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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 15 (2005) 3627–3631

Hydantoin derivative formation from oxidation of 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG) and incorporation of ^{14}C-labeled 8-oxodG into the DNA of human breast cancer cells

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Received 8 February 2005; revised 4 May 2005; accepted 10 May 2005

Abstract—One-electron oxidation of 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG) yielded a guanidinohydantoin derivative (dGh) and a spiroiminodihydantoin derivative (dSp), both putatively mutagenic products that may be formed in vivo. The nucleoside dGh was the major product at room temperature, regardless of pH. The results are contrary to previously published model studies using 2′,3′,5′-triacetoxy-8-oxo-7,8-dihydroguanosine (Luo, W.; Miller, J. G.; Rachlin, E. M.; Burrows, C. J. *Org. Lett*. **2000**, *2*, 613; Luo, W.; Miller, J.G.; Rachlin, E.M.; Burrows, C.J. *Chem. Res. Toxicol*. **2001**, *14*, 927), who observed a spiroiminodihydantoin derivative as the major product at neutral pH. Clearly, the functional groups attached to the ribose moiety of 8-oxodG influence the oxidation chemistry of the nucleobase derivative. To explore this chemistry in vivo, ^{14}C-labeled 8-oxodG was synthesized and incubated with growing MCF-7 human breast cancer cells, resulting in the incorporation of the compound into cellular DNA as measured by a novel accelerator mass spectrometry assay.

DNA oxidation is a field of interest due to the deleterious effects that oxidative damage promotes within cells.[1] Among the four bases, guanine has the lowest oxidation potential and constitutes a primary target of oxidants.[2] Reactive oxygen species (ROS) are known to damage genomic and mitochondrial DNA as well as the cellular nucleotide pool, and are therefore implicated in cancer, aging, and neurological disorders.[1] Among the numerous oxidation products of guanine, 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG) is the major product, which is generated by either hydration of the guanine radical cation formed by direct base oxidation or by hydroxy radical addition at the C-8 position of the imidazole ring of guanine.[1a] At present, 8-oxodG is recognized as a highly susceptible site for further oxidation compared to the parent guanine, and may serve as a genomic 'hot spot' for generation of further oxidative damage either by direct oxidation or indirectly via hole transfer from other initially generated radical base cation sites by one-electron oxidants, singlet oxygen, photochemical processes, and possibly peroxynitrite, as shown in Figure 1.[3] The redox potential for 8-oxodG is in the range of 0.58–0.75 V vs a nickel hydrogen electrode (NHE), compared to 1.29 V vs NHE for 2′-deoxyguanosine (dG).[4] It is now fully established that 8-oxodG is a much better substrate than dG for reaction with a wide set of oxidants.[1a,4] Such a low redox potential makes 8-oxodG a preferential target for numerous oxidizing agents. Among the one-electron oxidants, Ir^{IV} complexes are usually exploited for the selective oxidation of 8-oxodG because the redox potential for $IrCl_6^{2-}$ is 0.90 V vs NHE, just above that of 8-oxodG, but below the redox potentials of the four normal nucleobases, thus providing a reasonable mechanism[5] by which several 8-oxodG oxidation products are generated. These nucleoside derivatives include guanidinohydantoin[5a] (dGh), spiroiminodihydantoin[3a] (dSp), imidazolone,[3f] oxazolone,[3i] oxaluric acid,[6a] and urea.[6b] Recent work on these lesions in *Escherichia coli* has demonstrated that they are mutagenic in vivo.[7] For example, both dGh and dSp promote misincorporation of adenine and guanine opposite the oxidized bases, respectively, resulting in higher percentages of point mutations compared with 8-oxodG.

