

Quantification of the 2-Deoxyribonolactone and Nucleoside 5'-Aldehyde Products of 2-Deoxyribose Oxidation in DNA and Cells by Isotope-Dilution Gas Chromatography Mass Spectrometry: Differential Effects of γ -Radiation and Fe^{2+} -EDTA

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Abstract: The oxidation of 2-deoxyribose in DNA has emerged as a critical determinant of the cellular toxicity of oxidative damage to DNA, with oxidation of each carbon producing a unique spectrum of electrophilic products. We have developed and validated an isotope-dilution gas chromatography-coupled mass spectrometry (GC-MS) method for the rigorous quantification of two major 2-deoxyribose oxidation products: the 2-deoxyribonolactone abasic site of 1'-oxidation and the nucleoside 5'-aldehyde of 5'-oxidation chemistry. The method entails elimination of these products as 5-methylene-2(5H)-furanone (5MF) and furfural, respectively, followed by derivatization with pentafluorophenylhydrazine (PFPH), addition of isotopically labeled PFPH derivatives as internal standards, extraction of the derivatives, and quantification by GC-MS analysis. The precision and accuracy of the method were validated with oligodeoxynucleotides containing the 2-deoxyribonolactone and nucleoside 5'-aldehyde lesions. Further, the well-defined 2-deoxyribose oxidation chemistry of the enediyne antibiotics, neocarzinostatin and calicheamicin γ_1^1 , was exploited in control studies, with neocarzinostatin producing 10 2-deoxyribonolactone and 300 nucleoside 5'-aldehyde per 10^6 nt per μM in accord with its established minor 1'- and major 5'-oxidation chemistry. Calicheamicin unexpectedly caused 1'-oxidation at a low level of 10 2-deoxyribonolactone per 10^6 nt per μM in addition to the expected predominance of 5'-oxidation at 560 nucleoside 5'-aldehyde per 10^6 nt per μM . The two hydroxyl radical-mediated DNA oxidants, γ -radiation and Fe^{2+} -EDTA, produced nucleoside 5'-aldehyde at a frequency of 57 per 10^6 nt per Gy (G-value 74 nmol/J) and 3.5 per 10^6 nt per μM , respectively, which amounted to 40% and 35%, respectively, of total 2-deoxyribose oxidation as measured by a plasmid nicking assay. However, γ -radiation and Fe^{2+} -EDTA produced different proportions of 2-deoxyribonolactone at 7% and 24% of total 2-deoxyribose oxidation, respectively, with frequencies of 10 lesions per 10^6 nt per Gy (G-value, 13 nmol/J) and 2.4 lesions per 10^6 nt per μM . Studies in TK6 human lymphoblastoid cells, in which the analytical data were corrected for losses sustained during DNA isolation, revealed background levels of 2-deoxyribonolactone and nucleoside 5'-aldehyde of 9.7 and 73 lesions per 10^6 nt, respectively. γ -Irradiation of the cells caused increases of 0.045 and 0.22 lesions per 10^6 nt per Gy, respectively, which represents a \sim 250-fold quenching effect of the cellular environment similar to that observed in previous studies. The proportions of the various 2-deoxyribose oxidation products generated by γ -radiation are similar for purified DNA and cells. These results are consistent with solvent exposure as a major determinant of hydroxyl radical reactivity with 2-deoxyribose in DNA, but the large differences between γ -radiation and Fe^{2+} -EDTA suggest that factors other than hydroxyl radical reactivity govern DNA oxidation chemistry.

Introduction

DNA damage caused by a variety of endogenous oxidants and reactive chemicals generated by the innate immune system may play a significant role in the pathophysiology of inflam-

mation, cancer and degenerative diseases.¹⁻³ While studies of the chemistry of nucleobase damage have dominated the literature, there is growing evidence that oxidation of 2-deoxyribose in DNA plays a critical role in the toxicity of oxidative stress, whether as individual lesions, as part of complex DNA

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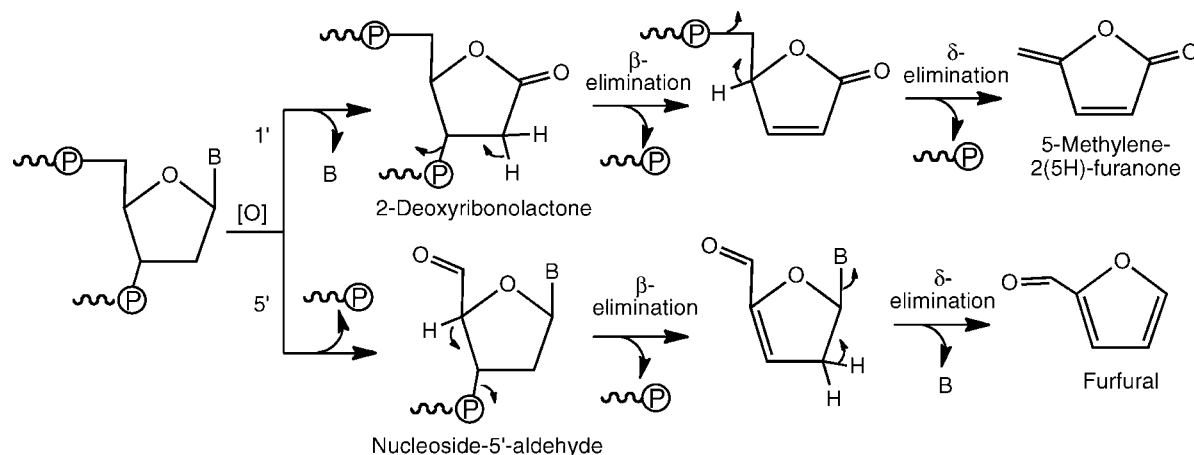
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Scheme 1. Elimination Products of the 2-Deoxyribonolactone and Nucleoside 5'-Aldehyde Products of 2-Deoxyribose in DNA

lesions with closely opposed strand breaks and oxidized abasic sites,^{4,5} or as participants in protein–DNA cross-links, and protein and DNA adducts.⁶ For hydroxyl radical and other reactive oxygen and nitrogen species, the oxidation of each of carbon in 2-deoxyribose in DNA occurs with an initial hydrogen atom abstraction to form a carbon-centered radical that, under normoxic conditions, adds molecular oxygen to form a peroxy radical;⁶ conditions of low oxygen concentration promote the formation of cyclic nucleotide products such as the 5',8-cyclopurine product of 5'-oxidation.⁷ The degradation of the peroxy radical results in the formation of a variety of electrophilic and genotoxic products that differ for each position.⁶ We have previously quantified hydroxyl radical-induced formation of several of these products, including the 3'-phosphoglycolate and 2-deoxypentose-4-ulose abasic site of 4'-oxidation,⁸ the 5'-(2-phosphoryl-1,4-dioxobutane) residue of 5'-oxidation⁹ and the 3-phosphoglycolaldehyde that arises in 3'-oxidation.^{6,10–12}

