Conformation and Proton Configuration of Pyrimidine Deoxynucleoside Oxidation Damage Products in Water

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Emerging data strongly suggest that the oxidation of DNA bases can contribute to genomic instability. Structural changes to DNA, induced by base oxidation, may reduce the fidelity of DNA replication and interfere with sequence-specific DNA−protein interactions. We have examined the structures of a series of pyrimidine deoxynucleoside oxidation damage products in aqueous solution. The modified nucleosides studied include the deoxynucleoside derivatives of 5-hydroxyuracil, 5-hydroxycytosine, 5-(hydroxymethyl)uracil, 5-(hydroxymethyl)cytosine, 5-formyluracil, and 5-formylcytosine. The influence of base oxidation on ionization constants, sugar conformation, and tautomeric configuration has been determined on the basis of UV, proton, and nitrogen NMR spectra of the ^{15}N-enriched derivatives. The potential biological consequences of the structural perturbations resulting from base oxidation are discussed.

Introduction

Oxidation of the DNA bases is considered an important source of both exogenous and endogenous genetic damage (*1*−*3*). Significant attention has been paid recently to mechanisms and rates of formation of oxidized bases in both normal and malignant tissue (*1*−*8*). Most of the focus to date has been on the formation of 8-oxoguanine. In a series of structural and biophysical studies, the mechanism of mispair formation of 8-oxoguanine during DNA replication has been determined (*9*−*12*). Parallel biochemical studies have revealed a series of pathways by which mutations resulting from the formation of 8-oxoguanine are prevented or repaired.

Significantly less is known about the biological consequences of pyrimidine oxidation. Pyrimidines are frequently modified in the 5-position. In thymine and 5-methylcytosine, the 5-methyl group may be oxidized to form the 5-hydroxymethyl and formyl derivatives (*13*−*16*). With cytosine, initial formation of cytosine glycol can yield 5-hydroxycytosine by dehydration or 5-hydroxyuracil by hydrolytic deamination followed by dehydration (*4*, *17*).

Among this group of oxidized pyrimidines, 5-hydroxycytosine (*18*, *19*) and 5-formyluracil (*20*−*22*) are known to induce mispair formation during DNA replication. The mechanisms by which oxidation of pyrimidines may increase the level of base mispair formation are as yet unknown. Oxidation of pyrimidines could also interfere with sequence-specific DNA−protein interactions (*23*, *24*), thus altering the control of gene expression. Repair enzymes which selectively remove the oxidized pyrimidine bases have been identified for most of these damage products in both prokaryotic and eukaryotic systems (*25*−*30*). The existence of these repair systems indicates that pyrimidine oxidation does occur in vivo, and that the existence of such damage products in DNA has negative biological consequences.

Recently, we reported the synthesis of a series of 5-substituted pyrimidine deoxynucleoside oxidation damage products containing stable isotopes (*31*). Conformational and tautomeric configurations of the neutral molecules were examined in DMSO. In this paper, we extend these studies to examination of conformational and equilibrium properties in water. The influence of base modification on ionization constants has been determined by measuring the pH dependence of UV spectra. Modifications of sugar conformation have been determined by examining proton−proton coupling constants. The predominant tautomeric forms of the neutral and selected ionized forms of these deoxynucleosides also have been determined by examining the ^{15}N NMR spectra of isotopically enriched derivatives. These properties are likely to be important in understanding the biological consequences of such modifications in DNA.

Materials and Methods

The synthesis and characterization of the pyrimidine deoxynucleoside analogues studied here have been reported previously (*31*). The 5-hydroxy derivative of dCMP (HOdCMP)[1] was prepared by the method of Eaton and Hutchinson (*32*) and

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[1] Abbreviations: dC, 2′-deoxycytidine; DMSO, dimethyl sulfoxide; dT, thymidine; FOdC, 5-formyl-2′-deoxycytidine; FOdU, 5-formyl-2′-deoxyuridine; HMdC, 5-(hydroxymethyl)-2′-deoxycytidine; HMdU, 5-(hydroxymethyl)-2′-deoxyuridine; HOdC, 5-hydroxy-2′-deoxycytidine; HOdCMP, 5-hydroxy-2′-deoxycytidine 5′-monophosphate; HOCDP, 5-hydroxycytidine 5′-diphosphate; HOdU, 5-hydroxy-2′-deoxyuridine; MOdC, N^4-methoxy-2′-deoxycytidine; QM, quantum mechanics.

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characterized by UV and NMR spectrometry. N^4-Methoxy-2′-deoxycytidine, enriched with ^{15}N in the ring N1 and N3 positions, was prepared from enriched 2′-deoxyuridine by the method of Lin and Brown (*33*).

UV spectra were recorded with a Perkin-Elmer lambda 3B ultraviolet/visible spectrophotometer. Ionization constants were determined by assessing the pH dependence of the UV spectra at a constant ionic strength of 0.1 M NaCl at room temperature (*34*).

^1H, ^{15}N, and ^{31}P NMR spectra were recorded with either a Varian 300 or a Varian Unity 500 Plus NMR spectrometer. The sugar conformation was examined by measuring proton−proton coupling constants as previously described (*35*, *36*).