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doi:10.1016/j.bmcl.2005.05.113

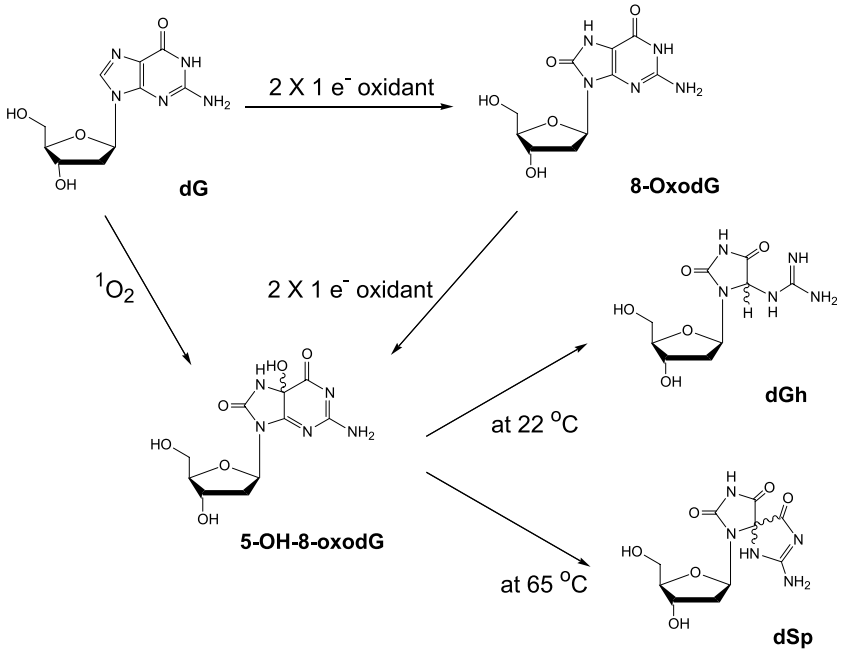
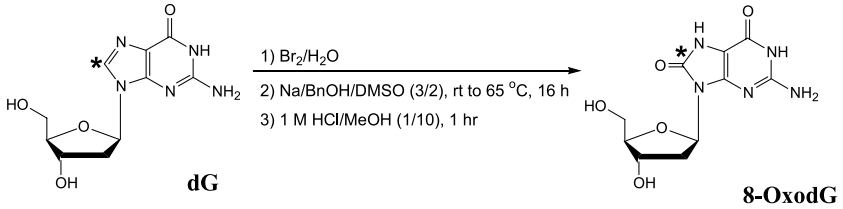


Figure 1. One-electron oxidation reactions of dG and 8-oxodG.

Importantly, dGh is well bypassed by DNA polymerase(s) and is nearly 100% mutagenic.

Major effort has been recently devoted to the delineation of mechanistic features of various oxidation reactions involving 8-oxodG and its analogs either as nucleoside derivatives or in oligonucleotides containing 8-oxodG lesion site-specifically.[6b,7b] For example, studies on oxidation of 2′,3′,5′-triacetoxy-8-oxo-7,8-dihydroguanosine (OG) with Ir^{IV} yielded two main products as the hydantoin derivatives, dGh and dSp. Importantly, the product distributions were highly pH-dependent, suggesting that deprotonation competes with direct rearrangement of the 5-hydroxyguanosine intermediate.[8]

To better understand 8-oxodG reactivity in the nucleotide pool at physiologically relevant concentrations, we prepared 8-oxodG and ^{14}C-labeled 8-oxodG as shown in Scheme 1.[9] The ^{14}C label allows monitoring of the incorporation of 8-oxodG and its derivatives into cellular DNA (vide supra). Briefly, the starting materials of dG (Chemgenes Corporation) and a mixture of 80% dG and 20% ^{14}C-labeled dG (specific activity 55 mCi/mmol, Moravek Biochemicals), respectively, were brominated with saturated bromine solution in water and the attached bromide was removed by the nucleophilic attack of benzylate, followed by oxidation with dilute HCl. We applied the same oxidation conditions previously described for OG studies,[5a,10] exploiting Ir^{IV} as a one-electron oxidant. Briefly, in a final volume of 300 μl of 75 mM potassium phosphate buffer (pH 4.6, 6.0, 7.2, and 8.4), 7.5 mM 8-oxodG was incubated with 7.5 mM Na_2IrCl_6 at 22 °C for 1 h to produce dGh as the major product. The product dSp was generated under identical conditions, except that reaction required 65 °C in a 10 mM sodium phosphate in 100 mM NaCl buffer at pH 7.2.[11] After filtration, each resulting reaction mixture (10 μl) was directly injected into the HPLC instrument for analysis or separation.[12] The purity of each isolated product was verified by HPLC for purity confirmation resulting in a single peak (~98% purity for both dGh and dSp, data not shown) and the products were further characterized by mass spectrometry.[13] Figure 2a shows an HPLC chromatogram of the 8-oxodG starting material with a mass spectrogram on the inset. Figure 2b shows the chromatogram run under the same conditions after the oxidation reaction was performed at pH 7.2—the most relevant pH value compared to in vivo conditions. The first two eluting peaks were separated and characterized by mass spectrometry, and assigned as dGh and dSp, respectively. Approximately 99% of the 8-oxodG starting material was consumed during the reaction and the first two peaks account for the majority of the total peak area for the