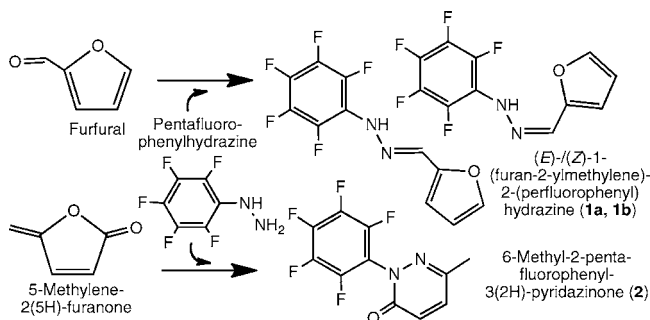
The present studies focus on the oxidation of the 1'- and 5'-positions in 2-deoxyribose in DNA, as shown in Scheme 1. Oxidation of the 1'-carbon produces a 2'-deoxyribonolactone abasic site that undergoes a rate-limiting β -elimination reaction to form a butenolide species with a half-life of 20 h in single-stranded DNA (32–54 h in duplex DNA) followed by a rapid δ -elimination to release 5-methylene-2(5H)-furanone (5MF).^{13–16} An analogous β -/ δ -elimination reaction with the 3-oxonucleotide product of 3'-oxidation yields 2-methylene-3(2H)-furanone

(2MF).^{17,18} The chemistry of 5'-oxidation partitions to form two sets of products: a 3'-formylphosphate-ended fragment and a 2-phosphoryl-1,4-dioxo-2-butane residue that undergoes β -elimination to form a *trans*-butenedialdehyde species; or a nucleoside 5'-aldehyde residue that can undergo β -/ δ -eliminations to produce furfural.^{19–21} Under conditions of low oxygen concentration, the 5'-radical can react to form 5',8-cyclopurine products.⁷

The goals of the present studies were two-fold. The first was to develop a method to rigorously quantify the 5MF and furfural elimination products of the 2-deoxyribonolactone and nucleoside 5'-aldehyde, respectively. With formation half-lives on the order of 25–100 h at 37 °C,^{13,22} these elimination products should arise spontaneously in cells and tissues and thus have potential for use as biomarkers of oxidative stress and as probes for the fate of DNA damage products in biological systems. The second goal was to rigorously define the contributions of these 2-deoxyribose oxidation products to the spectrum of products arising from hydroxyl radical reactions with DNA. While previous studies of the nucleoside 5'-aldehyde have been descriptive in nature, attempts to quantify the 2-deoxyribonolactone have led to the conclusion that the 2-deoxyribonolactone represents one of the most abundant DNA oxidation products caused by hydroxyl radicals.^{15,16,23} This stands in contrast to studies of 2-deoxyribose oxidation by putative hydroxyl radicals generated by Fe^{2+} –EDTA, in which deuterium isotope effects indicated relative reactivity in the order $5' > 4' > 3' \approx 2' \approx 1'$.²⁴ We sought to define the absolute and relative contributions of 2-deoxyribonolactone and nucleoside 5'-aldehyde to the chemistry of 2-deoxyribose oxidation in DNA by hydroxyl radical, with the conclusion that the level of 2-deoxyribonolactone varies significantly for Fe^{2+} –EDTA and γ -radiation. We have also performed studies in irradiated human cells, with

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Scheme 2. Reaction of 5MF and Furfural with Pentafluorophenylhydrazine

the observation of similar proportions of 2-deoxyribose oxidation products arising *in vitro* and in cells, and a ~250-fold protective effect against γ -radiation-induced 2-deoxyribose oxidation in DNA provided by the cellular environment.

Results

Development and Optimization of a GC–MS Method for the Quantification of 2-Deoxyribonolactone and Nucleoside 5'-Aldehyde in Oxidized DNA. As shown in Schemes 1 and 2, the overall strategy of quantifying the 2-deoxyribonolactone and nucleoside 5'-aldehyde products entails: (1) their elimination as 5MF and furfural, respectively, from oxidized DNA (Scheme 1); (2) derivatization of 5MF and furfural with pentafluorophenylhydrazine (PFPH) (Scheme 2); and (3) analysis of the derivatives by gas chromatography-coupled mass spectrometry (GC–MS) using isotopomeric internal standards.

The first step in method development involved synthesis and characterization of isotopically labeled and unlabeled forms of the PFPH derivatives of 5MF and furfural for use as standards. The reaction of PFPH with furfural led to the expected geometric isomers of the resulting hydrazone (**1a**, **1b** in Scheme 2), as observed previously by Ho and Yu.²⁵ **1a** and **1b** were well-resolved chromatographically (Figure 1) and showed identical electron ionization (EI) mass spectra (Supporting Information Figure 1A), with the combined signal for both isomers used to quantify the furfural-PFPH derivative from DNA samples. Reaction of PFPH with 5MF afforded an unexpected single product, 6-methyl-2-(perfluorophenyl)pyridazin-3(2H)-one (**2**), the structure of which is shown in Scheme 2. An attempt was

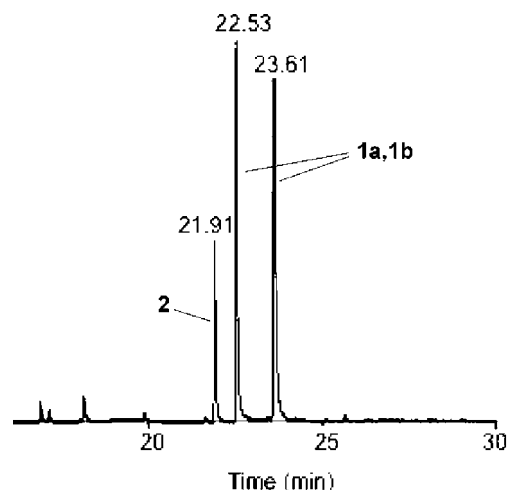


Figure 1. GC–MS chromatogram of **1a**, **1b**, and **2** with selected ion monitoring at m/z 276.

made to synthesize the PFPH derivative of 2MF, with the latter synthesized from thymidine by the method of Stubbe and co-workers.²⁶ A single product was identified by GC–MS, with a molecular ion at m/z 276 as the base peak, together with characteristic fragment ions at m/z 93 [C_3F_3]⁺, 117 [C_5F_3]⁺, 148 [C_6F_4]⁺, and 167 [C_6F_5]⁺. However, this conjugate proved to be too unstable for use as a standard, and we could not identify a species with the same retention time and m/z value in DNA samples exposed to γ -radiation or Fe^{2+} –EDTA (data not shown), perhaps due to the instability of 2MF–PFPH derivative. As shown in Supporting Information Figure 1, the chromatographically well resolved **1a**, **1b** and **2** all produced a strong molecular ion signal at m/z 276 that was used for subsequent quantitative analyses.

The next step was to define analytical parameters for the GC–MS quantification of **1a**, **1b**, and **2**. Following definition of the GC retention times and mass spectral behavior of **1a**, **1b**, and **2**, calibration curves were prepared by mixing heated, PFPH-treated samples of calf thymus DNA with fixed amounts of isotopically labeled **1a**, **1b**, and **2** and variable amounts of unlabeled forms and extracting **1a**, **1b**, and **2** into dichloromethane. Plots of the peak area ratios for unlabeled and labeled PFPH derivatives were linear with slopes of 0.0016 pmol^{-1} and 0.0033 pmol^{-1} for **1a+1b** and **2**, respectively (see Supporting Information Figure 2). Lines fitted by linear regression do not pass through the origin due to the presence of background levels of DNA oxidation products in the calf thymus DNA and other possible sample processing contaminants. The background level is equivalent to 1.65 pmol of **1a+1b** and 0.485 pmol of **2** in $250 \mu\text{g}$ of DNA or 2.2 1a+1b per 10^6 nt and 6.3 2 per 10^7 nt . These values represent the practical limit of quantification.

