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Oxidation Reactions of Cytosine DNA Components by Hydroxyl Radical and One-Electron Oxidants in Aerated Aqueous Solutions

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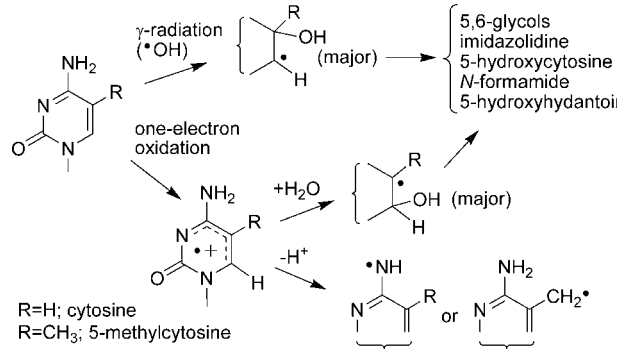
CONSPECTUS

Indirect evidence strongly suggests that oxidation reactions of cytosine and its minor derivative 5-methylcytosine play a major role in mutagenesis and cancer. Therefore, there is an emerging necessity to identify the final oxidation products of these reactions, to search for their formation in cellular DNA, and to assess their mutagenic features. In this Account, we report and discuss the main •OH and one-electron-mediated oxidation reactions, two of the most potent sources of DNA damage, of cytosine and 5-methylcytosine nucleosides that have been recently characterized. The addition of •OH to the 5,6-unsaturated double bond of cytosine and 5-methylcytosine generates final degradation products that resemble those observed for uracil and thymine. The main product from the oxidation of cytosine, cytosine glycol, has been shown to undergo dehydration at a much faster rate as a free nucleoside than when inserted into double-stranded DNA. On the other hand, the predominant •OH addition at C5 of cytosine or 5-methylcytosine leads to the formation of 5-hydroxy-5,6-dihydro radicals that give rise to novel products with an imidazolidine structure. The mechanism of the formation of imidazolidine products is accounted for by rearrangement reactions that in the presence of molecular oxygen likely involve an intermediate pyrimidine endoperoxide. The reactions of the radical cations of cytosine and 5-methylcytosine are governed by competitive hydration, mainly at C6 of the pyrimidine ring, and deprotonation from the exocyclic amino and methyl group, leading in most cases to products similar to those generated by •OH. 5-Hydroxypyrimidines, the dehydration products of cytosine and uracil glycols, have a low oxidation potential, and their one-electron oxidation results in a cascade of decomposition reactions involving the formation of isodialuric acid, dialuric acid, 5-hydroxyhydantoin, and its hydroxyketone isomer. In biology, GC → AT transitions are the most common mutations in the genome of aerobic organisms, including the *lacI* gene in bacteria, *lacI* transgenes in rodents, and the *HPRT* gene in rodents and humans, so a more complete understanding of cytosine oxidation is an essential research goal. The data and insights presented here shed new light on oxidation reactions of cytosine and 5-methylcytosine and should facilitate their validation in cellular DNA.



1. Introduction

Two of the most potent sources of DNA damage include the reaction of •OH with DNA components and one-electron oxidation of nucleobases. These reactions occur during the exposure of DNA to ionizing radiation (e.g., X-rays), Fenton reagents (e.g., $H_2O_2 + Fe^{2+}$), and photosensitization (e.g., 2-methyl-1,4-naphthoquinone) leading to a multitude of modifications.[1] As many as 30 modifications of cytosine, including diastereomers, have been characterized so far from studies of

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the nucleobase, nucleoside, and DNA.[2] The analysis of oxidized cytosine derivatives is complicated by the difficulty to separate the mixture of products by chromatography and because of the formation of numerous unstable compounds. Previous studies have focused on the chemistry and biology of the final and stable decomposition products of cytosine: 5,6-dihydroxy-5,6-dihydrouracil, the so-called "uracil glycols", 5-hydroxycytosine, 5-hydroxyuracil, 5-hydroxyhydantoin, and *N*-formamide.[3] Recent work in our laboratories has addressed the analysis of unstable and/or poorly characterized oxidation products of cytosine, including 5,6-dihydroxy-5,6-dihydrocytosine, "cytosine glycols", isodialuric acid, dialuric acid derivatives, and 1-carbamoyl-4,5-dihydroxy-2-oxo-imidazolidine.[4] The major stable and unstable products have been characterized for the nucleoside and in certain cases for oligodeoxynucleotides. In this Account, we emphasize our recent efforts to understand the mechanism of decomposition of cytosine derivatives induced by •OH and one-electron oxidation.