We determined the structure of HOdCMP using first-principle quantum mechanics, including solvation in water (Poisson−Boltzmann continuum-solvation model; *37*). In this solvation approach, the solute is described as a low-dielectric cavity ($\epsilon_1 = 1$) immersed in a high-dielectric continuum of solvent ($\epsilon_2 = 80$ for water). The solute−solvent boundary is described by the surface of closest approach as a sphere with a radius of 1.4 Å (probe radius for water) that is rolled over a van der Waals (vdW) envelope of the solute. On the basis of previous studies (*38*), the following atomic radii were used to build the vdW envelope of the solute: 2.0 Å for sp^2-hybridized C, 1.9 Å for sp^3-hybridized C, 1.50 Å for sp^2-hybridized N, 1.55 Å for sp^3-hybridized N, 1.55 Å for sp^2-hybridized O, 1.50 Å for sp^3-hybridized O, 1.45 Å for ether O, 1.25 Å for H attached to sp^2-hybridized C, 1.15 Å for other H's, and 1.8 Å for P. These calculations used Hartree−Fock (HF) theory with the 6-31G* basis set and were carried out with Jaguar 3.5 (*39*, *40*). The final structures are included as Supporting Information.

We also determined the structures and standard free energies for the N^1-methyl derivatives of uracil, thymine, and 5-formyluracil in their two tautomeric forms (4-keto and 4-enol) using the same method. However, more accurate levels of calculations were carried out for the N^1-methyl derivatives to increase the accuracy of the tautomer calculations. (1) The geometry was optimized further in the more accurate B3LYP/cc-pVTZ(-f) level (*41*−*43*), and (2) the vibration frequencies were calculated in the HF/6-31G* basis set in the gas phase to obtain the zero-point energy correction and the standard Gibbs free energy component after scaling the frequencies by 0.8953 (*44*). The initial structure of HOdCMP was built by replacing the 5-hydrogen of the crystallographic structure of 2′-deoxycytidine 5′-monophosphate (*45*) with a 5-hydroxy group. This structure was used as the starting point for optimizing the structure of the deprotonated form after removing the 5-hydroxy proton.

Results

The pH-dependent UV spectra of this series of nucleoside analogues were recorded, and the values of absorbance maxima and extinction coefficients are presented in Table 1. Ionization constants were determined by examining the pH dependence of the UV spectra, and these values are also presented in Table 1. As an example, the pH-dependent UV spectra of 5-hydroxy-2′-deoxyuridine are presented in Figure 1. A relationship between the apparent p*K* values for this series and the Hammett parameters (*46*) for the 5-substituents was observed and is presented in Figure 2.

For the deoxyuridine series, $pK_a = 9.47 + (-4.34)(\sigma_m) \pm 0.14$.

For the deoxycytidine series, $pK_b = 4.09 + (-3.98)(\sigma_m) \pm 0.19$.

The ^1H NMR spectra of this series in water were measured at a solution pH where the pyrimidine would be predominantly neutral. The values of the chemical shifts of the nonexchangeable proton resonances are presented in Table 2 and proton−proton coupling constants in Table 3. Coupling constants were used to examine sugar conformations, and the results of these determinations are presented in Table 4.

Table 1. Physical Characteristics of the Pyrimidine Deoxynucleosides[a]

	λ_{max} (nm)	ϵ ($\times 10^{-3}$)	pK_1	pK_2
dU	261	10.2	9.26	
HOdU	280	8.2	7.68	11.71
HMdU	264	10.6	9.33	
FOdU	280	13.4	8.12	
dC	271	9.0	4.31	
HOdC	291	7.2	3.75	7.37
HMdC	273	9.2	3.81	
FOdC	282	12.0	2.62	

[a] Values of absorbance maximum and molar absorbtivity are for the neutral molecule. Values of p*K* were determined at 25 °C with 0.1 M NaCl. For HOdU, the first p*K* corresponds to ionization of the 5-hydroxyl proton and the higher value to the dissociation of the N3 imino proton. The lower value for HOdC corresponds to the protonation of the N3 position, whereas the higher value corresponds to ionization of the 5-hydroxyl group.

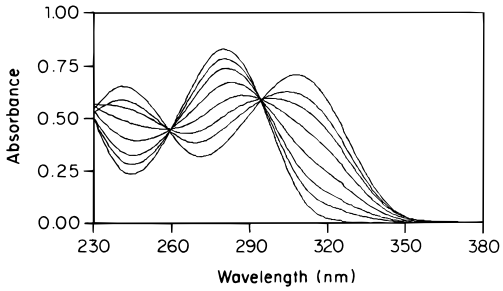

Figure 1. pH-dependent UV spectra of HOdU. The corresponding values of solution pH, with decreasing absorbance at 307 nm, are 9.71, 8.41, 8.11, 7.82, 7.50, 7.14, 6.81, and 5.21. Isosbestic points are observed at 259 and 294 nm.