The final step was to validate the analytical method and determine the overall efficiency of the elimination, derivatization and extraction steps using oligodeoxynucleotides containing defined quantities of 2-deoxyribonolactone and nucleoside 5'-aldehyde damage products. A 17-mer oligodeoxynucleotide, 5'-TGTGCCXAACTTACCGT-3', containing 2-deoxyribonolactone at **X** (**3**) was prepared with >92% purity by UV irradiation of a nitrobenzyl cyanohydrin nucleoside-containing precursor, as described by Zheng and Sheppard¹³ (Supporting Information Figure 3). A 3-mer oligodeoxynucleotide, 5'-TGC-3', with a nucleoside 5'-aldehyde terminus at T (**4**) was isolated in >95% purity by HPLC purification of the major product of a reaction of a self-complementary, duplex oligodeoxynucleotide, 5'-GCATGC-3', with the enediyne neocarzinostatin (Supporting Information Figure 3), as described by Sugiyama et al.²⁷ The nucleoside 5'-aldehyde-containing single-strand oligodeoxynucleotide had a half-life of 34 h at ambient temperature and pH 7.4 (Supporting Information Figure 4), which is in reasonable agreement with the half-life in double-stranded DNA of 101 h at 37 °C determined by Greenberg and co-workers²² given the stability conferred by duplex DNA for many damage products (e.g., 2-deoxyribonolactone¹³).

These well-characterized standards were used to validate the analytical method by spiking $250 \mu\text{g}$ of calf thymus DNA with **3** (88 pmol) or **4** (293 pmol) followed by heat and polylysine-catalyzed elimination of 5MF and furfural, respectively, derivatization with PFPH and GC–MS analysis of methylene chloride extracts, as described in Experimental Methods. The

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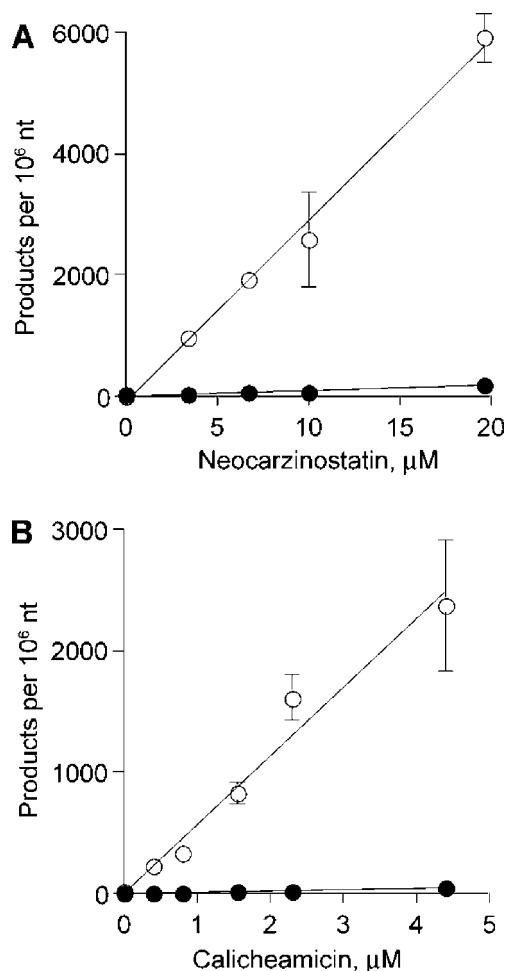


Figure 2. Quantification of 5MF (●) and furfural (○) in DNA exposed to neocarzinostatin (A), and calicheamicin γ_1^1 (B). DNA was treated with the oxidants and quantified by GC–MS as described in the Experimental Methods. The data represent mean \pm SD for three independent experiments. Fitting the data by linear regression yielded lines with the following equations: (A) 5MF: $y = 8.4x + 11$ ($r^2 = 0.96$); furfural: $y = 299x - 94$ ($r^2 = 0.99$); (B) 5MF: $y = 10x + 0.26$ ($r^2 = 1.0$); furfural: $y = 564x + 7.0$ ($r^2 = 0.99$).

yields of **1a+1b** and **2** were found to be $43 \pm 2.4\%$ and $21 \pm 0.7\%$ of the theoretical value, respectively, which represents the overall efficiency for the analytical method. Measurements of furfural and 5MF were thus corrected by a factor of 2.3 and 4.8, respectively, to arrive at the quantity of nucleoside 5'-aldehyde and 2-deoxyribonolactone, respectively.

Quantification of 2-Deoxyribonolactone and Nucleoside 5'-Aldehyde in DNA Oxidized by Eneidyne Antibiotics, γ -Radiation, and Fe^{2+} –EDTA. The validated method was applied to quantify 2-deoxyribonolactone and nucleoside 5'-aldehyde residues in DNA and cells exposed to the enediyne antibiotics, neocarzinostatin and calicheamicin, as positive controls and to γ -radiation and Fe^{2+} –EDTA. Neocarzinostatin is known to cause 1'-, 4'-, and 5'-oxidation of 2-deoxyribose in DNA,²⁸ which is consistent with the observation of $8.4 (\pm 0.99)$ 2-deoxyribonolactone and $298 (\pm 14)$ nucleoside 5'-aldehyde residues per 10^6 nt per μM (Figure 2A). Calicheamicin γ_1^1 has been proposed to produce only 4'- and 5'-oxidation,²⁸ yet we observed a low level of $10 (\pm 0.34)$ 2-deoxyribonolactone residues per 10^6 nt per μM in addition to the expected

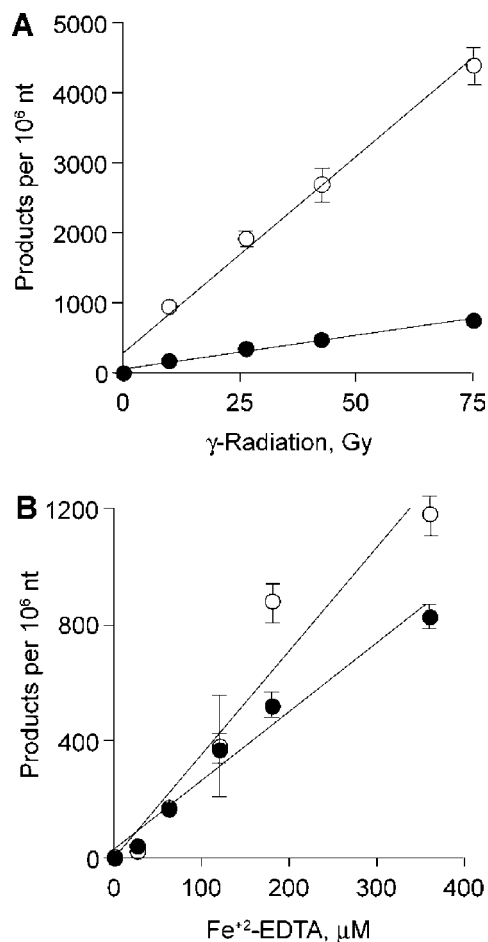


Figure 3. Quantification of 5MF (●) and furfural (○) in DNA exposed to γ -radiation (A), Fe^{2+} –EDTA (B). DNA was treated with the oxidants and quantified by GC–MS as described in the Experimental Methods. The data represent mean \pm SD for three independent experiments. Fitting the data by linear regression yielded lines with the following equations: (A) 5MF: $y = 9.7x + 59$ ($r^2 = 0.98$); furfural: $y = 57x + 268$ ($r^2 = 0.99$); (B) 5MF: $y = 2.4x + 30$ ($r^2 = 0.97$); furfural: $y = 3.5x + 1.7$ ($r^2 = 0.94$).