Scheme 1. Synthetic scheme for 8-oxodG and ^{14}C-labeled 8-oxodG (^{14}C position is asterisked).

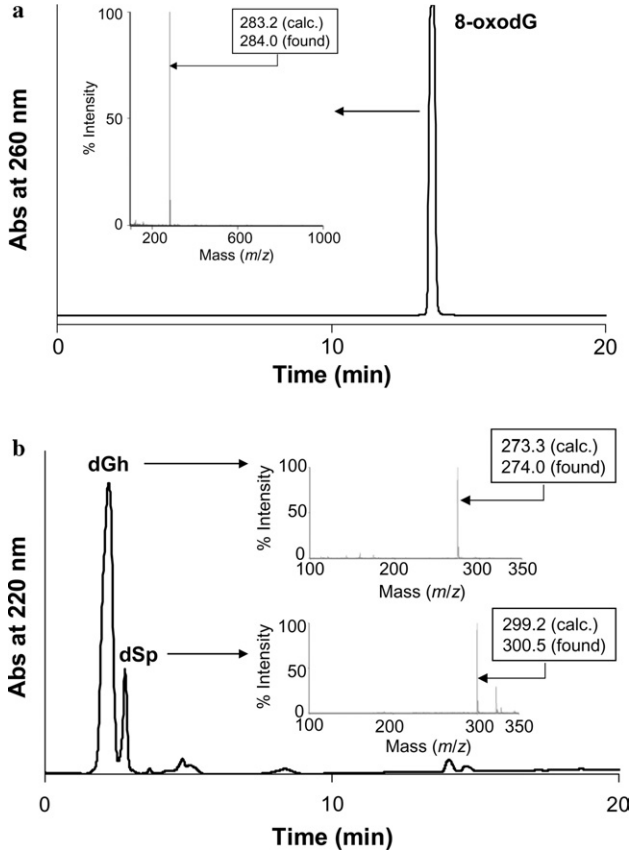


Figure 2. HPLC chromatograms showing: (a) 8-oxodG and (b) the 8-oxodG oxidation products dGh and dSp, from the reaction performed at 22 °C and pH 7.2. The insets are mass spectra for the purified peaks assigned 8-oxodG, dGh and dSp, respectively.

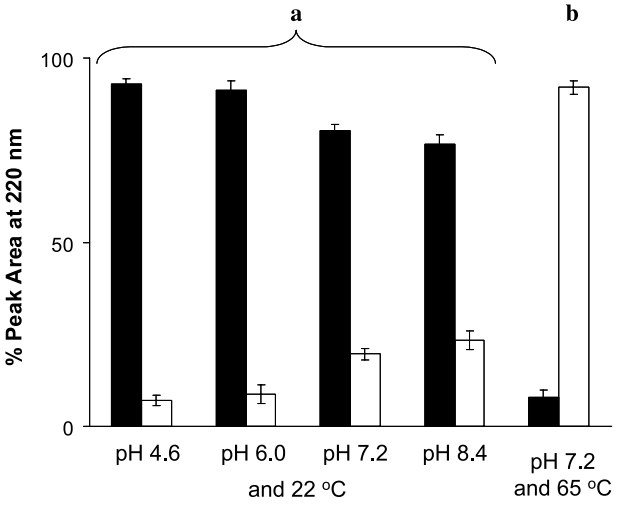


Figure 3. Relative ratios based on the peak areas corresponding to the two products of dGh (black bars) and dSp (white bars) formed at (a) 22 °C and pH 4.6, 6.0, 7.2, and 8.4, and at (b) 65 °C and pH 7.2. Experiments were performed in duplicate and the data shown were recorded at 220 nm.