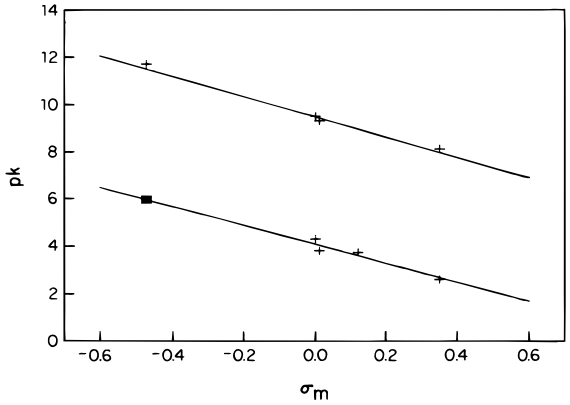

Figure 2. Relationship between the measured p*K* values and Hammett parameters. The p*K* values are listed in Table 1. The upper line corresponds to the dU series, whereas the lower line corresponds to the dC series. Hammett values are found in ref *46*. The black square corresponds to the pK_b that is expected for HOdC if the 5-hydroxy group is ionized.

To examine the predominant tautomeric forms, the proton-decoupled ^{15}N NMR spectra were recorded. The values of the ^{15}N chemical shifts are presented in Table 5. In selected cases, the nitrogen spectra were recorded for the ionized form of a derivative as shown for FOdU in Figure 3 and HOdC as shown in Figure 4.

The tautomeric form of FOdU could be stabilized by intramolecular hydrogen bond formation. Therefore, the relative energies of the keto and enol tautomeric forms in aqueous solution were calculated for the N^1-methyl derivatives (Figure 5). The 4-keto forms of both N^1-

Table 2. ^{1}H Chemical Shifts[a] for Pyrimidine Deoxynucleosides in D_2O

	pD	H6	5- substituent	H1′	H3′	H4′	H5′/H5″	H2′/H2″
dU	7.1	7.85	5.89	6.29	4.47	4.05	3.84, 3.76	2.43, 2.37
HOdU	5.6	7.38	−	6.25	4.40	3.97	3.79, 3.71	2.36, 2.36
HMdU	5.2	7.90	4.38	6.30	4.49	4.06	3.87, 3.78	2.44, 2.38
FOdU	6.2	8.80	9.67	6.26	4.49	4.13	3.92, 3.80	2.55, 2.44
dC	7.3	7.83	6.06	6.27	4.44	4.06	3.85, 3.76	2.44, 2.30
HOdC	6.0	7.44	−	6.28	4.44	4.04	3.85, 3.77	2.40, 2.27
HMdC	7.2	7.89	4.46	6.27	4.45	4.07	3.87, 3.77	2.45, 2.31
FOdC	5.1	8.87	9.57	6.25	4.49	4.20	3.98, 3.85	2.64, 2.43
MO^{4}dC	7.2	7.13	5.73	6.28	4.46	4.01	3.83, 3.75	2.33, 2.33

[a] Shifts are downfield of TMS in parts per million.

Table 3. Proton Coupling Constants for Pyrimidine Deoxynucleosides in D_2O

	H5−H6	H1′−H2′	H1′−H2″	H2′−H3′	H2″−H3′	H3′−H4′	H4′−H5′	H4′−H5″
dU	8.1	6.6	6.6	6.3	5.1	3.9	3.6	5.1
HOdU	−	6.9	6.9	6.3	4.2	3.9	3.6	5.1
HMdU	−	6.6	6.6	6.3	4.8	3.9	3.6	4.8
FOdU	−	5.7	6.3	6.6	5.1	4.5	3.3	4.5
dC	7.5	6.6	6.3	6.6	3.9	3.9	3.6	5.1
HOdC	−	6.6	6.6	6.6	4.5	4.5	3.6	5.4
HMdC	−	6.6	6.6	6.6	4.2	4.5	3.3	4.8
FOdC	−	6.3	6.4	6.3	4.9	4.8	3.3	5.1
MO^{4}dC	8.4	7.2	7.2	5.1	4.2	3.6	3.6	4.9

Table 4. Conformational Analysis of Pyrimidine Deoxynucleosides in D_2O

	% 2′-endo[a]	% gg[b]	predominant tautomeric form
dU	62.9	51.5	keto
HOdU	63.9	51.5	keto
HMdU	62.9	54.6	keto
FOdU	55.9	60.8	keto
dC	62.9	51.5	amino
HOdC	59.5	48.5	amino
HMdC	59.5	57.7	amino
FOdC	56.8	54.6	amino
MO^{4}dC	66.7	53.6	imino

[a] % 2′-endo = $J_{H1'-H2'}/(J_{H1'-H2'} + J_{H3'-H4'})$. [b] % gg = $[13.7 - (J_{H4'-H5'} + J_{H4'-H5''}) \times 100]/9.7$.

Table 5. ^{15}N Chemical Shifts[a] for Isotopically Enriched Pyrimidine Deoxynucleotides in 20% D_2O/H_2O

	pH	N1	N3	N exo	J_{N1-N3}	$J_{N3-N\ exo}$
dU	6.7	90.04	98.20	−	2.6	−
dU−	10.5	92.48	153.31	−	−	−
dC	7.0	96.48	141.56	32.57	−	−
dC+	1.5	96.89	82.82	42.57	2.6	−
HOdU	4.5	78.27	96.16	−	2.6	−
HOdU−	9.7	77.02	100.50	−	−	−
HOdC	5.5	87.92	139.15	27.12	−	−
HOdC+	1.5	86.05	81.75	38.65	2.9	−
HOdC−	10.2	85.99	138.94	25.12	−	−
HMdU	6.0	88.38	97.31	−	2.5	−
FOdU	5.8	100.97	98.79	−	2.6	−
FOdU−	10.2	106.00	152.30	−	−	−
HMdC	5.9	96.93	141.63	31.04	−	−
FOdC	5.8	110.36	140.23	36.52	−	5.4
MO4dC	7.2	75.52	61.76	*b*	2.9	−

[a] Chemical shifts are in parts per million downfield of [^{15}N]-aniline in DMSO (coaxial external standard). [b] The exo nitrogen was not enriched.