The importance of cytosine oxidation in biology is underlined by the fact that GC → AT transitions are the most common mutations in the genome of aerobic organisms, including the *lacI* gene in bacteria, the *lacI* transgenes in rodents, and the *HPRT* gene in rodents and humans.[5] An important source of GC → AT transitions involves reactive oxygen species in view of the similarities in the spectra of spontaneous mutations compared to those induced by oxidants, such as H_2O_2 and ionizing radiation.[6] Furthermore, mitochondrial DNA exposed to relatively abundant amounts of reactive oxygen species displays a strong bias toward GC → AT transitions (81%).[6b] The initial step in the formation of GC → AT transitions likely involves the deamination (loss of the exocyclic amino group) of cytosine, which greatly increases the likelihood that a mutation will occur during DNA replication. Although deamination takes place by thermal and enzymatic pathways, the oxidation of cytosine leads to saturation of the 5,6-double bond that enhances the rates of deamination by 5−6 orders of magnitude.[7] About half of the stable oxidation products of cytosine undergo deamination during their route to stable products. Interestingly, the percentage of GC → AT transitions at CpG dinucleotides[8] in human HPRT and cancer cells (25−50%) is several fold above the percentage of methylated CpG dinucleotides (1−2%). This suggests that the processing of oxidatively generated damage to 5-methylcytosine is exceedingly important in the formation of mutations.

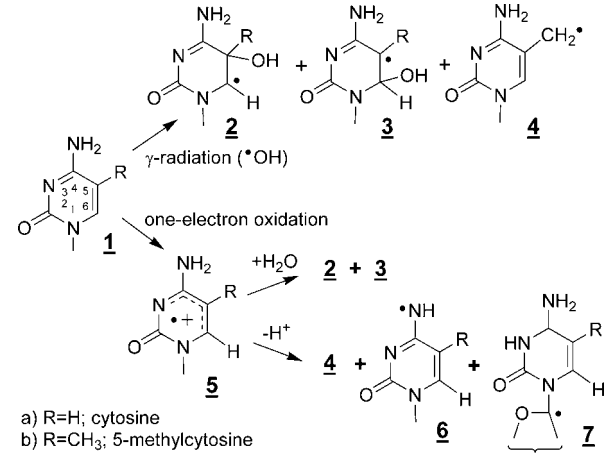

FIGURE 1. Radical oxidation of the cytosine moiety.

TABLE 1. Comparison of Initial Pathways of Damage for Hydroxyl Radical Reactions with Pyrimidines and Pyrimidine Radical Cations[a]

	•OH addition to C5	•OH addition to C6	H-abstraction from CH_3
thymine	60	30	10
cytosine	87	13	
5-methylcytosine	65	22	13

	H_2O addition to C5	H_2O addition to C6	deprotonation
thymine	~0	60	40[b]
cytosine	20	40	40[c]
5-methylcytosine	~0	40	60[b]

[a] Percentage of the total reaction for each pyrimidine base. [b] Deprotonation from the methyl group. [c] Deprotonation from the exocyclic amino group.

2. •OH-Mediated Oxidation of Cytosine Derivatives

2.1. Peroxyl Radicals and Hydroperoxides. The addition of •OH to the 5,6-double bond of cytosine (**1a**) leads to 5-hydroxy-5,6-dihydrocytos-6-yl (**2a**, 87%) and 6-hydroxy-5,6-dihydrocytos-5-yl (**3a**, 13%) as supported by theoretical and pulse radiolysis studies (Figure 1, Table 1; compounds denoted with the letter **a** refer to cytosine derivatives whereas those with **b** refer to 5-methylcytosine derivatives).[9] Similar to •OH adducts of thymine and uracil, **2a** and **3a** rapidly react with O_2 in aerated aqueous solutions to form the corresponding peroxyl radicals: 5(6)-hydroxy-6(5)-hydroperoxyl-5,6-dihydrocytosine radicals (e.g., **8a**); and in turn the corresponding hydroperoxides, 5(6)-hydroxy-6(5)-hydroperoxy-5,6-dihydrocytosine (e.g., **9a**) (Figure 2; the same pathway takes place for •OH-adducts at C6). The peroxy radicals of cytosine nucleosides (e.g., **8a**) undergo bimolecular decay via intermediate tetraoxides and oxyl radicals to form common pyrimidine oxidation products (**10**, **11a**, **12**, **13**). These are major stable products of the •OH-mediate oxidation of dCyd in aerated aqueous solutions (Table 2). The reduction of oxyl radicals explains the formation of 5-hydroxycytosine (**10**) and 5,6-

