Synthesis and Characterization of Isotopically Enriched Pyrimidine Deoxynucleoside Oxidation Damage Products

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Oxidative damage to DNA is an established source of genomic instability. In this paper, we describe the synthesis and characterization of several pyrimidine deoxynucleoside oxidation damage products, enriched with stable isotopes. These products include the 2′-deoxynucleoside derivatives of 5-(hydroxymethyl)uracil, 5-formyluracil, 5-hydroxyuracil, 5-(hydroxymethyl)-cytosine, 5-formylcytosine, and 5-hydroxycytosine. The common precursor is 2′-deoxy-2″-deutero[1,3-^{15}N]uridine. Additional stable isotopes are added during functional group conversions. Characterization of these derivatives includes mass spectrometry and ^{1}H and ^{15}N NMR spectroscopy. Proton and nitrogen NMR studies reported here allow an examination of the influence of the modification on sugar conformation and tautomeric equilibrium, properties likely to be important in understanding the biological consequences of these DNA damage products.

Introduction

The DNA of all organisms undergoes continuous cycles of damage and repair. Emerging studies have indicated the biological importance of endogenous damage to DNA, including both oxidation and hydrolysis (*1*−*3*). Exogenous agents including xenobiotics and radiation can significantly increase the formation of oxidation damage products. Biophysical studies are required to determine the mechanisms by which such damaged DNA bases perturb biological systems. Furthermore, careful analytical studies are required to accurately determine the relative contributions of endogenous and exogenous DNA damage to genomic instability.

A substantial volume of data has been generated on purine oxidation products; however, significantly fewer studies have appeared on the pyrimidine oxidation damage products. Biochemical and biophysical studies on the pyrimidine damage products have been limited by the availability of carefully characterized standards. Indeed, unambiguous chemical syntheses of several pyrimidine oxidation damage products described here have not appeared previously in the literature.

In addition, we describe here the synthesis of these pyrimidine deoxynucleoside oxidation damage products enriched with the stable isotopes ^{2}H, ^{15}N, and ^{18}O. Stable isotope enrichment is extremely useful for examining chemical properties, including tautomeric equilibrium, which are likely to be important in determining the biological consequences of these damaged bases. Furthermore, isotopically labeled modified bases incorporated into oligonucleotides should prove invaluable in examining the structures of damaged base pairs by NMR spectroscopy. Although the utility of heteronuclear NMR methods for structure determination is clear, few studies have been conducted on isotopically enriched nucleosides. Indeed, NMR studies on chemically synthesized and isotopically enriched normal pyrimidines such as 2′-deoxycytidine and 2′-deoxyuridine have not appeared in the literature. The few studies which have appeared to date have utilized chemically synthesized free bases or biosynthetic nucleotides.

In addition to their utility in biophysical studies, stable isotope-enriched nucleosides are important analytical standards. Several recent analytical (*4*−*9*) studies have demonstrated that artifactual damage may occur to bases during DNA isolation and that some damage products may be generated, modified, or destroyed during subsequent derivatization prior to analysis. The ideal standards for analytical studies of oxidative and hydrolytic DNA damage products, using mass spectrometric methods, would therefore be isotopically labeled markers which would undergo the same chemical reactions as would the damage products which are the subject of the analysis.

The focus of this paper is on a series of planar pyrimidine oxidation products which are known to occur in DNA. The oxidized thymidine derivatives 5-(hydroxymethyl)-2′-deoxyuridine and 5-formyl-2′-deoxyuridine can be formed by endogenous DNA oxidation, and increased levels are formed as a consequence of ionizing radiation (*10*−*14*). Both have been shown to be mutagenic in vitro (*15*−*18*). Oxidation of cytosine, initially forming the glycol, with subsequent hydrolytic deamination and dehydration generates 5-hydroxyuracil (*10*). All three of these oxidized pyrimidine derivatives are removed from DNA by base-selective glycosylases (*19*−*23*). The existence of repair systems in higher eucaryotes indicates that these derivatives are formed in vivo and that the presence of such derivatives in DNA has harmful biological consequences.

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The methyl group of 5-methylcytosine may be similarly oxidized forming 5-(hydroxymethyl)cytosine and 5-formylcytosine (*24*−*27*). Such derivatives have been less extensively studied. However, because of the potentially important role for cytosine methylation in the control of gene expression (*28*), 5-methylcytosine oxidation is likely to be biologically important. A base-specific glycosylase has been identified in vertebrates for 5-(hydroxymethyl)cytosine residues in DNA (*29*).

Oxidation of cytosine may result in the direct formation of 5-hydroxycytosine, or it may arise by formation of cytosine glycol followed by dehydration (*10*, *30*). This oxidation damage product has been shown recently to be highly mutagenic (*31*, *32*), and it is removed from DNA by endonuclease III (*23*, *33*). While the above-mentioned oxidized bases are likely mutagenic or lethal in vertebrate DNA, several of these oxidized bases, including 5-hydroxyuracil, 5-hydroxycytosine, and 5-formylcytosine, have been identified as minor components of RNA with apparently important structural and regulatory roles (*33*−*36*).

In this paper, we report on the synthesis of this series of oxidatively damaged pyrimidine deoxynucleosides containing stable isotopes prepared by unambiguous chemical syntheses. Such derivatives should prove valuable for analytical studies as well as biophysical studies aimed at understanding the biological consequences of such modified bases in DNA.

Experimental Section

Materials. Stable isotope precursors, urea-$^{15}N_2$ (98%+ ^{15}N-enriched), formaldehyde (98% ^{2}H-enriched), and water (95−98% ^{18}O-enriched) were obtained from Cambridge Isotope Laboratories, Andover, MA. Ammonium hydroxide (3.14 N, 99.9% ^{15}N-enriched) was obtained from Isotec, Inc., Miamisburg, OH, and tributyltin deuteride was obtained from Aldrich Chemical Co., Milwaukee, WI. All other synthetic chemicals were obtained commercially and were of the highest purity available.