5'-chemistry with $564 (\pm 49)$ nucleoside 5'-aldehyde residues per 10^6 nt per μM (Figure 2B). This is consistent with selectivity rather than specificity in the oxidation of 2-deoxyribose by enediynes in DNA, and with the minor-groove location of 1'-, 4'-, and 5'-hydrogen atom targets for all of the enediynes.⁶

Studies with the hydroxyl radical generators γ -radiation and Fe^{2+} –EDTA revealed dose-dependent formation of 2-deoxyribonolactone and nucleoside 5'-aldehyde damage products. As shown in Figure 3, γ -radiation produced $10 (\pm 0.75)$ 2-deoxyribonolactone and $57 (\pm 3.7)$ nucleoside 5'-aldehyde residues per 10^6 nt per Gy, which represents radiation chemical yields (G-values) of 13 and 74 nmol/J, respectively. Fe^{2+} –EDTA produced $2.4 (\pm 0.20)$ 2-deoxyribonolactone and $3.5 (\pm 0.46)$ nucleoside 5'-aldehyde per 10^6 nt per μM .

A more informative context for these results involves their expression as a percentage of total number of 2-deoxyribose oxidation events. This is achieved by quantifying total 2-deoxyribose oxidation by plasmid topoisomer analysis, which is a well established technique that accounts for all 2-deoxyribose oxidation events by exploiting the conversion of supercoiled plasmid DNA to nicked and linear forms following direct strand breaks and, after derivatization with agents such as putrescine,

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oxidized abasic sites.^{29–38} We previously established the frequency of total 2-deoxyribose oxidation at 141 events per 10^6 nt per Gy for γ -irradiation and at 10 events per 10^6 nt per μM for Fe^{2+} –EDTA using an identical irradiation source, DNA concentration, buffer and temperature conditions employed for the present studies.^{8,9,11,12} Linear regression analysis of plots of the quantities of 2-deoxyribonolactone and nucleoside 5'-aldehyde lesions versus calculated total 2-deoxyribose oxidation events at the various doses of γ -radiation revealed that 2-deoxyribonolactone accounted for $\sim 7\%$ of γ -radiation-induced 2-deoxyribose oxidation ($y = 0.069x + 0.059$, $r^2 = 0.98$), while nucleoside 5'-aldehyde residues accounted for $\sim 40\%$ of the damage ($y = 0.40x + 0.27$, $r^2 = 0.99$). On the other hand, $\sim 24\%$ of the 2-deoxyribose oxidation by Fe^{2+} –EDTA was composed of 2-deoxyribonolactone ($y = 0.24x + 0.03$, $r^2 = 0.97$), while $\sim 35\%$ was nucleoside 5'-aldehyde residues ($y = 0.35x + 0.0017$, $r^2 = 0.94$).

Quantification of 2-Deoxyribonolactone and Nucleoside 5'-Aldehyde in DNA in Human Cells Exposed to γ -Radiation. To assess the effects of the cellular environment on the formation of 2-deoxyribonolactone and nucleoside 5'-aldehyde in oxidized DNA, TK6 human lymphoblastoid cells were γ -irradiated and the quantity of 5MF and furfural determined in isolated DNA. An important facet of this experiment involved an assessment of the effect of the DNA isolation process on the level of 5MF and furfural measured in the isolated DNA. To this end, a control experiment was performed in which purified and irradiated DNA, containing a defined quantity of 2-deoxyribonolactone and nucleoside 5'-aldehyde, was added to cells during the DNA isolation process and the quantities of 5MF and furfural determined in the total isolated DNA. The measured quantities of 5MF and furfural were expressed relative to the quantity of purified and irradiated DNA that was added to the cells, with the background contribution of 5MF and furfural from the cellular genomic DNA removed by analysis of the slope of a γ -radiation dose–response curve. As shown in Supporting Information Figure 6, linear regression analysis revealed efficiencies of 5.3 5MF per 10^6 nt per Gy and 44 furfural per 10^6 nt per Gy. These yields correspond to 53% and 77%, respectively, of the yields obtained with irradiated purified DNA used to spike the cell samples (10 5MF and 57 furfural per 10^6 nt per Gy). These results reveal a tangible loss of 2-deoxyribonolactone and nucleoside 5'-aldehyde during the cell processing steps and provide correction factors of 1.9 and 1.3 for cellular yields of 5MF and furfural, respectively, in addition to the correction factors of 4.8 and 2.3, respectively, for the analytical method.

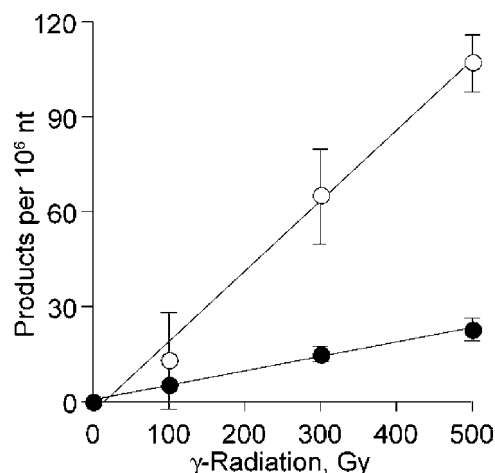


Figure 4. Formation of 5MF (●) and furfural (○) in γ -irradiated human TK6 cells. Cells were exposed to γ -radiation at ambient temperature and processed for GC–MS analysis as described in the Experimental Methods. Data were corrected for losses during DNA isolation and sample processing for GC–MS analysis as described in the text and background levels were subtracted. The data represent means \pm SD for three independent experiments. Fitting the data by linear regression yielded lines with the following equations: 5MF: $y = 0.045x + 1.4$ ($r^2 = 0.99$); furfural: $y = 0.22x + 1.1$ ($r^2 = 0.99$). The slopes are statistically significant at $p < 0.003$, and the results for 5MF and furfural at 300 and 500 Gy are significantly different from each other by t test with $p < 0.0045$ and $p < 0.0001$, respectively.

The results of the analysis of 2-deoxyribonolactone and nucleoside 5'-aldehyde in irradiated TK6 cells are shown in Figure 4. After correction for losses during DNA isolation and processing, we observed background levels of 2-deoxyribonolactone and nucleoside 5'-aldehyde of $9.7 (\pm 0.22)$ and $73 (\pm 1.5)$ lesions per 10^6 nt, respectively. Exposure of TK6 cells to γ -radiation resulted in corrected damage frequencies of $0.045 (\pm 0.0028)$ 5MF per 10^6 nt per Gy and $0.22 (\pm 0.012)$ furfural per 10^6 nt per Gy (Figure 4).

Discussion

The emerging appreciation for the biological importance of 2-deoxyribose oxidation in DNA has motivated efforts to define the quantities of the various DNA-bound and diffusible products, in an effort to identify the chemistry relevant to the biological situation.⁶ We have now expanded the repertoire of analytical methods with the development of an accurate, precise and sensitive method to quantify two major products of 2-deoxyribose oxidation: the 2-deoxyribonolactone abasic site arising from 1'-oxidation of DNA and the nucleoside 5'-aldehyde residue arising from 5'-oxidation of DNA. This approach exploits the simple chemical release of quantifiable electrophiles from oxidized DNA. The application of this method has revealed important quantitative features of 2-deoxyribose oxidation in purified DNA and in cells.