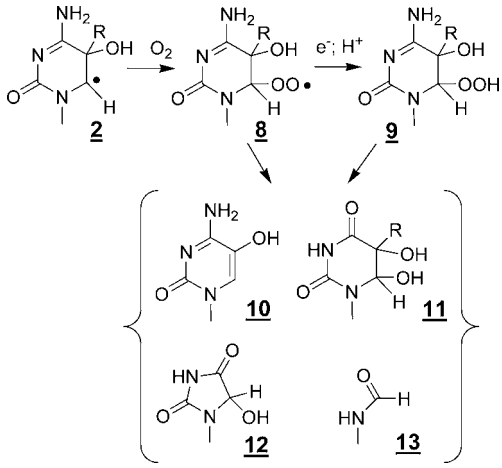

FIGURE 2. Decomposition of intermediate 5-hydroxy-6-peroxyl radicals and the corresponding 5-hydroxy-6-hydroperoxide of cytosine derivatives to common pyrimidine oxidation products.

TABLE 2. Yield (%) of Stable Products from γ-Irradiation and Photosensitization of dCyd in Aerated Aqueous Solutions[a]

product (dR = 2-deoxy-β-D-*erythro*-pentofuranosyl)	γ-irradiation[b]	UVA/MQ[c]
5,6-dihydroxy-5,6-dihydro-2′-deoxyuridine (**11a**)	28	27
5-hydroxy-2′-deoxycytidine (**10**)	12	7
N_1-(dR)-5-hydroxyhydantoin (**12**)	3	2
N_1-(dR)-formamide (**13**)	12[d]	2[d]
N_1-(dR)-biuret (**18**)	3	2
N_1-(dR)-1-carbamoyl-3,4-dihydroxy-2-oxoimidazolidine (**16a**)	17	6
aminocarbonyl[2-(dR)-amino]-2-oxomethylcarbamic acid (**17a**)	3	2
5′,6-cyclo-5-hydroxy-5,6-dihydro-2′-deoxyuridine	2	*e*
2′-deoxyuridine	*e*	36
cytosine	12	4
2-deoxyribono-1,4-lactone	*f*	4[g]
total	92	92

[a] Yields from normal-phase 2D-TLC and HPLC analyses with $^{14}C_2$-labeled dCyd. [b] Total loss of substrate (15%); $G = -85 \times 10^{-9}$ J mol^{-1}. [c] Total loss of substrate (22%). [d] Estimated by comparison of refractive index signal with that of **13**. [e] Not detected. [f] Trace amounts. [g] Produced in equal yield with cytosine.

dihydroxy-5,6-dihydrouracil (**11a**), which arise from the conversion of unstable 5,6-dihydroxy-5,6-dihydrocytosine (**19a**), as discussed in section 2.3. On the other hand, β-scission of oxyl radicals explains the formation of 5-hydroxyhydantoin (**12**) and *N*-formamide (**13**). In competition with bimolecular decay, peroxyl radicals are reduced to hydroperoxides (e.g., **9a**). This reaction may predominate in DNA because bimolecular decay pathways are not feasible. Although the hydroperoxides of cytosine are not stable under neutral conditions,[10] one expects that their decomposition is analogous to that of the well-characterized thymidine hydroperoxides.[11] Thus, the cytosine hydroperoxides may give rise to the same products that are formed by the bimolecular decay of peroxyl radicals. On the other hand, cytosine hydroperoxides may undergo additional reactions that change the type and

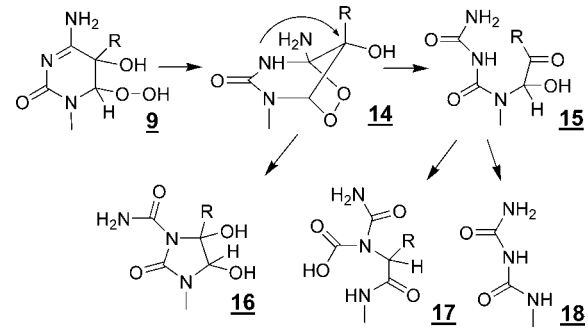

FIGURE 3. Formation of novel products by rearrangement of intermediate 5-hydroxy-6-hydroperoxides of cytosine derivatives.

distribution of products, such as deamination to uracil derivatives and rearrangement to endoperoxides (discussed below). Lastly, it should be noted that the fate of peroxyl radicals and hydroperoxides may induce alternative reactions in duplex DNA.[12] In particular, thymine peroxyl radicals or hydroperoxides react with guanine in duplex DNA to form tandem lesions involving side-by-side *N*-formamide and 8-oxo-7,8-dihydroguanine lesions.[12a] It is reasonable to propose that *N*-formamide in tandem lesions arises from •OH-induced decomposition of both cytosine and thymine.