Methods. Thin-layer chromatography was performed with glass-backed silica gel plates (Sigma). Thin-layer plates were developed with methanol in dichloromethane. Nucleosides were identified by UV absorbance as well as by reactions with functional group-specific reagents. The presence of deoxyribose was verified by spraying with the Dische diphenylamine reagent (*37*). The aldehyde group present on compounds **6** and **8** was identified with diphenylnitrohydrazine (*38*), and the phenolic group on compounds **2** and **4** was identified by spraying with ferric chloride (*39*).

Gas chromatography−mass spectrometry was performed with a Hewlett-Packard 5890 gas chromatograph interfaced with a 5970 mass-selective detector (*10*). ^{1}H and ^{15}N NMR spectra were recorded with either Varian Unity 500 Plus or 300 MHz NMR spectrometers. Proton NMR spectra in deuterated DMSO were recorded with tetramethylsilane as the internal standard. Nitrogen NMR spectra were recorded with ^{15}N-enriched aniline in deuterated DMSO placed in an external insert tube. The concentration of nucleoside was approximately 3 mg in 700 μL of DMSO. Ultraviolet spectra were recorded with a Perkin-Elmer lambda 3B ultraviolet−visible spectrophotometer.

Synthesis. All modified deoxynucleosides were characterized by TLC, UV, mass spectrometry, and NMR spectroscopy. Values obtained from exact mass measurements and NMR appear in the tables.

Uracil (1a). Uracil was prepared by the method of Harada and Suzuki (*40*, *41*). Polyphosphoric acid (28 g) and ^{15}N-enriched urea (2 g, 32.2 mmol) were placed in a 100-mL round-bottom flask. Propiolic acid (2.4 mL, 1.2 equiv) was added, and the solution was stirred at 80 °C overnight (20 h). The dark orange-brown solution was poured into 100 mL of ice-cold distilled water. Crystals of uracil began to form immediately, and the solution was maintained at 4 °C overnight. Crystalline ^{15}N-enriched uracil was recovered by filtration in 72% yield. With unlabeled materials, the yield for this conversion was as high as 80%.

Uridine (1b). Labeled uracil was converted to uridine using the method of Vorbrüggen (*42*). Labeled uracil and β-D-ribofuranose 1-acetate 2,3,5-tribenzoate were dried under vacuum for 3 h. Labeled uracil (0.82 g, 7.2 mmol) was placed in a 100-mL round-bottom flask with the protected ribose donor (4.7 g, 9.3 mmol, 1.3 equiv), and the flask was purged with argon. Acetonitrile (75 mL) was added to the flask and the flask again purged with argon with continuous stirring at room temperature for 10 min. The reaction flask was cooled in an ice bath, and HMDS (4.6 mL, 21.8 mmol, 3 equiv) was then added via a glass syringe followed by chlorotrimethylsilane (0.9 mL, 7.1 mmol, 1 equiv) and TMSTF[1] (3.3 mL, 18.2 mmol, 2.5 equiv). After addition of the TMSTF, the solution became clear and was stirred for an additional 30 min at 0 °C and then overnight at room temperature.

Solvents were removed under reduced pressure. The residue was dissolved in ethyl acetate and washed with saturated aqueous sodium bicarbonate followed by brine. The organic layer was dried over sodium sulfate and filtered, and the solvent was removed under reduced pressure. The residue was taken up into methanol (70 mL), and saturated aqueous ammonia (45 mL) was added. The resulting solution was stirred for 20 h. The crude product was purified by silica gel chromatography with methanol in dichloromethane. Labeled uridine was washed with hexanes and obtained as a white solid (90% yield). With unlabeled materials, the yield for this conversion was as high as 98%.

2′-Deoxyuridine (1c). Labeled uridine was converted to 2′-deoxyuridine by the method of Robins and co-workers (*43*). A 250-mL round-bottom flask was charged with labeled uridine (1.6 g, 6.5 mmol) which was dried by coevaporation with dry pyridine. Pyridine (60 mL) was then added, and the solution was stirred at 0 °C and purged with argon. To this solution was added TPDS-Cl_2 (2.5 mL, 7.8 mmol, 1.2 equiv) dropwise, and the mixture was stirred at 0 °C for an additional 2 h. Solvents were removed under reduced pressure, and the residue was dissolved in ethyl acetate and washed with distilled water, cold 1 M HCl, saturated aqueous sodium bicarbonate, and brine. The organic phase was dried with sodium sulfate and filtered, and the solvent was removed under reduced pressure.

The silylated product was taken up into acetonitrile (30 mL), cooled to 0 °C, and purged with argon. DMAP (2.4 g, 19.6 mmol, 3 equiv) was added followed by PTC-Cl (1.4 mL, 10.1 mmol, 1.5 equiv). The solution was stirred at 0 °C for 3 h. Solvents were removed under reduced pressure, and the residue was dissolved in ethyl acetate and washed as above. The residue was dried by coevaporation of toluene and then resuspended in toluene (130 mL) and the solution purged with argon. To this solution was added AIBN (213 mg, 1.3 mmol, 0.2 equiv) followed by tributyltin deuteride (5.3 mL, 19.6 mmol, 3 equiv), and the solution was stirred at 75 °C for 3 h. To remove silyl protecting groups, 1 M TBAF/THF (13 mL, 2 equiv) was added and the solution was stirred for an additional 30 min. The solvent was removed under reduced pressure, the residue suspended in dichloromethane/methanol, and the product isolated by silica gel chromatography. Labeled deoxyuridine was isolated in 59%