The isotope-dilution GC–MS approach developed here represents the most quantitative and chemically specific method for analysis of both the nucleoside 5'-aldehyde and 2-deoxyribonolactone products of 2-deoxyribose oxidation in DNA. We have established the overall efficiency of the elimination, derivatization and analytical steps using chemically and quantitatively well-defined substrates, which provides a critical correction for artifacts arising during sample processing. The accuracy of the methods was further validated in studies with the DNA-cleaving enediyne antibiotics, neocarzinostatin and calicheamicin. Both drugs undergo thiol-induced cyclization to

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produce a diradical species that, when positioned in the minor groove of DNA, abstracts hydrogen atoms from 2-deoxyribose on each strand.²⁸ Neocarzinostatin produces 2 single-strand lesions for every double-strand lesion, with 5'-oxidation occurring on one strand of a double-strand lesion and in most single-strand damage events.²⁸ Calicheamicin produces mainly double-stranded DNA damage, with 4'-oxidation on one strand and 5'-oxidation on the other.²⁸ Both drugs are also very efficient at producing DNA damage, with the concentration of damage events closely approximating the concentration of added drug.²⁸ These features of calicheamicin- and neocarzinostatin-induced formation of nucleoside 5'-aldehyde residues are consistent with our observation of high levels of furfural at 564 and 299 lesions per 10⁶ nt per μ M, respectively, which translates into 0.4 and 1 μ M concentrations of nucleoside 5'-aldehyde residues per μ M concentration of each drug. The lesser quantity of 1'-oxidation apparent with neocarzinostatin (8 5MF per 10⁶ nt per μ M) is consistent with the occurrence of 1'-chemistry only in double-stranded lesions and only in a small subset of damage sites (GC-containing damage sites).²⁸ The observation of a minor amount of 2-deoxyribonolactone associated with calicheamicin-induced damage suggests a degree of flexibility in the binding of the activated drug or in the selectivity of the diradical species for minor groove-accessible 2-deoxyribose hydrogen atoms. These results verify the accuracy of the methods.

Studies of the nucleoside 5'-aldehyde have to date been primarily descriptive in nature,^{19,22} as have earlier studies of the 2-deoxyribonolactone using various combinations of gas, liquid and other types of chromatography with mass spectrometric or UV spectroscopic detection.³⁹⁻⁴¹ More recent methods for quantifying 2-deoxyribonolactone have improved sensitivity, but were not chemically specific or rigorously quantitative.^{15,16,23} Greenberg and co-workers developed a biotinylated cysteine derivative that reacts with the butenolide β -elimination product of the 2-deoxyribonolactone (Scheme 1) to form a heat-stable cyclic amide.^{15,16} The method can detect the 2-deoxyribonolactone lesion in samples of oxidized DNA with an apparent limit of quantification in the high fmol/low pmol range. However, the accuracy of the method has not been rigorously established with quantitatively defined standards and the specificity of the probe has not been established exhaustively in reactions with the other α,β -unsaturated mono- and dicarbonyl species generated during DNA oxidation (e.g., nucleoside 5'-aldehyde and 1,4-dioxo-2-phosphorylbutane from 5'-oxidation, 3'-phosphoglycolaldehyde and the 3-oxonucleotide from 3-oxidation, etc.). These problems may be reflected in the conclusion that the 2-deoxyribonolactone represents 75–80% of all aldehyde- or ketone-containing 2-deoxyribose oxidation products arising with γ -radiation and Fe²⁺–EDTA.^{15,16} This would mean that aldehyde- and ketone-containing 2-deoxyribose oxidation products, which are produced from 2', 3', 4', and 5'-oxidation, represent less than one-quarter of total 2-deoxyribose oxidation products. Such a high proportion of the 2-deoxyribonolactone lesion is at odds with the site-specific deuterium isotope effect studies of Tullius and co-workers and with our chemically specific observation of the 2-deoxyribonolactone as 7% and 24% of 2-deoxyribose oxidation produced by γ -radiation and Fe²⁺–EDTA, respectively.

As the basis for our method, Razskazovskiy and co-workers took a different approach with greater specificity for the 2-deoxyribonolactone abasic site than the probe-based approach. This entailed elimination of the ribonolactone as 5MF (Scheme 1) followed by HPLC resolution and quantification of 5MF by UV spectroscopy.²³ They exploited their observation of a catalytic effect of polyamines and several metal ions for increasing the efficiency of heat-induced release of 5MF from irradiated DNA, with maximal release occurring with polylysine.²³ Our finding of 10 2-deoxyribonolactone abasic sites per 10⁶ nt per Gy of γ -radiation is significantly higher than the 1 lesion per 10⁶ nt per Gy measured by Roginskaya and co-workers using DNA subjected to 40 kVp X-rays.²³ However, when expressed as radiation chemical yield that takes into account energy deposition in the irradiated solution, the results are in better agreement, with G-values for 5MF formation of 13 nmol/J in the present studies and 6.7 nmol/J for Razskazovskiy and co-workers.²³ The variance is probably not due to the different radiation sources since the low energy X-rays employed by Roginskaya et al. would be expected to produce more damage per unit dose than ⁶⁰Co γ -rays due to the higher relative biological effectiveness of the X-rays.⁴² Further, the dose rates used in the two studies are similar (163 Gy/min in the present studies versus 180 Gy/min in the studies of Roginskaya et al.). One possible explanation is that the discrepancy is due to different product elimination conditions (shorter heating time, weaker catalyst), and the fact that no internal standard was employed to correct for the loss of 5MF during their workup process. Another discrepancy lies in the conclusion that 2-deoxyribonolactone represents 30% of total 2-deoxyribose oxidation in X-ray-irradiated DNA,²³ in comparison to our observation of a 7% frequency. This may be related to their use of free base release as a measure of total 2-deoxyribose oxidation, which misses sugar damage involving the nucleoside 5'-aldehyde, while we employed total strand breaks and abasic sites.

The studies in purified DNA reveal quantitative insights into mechanisms of 2-deoxyribose oxidation in DNA. Table 1 contains a compilation of the contributions of 2-deoxyribose oxidation products to total 2-deoxyribose oxidation in DNA by γ -radiation and Fe²⁺–EDTA, including data from the present studies. We observed that nucleoside 5'-aldehyde comprises 40% and 35% of the total 2-deoxyribose oxidation in DNA caused by γ -radiation and Fe²⁺–EDTA, respectively. In addition to our previous observation that a product of the other pathway of 5'-oxidation, the 2-phosphoryl-1,4-dioxobutane residue, represents ~4% and ~10% of total 2-deoxyribose oxidation by γ -radiation and Fe²⁺–EDTA, respectively,⁹ 5'-chemistry thus represents nearly half of 2-deoxyribose oxidation in DNA. This is consistent with theoretical and experimental evidence that the 5'-position represents a major target for oxidants as a result of solvent exposure.^{6,24} Solvent exposure may also account for the relatively high percentage of damage arising from 4'-oxidation (Table 1), with 13% of γ -radiation-induced 2-deoxyribose oxidation accounted for by products from the two pathways of 4'-chemistry: 3'-phosphoglycolate and the 2-deoxy-pentos-4-ulose abasic site.⁸