2.2. Unique 1-Carbamoyl-2-oxo-4,5-dihydroxyimidazolidine (16a). The formation of products **16a**−**18** constitutes a novel pathway of •OH-mediated decomposition for cytosine derivatives (Figure 3). The nucleoside of **16a** was recently examined in detail by NMR and MS analyses.[4f] These products consist of four diastereomers with chiral centers at C4 and C5 of the imidazolidine ring. Remarkably, the products interconverted via ring−chain tautomerism involving both N1−C5 and N3−C4 bonds of the ring, leading to epimerization of all four diastereomers. Starting with HPLC-purified compounds, the *trans* and *cis* diastereomers of **16a** converted at different rates into a mixture of four diastereomers depending on temperature and pH. The rate of epimerization was much greater for *cis* diastereomers (8.5×10^{-4} s^{-1} (half-life = 14 min) compared with *trans* diastereomers (5.8×10^{-6} s^{-1}; half-life = 33 h) at 37 °C and pH 7. The mechanism of formation of **16a** likely involves intramolecular rearrangement of 5-hydroxy-6-hydroperoxides (**9a**) into C6−C4 endoperoxides (**14a**) followed by O−O and C4−C5 bond cleavage. The mechanism of formation of **16a** is supported by chemical synthesis. The intermediate 5-hydroxy-6-hydroperoxides (**9a**) were specifically generated *in situ* by nucleophilic substitution of the halogen atom of 5-bromo-6-hydroxy-5,6-dihydro-2′-deoxycytidine by H_2O_2, a reaction that involves shift of the hydroxyl group from the C6 to C5 position with anchimeric assistance. The conversion of **9a** to **16a** appears to be concerted because it initially gives *cis* diastereomers rather than

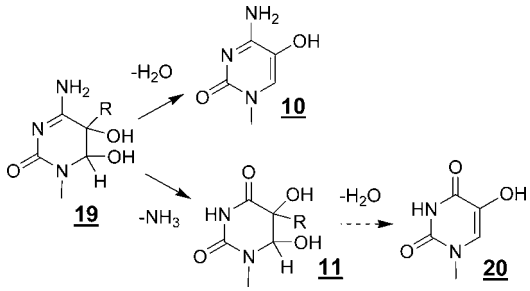

FIGURE 4. Competitive dehydration and deamination of cytosine 5,6-glycols.

a mixture of *cis* and *trans* diastereomers. Additional evidence for the formation of an intermediate endoperoxide (**14a**) was provided by $^{18}O_2$-labeling experiments showing the incorporation of two ^{18}O atoms into the structure of **16a**, that is, one label on each at the sides of O−O bond cleavage.[2d] In addition to **16a**, two other nucleosides with a related structure were identified in the mixture of products, aminocarbonyl[2-amino]-2-oxomethylcarbamic acid (**17a**) and biuret (**18**). The structure of **17a** was proposed on the basis of NMR and MS analyses, together with comparison of the corresponding oxidation product of 5-methylcytosine nucleoside. Formation of **17a** may be explained by cleavage of endoperoxide **14a** to an open ring intermediate (**15a**) followed by α-hydroxyketone rearrangement, dehydration, and subsequent hydrolysis at C2. This pathway is supported by $^{18}O_2$-labeling experiments showing the incorporation of two atoms of ^{18}O into the structure of **17a**, similar to **16a**. The acyclic structure of biuret products (**18a**) is expected to favor protonation at N1 and subsequent ring-opening of 2-deoxyribose moiety. This leads to isomerization of the 2-deoxyribose moiety of biuret (**18**) into different forms (α- and β-furanose, and α- and β-pyranose).[2d] The mechanism of formation of **18** may also be explained by cleavage of endoperoxide **14a** to an open ring intermediate (**15a**) followed by loss of glyoxal (CHO−CHO). The *trans* diastereomers of **16a** have been detected upon γ-irradiation of aerated aqueous solutions of dGpdC, dCpdG, and d(TpApCpG), as well as by the reaction of Fenton reagents with dCpC.[13] In contrast, the formation of **16a**, **17a**, and **18a** has not so far been clearly demonstrated in oxidized DNA.