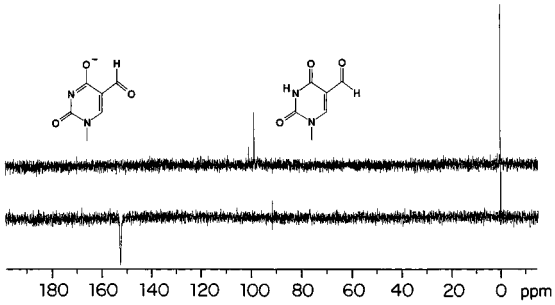

Figure 3. ^{15}N NMR spectra of FOdU in water: (top) neutral species and (bottom) ionized species.

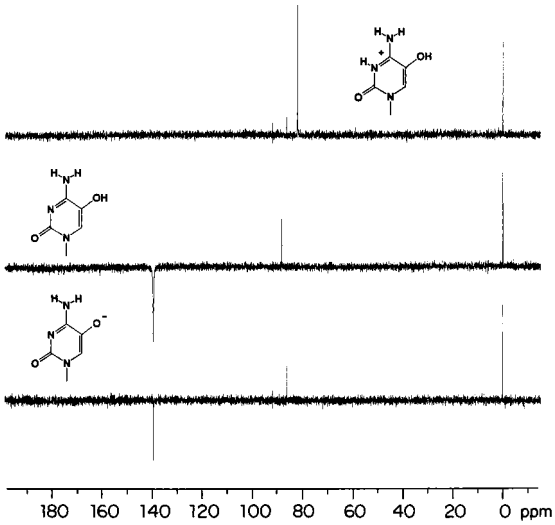

Figure 4. ^{15}N NMR spectra of HOdC in water: (top) protonated species, (middle) neutral species, and (bottom) anionic species.

methyluracil and N^1-methylthymine are more stable than the corresponding 4-enol tautomer by 9.4 and 9.2 kcal/mol, respectively.

The 5-formyl group of FOdU can be in two orientations because the formyl oxygen can be either cis or trans to the 4-keto group. The relative energies of the keto and enol tautomeric forms were calculated for both the cis and trans orientations of the 5-formyl substituent. The trans rotamer was found to be 0.7 kcal/mol more stable than the cis rotamer.

When in the trans orientation, the normal keto tautomer is more stable than the enol form by 10.5 kcal/mol. When the formyl group is in the cis orientation, the keto form still predominates; however, the relative stability of the keto form is reduced to 6.9 kcal/mol. Therefore, upon oxidation of the thymine methyl group, the tauto-

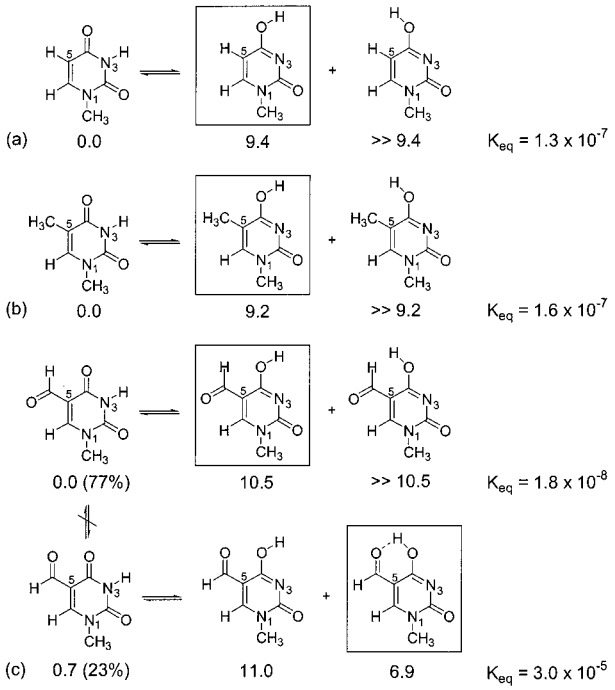

Figure 5. Tautomerization between the keto and enol forms of (a) 1-methyluracil, (b) 1-methylthymine, and (c) 1-methyl-5-formyluracil. The relative energies with respect to the more stable keto form of each compound are given in kilocalories per mole.

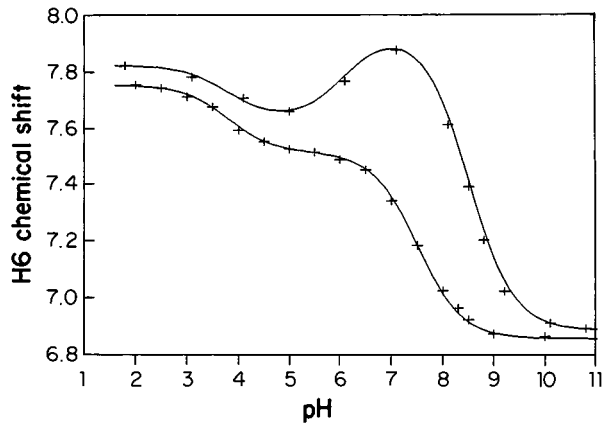

Figure 6. pH dependence of the chemical shift of the H6 proton of HOdC (lower curve) and HOdCMP (upper curve).

meric equilibrium constant (K_{eq} = [enol]/[keto]) can increase from 1.6 × 10^{-7} (thymine) to 3.0 × 10^{-5} when the formyl group is in the trans orientation. The potentially miscoding enol tautomer of 5-formyluracil is stabilized by formation of an intrabase hydrogen bond.