However, factors other than solvent exposure must account for the spectrum of 2-deoxyribose oxidation products. For example, 2-deoxyribonolactone accounts for 24% of total 2-deoxyribose oxidation by Fe²⁺–EDTA (Table 1), which

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Table 1. Relative Quantities of γ -Radiation- and Fe^{2+} -EDTA-Induced 2-Deoxyribose Oxidation in DNA

	% solvent accessible surface ^a	% reaction with hydroxyl radical ^a	2-deoxyribose oxidation product	% total 2-deoxyribose oxidation in isolated DNA		damage per 10 ⁶ nt per Gy in TK6 cells	ref
				γ -rad	Fe^{2+} -EDTA		
1'	1	11	2-deoxyribonolactone abasic site	7	24	0.045	present study
2'	11	13	erythrose abasic site	—	—	—	
3'	14	17	3'-phosphoglycolaldehyde	1	—	0.002	11, 12
			3'-oxo-nucleotide	—	—	—	
4'	28	22	3'-phosphoglycolate	10	—	—	8
			2-deoxypentos-4-ulose abasic site	3	—	—	8
5'	46	57	5'-nucleoside 5'-aldehyde	40	35	0.22	present study
			5'-(2-phosphoryl-1,4-dioxobutane)	4	10	—	9

^a Data taken from Balasubramanian et al.²⁴

reflects a significantly higher reactivity than solvent exposure (1%) and the isotope effect studies of Tullius and co-workers (11% relative reactivity) would predict.²⁴ This difference may reflect the higher stability of the 1'-radical, and thus the chemical reactivity of the site, which is calculated to fall in the order 1' > 4' > 2' > 3' > 5' by Sevilla and co-workers.⁴³ The observation of a 3.4-fold difference between γ -radiation and Fe^{2+} -EDTA in the proportion of 2-deoxyribonolactone (7% versus 24%, respectively) also suggests that factors other than formation of hydroxyl radical influence the reactivity of the oxidizing agents, such as the negative charge of the Fe^{2+} -EDTA complex as it affects interactions with the polyanionic DNA helix. Some caution is necessary in interpreting the data in Table 1, given the potential for contributions to total 2-deoxyribose oxidation from yet-to-be identified lesions and from damage not involving strand breaks and abasic sites, such as 5',8-cyclopurines.^{7,44}

The results of studies in cells subjected to ionizing radiation are also revealing. With corrections for losses during DNA isolation, the cellular environment caused a 260-fold decrease in the formation of nucleoside 5'-aldehyde from 57 to 0.22 lesions per 10⁶ nt per Gy and a 220-fold reduction in 2-deoxyribonolactone formation from 10 to 0.045 lesions per 10⁶ nt per Gy relative to purified DNA. This protective effect is similar to ~1000-fold reduction of γ -radiation-induced formation of 8-oxo-7,8-dihydro-dG in human cells⁴⁵ and of the 3-phosphoglycolaldehyde product of 3'-oxidation¹² (neither study corrected for artifactual losses), presumably due to the radical quenching effects of chromatin proteins and other species in the cell.⁴⁶ Some care must be exercised in interpreting the results of studies in cells due to the relatively labile nature of the 2-deoxyribonolactone (24 h half-life at 37 °C¹³) and the nucleoside 5'-aldehyde (30–100 h half-life²²) and the potential for DNA repair, which could potentially lead to loss of the lesions during the time between exposure and DNA isolation. However, the rapid irradiation and DNA workup (several hours), the correction for losses during isolation, and the consistency of the results with other types of DNA damage all support the quantitatively rigorous nature of the cellular data.

A comparison of the relative quantities of the 3'-phosphoglycolaldehyde, 2-deoxyribonolactone and nucleoside 5'-aldehyde products in isolated DNA and cells suggests that the cellular environment does not greatly alter the selectivity of hydroxyl radicals for the various positions in 2-deoxyribose. Irradiation dose response studies in isolated DNA caused

formation rates of 1.5,¹² 9.7 and 57 lesions per 10⁶ nt per Gy, respectively, which amounts to a ratio of 1:6.5:38. In cells, the rates were reduced to 0.002,¹² 0.045 and 0.22 lesions per 10⁶ nt per Gy, respectively, and a ratio of 1:22:110. The relative quantities are thus quite similar, especially in consideration of the fact that the data for 3'-phosphoglycolaldehyde quantification were not corrected for losses during DNA isolation from cells.¹² These results raise the possibility of consistent *in vitro* and *in vivo* results for other 2-deoxyribose oxidation products.

In conclusion, we have developed and validated a chemically specific and sensitive isotope-dilution GC-MS method for the quantification of DNA damage representing 1'- and 5'-oxidation chemistry *in vitro* and in cells: the 2-deoxyribonolactone and nucleoside 5'-aldehyde residues. Our observation that the 5'-carbon in 2-deoxyribose represents a major target for oxidants is in general agreement with the solvent-accessible surface area model of Balasubramanian et al.²⁴ The studies reveal quantitative insights into mechanisms of 2-deoxyribose oxidation as well as a comprehensive compilation of the contributions of 2-deoxyribose oxidation products to total 2-deoxyribose oxidation in DNA by γ -radiation and Fe^{2+} -EDTA. The GC-MS method provides a means for accurate determination of these elimination products as biomarkers of oxidative stress and as probes for the fate of DNA damage products in cells and tissues.

Experimental Methods

Materials. All chemicals and reagents were of the highest purity available and were used without further purification unless noted otherwise. Calf thymus DNA, pentafluorophenylhydrazine (PFPH), furfural, and poly-L-lysine were purchased from Sigma Chemical Co. (St. Louis, MO). 2-Deoxy-D-[¹³C₅]ribose was obtained from Omicron Biochemicals, Inc. (Southbend, IN), 3,4,5-[²H]-furfural from Medical Isotopes, Inc. (Pelham, NH), acetonitrile from Honeywell Burdick & Jackson (Muskegon, MI), and dichloromethane from Merck (Gibbstown, NJ). Deionized water was further purified with a Milli-Q system (Millipore Corporation, Bedford, MA) and was used in all experiments.

Instrumentation. Gas chromatography-mass spectrometry (GC-MS) analyses were performed on a Hewlett-Packard 6890 series GC system equipped with a Hewlett-Packard 5973 mass selective detector. High mass accuracy experiments were performed on an Agilent 6510 series Q-TOF mass spectrometer with a standard ESI interface. ¹H and ¹H-decoupled-¹³C NMR spectroscopic studies were performed on a Bruker 400 MHz spectrometer. Samples were irradiated in a Gammacell 220 Excel Irradiator (MDS Nordion, Canada) with an annular ⁶⁰Co source at a rate of 163.1 Gy/min.