2.3. Unstable 5,6-Dihydroxy-5,6-dihydrocytosine (19a). The decomposition of **19a** is governed by dehydration and deamination (Figure 4). The presence of a hydroxyl group at C6 of oxidized cytosine allows dehydration to 5,6-unsaturated cytosine derivatives (**19a** → **10**). Furthermore, saturation of the 5,6-double bond renders the molecule susceptible to hydrolytic deamination of the exocyclic amino group (**19a** → **11a**; conversion of 5,6-saturated cytosine to uracil derivatives). The nucleoside of **19a** was examined under various conditions by HPLC with UV detection (HPLC−UV).[4e] The half-life of **19a** was 50 min at 37 °C and 5 h at 22 °C in aqueous solution at pH 7. The rate of decomposition of **19a** was highest at pH 5 within a range of pH from 2 to 11 and increased with the concentration of phosphate anion (0−300 mM). The major pathway of decomposition for **19a** was dehydration as indicated by the formation of 5-hydroxycytosine (**10**, >90%). The deamination of cytosine 5,6-glycols to uracil 5,6-glycols (**19a** → **11a**) was a minor pathway for the isolated nucleoside (<10%, pH 2−8), although this pathway increased substantially above pH 8. The stability of **19a** was examined indirectly in oxidized calf-thymus DNA by strong acid hydrolysis, which leads to cleavage of the *N*-glycosidic bond and quantitatively converts **19a** to **10**; similarly, acid hydrolysis converts uracil 5,6-glycols (**11a**) to 5-hydroxyuracil (**20**). The base derivatives of **10** and **20** were subsequently measured by HPLC with electrochemical detection (HPLC−EC). Thereby, the yield of **19a** in irradiated DNA decreased as a function of time during post-treatment incubation at 37 °C and pH 7, whereas the yield of **11a** increased with matching kinetics. From these studies, the half-life of **19a** in DNA was estimated to be 28 h at 37 °C and pH 7, and the ratio of dehydration to deamination was 1.5:1 in favor of dehydration. These studies were recently extended to double-stranded poly(dG−dC) containing **19a**.[4g] The oxidation of poly(dG−dC) by $KMnO_4$ led mainly to the formation of **19a** as inferred by HPLC−EC or GC−MS analyses. With oxidized poly(dG−dC), the half-life of **19a** was estimated to be 6.5 h at 37 °C and pH 7, while the ratio of dehydration and deamination was 5:1. In comparison to oxidized calf-thymus DNA, the behavior of **19a** in poly(dG−dC) resembled more that of the nucleoside in aqueous solution; that is, the half-life was reduced and the ratio of deamination was lower. Changes in these properties may reflect a different mixture of *cis* and *trans* diastereomers of **19a** depending on the method of oxidation (i.e., ionizing radiation vs $KMnO_4$), as well as differences in the secondary structure of the polymers. Together, the results with oxidized calf-thymus and poly(dG−dC) indicate that double-stranded DNA extends the lifetime of **19a**. Interestingly, the properties of **19a** are similar to those of 6-hydroxy-5,6-dihydrocytosine, the so-called "cytosine photohydrate", which contains a hydroxyl group at C6 of cytosine, allowing the β-elimination reaction to occur. The half-life of cytosine photohydrates was 18 h in poly(dG−dC) compared with 25 min at 37 °C and pH 7.[14]

Exposure of DNA to ionizing radiation in aqueous aerated solutions leads to the formation of 5-hydroxycytosine (**10**), uracil 5,6-glycols (**11a**), and 5-hydroxyuracil (**20**) to a level of 19, 16, and 24 lesions per million cytosine per Gy, respec-

tively, as determined by enzymatic digestion of DNA and HPLC−EC analysis.[2e] Likewise these products are formed in DNA treated with Fenton reagents (e.g., Fe^{2+}/H_2O_2). Interestingly, there was no evidence for the formation of **20** following incubation of MnO_4-oxidized poly(dG−dC), suggesting that cytosine glycols **19a** and uracil glycols **11a** are not primary precursors of 5-hydroxyuracil **20** in double-stranded DNA.[4e,g] In contrast, •OH-induced decomposition of cytosine in DNA produces relatively large amounts of **20** that may arise by the deamination and subsequent elimination of H_2O_2 from intermediate 6-hydroxy-5-hydroperoxides.

2.4. •OH Induced Damage to 5-Methylcytosine (1b). The chemistry of •OH-induced decomposition of 5-methylcytosine derivatives is similar in many respects to that of cytosine derivatives. The main products observed after γ-irradiation of an aerated aqueous solution of the nucleoside (**1b**, R = CH_3; Figure 1) included the corresponding derivatives of 5,6-dihydroxy-5,6-dihydro-5-methylcytosine (**19b**), 1-carbamoyl-4,5-dihydroxy-5-methyl-2-oxo-imidazolidine (**16b**), aminocarbonyl[2-amino]-carbamic acid (**17b**), and *N*-formamide (**13**).[15] The four *cis* and *trans* diastereomers of 5-methylcytosine 5,6-glycols (**19b**) were prepared by bromination of the parent nucleoside followed by hydrolysis in aqueous solution.[15b] Deamination of **19b** to thymidine 5,6-glycols (**11b**) takes place with a half-life of 17 and 22 h for *cis*-(5*S*,6*S*) and *cis*-(5*R*,6*R*) diastereomers, respectively, at 37 °C and pH 7; in contrast, the *trans* diastereomers undergo deamination more efficiently than the *cis* diastereomers. Thus, **19b** isomers are more stable than cytosine glycols **19a** by about 20-fold for the corresponding nucleosides. Other similarities in the pathway of decomposition of cytosine and 5-methylcytosine include the formation of imidazolidine **16b** and associated carbamate modifications (**17b**).[15a] Interestingly, the γ-irradiation of aerated aqueous solutions of 5-methylcytosine nucleoside gives rise to a novel product consisting of two diastereomers, 4-amino-1-5-dihydro-5-methyl-2-*H*-imidazol-2-one (not shown).[15a] This product likely forms by a similar pathway as that for 5-hydroxyhydantoin (**12**) except for retention of the exocyclic amino group.