Additional experimental studies were performed with the 5′-phosphate derivative of HOdC, HOdCMP. We observed that the p*K* value of the 5-hydroxy group increases from 7.4 in the deoxynucleoside (HOdC) to 8.5 in the deoxynucleoside 5′-monophosphate (HOdCMP). Changes in p*K* values were measured by titration of both UV and proton NMR spectra (Figure 6). In contrast to HOdC, the line width of the H6 proton of HOdCMP broadened significantly in the vicinity of the p*K* value, indicating an intermediate rate of exchange between two conformationally distinct species (Figure 7).

The ionization constants of the phosphate for dCMP and HOdCMP were measured to be 6.6 and 6.0, respectively, by monitoring the pH-dependent changes in phosphorus chemical shifts.

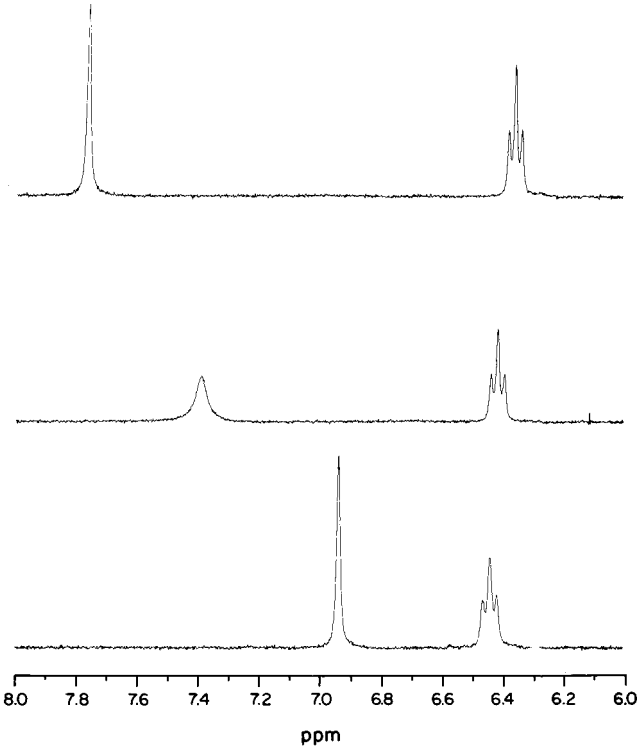

Figure 7. H6 and C1′ region of the ^{1}H NMR spectra of HOdCMP at pH 7 (top), 8.9 (middle), and 10.5 (bottom).

The energy-minimized conformations of HOdCMP in aqueous solution with and without the 5-hydroxyl proton were determined from ab initio QM (HF/6-31G*) and are shown in Figure 8. The energy (without zero-point energy correction) of HOdCMP (Figure 8A) in the folded conformation, in which the phosphate and 5-hydroxyl substituent are on the same side of the sugar, is 0.5 kcal/mol more stable than the extended conformation. However, when the 5-hydroxy substituent is ionized, the folded conformation is 29 kcal/mol less stable than the extended conformation (Figure 8B).

Discussion

Pyrimidine Oxidation and Changes in p*K* Values. The modification of DNA bases by oxidation can substantially alter acid/base properties. Changes in ionization constants, which result in increased amounts of ionized forms near physiological pH, could potentially alter the structure and biological function of DNA. The p*K* values for a series of oxidized pyrimidine damage products have been measured by spectrophotometric titration as reported in Table 1. In the case of the 5-hydroxy derivatives, an additional acidic proton is introduced into the 5-position. The pK_a values for this proton were determined to be 7.37 and 7.68 for HOdC and HOdU, respectively. Because the pK_a values of the 5-hydroxypyrimidines are near physiological pH, both neutral and ionized forms must be considered when examining the biological consequences of these derivatives.

The oxidation of the base moiety in the 5-position can also affect ionization of the N3 position. The electronic-inductive properties of a substituent in the 5-position can influence strongly the ionization of the N3 position.