Synthesis of (E)- and (Z)-1-(Furan-2-ylmethylene)-2-(perfluorophenyl)hydrazine (1a and 1b). The PFPH derivatives of furfural and 3,4,5-[²H]₃-furfural (**1a**, **1b**) were prepared in overnight reactions with a 10-fold molar excess of PFPH in hexane at ambient temperature, as described by Ho and Yu.²⁵ The products were characterized by GC-MS and high mass accuracy mass spectrom-

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etry. The reaction of furfural with PFPH leads to a hydrazone with *cis*- and *trans*- isomers (**1a**, **1b**) that were well resolved by GC–MS (Figure 1) and showed identical EI mass spectra. As shown in Supporting Information Figure 1A, the molecular ion $[M]^+$ at m/z 276 was detected as the base peak, with abundant fragment ions at m/z 117, 155, and 182 that corresponded to $[C_5F_3]^+$, $[C_5F_5]^+$, and $[C_5F_5NH]^+$, respectively. Standard stock solutions of defined concentration were prepared as described elsewhere.²⁵

Synthesis of 6-Methyl-2-(perfluorophenyl)pyridazin-3(2H)-one (2). The synthesis of **2** was achieved by reaction of 5MF, prepared from 2-deoxy-D-ribose as described elsewhere,⁴⁷ with a 5-fold molar excess of PFPH at 21 °C for 15 h. The reaction mixture was resolved by reverse-phase HPLC on a Phenomenex Synergi Column (250 mm × 10 mm, 4 μ M) using water and acetonitrile as the mobile phase, with a single product eluting at 11.6 min. Following repurification with a shallower acetonitrile gradient (24.4 min), the chromatographically pure product was determined to be 6-methyl-2-pentafluorophenyl-3(2H)-pyridazinone (**2**) (Scheme 2). GC–MS analysis revealed a molecular ion $[M]^+$ at m/z 276 as the base peak (Supporting Information Figure 1B) and characteristic fragment ions at m/z 93 $[C_5F_3]^+$, 117 $[C_5F_5]^+$, 167 $[C_6F_5]^+$, 229 $[M - CO - F]^+$, 248 $[M - CO]^+$ and 257 $[M - F]^+$. ¹H NMR (CDCl₃): δ 7.20 (d, 1H, $J = 9.7$ Hz), 6.99 (d, 1H, $J = 9.7$ Hz), 2.38 (s, 3H, CH₃). ¹³C NMR (CDCl₃): δ 158.7 (s), 146.9 (s), 143.9 (d, $J_{CF} = 260.3$ Hz), 142.2 (d, $J_{CF} = 259.5$ Hz), 138.1 (d, $J_{CF} = 259.9$ Hz), 135.0 (s), 130.8 (s), 116.7 (s), 21.0 (s). UV absorbance spectrophotometry revealed a $\lambda_{max} = 295$ nm, with an extinction coefficient of 4000 M⁻¹ cm⁻¹. High mass accuracy mass spectrometry revealed a close correlation between theoretical (277.0400) and measured (277.0396) m/z values of the $[M + H]^+$ ion, which is consistent with a molecular formula of C₁₁F₅H₅N₂O. Using a similar strategy, the ¹³C₅-labeled version of **2** was synthesized from 2-deoxy-D-[¹³C₅]ribose. Standard stock solutions of defined concentration were prepared by weighing and verified by UV absorbance.

Synthesis and Characterization of the PFPH Derivatives of 2MF. 2MF was synthesized from thymidine by the method of Stubbe and co-workers,²⁶ which entails oxidation of 5'-*O*-p-tolylsilylthymidine to 3'-keto-5'-*O*-p-tolylsilylthymidine with pyridinium dichromate and release of 2MF by base-catalyzed elimination reaction. 2MF was reacted with a 5-fold molar excess of PFPH in dichloromethane for 15 h at 21 °C. Analysis of the reaction mixture by GC–MS revealed a single product with a molecular ion at m/z 276 as the base peak and characteristic fragment ions at m/z 93 $[C_5F_3]^+$, 117 $[C_5F_5]^+$, 148 $[C_6F_4]^+$, and 167 $[C_6F_5]^+$. The product proved to be unstable during storage at -80 °C, so an internal standard could not be prepared for GC–MS analyses.

Synthesis of 2-Deoxyribonolactone- and 5'-Aldehyde-Containing Oligonucleotides. A 17-mer oligodeoxynucleotide, 5'-TGTGCCXAACTTACCGT-3', containing a 2-deoxyribonolactone at **X** (**3**) was prepared by UV irradiation of a precursor containing a nitrobenzyl cyanohydrin nucleoside analogue, as described by Zheng and Sheppard.¹³ The conversion efficiency was determined to be 92% by reverse-phase HPLC with a system consisting of a Thermo Hypersil GOLD aQ 150 mm × 2.1 mm column with 3 μ M particle size eluted at 200 μ L/min with a gradient of acetonitrile in 10 mM ammonium acetate (1% acetonitrile for 2 min, 1–20% over 23 min, 20–100% over 3 min, held at 100% acetonitrile for 10 min); the retention times of the oligodeoxynucleotides were 23.9 and 25.4 min for the deoxyribonolactone-containing and parent species, respectively. The final products were identified by high mass accuracy mass spectrometry (Supporting Information Figure 3) and by polyacrylamide gel electrophoretic analysis (Supporting Information Figure 5).

An oligodeoxynucleotide standard containing a nucleoside 5'-aldehyde terminus, 5'-TGC-3' (**4**), was prepared as described by Sugiyama et al.²⁷ Briefly, a 90 μ M solution of a self-complementary

hexadeoxynucleotide, 5'-GCATGC-3', in potassium phosphate buffer (50 mM, pH 7.4) was treated with 46 μ M neocarzinostatin and 10 mM glutathione, the latter to activate the drug for DNA cleavage. After 12 h at 0 °C, the cleavage mixture was resolved by reverse-phase HPLC using the conditions described above, with collection of the nucleoside 5'-aldehyde-containing TGC fragment eluting at 15.2 min. The product was characterized by high mass accuracy mass spectrometry with an $[M + H]^+$ ion detected at m/z 859.1850 (Supporting Information Figure 3). The presence of nucleoside 5'-aldehyde was further confirmed by converting it to a hydrazone derivative with a 10-fold molar excess of PFPH. The resulting PFPH hydrazone derivative had an HPLC elution time of 28.5 min and an expected $[M + H]^+$ at 1039.2 m/z (data not shown). The oligodeoxynucleotide was stable at -80 °C for more than 1 mo and was determined to have a half-life of 34 h at 21 °C (Supporting Information Figure 4), which compares favorably with the half-life of 100 h at 37 °C in the studies of Greenberg and co-workers.²²

Oxidation of Purified DNA. Buffered solutions of calf thymus DNA were subjected to oxidation by various agents as follows. Solutions of DNA (0.6 mL, 420 μ g/mL) in Chelex-treated potassium phosphate buffer (50 mM, pH 7.4) were subjected to γ -irradiation at a dose-rate of 161.3 Gy/min over a range of 10–108 Gy. DNA damaging reactions by neocarzinostatin, calicheamicin γ_1 , and Fe²⁺-EDTA were conducted as reported elsewhere by Chen et al.^{9,48} In brief, DNA samples were exposed to neocarzinostatin and calicheamicin and freshly prepared Fe²⁺-EDTA at concentrations of 3.4–19.6 μ M, 0.4–4.4 μ M, and 25–360 μ M, respectively, with 10 mM glutathione used to activate neocarzinostatin and calicheamicin. Following Sephadex G25 chromatography (GE Healthcare), the DNA was processed for GC–MS analysis.