3. One-Electron Oxidation of Cytosine Derivatives

3.1. Hydration and Deprotonation. The radical cation of cytosine **5a** is efficiently generated in aqueous solution by near-UV photosensitization of the nucleoside with 2-methyl-1,4-naphthoquinone (menadione, MQ).[2g] Triplet excited MQ undergoes charge transfer with the cytosine moiety of the 2′-deoxyribonucleoside to give the corresponding radical cation (**5a**; Figure 1) with a quantum yield of 0.33. The mixture of products induced by MQ photosensitized oxidation was characterized by detailed NMR and mass spectrometry. The mechanism of formation of stable products may be divided into two competitive reaction pathways: hydration (**5a** → **2a** + **3a**) and deprotonation (**5a** → **6a** + **7a**; Figure 1).

Hydration of **5a** leads to OH-substituted radicals that are identical to those generated by the addition of •OH to cytosine (**2a** and **3a**). Thus, the majority of products for the nucleoside are the same from both one-electron oxidation and •OH-induced reactions, including 5-hydroxycytosine (**10**), uracil 5,6-glycols (**11a**), 5-hydroxyhydantoin (**12**), *N*-formamide (**13**), and imidazolidine 4,5-glycols (**16a**) (Table 2). Experiments in the presence of $^{18}O_2$ showed that label is incorporated at both C5 and C6 of the oxidation products of cytosine in a ratio of about 40% and 60%, respectively.[2d] These results suggest that the addition of H_2O occurs with similar efficiency at C5 and C6 positions of **5a**. Thus, the addition of •OH to **1a** leads mainly to C5-OH adducts while the hydration of **5a** gives rise to a mixture of both radicals with a preference for C6-OH adducts. This difference probably explains changes in the relative yield of common oxidation products. Clearly, the major stable products of both hydroperoxides are **11a**. In contrast, the greater yield of **16a** in the reactions of •OH compared with those of **5a** suggests that 5-hydroxy-6-hydroperoxides, the main intermediates in •OH reactions, transform more efficiently than 6-hydroxy-5-hydroperoxides into **16a**. In support of this pathway, the yield of **16a** was negligible after direct generation of 6-hydroxy-5,6-dihydrocytos-5-yl radicals by UV photolysis of 5-bromo-6-hydroxy-5,6-dihydro-2′-deoxycytidine.

The deprotonation of **5a** leads to uracil as a major pathway in the MQ-photosensitized oxidation of the nucleoside.[2d] The formation of cytosine N4-aminyl radicals (**6a**) was confirmed by EPR experiments in the photosensitization of 1-methylcytosine by triplet anthraquinone-2,6-disulfonic acid (AQ).[16] In a computational study, the pathway of deamination with the lowest free energy barrier involved the addition of water to the N3−C4 imine bond followed by cleavage of the C4−N4 bond.[17] Finally, **5a** was observed to undergo deprotonation from the C1′ position of the sugar moiety (**7a**) leading to the release of free base **1a** and 2-deoxyribono-1,4-lactone in equal amounts.[2a]

3.2. Studies of Dinucleotides and Oligonucleotides. The photosensitized one-electron oxidation of d(CpC) was shown to produce tandem lesions in which the N3 of cytosine is attached to the C5 of another cytosine moiety in the dinucleotide.[18] The formation of these lesions was proposed to

involve attack of the aminyl radical of cytosine at either C5 or C6 of an undamaged cytosine rather than the attack of cytosine radical cations or its hydration product. Interestingly, the maximum yield of cross-link was observed at pH 5.5, at the pK_a of MQ, suggesting that proton transfer from cytosine radical cations to MQ enhanced the yield of aminyl radicals. Another cross-link was detected in the oxidation of d(CpC) in which the C6 of uracil was linked to the N3 of cytosine, pointing to a similar mechanism of cross-linking as above except that the 5,6-saturated moiety undergoes deamination before dehydration to the 5,6-unsaturated uracil derivative.[18b]