[1] Abbreviations: HMdU, 5-(hydroxymethyl)-2′-deoxyuridine; HMdC, 5-(hydroxymethyl)-2′-deoxycytidine; FOdU, 5-formyl-2′-deoxyuridine; FOdC, 5-formyl-2′-deoxycytidine; HOdU, 5-hydroxy-2′-deoxyuridine; HOdC, 5-hydroxy-2′-deoxycytidine; AIBN, 2,2′-azobis(2-methylpropionitrile) (78-67-1); DMSO, dimethyl sulfoxide (2206-27-1); DMAP, 4-(dimethylamino)pyridine (1122-58-3); HMDS, 1,1,1,3,3,3-hexamethyldisilazane (999-97-3); PTC-Cl, phenyl chlorothionoformate (1005-56-7); TBAF, tetra-*n*-butylammonium fluoride (429-41-4); TMSTF, trimethylsilyl trifluoromethanesulfonate (27607-77-8); TPDS-Cl_2, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (69304-37-6).

yield. With unlabeled materials, the yield for this conversion was as high as 60%.

5-Hydroxy-2′-deoxyuridine (2). Labeled 2′-deoxyuridine was converted to 5-hydroxy-2′-deoxyuridine by the method of Podrebarac and Cheng (*44*). All glassware was oven-dried overnight, and all handling of materials was done in an argon-purged glovebag. A 100-mL round-bottom flask was charged with labeled 2′-deoxyuridine (200 mg, 0.87 mmol) and coevaporated from 95% ^{18}O-enriched water. Enriched water (^{18}O, 99%, 1 mL) was then added to the flask in an argon-purged glovebag. Bromine-saturated ^{18}O-enriched water (1 mL) was added to the solution, and the solution was stirred until it became clear. Dry pyridine (2 mL) was then added to the flask at 0 °C, and stirring was continued at room temperature overnight. Solvent was removed under reduced pressure, and the product was isolated by silica gel chromatography in 42% yield. With unlabeled materials, the yield for this conversion was as high as 87%. Analysis by GC/MS indicated that enrichment with ^{18}O was 85%.

2′-Deoxycytidine (3). Labeled 2′-deoxyuridine was converted to 2′-deoxycytidine by the method of Divakar and Reese (*45*). A 100-mL round-bottom flask was charged with 2′-deoxyuridine (600 mg, 2.6 mmol) which was dried by coevaporation of dry pyridine. Pyridine was added followed by acetic anhydride. The solution was stirred at room temperature for 3 h. The solvent was removed under reduced pressure, and the residue was dried by coevaporation of toluene.

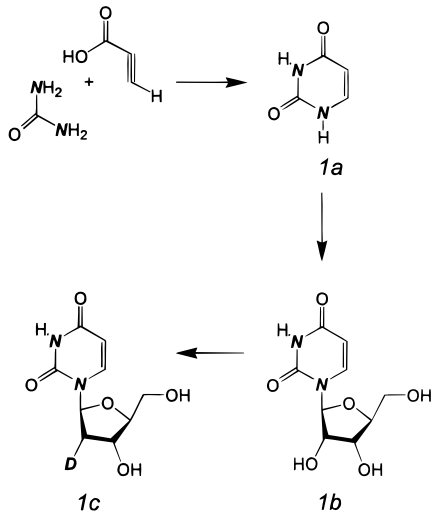
The dry, acetylated nucleoside was suspended in dry acetonitrile. The solution containing 1,2,4-triazole, $POCl_3$, and triethylamine in acetonitrile was then added dropwise. The solution was stirred for 1 h at which time water and triethylamine were added to quench the reaction. The solvent was removed under reduced pressure, and the residue was dissolved in dichloromethane and washed as above. Solvent was removed under reduced pressure, and the residue was suspended in 1,4-dioxane to which ^{15}N-enriched ammonium hydroxide was added. The solution was allowed to stir at room temperature overnight. Conversion of the intermediate triazalo nucleoside to the cytidine derivative was monitored by changes in the UV spectrum of an aliquot of the reaction.

Deprotection was completed by slow addition of sodium ethoxide which was neutralized upon the addition of acetic acid. Solvent was removed under reduced pressure, and the product was isolated by silica gel chromatography in 65% yield.

5-Hydroxy-2′-deoxycytidine (4). Labeled 2′-deoxycytidine was converted to 5-hydroxy-2′-deoxycytidine by the method of Eaton and Hutchinson (*46*). A 100-mL round-bottom flask was charged with labeled 2′-deoxycytidine (200 mg, 0.86 mmol) and coevaporated from 95% ^{18}O-enriched water. Enriched water (^{18}O, 99%, 1 mL) was then added to the flask in an argon-purged glovebag. Bromine-saturated ^{18}O-enriched water (1 mL) was added to the solution, and the solution was stirred until it became clear. Collidine (2 mL) was then added to the flask at 0 °C, and stirring was continued at room temperature overnight. The solvent was removed under reduced pressure, and the product was isolated by silica gel chromatography in 23% yield. Analysis by GC/MS indicated that ^{18}O-enrichment was 82%. With unlabeled materials, the yield for this conversion was as high as 86%.

5-(Hydroxymethyl)-2′-deoxyuridine (5). Labeled 2′-deoxyuridine was converted to 5-(hydroxymethyl)-2′-deoxyuridine with aqueous formaldehyde as previously described (*47*, *48*); however, potassium hydroxide was replaced by triethylamine (*49*). The labeled 2′-deoxyuridine (500 mg, 2.2 mmol) was placed in a 10 mL Teflon-lined screw-cap vial. Aqueous paraformaldehyde (^{2}H, 99% enriched, 20% solution, 3 mL) and 4 mL of triethylamine were added, and the vial was vortexed until the nucleoside had dissolved. The vial was then heated at 60 °C for 6 days. Solvent was removed under reduced pressure, and the product was isolated by silica gel chromatography in 39% yield. With unlabeled materials, the yield for this conversion was as high as 90%.