entire chromatogram. Although dGh and dSp exist as diastereomers, we observed only one peak for each nucleoside derivative, which was likely due to poor resolution of the highly polar species that elute from the HPLC column quickly under reverse-phase conditions compared to protected nucleosides such as OG and oligonucleotides containing the lesions, in which cases the diastereomers are resolvable.[7b] Although dGh eluted at or near the retention time expected for the solvent front, the small injection volume of 10 μl produced an essentially negligible salt peak, which was not discernable from the background on the 8-oxodG control chromatogram as shown in Figure 2a. Contamination of the dGh peak with dSp, or vica versa, was not observed in the mass spectrometry data. Other minor products formed were not characterized. Figure 3 shows the percent peak areas of dGh and dSp derived from the one-electron oxidation reaction of 8-oxodG, and that dGh is the major product, regardless of pH. The peak areas of dG and dSp from each chromatogram were added up and normalized to a value of 100%. The proportion of dSp and dGh contained in the peaks was plotted as a percentage of the total peak area of the two products. The percent peak area from dGh contained in the two peaks at pH 4.6, 6.0, 7.2, and 8.4 was measured at 220 nm to be 92.8 ± 1.4, 91.3 ± 2.6, 80.4 ± 1.6, and $76.6 \pm 2.6\%$ (Fig. 3). The reaction at pH 7.2 and 65 °C afforded $8.0 \pm 0.8\%$ dGh, with the remainder of the major product as dSp. The measured peak area values gave a conservative estimate of the percent dSp in each reaction mixture, since the published molar absorptivity is ~30% greater for dSp than for dGh based on the known extinction coefficients for these derivatives formed from OG oxidation at 220–230 nm.[5c] Percent peak areas measured at 260 nm, although several fold lower, resulted in a close correlation with those measured at 220 nm for dGh at 97.3 ± 0.7, 97.3 ± 0.7, 87.6 ± 1.0, and $86.4 \pm 0.5\%$, respectively. The similar percentages from measurement at both 220 and 260 nm indicate a minimal contribution from the solvent front to the Gh peak areas. These data are consistent with a recent study using the nucleobase 8-oxoG, which also showed a similar product distribution in the same pH range.[8] However, this report shows that the structurally similar uric acid undergoes deprotonation at N9, which disfavors spirocyclization. Therefore, this comparison must be made tenuously considering that N9 of 8-oxodG is the site of the glycosidic bond to deoxyribose. Clearly, dGh was the major product at physiological pH, which was formed with a minor, but significant, yield of dSp.

Importantly, though the previously reported one-electron oxidation reactions of OG were highly pH-dependent and dSp derivative was the major product,[5a] it was also reported that the dGh derivative was the major product in the one-electron oxidation of 8-oxodG in single-stranded and duplex DNA under the same conditions employed in this study.[3g] These differences might originate from the use of ribose instead of deoxyribose, or from the use of protecting groups. The free hydroxy groups in our study may play a role either in the hydrolysis step at C-5 or C-4 site by a general base mechanism[8] for the production of dGh from 8-oxodG. We were also able to utilize pH and temperature to synthesize ^{14}C-labeled dGh and dSp from ^{14}C-labeled 8-oxodG for use in experiments with cultured human cells.[13]