GC–MS Quantification of the PFPH Derivatives of 5MF and Furfural in Oxidized DNA. The general strategy for quantification of dRL and NA lesions in oxidized purified DNA involves release of their β - δ -elimination products, 5MF and furfural, respectively, derivatization of the products with PFPH, addition of internal standards, extraction of the PFPH derivatives, and quantification by GC–MS. To each DNA sample (0.6 mL) was added an aliquot of an aqueous solution of poly-L-lysine (15 μ L, 0.1 M) followed by incubation at 90 °C for 1 h. After cooling to ambient temperature, an aliquot of an aqueous solution of PFPH (30 μ L, 0.2 M) was added and the reaction allowed to proceed overnight at ambient temperature. Following addition of a 20 μ L mixture of isotope-labeled internal standards (9 μ M [²H]₃-**1a/1b**, 15 μ M [¹³C]₅-**2**), the solutions were extracted three times with 300 μ L of dichloromethane. The combined organic layers were dried under N₂ and the residue was dissolved in 20 μ L of dichloromethane.

GC–MS analysis was performed with 1 μ L of the dichloromethane solution of each sample using positive electron impact ionization (EI; 70 eV). Samples were injected onto a DB-35MS capillary column (30 m × 0.25 mm × 0.25 μ m film thickness) in a Hewlett-Packard 6890/5973 GC–MS system operating with the following parameters: injector temperature set at 250 °C in the splitless mode for the first 1.5 min, and transfer line temperature at 280 °C; carrier gas (He) 1 mL/min; GC oven at 40 °C for 2 min, increased to 200 at 7 °C/min, ramped to 310 at 20 °C/min, and held at 310 °C for 5 min; solvent delay, 16 min (to allow the elution of excess PFPH reagent prior to mass spectrometer scan). Data were acquired by an HP5973 Mass Selective Detector operated in selected ion monitoring mode (SIM) at m/z values of 276, 279, and 281. Using calibration curves that were validated daily by running a standard mixture before the samples, quantities of **1a+1b** were determined from the sum of the peak area ratios of **1a** and **1b** (m/z 276) to [²H]₃-**1a** and [²H]₃-**1b** (m/z 279), respectively, while **2** was quantified from the peak area ratio of **2** (m/z 276) to [¹³C]₅-**2** (m/z 281). Calibration curves were prepared using mixtures of

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variable quantities of **1a+1b** (2.92–2920 pmol) and **2** (0.432–432 pmol) and fixed quantities of $^2\text{H}_3$ -(**1a+1b**) (180 pmol) and **2** (300 pmol) with calf thymus DNA (0.6 mL, 420 $\mu\text{g}/\text{mL}$) in Chelex-treated potassium phosphate buffer (50 mM, pH 7.4).

Validation of the GC–MS Method. The efficiency of the elimination, derivatization, and analytical steps was determined using the oligodeoxynucleotides **3** and **4**, which provides a critical correction for artifacts arising during sample processing. The validation entailed spiking 250 μg of calf thymus DNA with **3** (88 pmol) or **4** (293 pmol) followed by heat and polylysine-catalyzed elimination of 5MF or furfural, respectively, derivatization with PFPH, and GC–MS analysis of extracts, as described above. The overall efficiency of the analytical method was calculated as the measured quantities of **2** or **1a + 1b** divided by the quantity of added **3** or **4**, respectively. The yields of **1a+1b** and **2** were found to be $43 \pm 2.4\%$ and $21 \pm 0.7\%$ of the theoretical value, respectively. All data were subsequently corrected by factors of 2.3 and 4.8, respectively.

Quantification of the PFPH Derivatives of 5MF and Furfural in γ -Irradiated TK6 Cells. Studies were undertaken to quantify the formation of 2-deoxyribonolactone and nucleoside 5'-aldehyde in TK6 human lymphoblastoid cells exposed to γ -radiation. Cultures of TK6 human lymphoblastoid cells, grown to 1×10^6 cells per milliliter as described previously,¹² were washed three times with PBS and resuspended in PBS at a concentration of 5×10^6 cells per milliliter. The suspensions (10 mL) were exposed to γ -radiation (0–500 Gy), at ambient temperature at a dose-rate of 161.3 Gy/min. Genomic DNA was isolated using a Qiagen DNA isolation kit and quantified by UV absorption at 260 nm. The DNA was processed for GC–MS analysis of 5MF and furfural as described earlier.

To control for effects of the cellular environment on the quantities of 2-deoxyribonolactone and nucleoside 5'-aldehyde during DNA isolation, a control experiment was performed in which purified, irradiated DNA containing defined quantities of 2-deoxyribonolactone and nucleoside 5'-aldehyde was added to lysed cells during the DNA isolation procedure and 5MF and furfural were quantified as described earlier. Samples of purified genomic DNA from TK6 cells, isolated using the Qiagen kits described earlier, were γ -irradiated with 0, 8.4, 22.4, or 36.4 Gy. A 122 μg quantity of the irradiated DNA was added to 5×10^7 unexposed TK6 cells ($\sim 250 \mu\text{g}$ DNA) at the nucleus isolation stage of the Qiagen genomic DNA isolation protocol. Following purification of the total DNA mixture, 5MF and furfural were quantified by GC–MS in the purified DNA and spiked-cell DNA, as described earlier. The quantities of 5MF and furfural in the spiked-cell DNA were expressed as lesions per

nucleotide of the purified, irradiated DNA added to the cell sample. As shown in the radiation dose–response curve in Supporting Information Figure 6, fitting the data by linear regression yielded lines for 5MF and furfural with slopes of 5.3 5MF per 10^6 nt per Gy ($r^2 = 0.99$) and 44 furfural per 10^6 nt per Gy ($r^2 = 1.0$). On the basis of analyses in purified DNA (10 5MF and 57 furfural per 10^6 nt per Gy), all data from cellular experiments were subsequently corrected by factors of 1.9 for 5MF and 1.3 for furfural to account for losses during DNA isolation.

Acknowledgment. We extend thanks to Dr. Terry Sheppard (Nature Publications) for providing the precursor to 2-deoxyribonolactone-containing oligonucleotide, to Prof. Jacquelyn Yanch (Dept. of Nuclear Science and Engineering, MIT), Dr. Dennis H. W. Chan (Dept. of Chemistry, Hong Kong University of Science and Technology), and Dr. Liang Cui (Dept. of Biological Engineering, MIT) for helpful discussions and insights into data interpretation, to Drs. Jeff Simpson and Bob Kennedy for help with NMR experiments (Spectroscopy Laboratory, Dept. of Chemistry, MIT), and to Ms. Bahar Edrissi for the cell culture. Special thanks are extended to Prof. Yuriy Razskazovskiy (East Tennessee State University) for providing samples of 5MF and for critical insights into radiation chemistry. The chromatographic and mass spectrometric analyses were performed in the Bioanalytical Facilities Core of the MIT Center for Environmental Health Sciences. This work was supported by grants from the National Institute of Environmental Health Sciences (ES002109), the National Center for Research Resources (RR023783-01, RR017905-01), and the National Cancer Institute (CA103146).

Supporting Information Available: Electron impact ionization mass spectra of the PFPH derivatives of furfural (**1a**, **1b**) and 5MF (**2**); calibration curves for the gas chromatography–mass spectrometry analysis of **1a**, **1b**, and **2**; reverse-phase HPLC analysis of the 2-deoxyribonolactone- and nucleoside 5'-aldehyde-containing oligodeoxynucleotides; reverse-phase HPLC analysis of the stability of the 5'-TGC-3' containing nucleoside 5'-aldehyde terminus (**4**); polyacrylamide gel electrophoretic analysis of the 2-deoxyribonolactone-containing oligodeoxynucleotide (**3**). This material is available free of charge via the Internet at <http://pubs.acs.org>.

JA910928N