Scheme 1. Preparation of Labeled 2′-Deoxyuridine



5-Formyl-2′-deoxyuridine (6). Labeled 5-(hydroxymethyl)-2′-deoxyuridine (100 mg, 0.38 mmol) was converted to 5-formyl-2′-deoxyuridine with rigorously stirred excess activated MnO_2 in DMSO (*13*) overnight at room temperature. The product was isolated in 74% yield.

5-(Hydroxymethyl)-2′-deoxycytidine (7). Labeled 5-(hydroxymethyl)-2′-deoxyuridine (540 mg, 2.0 mmol) was converted to 5-(hydroxymethyl)-2′-deoxycytidine in the same manner described above by which 2′-deoxyuridine was converted to 2′-deoxycytidine, and the product was isolated with a yield of 48%. With unlabeled materials, the yield for this conversion was as high as 50%.

5-Formyl-2′-deoxycytidine (8). Labeled 5-(hydroxymethyl)-2′-deoxycytidine (100 mg) was converted to 5-formyl-2′-deoxycytidine with activated MnO_2 in DMSO as with 5-formyl-2′-deoxyuridine. The product was isolated by silica gel chromatography in 67% yield.

Exact Mass Measurements. Exact mass measurements were performed at the University of California, Riverside on a ZAB-SE4F instrument operating in either the positive or negative FAB mode using a water, glycerol, poly(ethylene glycol) matrix.

Determination of Sugar Conformation. Sugar conformations were determined based upon measurement of proton−proton coupling constants as described previously (*50*, *51*). The percentage of the 2′-endo conformation was determined as follows:

$$\text{percentage C2′-endo} = [100 \times J(1'2')]/[J(1'2') + J(3'4')]$$

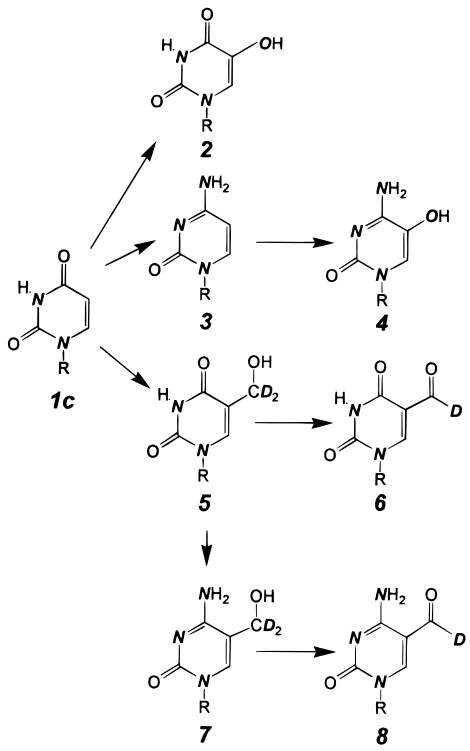
The percentage of the gauche−gauche (gg) rotamer was determined as follows:

$$\text{percentage gg} = \{[13.7 - (J(4'5') + J(4'5''))] \times 100\}/9.7$$

Results and Discussion

Uracil (**1a**), enriched in both the N1 and N3 ring nitrogens (Scheme 1), is conveniently and efficiently obtained from enriched urea as previously described (*40*, *41*). Uracil is converted to the ribonucleoside **1b** in high yield by the method of Vorbrüggen (*42*). Labeled uridine (**1b**), converted to labeled 2′-deoxyuridine (**1c**) as described by Robins and co-workers for unlabeled uridine (*43*), serves as the deoxynucleoside precursor for the remaining pyrimidine oxidation products. Reduction of uridine to 2′-deoxyuridine provides the opportunity to introduce deuterium into the 2″ position thus increasing the mass of the deoxynucleoside by 1 mass unit. Deuteration of the 2″ position also reduces the complexity of

Scheme 2. Preparation of the Deoxynucleoside Derivatives



the observed proton−proton couplings for the remaining sugar protons which simplifies examination of the sugar pucker equilibrium.

Enriched 2′-deoxyuridine (**1c**) is then converted to the remaining pyrimidine deoxynucleosides as shown in Scheme 2. Deoxyuridine is converted to 2′-deoxcytidine (**3**) via the 4-triazole intermediate and ammonolysis with enriched ammonia as described by Divakar and Reese (*45*). The 5-hydroxy derivatives **2** and **4** are generated in high yield from the parent deoxynucleosides **1c** and **3** by bromination followed by hydrolysis and dehydration in pyridine/water and collidine/water, respectively (*44*, *46*).

The derivatives resulting from oxidation of the 5-methyl group of thymidine (**5** and **6**) and 5-methyl-2′-deoxycytidine (**7** and **8**) have a common precursor, 5-(hydroxymethyl)-2′-deoxyuridine (**5**), which is generated from deoxyuridine (**1c**) in alkaline aqueous formaldehyde. Previously, hydroxymethylation of deoxyuridine was performed by constant addition of potassium hydroxide (*47*, *48*). The overall yield of the reaction was reduced by the presence of large quantities of salt. Alternatively, replacement of potassium hydroxide by triethylamine (*49*) considerably reduces problems associated with purification and subsequently increases overall yields. The 5-hydroxymethyl derivative of deoxycytidine (**7**) is produced more efficiently by amination of **5** rather than hydroxymethylation of **3**. The corresponding aldehyde derivatives (**6** and **8**) are generated by oxidation of the hydroxymethyl derivatives with activated manganese dioxide in dimethyl sulfoxide (*13*).