Most of the previous model studies on the oxidation of 8-oxodG were performed using protected 8-oxodG derivatives, such as OG, to prevent oxidation of 5′-OH and to allow longer retention times and better resolution by HPLC owing to higher hydrophobicity compared to the unprotected 2′-deoxynucleosides. Because of the possibility of oxidation of 5′-OH by Ir^{IV} complexes in this study, the silver mirror experiment for detection of aldehydes was used, in which the presence of the functional group reduces $Ag(NH_3)_2^+$ to metallic silver. No aldehyde was detected in the purified products, dGh and dSp. In contrast, the oxidation of 8-oxodG with peroxynitrite, a potent oxidant that is probably formed in vivo, led to the production of several aldehydes, likely resulting from the oxidation of 5′-OH of the deoxyribose ring (data not shown). Clearly, exploitation of the proper oxidant for 8-oxodG oxidation can lead to selective oxidation of the purine ring instead of the OH groups of the ribose ring. Any aldehyde product(s) may be of little biological consequence since their 5′-OH group(s) would no longer be available for phosphorylation by kinases that participate in the nucleoside salvage pathway.

To explore the in vivo oxidation of 8-oxodG, MCF-7 human breast cancer cells were grown in the presence of ~10 pmol of ^{14}C-labeled 8-oxodG for 0, 1, 2, and 4 days, respectively. [14] Isolated DNA was assayed for the presence of the radiolabel using accelerator mass spectrometry, a method for the quantitation of small amounts of radiocarbon (zeptomol sensitivity) with high precision.[15] Figure 4 shows that radiolabeled nucleoside incorporation into DNA reached a maximum of ~10,000 amol/100 μg of DNA after 2 days and then decreased ~2-fold by day 4, presumably due to DNA repair competing with radiolabeled 2′-deoxynucleoside incorporation. Clearly, this experiment demonstrated that modified 2′-deoxynucleoside(s) can be incorporated into cellular DNA at biologically relevant levels. This experiment, to our knowledge, represents the first report of direct measurement of 8-oxodG incorporation into DNA in vivo. The ^{14}C-labeled 8-oxodG may have, in part, been converted to additional products in vivo.

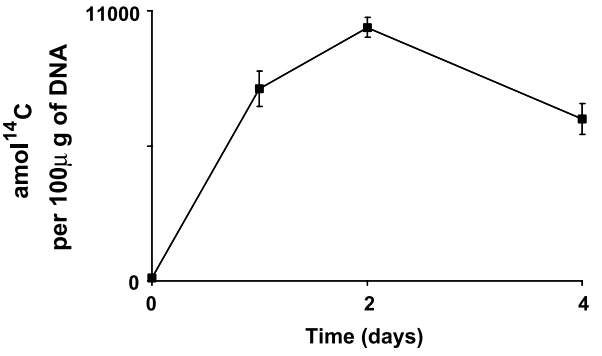


Figure 4. Incorporation of ^{14}C-labeled 8-oxodG into the DNA of growing MCF-7 human breast cancer cells. The ratio of ^{14}C to total carbon contained in the purified genomic DNA was measured with accelerator mass spectrometry. Experiments were performed in triplicate.

We are now investigating enzymatic digestion of the radiolabeled DNA to 2′-deoxynucleosides to determine to what extent the 8-oxodG is oxidized in vivo, either in the nucleotide pool or in the newly synthesized DNA. These data indirectly support the formation of the triphosphate of 8-oxodG or its oxidation products via the nucleotide salvage pathway which serves as a substrate for DNA polymerase(s) to enable incorporation of the modified nucleoside derivative into DNA.

It is tempting to speculate that dG can be first oxidized to 8-oxodG via a variety of mechanisms, and can then be oxidized to dGh and dSp via a one-electron oxidation mechanism. Alternatively, these nucleoside derivatives may be formed directly from dG. The nucleosides dGh and dSp, and their corresponding mono-, di-, and triphosphates, if formed in vivo, are likely to be available in the nucleotide pool for subsequent promutagenic incorporation into DNA. The proportion of the dSp and dGh that is ultimately present in DNA may be governed by the chemistry of their formation in a substituent-dependent fashion.

