODN 1(AQ_2) and ODN 2(Y) upon 312 nm photoirradiation for 0 h (lanes 2 and 5), 1 h (lanes 3 and 6), and 2 h (lanes 4 and 7) in 10 mM sodium cacodylate buffer containing 100 mM NaCl (pH 7.0) at 20 °C. After treatment with hot piperidine (90 °C, 20 min), the samples were electrophoresed through denaturing 20% polyacrylamide/7 M urea: lanes 2–4: ODN 1(AQ_2)/ODN 2(^{m}C) duplex; lanes 5–7: ODN 1(AQ_2)/ODN 2(C) duplex; lane 1: Maxam–Gilbert G+A sequencing lanes.

strand cleavage at the ^{m}C site in the ODN 1(AQ_2)/ODN 2(^{m}C) duplex after 2 h photoirradiation, whereas only a background level of cleavage at the corresponding C residue was observed for the ODN 1(AQ_2)/ODN 2(C) duplex. In addition, strand cleavages at adjacent G sites were substantially suppressed. These results strongly suggest that the $^{m}C^{\bullet+}$ species formed by eventual one-electron transfer to $^{3}AQ_2$* is attributable for the photosensitized oxidative strand cleavage at ^{m}C in the ODN 1(AQ_2)/ODN 2(^{m}C) duplex. It is thus concluded that selective strand cleavage at the ^{m}C site is likely to occur through one-electron transfer oxidation of ^{m}C by intercalated ^{3}AQ* into $^{m}C^{\bullet+}$ and $AQ^{\bullet-}$

species, but not through direct hydrogen atom abstraction from ^{m}C by ^{3}AQ*.

In light of the kinetic isotope effects observed in the AQS-photosensitized oxidation of d^{m}C in aqueous solution, we further examined the AQ-photosensitized oxidation reactivity of the DNA duplex containing 5-trideuteriomethylcytosine ([D_3]^{m}C) instead of ^{m}C at the target site. The synthesis of the ODN containing [D_3]^{m}C is outlined in Scheme 4. *N*-Benzoyl-5-([D_3]methyl)-2′-deoxycytidine (**5**) was prepared from **3** by standard benzamide protection of the exocyclic amine and subsequent desilylation of the hydroxy groups. The resulting N4-protected 2′-deoxycytidine analogue **5** was converted into 4,4′-dimethoxytrityl derivative **6** and then converted into the corresponding phosphoramidite derivative **7**; this was followed by incorporation into DNA by using a DNA synthesizer and conventional β-cyanophosphoramidite chemistry. Photoirradiation at 312 nm of the ODN 1(AQ$_1$)/ODN 2([D_3]^{m}C) duplex and subsequent piperidine treatment induced selective strand cleavage at the [D_3]^{m}C site, as in a similar photooxidation of ^{m}C in the ODN 1(AQ$_1$)/ODN 2(^{m}C) duplex (Figure S6 in the Supporting Information). Figure 6 shows the quantitative analysis of AQ-photosensitized strand cleavages at the ^{m}C and [D_3]^{m}C sites in the ODN 1(AQ$_1$)/ODN 2(^{m}C) duplex and the ODN 1(AQ$_1$)/ODN 2([D_3]^{m}C) duplex, respectively. The efficiency of strand cleavage at [D_3]^{m}C was slightly suppressed in comparison with that at ^{m}C. Whereas the kinetic isotope effect on the net strand cleavage was small, it is presumable that deprotonation of the ^{m}C$^{\bullet+}$ intermediate into a C5-methyl carbon-centered radical is involved in the formation of alkali-labile oxidation products, such as 5-(hydroxymethyl)cytosine and/or 5-formylcytosine,[12,15] which lead to selective DNA strand cleavage at the ^{m}C site. The occurrence of bimolecular reaction processes such as internal α-hydrogen transfer in an ^{m}C-tetroxide intermediate, as in the case of the AQS-photosensitized oxidation of d^{m}C, may be ruled out in the AQ-photosensitized oxidation of the ^{m}C site in the restricted structure of the DNA duplex. Similar reaction pathways were recently proposed to explain strand cleavage at consecutive T residues during hole transfer in DNA.[11]