Models of charge transfer in DNA have shown that near-UV photolysis of oligonucleotides containing AQ induces damage mainly at GG dinucleotides as indicated by the formation of strand breaks on electrophoresis.[19] More recent studies indicate that damage also occurs at thymine under certain conditions, that is, in the lack of oxygen or in sequences poor in guanine.[20] In MQ tethered oligonucleotides, damage at individual bases was estimated to follow the order, A > T > G > C, as inferred by enzymatic digestion and HPLC−UV analyses.[20a] The loss of A and T in these experiments may be explained in part by the combination of quinone with either A or T radicals as inferred the formation of quinone base adducts.[20a−c] Thus, guanine is the principal site of oxidatively generated damage excluding secondary reactions of the photosensitizer. On the other hand, 5-methylcytosine appears to be an excellent target for the induction of damage in MQ tethered oligonucleotides.[21] Efficient piperidine-induced cleavage was observed with 5-methylcytosine opposite MQ. Surprisingly however, no cleavage was observed in sequences with neighboring GG doublets, which have a lower oxidation potential than 5-methylcytosine. These results suggest that the semiquinone radical anion efficiently undergoes recombination with radical cations centered at GG dinucleotides, whereas the recombination with radical cations of 5-methylcytosine is inefficient, probably because it undergoes rapid deprotonation from the methyl group.

3.3. One-Electron Oxidation of 5-Methylcytosine (1b). The main site of deprotonation of 5-methylcytosine radical cations (**5b**, R = CH_3; Figure 1) for the nucleoside generated by MQ-photosensitized oxidation was the methyl group (Table 1).[15c] The resulting 5-methyl-(2′-deoxycytidylyl) radical (**4**) likely transforms into the corresponding peroxyl radical and hydroperoxide, which in turn decompose into stable methyl oxidation products, namely, 5-(hydroxymethyl)-2′-deoxycytidine (**23**) and 5-formyl-2′-deoxycytidine (**24**) (Figure 5). The half-life of **22** was estimated to be 9.5 h at 24 °C. This pathway is analogous to that of methyl oxidation products of thymidine.[22] It should be added that **23** is susceptible to secondary oxidation; for example, **23** is converted into **24** during MQ photosensitization. The photosensitized one-electron oxidation of **1b** by MQ attached to oligonucleotides displayed an unusual pH dependence with a maximum production of **24** observed at pH 5−6 and lower yields at both extremes of pH (pH 4 and 8).[21b] One would expect that high pH increases deprotonation at the methyl group similar to thymine radical cations.[2g] A decrease in the yield of **24** at high pH in the case of **1b** may be explained by competitive deprotonation from the exocyclic amino and methyl groups. In support of this pathway, the initial yield of **24** dramatically increased (5-fold) and did not show a dependence on pH above pH 5 when the exocyclic amino group was substituted with methyl groups, thereby preventing deprotonation at this position.[21b] These results suggest that deprotonation from the exocyclic amino group is favored over that from the methyl group at neutral pH.

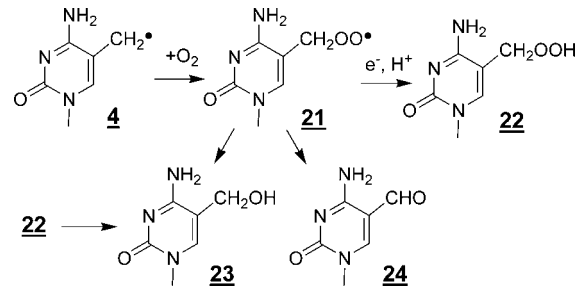

FIGURE 5. Formation of methyl oxidation products from 5-methylcytosine oxidation.

The formation of 5,6-dihydroxy-5-methyl-5,6-dihydrocytosine (**19b**, R = CH_3) was a major pathway in the MQ-photosensitized oxidation of the nucleoside of **1b**.[15a] When the reaction was carried out in $^{18}O_2$, the label was incorporated at C5 in the structure of the 5,6-glycols **11b** as determined by mass spectrometry analysis. This indicates that H_2O exclusively adds to C6 and oxygen to C5 of transient **5b**. In contrast, the addition of H_2O takes place at both C5 and C6 in the case of **5a**. This pathway leads to common pyrimidine oxidation products as described for •OH reactions. The lack of detection of 1-carbamoyl-4,5-dihydroxy-5-methyl-2-oxo-imidazolidine (**16b**, R = CH_3; Figure 3) in MQ-photosensitized oxidation of **1b** suggests that these products are exclusively formed by the decomposition of the corresponding 5-hydroxy-6-hydroxperoxides rather than 6-hydroxy-5-hydroperoxides.