Acknowledgments

We acknowledge Kurt Hack for preparation of AMS samples and John Vogel, Karen Dingley, and Kenneth Turteltaub for helpful advice and interpretation of the data. This work was performed at the Research Resource for Biomedical Accelerator Mass Spectrometry, operated at University of California Lawrence Livermore National Laboratory under the auspices of the U.S. DOE Contract W-7405-ENG-48 and partially supported by NIH/NCRR, Biomedical Technology Program Grant P41 RR13461, NIH Grant CA55861, and by the California Breast Cancer Research Program Grant 9KB-0179.

References and notes

1. (a) Burrows, C. J.; Muller, J. G. *Chem. Rev.* **1998**, *98*, 1109; (b) Burrows, C. J.; Muller, J. G.; Kornyushyna, O.; Luo, W.; Duarte, V.; Leipold, M. D.; David, S. S. *Environ. Health Perspect.* **2002**, *110*, 713; (c) Sugden, K. D.; Martin, B. D. *Environ. Health Perspect.* **2002**, *110*, 725; (d) Cadet, J.; Douki, T.; Gasparutto, D.; Ravanat, J. L. *Mutat. Res.* **2003**, *531*, 5.
2. Steenken, S.; Jovanovic, S. V. *J. Am. Chem. Soc.* **1997**, *119*, 617.
3. (a) Duarte, V.; Muller, J. G.; Burrows, C. J. *Nucleic Acids Res.* **1999**, *27*, 496; (b) Niles, J. C.; Burney, S.; Singh, S. P.; Wishnok, J. S.; Tannenbaum, S. R. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 11729; (c) Henderson, P. T.; Jones, D.; Hampikian, G.; Kan, Y. Z.; Schuster, G. B. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 8353; (d) Kasai, H.; Yamaizumi, Z.; Berger, M.; Cadet, J. *J. Am. Chem. Soc.* **1992**, *114*, 9692; (e) Ravanat, J. L.; Cadet, J. *Chem. Res. Toxicol.* **1995**, *8*, 379; (f) Raoul, S.; Berger, M.; Buchko, G. W.; Prakash, C.; Joshi, P. C.; Morin, B.; Weinfeld, M.; Cadet, J. *J. Chem. Soc., Perkin Trans. 2* **1996**, 371; (g) Gasparutto, D.; Ravanat, J. L.; Gerot, O.; Cadet, J. *J. Am. Chem. Soc.* **1998**, *120*, 10283; (h) Muller, J. G.; Duarte, V.; Hickerson, R. P.; Burrows, C. J. *Nucleic Acids Res.* **1998**,

26, 2247; (i) Vialas, C.; Pratviel, G.; Claparols, C.; Meunier, B. *J. Am. Chem. Soc.* **1998**, *120*, 11548; (j) Leipold, M. D.; Muller, J. G.; Burrows, C. J.; David, S. S. *Biochemistry* **2000**, *39*, 14984.