To compare the one-electron oxidizing abilities between photoirradiated AQ$_1$ and NQ$_1$ sensitizers as tethered to ODN, a duplex of ODN 1(NQ$_1$), bearing NQ instead of AQ, with the complementary ODN 2(^{m}C) containing ^{m}C was also prepared and photoirradiated at 312 nm, by reference to the AQ$_1$ photosensitization. As shown in Figure 7, whereas an intense spot corresponding to strand cleavage at the ^{m}C site was clearly observed in the AQ-sensitized photooxidation, a smaller amount of strand cleavage occurred at the ^{m}C site in the NQ-sensitized photooxidation: the apparent efficiencies (relative values) of strand cleavage at the ^{m}C site were 12% in the ODN(AQ$_1$) duplex and 4% in the ODN(NQ$_1$) duplex. More quantitatively, by using a phenylglyoxylic acid chemical actinometer,[38] the relative quantum yields of AQ- and NQ-photosensitized strand cleavage at the ^{m}C site were estimated to be 0.9×10^{-5} and 0.6×10^{-5}, respectively, which indicated that the AQ photosensitization shows 1.5-times higher efficiency in the one-electron oxidation of ^{m}C than the NQ photosensitization. The relatively more efficient strand cleavage at the ^{m}C site in the ODN 1(AQ$_1$)/ODN 2(^{m}C) duplex is attributable to a considerably larger quantum yield of intersystem crossing for AQ ($\Phi_{isc}=0.9$)[39] relative to NQ ($\Phi_{isc}=0.66$).[40] Recently, Lewis, Wasielewski and co-workers reported that one-electron oxidation of DNA bases by singlet excited AQ (^{1}AQ*) and charge recombination of the resulting singlet radical ion pair is more efficient than the for-



Scheme 4. Reagents and conditions: a) Benzoyl chloride, 4-(dimethylamino)pyridine, CH_2Cl_2, 94%; b) triethylamine trihydrogenfluoride, THF, quant.; c) 4,4′-dimethoxytritylchroride, pyridine, 92%; d) *N*,*N*-diisopropylmethyl phosphonamidic chloride, diisopropylethylamine, CH_3CN, quant.; e) automated DNA synthesis. Bz: benzoyl; DMTr: 4,4′-dimethoxytrityl.

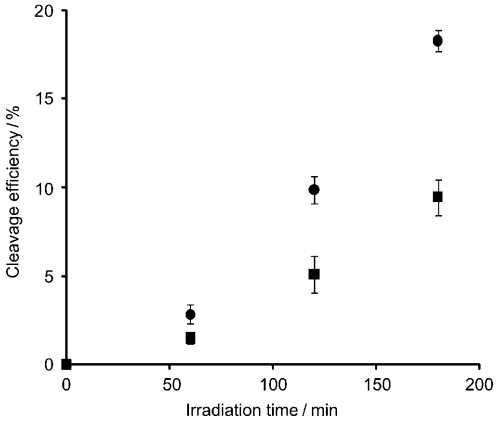


Figure 6. Photooxidative strand cleavages at the ^{m}C and [D_3]^{m}C sites as determined by polyacrylamide gel electrophoresis. The ODN 1(AQ$_1$)/ODN 2(Y) duplexes (0.5 μM) were photoirradiated (312 nm, 0–3 h) in 10 mM sodium cacodylate buffer containing 100 mM NaCl (pH 7.0) at 0°C. After treatment with hot piperidine (90°C, 20 min), the samples were electrophoresed through denaturing 20% polyacrylamide/7 M urea. The cleavage efficiencies were calculated from the intensities of the respective strand cleavages at the ^{m}C (●) and [D_3]^{m}C (■) sites relative to the total intensities. Each error bar represents the standard deviation calculated from three experimental results.