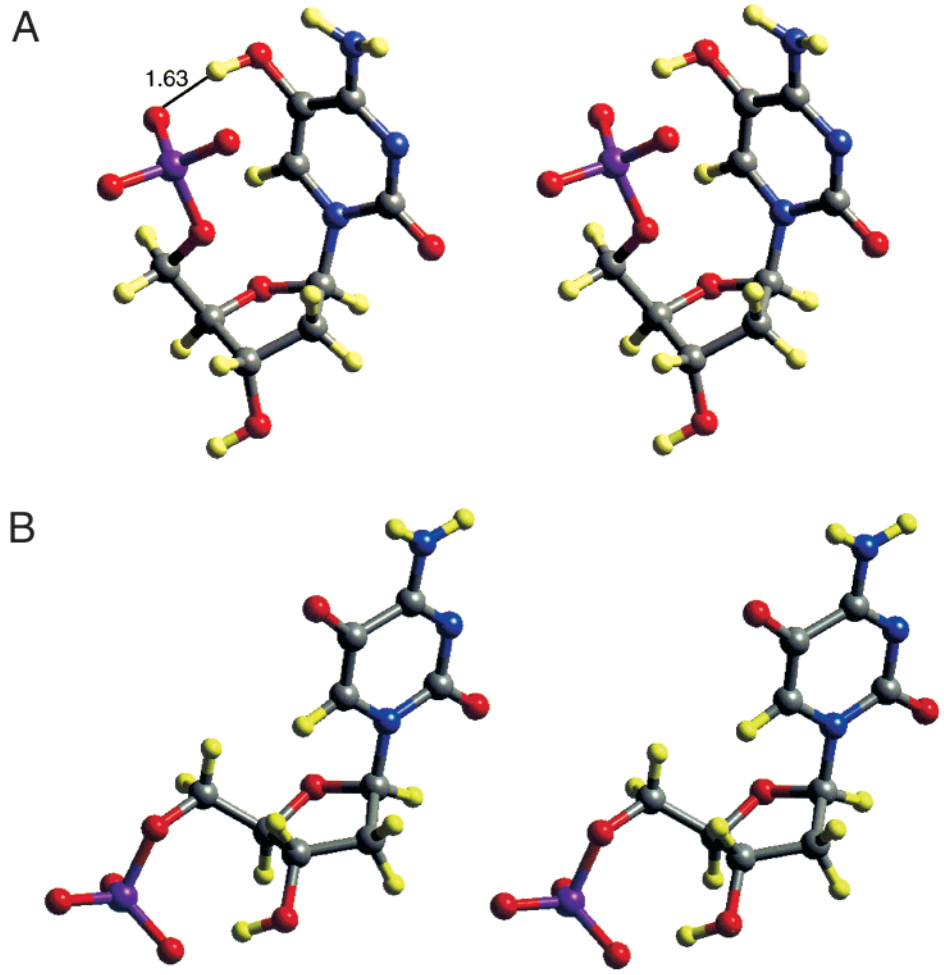

Figure 8. Stereoview of the energy-minimized structures of HOdCMP in aqueous solution (A) in which the 5-hydroxy proton is attached and (B) in the ionized form from ab initio quantum mechanics calculations (HF/6-31G*).

Electron-withdrawing substituents decrease p*K* values, whereas electron-donating substituents increase p*K* values. Previously, we demonstrated a linear Hammett relationship for the 5-substituted deoxyuridine series (*20*). Here, we show that this relationship can be extended to the deoxycytidine series (Figure 2). The observation of a linear Hammett relationship confirms the fact that the modification-induced changes in the p*K* of the N3 position, within the pyrimidine series, can be attributed primarily to the inductive properties of the 5-substituent. Further, the existence of this relationship can be used to estimate p*K* values in cases where experimental values have not been measured.

Among the most profound changes in p*K* values resulting from pyrimidine oxidation is observed upon the oxidation of thymidine to FOdU. The electron-donating methyl group of thymidine decreases the level of ionization at N3 relative to that of deoxyuridine. Conversely, oxidation of thymidine to FOdU results in a reversal of the inductive properties of the 5-substituent from donating to withdrawing. As a consequence, the acidity of the N3 proton increases, as indicated by the drop in the N3 pK_a value from 9.69 (*20*) to 8.12 (Table 1). Relative to thymidine, FOdU would exist as the ionized form to a substantially greater extent at physiological pH. It has been proposed that the ionized form of FOdU could mispair with guanine during polymerase-mediated DNA replication, resulting in a transition mutation (*20*). As shown in Figure 3, ionization of FOdU results in a significant downfield shift of the N3 ^{15}N resonance, consistent with a change in the N3 position from a hydrogen bond donor to acceptor. These data demonstrate that ^{15}N NMR is a powerful tool for examining the hydrogen-bonding potential of modified nucleosides.

In the case of the 5-hydroxy derivatives, HOdU and HOdC, the 5-substituent could be either electron-donating or -withdrawing, depending upon whether the substituent is neutral or ionized. As the pK_a for the 5-hydroxy group is near physiological pH, this equilibrium must be considered important. With HOdU, the 5-hydroxy proton is more acidic than the N3 proton. The ionized 5-hydroxy group is strongly electron-donating with respect to the pyrimidine ring, resulting in an increase in the pK_a to 11.71 for the N3 position. Conversely, the 5-hydroxy substituent of HOdC would be neutral, and therefore electron-withdrawing, below neutral pH. The pK_b of the N3 position of HOdC is decreased from 4.31 (dC) to 3.75 (HOdC). A decrease in the acidity of the N3 proton of HOdU or a decrease in the basicity of the N3 position of HOdC could result in a diminished level of base-pairing interactions in DNA.

Pyrimidine Oxidation and Potential Changes in Tautomeric Form. The oxidation of the pyrimidine base could shift the tautomeric equilibrium toward the unpreferred or minor form. The potential role of rare tautomeric forms in base mispair formation and mutagenesis was first proposed by Watson and Crick (*47*). Whereas the ionization constants of the pyrimidines may

be easily measured by titration, determining the tautomeric form of a pyrimidine derivative is more difficult.