4. Hickerson, R. P.; James, F. P.; Muller, J. G.; Foote, C. S.; Burrows, C. J. *J. Am. Chem. Soc.* **1999**, *121*, 9423.
5. (a) Luo, W.; Muller, J. G.; Rachlin, E. M.; Burrows, C. J. *Org. Lett.* **2000**, *2*, 613; (b) Luo, W.; Muller, J. G.; Rachlin, E. M.; Burrows, C. J. *Chem. Res. Toxicol.* **2001**, *14*, 927; (c) Luo, W.; Muller, J. G.; Burrows, C. J. *Org. Lett.* **2001**, *3*, 2801.
6. (a) Duarte, V.; Gasparutto, D.; Jaquinod, M.; Ravanat, J.-L.; Cadet, J. *Chem. Res. Toxicol.* **2001**, *14*, 46; (b) Henderson, P. T.; Neeley, W. L.; Delaney, J. C.; Gu, F.; Niles, J. C.; Hah, S. S.; Tannenbaum, S. R.; Essigmann, J. M. *Chem. Res. Toxicol.* **2005**, *18*, 12.
7. (a) Henderson, P. T.; Delaney, J. C.; Gu, F.; Tannenbaum, S. R.; Essigmann, J. M. *Biochemistry* **2002**, *41*, 914; (b) Henderson, P. T.; Delaney, J. C.; Muller, J. G.; Neeley, W. L.; Tannenbaum, S. R.; Burrows, C. J.; Essigmann, J. M. *Biochemistry* **2003**, *42*, 9257.
8. Yu, H.; Niles, J. C.; Wishnok, J. S.; Tannenbaum, S. R. *Org. Lett.* **2004**, *6*, 3417.
9. (a) Lin, T.-S.; Cheng, J.-C.; Ishiguro, K.; Sartorelli, A. C. *J. Med. Chem.* **1985**, *28*, 1194; (b) Nampalli, S.; Kumar, S. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1677(c) High-resolution MS (HR-MS) experiments were performed in ESI mode on a Micromass Fourier transform MS (Micromass UK Limited). The purified sample was dissolved in 10% methanol containing 0.1% formic acid. The sample was directly infused into the MS system by a syringe pump without a column at a flow rate of 10 μl/min. All ESI-MS spectra were obtained in positive ion mode with an electrospray capillary potential of 3.0 kV and a cone potential of 21 V.; (d) The mass of each final compound was determined using mass spectrometry with 8-oxodG [*m*/*z* 283.2 (calculated), 284.0 (observed)] and ^{14}C-labeled 8-oxodG [*m*/*z* 285.2 (calculated), 285.9 (observed)], resulting in the expected mass.
10. Ye, Y.; Muller, J. G.; Luo, W.; Mayne, C. L.; Shallop, A. J.; Jones, R. A.; Burrows, C. J. *J. Am. Chem. Soc.* **2003**, *125*, 13926.
11. Kornyushyna, O.; Berges, A. M.; Muller, J. G.; Burrows, C. J. *Biochemistry* **2002**, *41*, 15304.
12. The reaction mixture was analyzed by reversed-phase HPLC using an analytical (250 × 4.6 mm i.d.) Hypersil ODS column (Thermo Electron Corporation, Bellefonte, PA). A 20% linear gradient of 50% acetonitrile in water for 30 min was employed at a flow rate of 1 mL/min (detection of products both at 220 and 260 nm).
13. dGh was identified by the signal at *m*/*z* 274.0 (calcd *m*/*z* 273.3) and dSp at *m*/*z* 300.5 (calcd *m*/*z* 299.2). The corresponding ^{14}C-labeled dGh and dSp were also identified by the signal at *m*/*z* 275.9 (calcd *m*/*z* 275.3) and at *m*/*z* 302.3 (calcd *m*/*z* 301.2), respectively.
14. MCF-7 human breast cancer cells were cultured following standard procedures until approximately 50% confluence was reached. Each plate was dosed with 300 dpm of ^{14}C-labeled 8-oxodG, referring to ~10 pmol of compound (^{14}C-labeled 8-oxodG comprises 20% of the total 8-oxodG dosed) with 1% DMSO. Triplicate plates were incubated with the labeled nucleoside for 0, 1, 2, and 4 days. DNA was isolated using a Qiagen kit to yield ~50–150 μg DNA per plate. The ratio of ^{14}C to total carbon in each sample was measured in Modern units by Accelerator Mass Spectrometry in Lawrence Livermore National Laboratory. Calculation of the number of attomole (amol) of ^{14}C per 100 μg of extracted cellular DNA was performed using the conversion factor of 1 Modern equals 97.8 amol of ^{14}C per mg of total carbon.
15. (a) Ognibene, T. J.; Bench, G.; Vogel, J. S.; Peaslee, G. F.; Murov, S. *Anal. Chem.* **2003**, *75*, 2192; (b) Li, Y.; Shawgo, R. S.; Tyler, B.; Henderson, P. T.; Vogel, J. S.; Rosenberg, A.; Storm, P. B.; Langer, R.; Brem, H.; Cima, M. J. *J. Controlled Release* **2004**, *100*, 211; (c) Sandhu, P.; Vogel, J. S.; Rose, M. J.; Ubick, E. A.; Brunner, J. E.; Wallace, M. A.; Adelsberger, J. K.; Baker, M. P.; Henderson, P. T.; Pearson, P. G.; Baillie, T. A. *Drug Metab. Dispos.* **2004**, *32*, 1254.