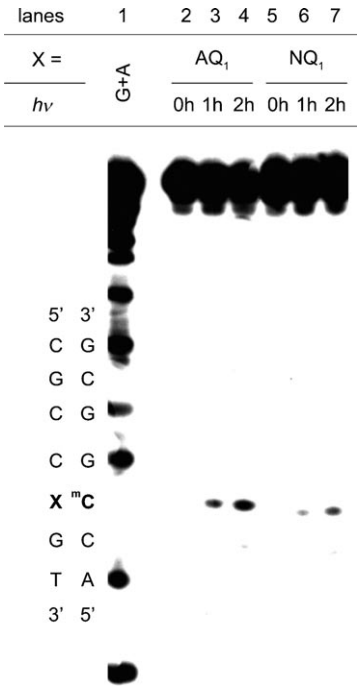


Figure 7. Comparison of AQ- and NQ-photosensitized oxidations of DNA duplexes bearing ^{m}C. A representative autoradiogram of denaturing gel electrophoresis for ^{32}P-5′-end-labeled AQ- or NQ-tethered duplexes possessing an ^{m}C site. The ODN 1(X)/ODN 2(^{m}C) duplexes (0.5 μM) were photoirradiated (312 nm, 0–2 h) in 10 mM sodium cacodylate buffer containing 100 mM NaCl (pH 7.0) at 20 °C. After treatment with hot piperidine (90 °C, 20 min), the samples were electrophoresed through denaturing 20 % polyacrylamide/7 M urea: lane 1: Maxam–Gilbert G+A sequencing lanes; lanes 2–4: ODN 1(AQ_1)/ODN 2(^{m}C) duplex; lanes 5–7: ODN 1(NQ_1)/ODN 2(^{m}C) duplex.

mation of the long-lived triplet charge separated state that is responsible for the oxidative DNA strand cleavage.[41] In this light, the considerably low quantum yield of strand cleavage at the ^{m}C base in the present AQ-photosensitized oxidation may be accounted for by the concomitant occurrence of fast singlet charge separation and recombination processes between ^{1}AQ* and ^{m}C. Another possibility cannot be ruled out that hole transfer from the primary radical cation intermediate, $^{m}C^{\bullet+}$, to the neighboring G sites and/or back electron transfer from $AQ^{\bullet-}$ to $^{m}C^{\bullet+}$ may occur to lower the efficiency of strand cleavage at the ^{m}C site.[9g,14c]

Conclusion

In this study, we have demonstrated that photosensitized one-electron oxidation of d^{m}C by ^{3}AQS* affords the characteristic final oxidation products of d^{f}C and d^{hm}C. The deuterium isotope effects on the AQS-photosensitized one-electron oxidation of [D_3]d^{m}C suggest that internal α-hydrogen transfer in an ^{m}C-tetroxide intermediate is a possible rate-determining step to produce d^{f}C and d^{hm}C. Photooxidative strand cleavage at an ^{m}C site in a DNA duplex was also investigated by using a modified ODN that covalently incorporated an AQ sensitizer into the backbone of the DNA strand. The AQ photosensitization of the DNA duplex induced efficient one-electron oxidation of ^{m}C and thus resulted in exclusive strand cleavage at the target ^{m}C site upon treatment with hot piperidine. In contrast to the efficient strand cleavage at the ^{m}C site, the adjacent GG site, which would normally be a hotspot for photosensitized oxidation, underwent a smaller amount of such oxidative strand cleavage, presumably due to the occurrence of rapid charge recombination processes between the $G^{\bullet+}$ and $AQ^{\bullet-}$ species. Evidence was also obtained that similar AQ-photosensitized strand cleavage at the [D_3]^{m}C site was slightly suppressed relative to cleavage at the ^{m}C site, which suggests that the AQ-photosensitized strand cleavage at the ^{m}C site potentially involves a pathway of deprotonation at the C5-methyl group on the $^{m}C^{\bullet+}$ species into a methyl carbon-centered radical intermediate. The more efficient AQ-sensitized photooxidative strand cleavage at the ^{m}C site is probably attributable to an enhanced intersystem crossing of photoexcited AQ to generate a larger yield of the primary radical cation intermediate, $^{m}C^{\bullet+}$. The results presented herein may provide a guide for the molecular design of highly sensitive photochemical methods for the identification and analysis of methylated cytosine in DNA.