The most unequivocal method for the determination of the tautomeric form for the pyrimidines requires an examination of ^{15}N NMR spectra. The parent pyrimidine deoxynucleosides, dU and dC, are found predominantly in the 4-keto and 4-amino forms, respectively. The essential difference between the two is that the N3 position of dU acts as a hydrogen bond donor whereas the N3 position of dC acts as a hydrogen bond acceptor. A change from "uracil-like" to "cytosine-like" would be accompanied by large changes in the N3 ^{15}N chemical shift.

To verify the relationship between tautomeric form and ^{15}N chemical shift, we prepared the ^{15}N-enriched derivative of N4-methoxy-2′-deoxycytidine (MOdC) and examined its ^{15}N NMR spectrum in water. MOdC is a known mutagenic analogue which is found predominantly in the imino or uracil-like tautomeric form (*48*−*51*). As shown in Table 5, we observed the N3 chemical shift to be 61.76 ppm, significantly upfield of N3 within the dC series, and even further upfield than N3 in the dU series. These data confirmed that base modification could induce a tautomeric shift, and that such a shift could be verified by the measurement of ^{15}N NMR spectra.

Previously, we reported that the N3 resonance in DMSO of the 5-substituted deoxyuridine derivatives was observed at 97.3 ± 1.2 ppm, and for the deoxycytidine series at 150.9 ± 1.0 ppm (*31*). These data indicated that, in DMSO, conversion of the parent pyrimidine to the oxidized derivative had no observable effect upon the preferred tautomeric form.

A tautomeric equilibrium is known to be influenced by changes in solvent (*52*). We therefore examined the ^{15}N NMR spectra of the ^{15}N-enriched derivatives in aqueous solution. A solution pH was selected on the basis of the p*K* values discussed above at which each derivative would be predominantly neutral. Within the dU series, the N3 resonance is observed at 97.6 ± 1.5 ppm, and within the dC series, at 140.6 ± 1.0 ppm (Table 5). The N3 resonance chemical shift for the dU derivatives changes by less than 1 ppm upon moving from DMSO to water. In contrast, the N3 resonance in the dC series uniformly moves upfield by approximately 10 ppm when comparing values in DMSO and water, likely due to formation of a hydrogen bond with water. In accord with the data obtained in DMSO, the results of this study indicate that the formation of the oxidized pyrimidine damage products examined here does not result in an observable change in tautomeric form.

Although the formation of the oxidized pyrimidines studied here does not result in observable tautomeric shifts for the neutral species in aqueous solution, we considered the possibility that ionization and tautomeric changes might be coupled in the case of HOdC. The 5-hydroxy substituent of HOdC ionizes near physiological pH (pK = 7.37), becoming strongly electron-donating to the N3 position. Experimentally, we cannot measure the pK_b of the N3 position when the 5-hydroxy substituent is ionized because of the relative p*K* values. In the case of HOdU, the negatively charged 5-hydroxy substituent increases the pK_a of HOdU by approximately 1.5 units. On the basis of the Hammett plot shown in Figure 2, we would estimate that the pK_b of the N3 position of HOdC, with an ionized 5-substituent, would be approximately 6 (black square in Figure 2). An increase in the basicity of the N3 position could result in a tautomeric shift for anionic HOdC from the amino to the imino tautomeric form. In the case of MOdC discussed above, an electron-withdrawing substituent on the exocyclic amino group results in a tautomeric shift. Recently, Fresco and co-workers presented resonance Raman spectral data supporting the formation of an imino tautomer for anionic HOdC (*53*).

In Figure 4, we present the ^{15}N NMR spectra of HOdC for the cationic, neutral, and anionic forms. At low pH, the N3 position is protonated, and the N3 position becomes uracil-like as indicated by the upfield shift of the N3 resonance. At higher pH, the 5-hydroxy substituent ionizes. Relative to the neutral form, the N3 resonance of anionic HOdC changes by less than 0.25 ppm. These data indicate that the predominant tautomeric form of HOdC, for either the neutral or anionic species, is the normal amino form. A tautomeric shift of the order of magnitude reported by Fresco and co-workers (*53*) is likely below the limit of detection for this method.

Pyrimidine Oxidation and Changes in Sugar Conformation. Previously, it was reported that, within the ribonucleoside series, certain 5-substituents perturbed sugar conformation (*54*). Changes in sugar conformation, induced by oxidation of the pyrimidine, could alter DNA structure and potentially facilitate base mispair formation. Changes induced in the 2′-endo and gauche−gauche equilibrium, based upon proton−proton coupling constants, are shown in Table 4. Within both the dU and dC series, the 5-formyl substituent has the greatest effect upon sugar conformation. We cannot at this point explain the relationship between the pyrimidine modification and the changes in sugar conformation.

Pyrimidine Oxidation and Base Mispair Formation. Ultimately, we explain why specific structural modifications to DNA enhance miscoding during polymerase-mediated DNA replication. In the case of FOdU, modification-induced ionization appears to be the best explanation (*20*). Oxidation of the thymidine methyl group significantly enhances ionization of the N3 position, allowing formation of an ionized FOdU mispair with guanine in a pseudo-Watson−Crick geometry. Recent studies with DNA polymerase, demonstrating an increased level of mispairing with increasing solution pH, are in accord with this model (*55*, *56*). Alternatively, the proximity of the 5-formyl substituent to the 4-keto group could potentially stabilize the aberrant tautomeric form through formation of an intramolecular hydrogen bond.