The conversion reactions are easily followed by TLC analysis as each modification results in significant changes in R_f. Several functional group tests which are performed on the TLC plates assist in monitoring the conversions, including the diphenylamine reaction for the deoxyribose sugar present in **1c**−**8** (*37*), dinitrophenylhydrazine for the aldehyde derivatives **6** and **8** (*38*), and ferric chloride for the 5-hydroxy derivatives **2** and **4** (*39*). All of the pyrimidine deoxynucleoside oxidation products were purified by flash chromatography.

Prior to synthesis of the labeled derivatives, a series of unenriched derivatives was prepared and characterized. The identity of each compound was confirmed by the UV spectrum and comparison with literature values where possible, exact mass measurements (Table 1), and ^{1}H NMR spectra (Table 2). Derivatives enriched with stable isotopes were then prepared and characterized in a similar fashion. Derivatives enriched with ^{15}N were also characterized by measurement of the ^{15}N NMR spectra as described below.

The N1 resonance of enriched dU (**1c**) is observed at 87.46 ppm downfield from external ^{15}N-enriched aniline. The corresponding N1 resonance of dC (**3**) is observed at 96.47 ppm. The N3 resonance of dU is observed at 98.12 ppm, whereas the corresponding N3 resonance of dC is observed at 151.66 ppm. The significant difference in the chemical shift of these two N3 resonances is attributed to the differences in the predominant tautomeric forms of dU and dC. The amino nitrogen resonance of dC is observed at 33.48 ppm. For dU, a coupling of 2.8 Hz is observed between the N1 and N3 resonances. For dC, no coupling is observed between N1 and N3; however, a coupling of 6.2 Hz is observed between N3 and the exocyclic amino nitrogen. Reciprocal proton−nitrogen couplings are observed in the proton-coupled spectra as described below.

The resonances observed in the proton NMR spectrum of the unenriched naturally occurring deoxynucleosides dU and dC are presented in Table 2. Upon substitution of ^{15}N into the N1 and N3 positions of dU and dC as well as the exocyclic N4 amino group of dC, the complexity of the proton NMR spectrum increases significantly. The imino resonance of dU at 11.26 ppm is split into a doublet with a coupling constant of 89.7 Hz (Table 3). Similarly, the amino protons of dC which resonate at 7.17 and 7.05 ppm are split by 89.9 Hz. The magnitude of these one-bond proton−nitrogen coupling constants indicates direct attachment of the protons and confirms the keto and amino tautomeric conformations of dU and dC, respectively, as shown in Scheme 2.

Enrichment with ^{15}N also increases the complexity of the nonexchangeable proton resonances for dU and dC as shown in Figure 1. In the unenriched derivatives, the H5 and H6 resonances of dU and dC are coupled to one another and appear as doublets with coupling constants of 8.1 and 7.4 Hz, respectively. Upon ^{15}N enrichment, the low-field H6 resonance of dU is split by coupling to N1 with a coupling constant of 2.1 Hz. In the case of dC, however, no coupling is observed between H6 and N1. The H5 resonance of dU is observed as a doublet of doublet of doublets corresponding to coupling to H6 (8.1 Hz), N1 (4.5 Hz), and N3 (2.4 Hz), respectively. For dC, the H5 resonance is observed as a doublet of doublets with coupling to H6 (7.4 Hz) and N3 (3.6 Hz).

For both dU and dC, the magnitude of the N1−H1′ coupling is sufficiently small that no apparent coupling is observed. However, as shown in the two-dimensional proton−nitrogen spectrum of dU (Figure 2), for which the acquisition parameters were set for small magnitude couplings (J = 3 Hz), cross-peaks are observed between N1 and the imino proton H3 as well as H6, H1′, H5, and H2′. Three-bond coupling is also confirmed between N3

Table 1. Exact Mass Measurements for Deoxynucleosides

		natural abundance			enriched		
compd	mode		theor	exptl		theor	exptl
dU	−	$C_9H_{11}N_2O_5$	227.0668	227.0679	$C_9H_{10}O_5{}^2H^{15}N_2$	230.0670	230.0663
HOdU	−	$C_9H_{11}N_2O_6$	243.0617	243.0618	$C_9H_{10}O_5{}^2H^{15}N_2{}^{18}O$	248.0662	248.0655
HMdU	−	$C_{10}H_{13}N_2O_6$	257.0773	257.0782	$C_{10}H_{10}O_6{}^2H_3{}^{15}N_2$	262.0899	262.0903
FOdU	−	$C_{10}H_{11}N_2O_6$	255.0617	255.0608	$C_{10}H_9O_6{}^2H_2{}^{15}N_2$	259.0683	259.0696
dC	+	$C_9H_{14}N_3O_4$	228.0984	228.0975	$C_9H_{13}O_4{}^2H^{15}N_3$	232.0957	232.0969
HOdC	+	$C_9H_{14}N_3O_5$	244.0933	244.0942	$C_9H_{13}O_4{}^2H^{15}N_3{}^{18}O$	250.0949	250.0964
HMdC	+	$C_{10}H_{16}N_3O_5$	258.1090	258.1085	$C_{10}H_{13}O_5{}^2H_3{}^{15}N_3$	264.1186	264.1191
FOdC	+	$C_{10}H_{14}N_3O_5$	256.0933	256.0923	$C_{10}H_{12}O_5{}^2H_2{}^{15}N_3$	261.0968	261.0965

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

compd	HN/NH$_2$	H6	5 substituent	H1′	H3′	H4′	H5′,5″	H2′,2″
dU	11.26	7.85	5.63	6.15	4.23	3.78	3.58, 3.53	2.10, 2.05
HOdU	11.41	7.33	8.64	6.18	4.22	3.75	3.54, 3.54	2.06, 2.00
HMdU	11.30	7.72	4.13	6.19	4.22	3.86	3.57, 3.52	2.09, 2.07
FOdU	11.73	8.71	9.76	6.09	4.24	3.86	3.64, 3.57	2.24, 2.18
dC	7.05, 7.17	7.79	5.73	6.15	4.20	3.76	3.56, 3.51	2.10, 2.18
HOdC	6.71, 7.31	7.21	8.88	6.18	4.19	3.74	3.54, 3.53	2.05, 1.90
HMdC	6.58, 7.29	7.74	4.17	6.17	4.20	3.76	3.58, 3.53	2.10, 1.94
FOdC	7.86, 8.11	8.84	9.46	6.07	4.23	3.87	3.68, 3.60	2.31, 2.10

[a] Shifts are downfield of TMS in ppm.