Experimental Section

General methods: 9,10-Anthraquinone 2-sulfonate (AQS) was purchased from Tokyo Chemical Industry and was used without further purification. 5-Methyl-2′-deoxycytidine (d^{m}C) was obtained commercially from MP Biomedicals. 5-Formyl-2′-deoxycytidine (d^{f}C)[42] and 5-(hydroxymethyl)-2′-deoxycytidine (d^{hm}C)[43] were prepared as described previously. Anthraquinone 2-carboxylic acid *N*-hydroxysuccinimidyl ester (AQ-NHS) and 2-(3-bromopropionamido)anthraquinone were prepared following reported methods.[27,37] The reagents for the DNA synthesizer were purchased from Glen Research. Oligodeoxynucleotides (ODNs) containing an amino linker were synthesized on an Applied Biosystems Model 3400 DNA/RNA synthesizer with BD Uni-Link AminoModifier (BD Biosicences Clontech). NQ-tethered ODNs were prepared by conjugation of 3-(*N*-hydroxysuccinimidylethyl)-NQ with ODNs containing an amino linker.[12a,16] All complementary ODNs were purchased from Invitrogen. Mass spectrometry analyses of ODNs were performed with a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Perseptive Voyager Elite, acceleration voltage 21 kV, negative mode) with 2′,3′,4′-trihydroxyacetophenone as the matrix, by using T_8 ([*M*−H]$^-$ 2370.61), T_{17} ([*M*−H]$^-$ 5108.37), and T_{27} ([*M*−H]$^-$ 8150.33) as the internal standards. Calf intestinal alkaline phosphatase (AP), nuclease P1 (P1), and phosphodiesterase I were purchased from PROMEGA, YAMASA, and ICN, respectively. [γ-^{32}P]Adenosine triphosphate (ATP; 6000 Ci mmol^{-1}) and T4 polynucleotide kinase (10 units μL^{-1}) were obtained from Perkin–Elmer and Nippon Gene, respectively. All aqueous solutions were prepared by using purified water (YAMATO, WR600A).

4-(*N*-1-Triazoyl)-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-5-([D_3]methyl)-2′-deoxyuridine (2): 3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-5-([D_3]methyl)-2′-deoxyuridine (**1**) was prepared as reported previously.[44] 1,2,4-Triazole (848 mg, 12.3 mmol) was suspended in acetonitrile (20 mL) cooled to 0 °C, to which $POCl_3$ (0.27 mL, 2.96 mmol) was slowly added. Triethylamine was then added dropwise and the suspension was stirred for 20 min. Compound **1** (290 mg, 0.6 mmol) was dissolved in acetonitrile (8 mL) and added to the solution, and the solution was continuously stirred overnight. The reaction was quenched with water and extracted with ethyl

acetate. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by column chromatography (SiO_2, 30% ethyl acetate/hexane) to give **2** (229 mg 72%) as a colorless oil: ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 9.26 (s, 1H; azole), 8.22 (s, 1H; azole), 8.08 (s, 1H; C6−H), 6.27 (t, 1H, J = 6.2 Hz; C1′−H), 4.39–4.35 (1H; C4′−H), 4.04 (1H; C3′−H), 3.94 (dd, 1H, J = 11.6, 2.6 Hz; C5′−H), 3.78 (dd, 1H, J = 11.6, 2.6 Hz; C5′−H), 2.62 (ddd, 1H, J = 13.5, 6.2, 3.7 Hz; C2′−H), 2.09–2.02 (1H; C2′−H), 0.89 (s, 9H; TBS), 0.88 (s, 9H; TBS), 0.09 (d, 6H, J = 5.1 Hz; TBS), 0.06 ppm (d, 6H, J = 4.9 Hz; TBS); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 158.1, 153.9, 153.4, 146.6, 145.0, 105.1, 88.8, 87.8, 71.6, 62.6, 42.6, 25.9, 25.7, 18.4, 18.0, −4.5, −4.9, −5.3, −5.4 ppm; FABMS (matrix: 3-nitrobenzyl alcohol): *m/e*: 525 [$(M+H)^+$]; HRMS: calcd for $C_{24}H_{41}D_3N_3O_4Si_2$: 525.3120; found: 525.3132.