The data reported here indicate that either ionization or tautomerization, promoted by interbase hydrogen bond formation, could increase the miscoding potential of FOdU. However, at physiological pH, ionization could occur several orders of magnitude more frequently than enol tautomer formation (Figure 5). In contrast to FOdU, the oxidation of thymidine to HMdU does not induce unusual ionization or tautomeric forms. These data are in accord with in vitro polymerase studies that demonstrate that HMdU is not a miscoding lesion (*57*).

The dC derivative, HOdC, is also a mutagenic base analogue (*17*−*19*). The mechanism for the mutagenicity of HOdC is as yet unclear. Fresco and co-workers reported that anionic HOdC could assume a potentially miscoding tautomeric form (*53*). It is possible that the presence of a rare form at a frequency of 1% would not be observed by the methods reported here.

Formation of an Unusual Hydrogen Bond by HOdCMP. In the model reported by Fresco and co-workers, the unusual tautomeric form requires ionization of the 5-hydroxy substituent. The reported pK_a for the 5′-diphosphate of 5-hydroxycytidine (HOCDP) is substantially higher (*32*) than that of HOdC reported here, suggesting that the presence of the 5′-phosphate might alter the ionization of the 5-hydroxy group. As ionization is needed for the proposed tautomeric shift, we conducted further studies with HOdCMP in aqueous solution.

The pK_a value of the 5-hydroxyl proton of HOdCMP was determined to be 8.5 by NMR titration (Figure 6), an increase of 1.1 pH units, as a consequence of the addition of the 5′-phosphate. The H6 proton of HOdCMP is observed to shift downfield during the titration when the pH approaches the phosphate p*K* due to the deshielding effects of the nearby anionic phosphate group. The p*K* of the phosphate group, assessed by the pH dependence of the phosphorus NMR chemical shift, was observed to be 6.0 for HOdCMP, whereas for dCMP, it was 6.6. These data demonstrate that the addition of a 5′-phosphate group can substantially alter the ionization of the pyrimidine 5-hydroxy group.

The H6 resonance of HOdCMP broadens significantly near the p*K* (Figure 7). In contrast, the line width of the H6 resonance of HOdC does not change throughout the titration of the 5-hydroxy group (data not shown). The observed line broadening indicates that the neutral and ionized forms are no longer in fast exchange, but rather that they are approaching intermediate exchange on the NMR time scale. A significantly lower rate of exchange could be explained by a substantial conformational change which accompanies ionization of HOdCMP.

The significant increase in the pK_a of the 5-hydroxy group, the decrease in the pK_a of the phosphate relative to dCMP, and the deshielding of the H6 proton indicate a strong interaction, and likely a hydrogen bond, between the 5-hydroxy substituent and 5′-phosphate of HOdCMP. Such an interaction is also predicted from QM calculations (Figure 8). In HOdC, the normal gauche−gauche conformation predominates, consistent with the model. However, due to significant line broadening observed in the resonances of the 5′- and 4′-protons of HOdCMP, changes in the sugar equilibrium could not be directly assessed.

Ionization of the 5-hydroxy group would eliminate a hydrogen bond with the 5′-phosphate, leaving a substantial electrostatic repulsion, which is consistent with the observed p*K* changes. The line broadening of the H6 resonance at the midpoint of the titration (Figure 7) suggests that the ionization of the 5-hydroxy group must be accompanied by other significant conformational changes.

In the preferred gauche−gauche conformation, which would be enhanced by formation of the 5-hydroxy-5′-phosphate hydrogen bond, the zigzag arrangement of the phosphate through H4′ would maximize the magnitude of the phosphorus H4′ coupling. Due to line broadening, phosphate−proton couplings cannot be measured accurately. However, at pH 10, when the 5-hydroxy is ionized, the line widths become substantially more narrow. At pH 10, no coupling is observed between H4′ and the phosphate, indicating that the phosphate and H4′ are no longer in the zigzag pattern (*58*). The structure predicted from QM of the high-pH form of HOdCMP (Figure 8) suggests that the repulsion between the negatively charged 5-enolate and 5′-phosphate is diminished by rotation around the C4−C5 and C5−O bonds. Such movement is consistent with the observed line broadening of the H6 resonance during the pH transition. Previously, Sarma and co-workers (*58*) presented data that electron-rich atoms in the 6-position of the pyrimidine ring can disfavor the gauche−gauche orientation of pyrimidine nucleoside phosphates, reducing the magnitude of the phosphate−H4′ coupling as observed here.

In a model B-form DNA duplex, the 5-hydroxy proton of a 5-hydroxycytosine residue would be too far to form such a hydrogen bond. If such a hydrogen bond were to form in DNA, it might hold the 5-hydroxycytosine in an aberrant geometry which would promote mispair formation. Alternatively, if such a hydrogen bond cannot form in DNA, the 5-hydroxycytosine residue could be substantially ionized in a DNA template at physiological pH, thereby perturbing other interactions with adjacent bases, including base stacking. Currently, studies are in progress to examine the structure of HOdC in DNA.

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Supporting Information Available: Coordinates for the structures shown in Figure 8. This material is available free of charge via the Internet at http://pubs.acs.org.

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