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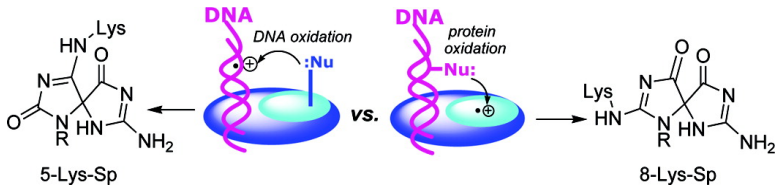

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DNA−Protein Cross-links between Guanine and Lysine Depend on the Mechanism of Oxidation for Formation of C5 Vs C8 Guanosine Adducts

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Abstract: The reaction between N^{α}-acetyllysine methyl ester (Lys) and 2′-deoxyguanosine (dGuo) was used to study structural aspects of DNA−protein cross-link (DPC) formation. The precise structure of DPCs depended on the nature of the oxidant and cross-linking reactions in which a series of different oxidation conditions generated a distribution of adducts, principally spirodiiminodihydantoins with lysine appended at the purine position of C5 (5-Lys-Sp), C8 (8-Lys-Sp), or both C5 and C8 (5,8-diLys-Sp). Singlet oxygen oxidation of dGuo produced 5-Lys-Sp exclusively when Rose Bengal or methylene blue was used to photochemically generate $^1O_2$ in the presence of Lys, whereas riboflavin or benzophenone-mediated photochemistry generated lysine radicals and led to C8 adduct formation, yielding 8-Lys-Sp and 5,8-diLys-Sp. Notably, the yield of dGuo modifications from riboflavin photooxidation increased dramatically in the presence of lysine. Oxidation of deoxyguanosine/lysine mixtures with Na_2IrCl_6 or sulfate radicals produced both 5-Lys-Sp and 8-Lys-Sp. The same adducts were formed in single and double-stranded oligodeoxynucleotides, and these could be analyzed after nuclease digestion. Adduct formation in duplex DNA was somewhat dependent on the accessibility of lysine to C5 vs C8 of the purine. No adduct formation was detected between lysine and the other nucleobases T, C, or A. Overall, the precise location of adduct formation at C5 vs C8 of guanine appears to be diagnostic of the oxidation pathway.

Introduction

DNA−protein cross-links (DPCs) are a common outcome of oxidative damage to cells, but still remain, from a chemical point of view, the least-well understood DNA lesion.[1] DPC levels in human white blood cells range from 0.5 to 4.5 per 10^7 bases, and they were observed to accumulate as a function of age in mouse organs. They also strongly correlated with the presence of 8-oxo-7,8-dihydroguanosine (OG), a biomarker of oxidative stress.[2]

As an abundant and significant type of DNA damage, DPC formation has been observed under diverse conditions; therefore, a wide range of structures and reaction mechanisms are anticipated depending on the nature of the oxidant, the medium, and the proximity of reactive DNA bases to protein side chains. In early studies, several groups observed DPC formation between pyrimidine bases and the amino acids tyrosine, phenylalanine, or lysine in photochemical or radical-induced cross-linking reactions.[3−6] More recently, Stemp and co-workers identified covalent adducts between DNA and histones or other proteins when guanine (G) residues in duplex DNA were oxidized with the DNA-bound intercalator Ru(1,10-phenanthroline)$_2$dipyridophenazine^{+3}.[7,8] Morin and Cadet characterized an adduct formed between guanine and lysine that was attributed to the initial generation of a guanine radical followed by nucleophilic trapping by lysine.[9−11] Recently, Ravanat and co-workers investigated the efficient formation of guanine−lysine cross-links upon riboflavin-mediated photooxidation of the trinucleotide TGT in the presence of the tripeptide KKK and have identified the nucleophilic addition of the ϵ-amino group of the central lysine residue to C8 of guanine.[12]

Covalent cross-links are also readily formed between DNA and proteins when the oligonucleotide contains an easily oxidized base such as 8-oxo-7,8-dihydroguanosine. For example, we have previously observed DPC formation from the two-electron oxidation of an OG-containing DNA oligomer bound to either *Escherichia coli* MutY or single-stranded binding protein (SSB) and have shown that lysine adds to the C5 position

(1) Barker, S.; Weinfeld, M.; Murray, D. *Mutat. Res.* **2005**, *589*, 111−135.
(2) Izzotti, A.; Cartiglia, C.; Taningher, M.; De Flora, S.; Balansky, R. *Mutat. Res.* **1999**, *446*, 215−223.
(3) Sun, G.; Fecko, C. J.; Nicewonger, R. B.; Webb, W. W.; Begley, T. P. *Org. Lett.* **2006**, *8*, 681−683.
(4) Margolis, S. A.; Coxon, B.; Gajewski, E.; Dizdaroglu, M. *Biochemistry* **1988**, *27*, 6353−6359.
(5) Dizdaroglu, M.; Gajewski, E. *Cancer Res.* **1989**, *49*, 3463−3467.
(6) Gajewski, E.; Dizdaroglu, M. *Biochemistry* **1990**, *29*, 977−980.
(7) Kurbanyan, K.; Nguyen, K. L.; To, P.; Rivas, E. V.; Lueras, A. M. K.; Kosinski, C.; Steryo, M.; Gonzalez, A.; Mah, D. A.; Stemp, E. D. A. *Biochemistry* **2003**, *42*, 10269−10281.
(8) Nguyen, K. L.; Steryo, M.; Kurbanyan, K.; Nowitzki, K. M.; Butterfield, S. M.; Ward, S. R.; Stemp, E. D. A. *J. Am. Chem. Soc.* **2000**, *122*, 3585−3594.
(9) Morin, B.; Cadet, J. *Photochem. Photobiol.* **1994**, *60*, 102−109.
(10) Morin, B.; Cadet, J. *J. Am. Chem. Soc.* **1995**, *117*, 12408−12415.
(11) Morin, B.; Cadet, J. *Chem. Res. Toxicol.* **1995**, *8*, 792−799.
(12) Perrier, S.; Hau, J.; Gasparutto, D.; Cadet, J.; Favier, A.; Ravanat, J. L. *J. Am. Chem. Soc.* **2006**, *128*, 5703−5710.