3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-5-([D_3]methyl)-2′-deoxycytidine (3): 25% Ammonium deuteroxide (10 mL) was added to a solution of **2** (732 mg, 1.4 mmol) in acetonitrile (20 mL) and the mixture was stirred at room temperature for 4 h. The reaction mixture was concentrated in vacuo, then diluted with water and extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by column chromatography (SiO_2, 33% acetone/chloroform) to give **3** (730 mg, quant.) as a colorless oil: ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.57 (s, 1H; C6−H), 6.30 (t, 1H, J = 6.5 Hz; C1′−H), 4.35–4.32 (1H; C4′−H), 3.92–3.84 (2H; C3′−H, C5′−H), 3.74 (dd, 1H, J = 11.3, 2.6 Hz; C5′−H), 2.36 (1H; C2′−H), 1.95 (dd, 1H, J = 13.4, 6.6 Hz; C2′−H), 0.88 (18H; TBS), 0.06 ppm (12H; TBS); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 165.2, 155.6, 138.4, 101.2, 87.7, 85.9, 71.8, 62.7, 42.2, 29.3, 25.9, 25.7, 18.4, 18.0, −4.6, −4.9, −5.4 ppm; FABMS (matrix: 3-nitrobenzyl alcohol): *m/e*: 473 [$(M+H)^+$]; HRMS: calcd for $C_{22}H_{41}D_3N_3O_4Si_2$: 473.3059; found: 473.3045.

5-[D_3]Methyl-2′-deoxycytidine ([D_3]d^{m}C): Acetic acid (32.5 μL) and tetrabutylammonium fluoride (1.2 mL, 1.2 mmol) were added to a solution of **3** (179 mg, 0.38 mmol) in anhydrous tetrahydrofuran (3.8 mL) and the mixture was stirred at room temperature for 7 h. The reaction mixture was concentrated. The crude product was roughly purified by column chromatography (SiO_2, 20% methanol/chloroform with 2% triethylamine) and then purified by reversed-phase HPLC (7% methanol/water) to give [D_3]d^{m}C (58 mg, 63%) as a white solid: ^{1}H NMR (CD_3OD, 400 MHz): δ = 7.76 (s, 1H; C6−H), 6.16 (t, 1H, J = 6.5 Hz; C1′−H), 4.30–4.26 (1H; C4′−H), 3.82 (1H; C3′−H), 3.74–3.62 (2H; C5′−H), 2.23 (ddd, 1H, J = 13.5, 6.2, 3.7 Hz; C2′−H), 2.07–2.01 ppm (1H; C2′−H); ^{13}C NMR (CD_3OD, 100 MHz): δ = 167.1, 158.0, 140.2, 104.2, 88.8, 87.3, 71.9, 62.7, 42.0, 30.7 ppm; FABMS (matrix: glycerol): *m/e*: 245 [$(M+H)^+$]; HRMS: calcd for $C_{10}H_{13}D_3N_3O_4$: 245.1329; found: 245.1329.

3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-*N*-benzoyl-5-([D_3]methyl)-2′-deoxycytidine (4): Benzoyl chloride (40 μL, 0.33 mmol) was added to a solution of **3** (100 mg, 0.21 mmol) and 4-dimethylaminopyridine (55 mg, 0.45 mmol) in anhydrous dichloromethane (9.5 mL). The reaction was stirred at room temperature overnight. The reaction was quenched with methanol (1 mL) and concentrated under reduced pressure. The crude product was purified by column chromatography (SiO_2, 20% ethyl acetate/hexane) to give **4** (114 mg, 94%) as a white foam: ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.30 (2H; Bz), 7.67 (s, 1H; C6−H), 7.49 (3H; Bz), 6.32 (dd, 1H, J = 7.6, 6.0 Hz; C1′−H), 4.39 (1H; C4′−H), 3.96 (1H; C3′−H), 3.88 (dd, 1H, J = 11.4, 2.4 Hz; C5′−H), 3.76 (dd, 1H, J = 11.4, 2.4 Hz; C5′−H), 2.34–2.29 (1H; C2′−H), 2.01 (1H; C2′−H), 0.91 (18H; TBS), 0.11 ppm (12H; TBS); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 179.6, 159.8, 147.9, 137.3, 136.8, 132.3, 129.9, 128.1, 111.5, 88.1, 85.5, 72.3, 63.0, 41.7, 27.6, 25.9, 25.7, 18.4, 18.0, −4.6, −4.8, −5.4 ppm; FABMS (matrix: 3-nitrobenzyl alcohol): *m/e*: 577 [$(M+H)^+$]; HRMS: calcd for $C_{29}H_{45}D_3N_3O_5Si_2$: 577.3321; found: 577.3310.