Table 3. Chemical Shifts[a] and Coupling Constants (Hz) for Isotopically Enriched Pyrimidine Deoxynucleotides in DMSO

compd	N exo	N1	N3	J_{N-H}	J_{N1-N3}	$J_{N3-N\ exo}$	J_{N1-N6}
dU		87.46	98.12	89.7	2.8		2.1
HOdU		74.50	95.87	89.7	2.8		1.6
HMdU		84.70	96.75	89.7	2.4		2.1
FOdU		99.42	98.51	89.7	2.6		1.0
dC	33.48	96.47	151.66	89.9		6.2	
HOdC	27.53	84.94	151.82	91.6, 91.3		6.1	
HMdC	31.38	95.50	151.53	89.9, 88.1		6.4	
FOdC	35.66	113.13	148.44	91.5, 90.0		6.1	

[a] Chemical shifts are in ppm downfield of [^{15}N]aniline in DMSO.

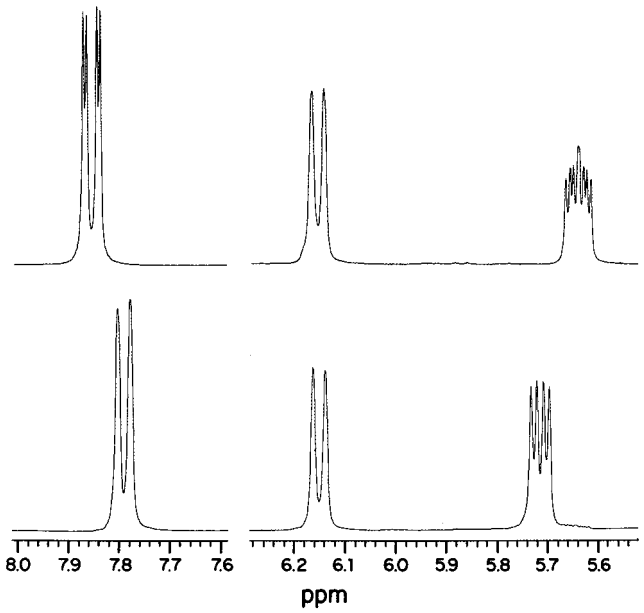


Figure 1. ^{1}H NMR spectrum of the nonexchangeable base and H1′ proton of isotopically enriched dU (upper) and dC (lower).

and H5. These data suggest that, although small in magnitude, the coupling between ring nitrogens and nonexchangeable protons is sufficient for unequivocal proton assignments in selectively enriched oligonucleotides which may be of particular importance with unusual DNA structures where proton assignment via through-space connectivities is not possible. The above data presented for dU and dC are consistent with previously published data on free base and ribonucleoside derivatives of uracil and cytosine (*52*−*56*). To our knowledge, such data on the oxidized pyrimidine nucleosides described below have not previously appeared in the literature.

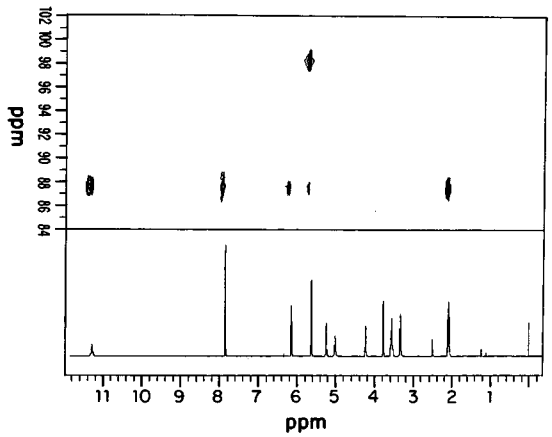


Figure 2. Two-dimensional ^{1}H−^{15}N HMQC spectrum of enriched dU. The magnitude of the delay was optimized for observation of small-magnitude proton−nitrogen couplings (J = 3 Hz).

The 5-hydroxyl derivatives HOdU (**2**) and HOdC (**4**) are known DNA oxidation products, and both can result in base mispair formation during DNA replication. The mechanism of mispair formation is as yet unknown. Conversion from the parent to the 5-hydroxyl derivative can be conducted in ^{18}O-enriched water permitting an increase in the mass of the product by an additional 2 mass units. The enriched derivatives reported here are a total of 5 and 6 mass units higher than the corresponding unenriched derivatives.

In both cases, substitution with the 5-hydroxyl group induces an upfield change in the chemical shift of the H6 resonance by 0.52 and 0.58 ppm relative to the parent derivatives dU and dC, respectively. For both HOdU and HOdC, substitution results in relatively small changes in the chemical shifts of the N3 resonances; however,

Table 4. Conformational Analysis of Pyrimidine Deoxynucleosides in DMSO

compd	% 2′-endo	% gg	predominant tautomeric form
dU	74.4	60.8	keto
HOdU	75.9	66.4	keto
HMdU	74.8	61.6	keto
FOdU	64.8	72.2	keto
dC	73.3	63.5	amino
HOdC	77.2	56.5	amino
HMdC	73.3	58.8	amino
FOdC	62.3	67.0	amino

such substitution results in significant upfield changes in the chemical shifts of the N1 resonances of 12.96 and 11.53 ppm, respectively. The N3 nitrogen resonance as well as the N3 imino proton resonance of enriched HOdU is split by 89.7 ppm. Similarly, the amino protons of enriched HOdC are observed as doublets with coupling constants of 91.6 and 91.3 Hz. As with the parent derivative dU, coupling is observed between N1 and N3 as well as N1 and H6 for HOdU. For HOdC, coupling is observed between N3 and the exocyclic amino nitrogen (6.1 Hz).