3.4. Oxidation of 5-Hydroxypyrimidines. The nucleosides of 5-hydroxycytosine (**10**) and 5-hydroxyuracil (**20**) are susceptible to secondary oxidation because of their relatively low oxidation potential. The oxidation potential of **10** is 70 mV, while that of **20** is 150 mV versus Pd reference electrode (in comparison, the oxidation potential of normal bases is

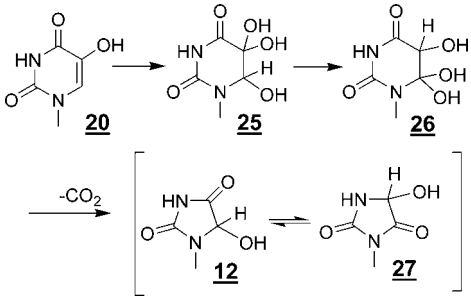

FIGURE 6. Decomposition of dialuric and isodialuric acid intermediates.

greater than 700 mV).[2e,4c] The oxidation of the nucleoside of **20** by either Br_2 or Na_2IrCl_6 leads to the quantitative formation of the corresponding isodialuric acid modification (**25**, Figure 6).[4a,b] These studies permitted a comprehensive assessment of the decomposition of isodialuric acid nucleoside derivatives. Remarkably, the decomposition of **25** involves a sequence of three transformations in which each product (**25**, **26**, **12**, and **27**) exists as a pair of diastereomers. The isodialuric acid product **25** transforms into the corresponding dialuric acid product **26** after 2 h at 37 °C in neutral aqueous solution. This transformation (**25** → **26**) occurs in a stereospecific manner, indicating a mechanism involving α-hydroxyketone rearrangement with concerted transfer of a proton from C6 to C5. Both **25** and **26** assumed a fully hydrated hemiacetal form in aqueous solution. When purified **26** was incubated at 37 °C for 72 h, it completely transformed to a mixture of four additional products, which included two diastereomers of 5-hydroxyhydantoin (**12**) and two diastereomers of the α-hydroxyketone isomer of **12**, 5-hydroxy-3-methylimidazolidine-2,4-dione (**27**). The mechanism of decomposition of **26** probably involves initial ring opening between N1−C6 followed by decarboxylation of an intermediate ureide containing a terminal α-hydroxycarboxylic acid group. The interconversion of the two pairs of diastereomers of **12** and **27** may be accounted for by the following reactions: (1) α-hydroxyketone rearrangement (**12** → **27**) and (2) ring−chain tautomerism at N1−C5 (conversion between diasteromers of **12**) and ring−chain tautomerism at N3−C4 (conversion between diastereomers of **27**). The rates of α-hydroxyketone rearrangement at 37 °C and pH 7 varied from 1.6×10^{-6} to 2.4×10^{-6} s^{-1} (half-life = 80−120 h), whereas the rates of ring−chain tautomerism were about 12-fold slower. When the individual diastereomers were incubated at 37 °C under neutral conditions, they reached equilibrium after about 1 week to give a 50:50 mixture of diastereomers of **12** and **27**. The oxidation of **10** as the nucleoside by Br_2 resulted in the formation of **25** and **26** derivatives bearing an exocyclic amino group.[4b] These results indicate that **10** undergoes oxidation by a similar pathway as **20** and that the deamination of intermediate products takes place on the same time scale as the decomposition of **25** and **26** (several hours). Treatment of trimers as well as longer oligonucleotides (<15-mer) containing **20** with Na_2IrCl_6 led to the quantitative formation of **25** within the oligomer as inferred by MALDI-TOF analysis.[4d] Interestingly, whereas the fully hydrated form of **25** existed as a free nucleoside in aqueous solution, the dehydrated carbonyl form appeared to prevail in single-stranded oligomers. Similar to the modified nucleoside, the trimer containing **25** was observed to undergo a series of transformations from **25** to **26** at initial times (0−10 h) and then appeared to reach equilibrium at later times (30 h) with the formation of **12** and **27**, as monitored by HPLC.

4. Perspectives: Extension of Studies to Cellular DNA

There is still a paucity of relevant information on the formation of cytosine oxidation products in cellular DNA. Efforts should be made by using the highly sensitive and accurate HPLC−MS/MS in the multiple reaction monitoring mode to detect stable cytosine oxidation products such as the 2′-deoxyribonucleoside derivatives of **10**, **11**, and **16a**. With further studies, one should be able to isolate and characterize oxidized cytosine products in cellular DNA upon exposure to •OH and one-electron oxidants, as previously was the case for several other modifications of thymine, guanine, and adenine.[1b]

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FOOTNOTES

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