***N*-Benzoyl-5-([D_3]methyl)-2′-deoxycytidine (5)**: Triethylamine trihydrogenfluoride (43 μL, 0.26 mmol) was added to a solution of **4** (50 mg, 0.087 mmol) in anhydrous tetrahydrofuran (0.9 mL). The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. The crude product was purified by column chromatography (SiO_2, 0–10% methanol/chloroform) to give **5** (30 mg, quant.) as a white foam: ^{1}H NMR ([D_6]DMSO, 400 MHz): δ = 8.19 (d, 2H, J = 7.1 Hz; Bz), 8.06 (s, 1H; C6−H), 7.64–7.47 (3H; Bz), 6.16 (t, 1H, J = 6.6 Hz; C1′−H), 5.27 (d, 1H, J = 4.3 Hz; C3′−OH), 5.11 (t, 1H, J = 5.1 Hz; C5′−OH) 4.28–4.24 (1H; C4′−H), 3.81 (1H; C3′−H), 3.67–3.55 (2H; C5′−H), 2.31–2.06 ppm (2H; C2′−H); ^{13}C NMR ([D_6]DMSO, 100 MHz): δ = 178.1, 159.2, 147.4, 138.9, 136.7, 132.5, 129.3, 128.3, 109.7, 104.8, 87.7, 84.9, 70.0, 53.5, 20.5 ppm; FABMS (matrix: glycerol): *m/e*: 349 [$(M+H)^+$]; HRMS: calcd for $C_{17}H_{17}D_3N_3O_5$: 349.1591; found: 349.1591.

5′-Dimethoxytrityl-*N*-benzoyl-5-([D_3]methyl)-2′-deoxycytidine (6): 4,4′-Dimethoxytritylchroride (420 mg, 1.24 mmol) was added to a solution of **5** (300 mg, 0.86 mmol) in anhydrous pyridine and the mixture was stirred at room temperature for 2.5 h. The reaction was quenched with methanol (1 mL) and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography (SiO_2, 0–5% methanol/dichloromethane with 1% triethylamine) to give **6** (547 mg, 92%) as a foam: ^{1}H NMR ([D_6]DMSO, 400 MHz): δ = 8.17 (d, 2H, J = 7.1 Hz; Bz), 7.79 (s, 1H; C6−H), 7.60–7.22 (13H; DMTr, Bz), 6.90 (dd, 4H J = 8.8, 2.0 Hz; DMTr), 6.20 (t, 1H, J = 6.5 Hz; C1′−H), 5.36 (d, 1H, J = 4.6 Hz; C3′−OH), 4.34 (1H; C4′−H), 3.94–3.93 (1H; C3′−H), 3.72 (s, 6H; OCH_3), 3.31–3.23 (2H; C5′−H), 2.32–2.24 ppm (2H; C2′−H); ^{13}C NMR ([D_6]DMSO, 100 MHz): δ = 178.1, 161.9, 158.9, 158.6, 158.1, 149.6, 147.4, 144.7, 138.3, 137.3, 136.6, 135.4, 135.2, 132.5, 129.7, 129.3, 128.9, 128.3, 128.2, 127.9, 127.7, 126.8, 125.3, 123.9, 113.2, 110.0, 85.9, 84.9, 70.2, 63.5, 55.0, 21.0 ppm; FABMS (matrix: 3-nitrobenzyl alcohol): *m/e*: 651 [$(M+H)^+$]; HRMS: calcd for $C_{38}H_{35}D_3N_3O_7$: 651.2898; found: 651.2888.