The proximity of the 5-hydroxyl substituent to the 4-keto and 4-amino groups of HOdU and HOdC suggested that intrabase hydrogen bonding may offset the tautomeric equilibrium in favor of a minor enol or imino conformation. The data presented here on the ^{15}N chemical shifts and nitrogen−proton coupling constants, however, demonstrate that substitution with the 5-hydroxyl group does not substantially alter the tautomeric equilibrium of the neutral derivatives in DMSO.

Minor changes are observed in other conformational parameters for HOdU and HOdC determined from the magnitudes of proton−proton couplings. With both of the 5-hydroxyl derivatives, the proportion of the normal 2′-endo conformation increases slightly as observed in the ribonucleoside series (*57*). With HOdU, the percentage of the normal gauche−gauche conformation about the C4′−C5′ bond increases relative to the parent dU; however, a change of a similar magnitude, but opposite in direction, is observed with HOdC.

Both HMdU (**5**) and HMdC (**7**) may result from oxidation of the 5-methyl group of thymidine and 5-methyl-2′-deoxycytidine, respectively. Both derivatives are also found as normal constituents in the DNA of certain bacteriophages (*58*). Conversion of dU to HMdU can be accomplished using deuterated formaldehyde in alkaline aqueous solution, increasing the mass of the products by 2 additional mass units. Replacement of potassium hydroxide with triethylamine considerably simplifies isolation of reaction products. The hydroxymethyl methylene protons observed at 4.13 and 4.17 ppm in the unenriched derivatives of HMdU and HMdC, respectively, are absent in the ^{1}H NMR spectrum of the ^{2}H-enriched derivatives.

Substitution with the 5-hydroxymethyl group induces only minor changes in the chemical shifts of the nitrogen resonances of both HMdU and HMdC (Table 3). The N3 resonance of HMdU and the amino nitrogen resonances of HMdC are coupled by approximately 90 Hz to the corresponding imino and amino protons, and reciprocal splitting is observed in the proton NMR spectrum. Both HMdU and HMdC in DMSO are therefore predominantly in the normal keto and amino tautomeric forms, respectively.

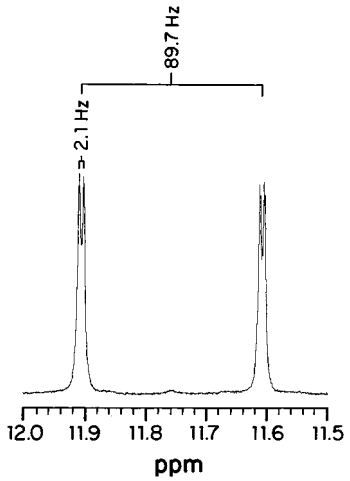


Figure 3. ^{1}H NMR spectrum of the imino region of enriched 5-formyl-2′-deoxyuridine (FOdU). The large coupling (89.7 Hz) results from the one-bond coupling of the imino proton to N3, whereas the smaller coupling (2.1 Hz) results from three-bond coupling to N1.

Substitution with the hydroxymethyl group induces little or no changes in the sugar pucker; however, as with the 5-hydroxyl derivatives, 5-hydroxymethyl substitution increases the proportion of the gauche−gauche rotamer in the dU series but results in a decrease in the dC series (Table 4). The potential for hydrogen bonding between the 5-hydroxymethyl group and the 4-keto or 4-amino group also exists, and therefore, such hydrogen bonding may explain the observed perturbations in the rotameric equilibrium for the 5-hydroxymethyl derivatives as well.

Oxidation of the 5-hydroxymethyl to 5-formyl derivatives **6** and **8** is accomplished in high yield with activated manganese dioxide in DMSO. Both FOdU (**6**) and FOdC (**8**) have been identified as oxidation damage products of thymidine and 5-methyl-2′-deoxycytidine, respectively. Little is currently known about the consequences of FOdC formation in DNA; however, FOdU is known to be mutagenic and can result in DNA−protein cross-links due to its capacity to condense with amines (*59*).

The aldehydic protons of FOdU and FOdC resonate at 9.76 and 9.46 ppm, respectively, and these signals are lost in the proton spectrum of the deuterated derivative. Substitution with the 5-formyl group shifts the H6 resonance downfield by 0.86 and 1.05 ppm relative to the unsubstituted parent deoxynucleosides dU and dC. In both FOdU and FOdC, the chemical shift of the N3 resonance changes little from that of the parent dU and dC derivatives. However, the chemical shift of the N1 resonance of FOdU has moved 11.96 ppm downfield and that of FOdC by 16.66 ppm.