3′-(*N,N*-Diisopropylmethylphosphonamidite)-5′-dimethoxytrityl-*N*-benzoyl-5-([D_3]methyl)-2′-deoxycytidine (7): Compound **6** was coevaporated twice with anhydrous acetonitrile (1 mL). *N,N*-Diisopropylmethyl phosphonamidic chloride (16 μL, 0.082 mmol) was added to a solution of **6** (50 mg, 0.077 mmol) and diisopropylethylamine (39 μL, 0.23 mmol) in anhydrous acetonitrile (0.75 mL) and the mixture was stirred at room temperature for 1.5 h. The reaction mixture was filtered and then placed on a DNA synthesizer.

Synthesis of ODNs containing a C5-deuterated 5-methylcytosine base: ODNs containing 5-[D_3]methyl-2′-deoxycytidine were synthesized on an Applied Biosystems Model 3400 DNA/RNA synthesizer by using standard β-cyanoethylphosphramidite chemistry. Synthesized ODNs were purified by reversed-phase HPLC on an Inertsil ODS-3 column (10× 250 mm, elution with a solvent mixture of 0.1 M triethylammonium acetate (TEAA) at pH 7.0, linear gradient over 60 min from 0% to 30% acetonitrile at a flow rate of 3.0 mL min^{-1}). The purity and concentration of the modified ODN was determined by complete digestion with AP, P1, and phosphodiesterase I. The synthesized ODN ([D_3]^{m}C) was identified by MALDI-TOF mass spectrometry ([$M-H$]$^-$; calcd: 5583.67; found: 5585.5).

Synthesis of ODNs possessing 9,10-anthraquinone (AQ) sensitizer:

Synthesis of ODN 1(AQ_1): 10 mM AQ-NHS in acetonitrile solution containing 20 vol% dimethylformamide (45 μL) and saturated $NaHCO_3$ (30 μL) were added to a 1.2 mM solution (total volume: 75 μL) of ODNs possessing an aminohexyl linker internally and incubated at 37°C overnight. The reaction mixture was first subjected to gel filtration by using MicroBio-spin 6 columns (BioRad) and then purified by reversed-phase HPLC with a 0–30% linear gradient (over 30 min) of acetonitrile/0.1 M TEAA buffer solution at pH 7.0. The purity and concentration of the AQ-modified ODN were determined by complete digestion with AP, P1, and phosphodiesterase I. The synthesized ODN 1(AQ_1) was identified by MALDI-TOF mass spectrometry ([$M-H$]$^-$; calcd: 5546.68; found: 5546.84).

Synthesis of ODN 1(AQ_2): Saturated 2-(3-bromopropionamido)anthraquinone (approximately 5 mM) in ethanol solution (250 μL) and saturated $NaHCO_3$ (30 μL) were added to a 0.6 mM solution (total volume: 480 μL) of ODNs possessing an aminohexyl linker internally and incubated at 60°C for 20 h. The reaction mixture was purified by reversed-phase HPLC. The synthesized ODN 1(AQ_2) was identified by ESI-TOF mass spectrometry ([$M-4H$]$^{4-}$; calcd: 1392.4; found: 1392.5).

Photosensitized oxidation of 5-methyl-2′-deoxycytidine by anthraquinone 2-sulfonate: A solution of d^{m}C (200 μM) in 2 mM sodium cacodylate buffer (pH 7.0) containing 20 mM NaCl was added to an aqueous solution

of AQS (200 μM). The solution (100 μL) was exposed to 312 nm UV light with a Lourmat TFX-20 M transilluminator (Vilber Lourmat, France) at 0 °C. Analytical HPLC was performed with a HITACHI Lachrom Elite HPLC system. Sample solutions (10 μL) were injected onto a reversed-phase column (Inertsil ODS-3, GL Sciences Inc., 4.6×250 mm). A solvent mixture of TEAA buffer solution (0.1 M, pH 7.0) containing 5 vol% acetonitrile was delivered as the mobile phase. The column eluents were monitored by UV absorbance at 260 nm. In the deuterium isotope experiment, aqueous solutions of d^mC (200 μM) or $[D_3]d^mC$ (200 μM) containing AQS (200 μM) were photoirradiated under the conditions described above. After 312 nm photoirradiation, the solution was immediately subjected to HPLC analysis. Each deuterium isotope effect (k_H/k_D) of degradation and formation was estimated from the initial rates of d^mC and $[D_3]d^mC$ degradation and those of d^fC and deuterated d^fC formation.

Nanosecond laser flash photolysis: The laser flash photolysis experiments were carried out with a Unisoku TSP-601 flash spectrometer. A Continuum Surelite-I Nd:YAG (*Q*-switched) laser with the third harmonic at 355 nm (approximately 50 mJ per 6 ns pulse) was employed for the flash photoirradiation. Further details of the laser flash photolysis system have been described previously.[45] Aqueous acetonitrile solutions (acetonitrile/water, 9:1) of d^mC (500 μM) containing AQS (50 μM) were deaerated by passing argon through the solution prior to the laser flash photolysis experiments.

Photooxidative cleavage reaction and PAGE analysis: ODNs were 5′-^{32}P-labeled by phosphorylation with [γ-^{32}P]ATP (4 μL) and T4 polynucleotide kinase (4 μL). The reaction mixtures were purified by using a QIAquick Nucleotide Removal Kit (QIAGEN) to remove excess unincorporated nucleotide. The ^{32}P-5′-end labeled ODNs (<0.4 μM strand concentration) were hybridized by their complementary ODNs possessing the AQ_1 or NQ_1 chromophore (0.5 μM) in 2 mM sodium cacodylate buffer (pH 7.0) containing 20 mM NaCl. Hybridization was achieved by heating the sample at 90 °C for 5 min and slowly cooling to room temperature. The solutions of ^{32}P-5′-end labeled duplex were irradiated at 312 nm UV light at 20 °C on exposure to air. After irradiation, all reaction mixtures were subjected to precipitation by ethanol. The precipitated DNA was resolved in 10% piperidine (50 μL), heated at 90 °C for 20 min, and concentrated. The resulting samples were then analyzed by PAGE, for which the experimental details were described previously.[12]

Determination of quantum yields of photosensitized oxidation and strand cleavage reaction: Quantum yield measurements were carried out by using a phenylglyoxylic acid actinometer.[38] The light flux was estimated to be 1.66×10^{15} photon s^{-1}. The relative quantum yields of strand cleavage at the ^mC site were calculated from the gel electrophoresis study. In determining the quantum yields, molar extinction coefficients (ε) of the respective photosensitizers at their absorption wavelengths were estimated: AQ 2-carboxylic acid: $\varepsilon_{312}=3605\,M^{-1}cm^{-1}$; NQ: $\varepsilon_{312}=1793\,M^{-1}cm^{-1}$.

Melting temperature (T_m) of hybridized ODNs: 1 μM appropriate ODNs were dissolved in 10 mM sodium cacodylate buffer (pH 7.0) containing 100 mM NaCl. UV melting curves were recorded on a JASCO-V630 spectrophotometer equipped with a multicell block and a Peltier temperature controller. Melting curves were obtained by monitoring the UV absorbance at 260 nm with elevating temperature from 4 to 80 °C at a rate of 1 °C min^{-1}.

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[31] UV-B (290–320 nm) radiations can induce the formation of dimeric photoproducts between adjacent pyrimidine bases in DNA.[31a] Among the dimeric pyrimidine photooxidation products, pyrimidine (6–4) photodimers and their Dewar valence isomers may be alkali-labile lesions.[31b] We could not identify the formation of such dimeric photoproducts, so it is plausible that the present photoirradiation conditions did not produce the alkali-labile dimeric photoproducts, in accordance with the suppression of piperidine-induced strand cleavage at ^mC bases in the control duplex (ODN 1(G)/ODN 2(^mC)). See: a) *Photochemistry and Photobiology of Nucleic Acids: Chemistry Vol. I* (Ed. S. Y. Wang), Academic Press, New York, **1976**; b) T. Douki, J. Cadet, *Biochemistry* **1994**, *33*, 11942–11950.
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