The mispairing potential of FOdU (**6**) during DNA replication might be attributed to a shift of the tautomeric equilibrium in favor of the enol form stabilized by a hydrogen bond between the proton at the 4-position of the enol tautomer with the carbonyl of the 5-formyl group. However, the imino proton at 11.73 ppm is split by 89.7 ppm due to one-bond coupling to N3 and 2.1 Hz due to three-bond coupling to N1 (Figure 3), establishing that the predominant tautomeric form of FOdU in DMSO is the normal keto configuration. Similarly, the predominant tautomeric form of FOdC in DMSO is the normal amino configuration. In contrast to the other substituents, however, we observe that for both FOdU and FOdC,

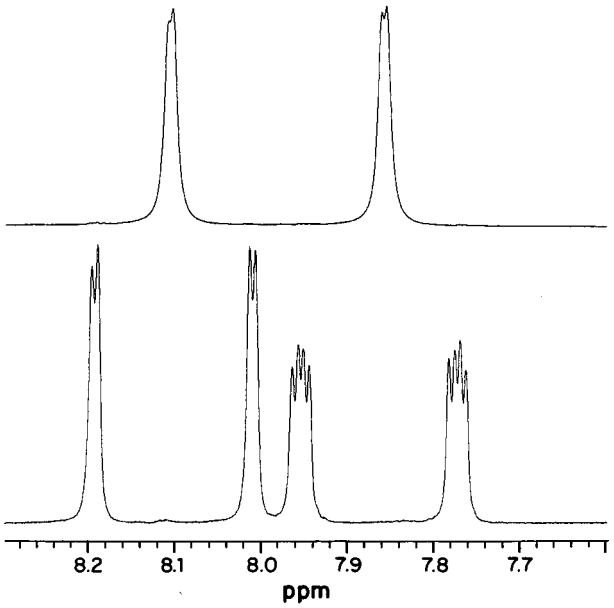


Figure 4. 500-MHz ^{1}H NMR spectrum of the amino proton region of 5-formyl-2′-deoxycytidine (FOdC) unenriched (upper) and enriched (lower). Both amino protons of the enriched derivative are directly coupled to N4; however, the upper field amino resonance is additionally coupled to N3.

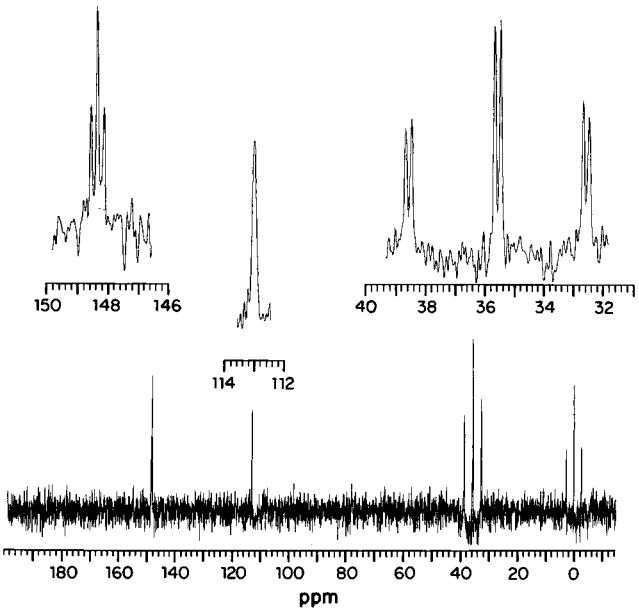


Figure 5. Proton-coupled ^{15}N spectrum of labeled 5-formyl-2′-deoxycytidine. From lower field, the resonances correspond to N3, N1, exocyclic amino group, and external [^{15}N]aniline, respectively. The resonances are shown enlarged to show the smaller magnitude couplings.

the sugar pucker equilibrium is shifted away from the normal 2′-endo conformation. In both cases, however, the rotameric equilibrium is shifted in favor of the gauche−gauche form, perhaps due to the formation of a hydrogen bond between the 5-formyl carbonyl oxygen and the 5′-hydroxyl.

With FOdC, hydrogen bonding is also possible with the 4-amino group when the 5-formyl oxygen is trans to the H6 proton. Evidence supporting such a hydrogen bond is the unusually narrow line width of the amino protons observed at 7.86 and 8.11 ppm (Figure 4). Indeed, the resonances are sufficiently narrow that small coupling (3.6 Hz) is observed between the amino protons. Upon substitution with ^{15}N, both amino resonances are split by approximately 90 Hz, confirming the assignment of the predominant amino tautomeric form. The amino resonance to higher field, however, shows additional coupling of 6.6 Hz. An examination of the proton-coupled ^{15}N spectrum reveals that this additional coupling is with the N3 nitrogen. Three resonances are seen in the nitrogen spectrum of FOdC (Figure 5) corresponding to the N3, N1, and amino nitrogens. The amino nitrogen is coupled to each of the amino protons as well as to N3 (6.1 Hz). The N1 nitrogen is observed as a singlet. The N3 nitrogen is observed as a triplet due to coupling to N4 as well as to only one of the amino protons. It is somewhat surprising that only one of the amino protons couples to N3; however, selective coupling of only one of the amino protons has been previously reported for cytosine hydrochloride, enriched in both the amino group and ring nitrogen, in DMSO (*52*).

Conclusions

The synthetic scheme presented in this paper provides several of the biologically important pyrimidine deoxynucleoside oxidation damage products identified thus far by routes sufficiently unambiguous to generate standards for analytical studies. The conversions are efficient enough to be used for the generation of the isotopically labeled derivatives, and isotopic enrichment of up to 6 additional mass units has been obtained from relatively inexpensive enriched reagents.

Examination of the NMR spectra of these isotopically enriched derivatives provides perhaps the most unambiguous determination of the predominant tautomeric form. The 5-substituents studied have substantial electronic effects upon the pyrimidines as indicated by significant changes in both ^{1}H and ^{15}N chemical shifts. Further, the proximity of the 5-substituent to the 4-keto or 4-amino groups introduces the possibility of changes in tautomeric form stabilized by intrabase hydrogen bonding. In all of the cases examined here, however, substitution did not shift the predominant tautomeric form. When incorporated in oligonucleotides, however, the local base-pairing environment may induce tautomeric shifts (*60*). Isotopically labeled derivatives have been previously used to establish the mode of hydrogen-bonding and base-pairing interactions in DNA (*61*, *62*), and similar studies are currently in progress with the pyrimidine damage products reported here.

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