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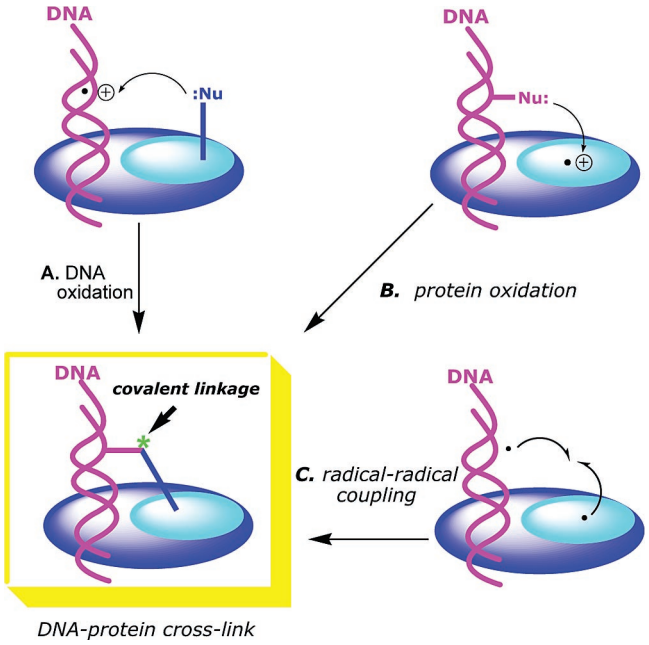
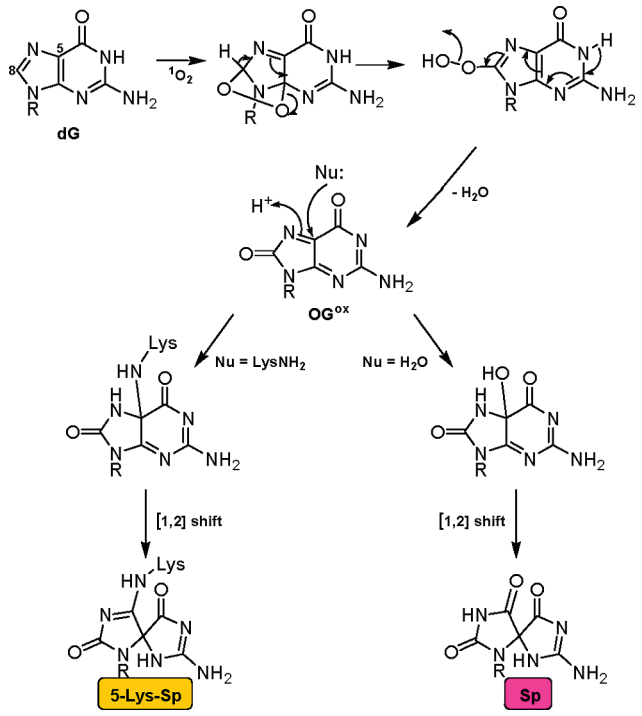


Figure 1. Covalent bond formation between DNA and protein components under oxidative stress can originate from an electron-deficient species on a DNA base being trapped by a protein nucleophile (A), from oxidation of an amino acid side chain that reacts with a DNA base (B), or less likely, from coupling of two radical species generated on both the DNA and protein components (C).

Scheme 1. $^1O_2$-Mediated Guanine Oxidation in the Presence of Lys[36]



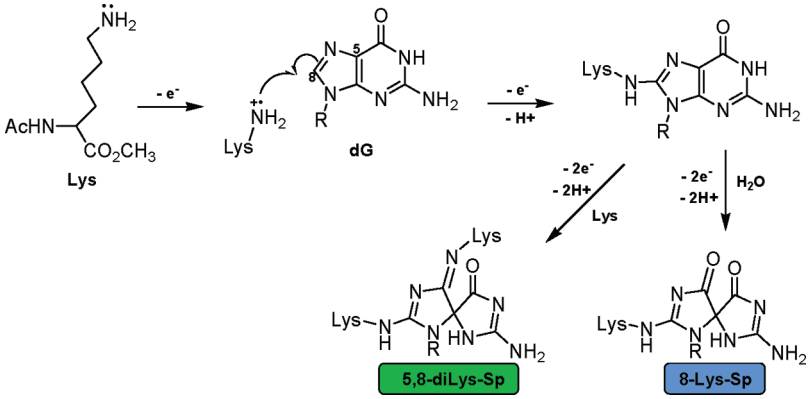
of oxidized OG.[13,14] When OG is present in the DNA−protein complex, the OG base is clearly the most easily oxidized site among the two biopolymers; its one-electron reduction potential is approximately 0.7 V. vs NHE,[15] substantially lower than the amino acid side chains or other bases. However, in a more typical case, where DNA is largely devoid of OG, oxidized adducts forming at G might occur either through initial oxidation at G, followed by trapping of a guanine-centered electron-deficient intermediate by a protein nucleophile (see Figure 1, path A), or alternatively, via initial formation of a protein radical that is subsequently covalently trapped by a G residue in DNA (see Figure 1, path B). In support of the latter case, Davies and co-workers proposed that oxidation of nucleosomes with HOCl occurs predominantly through histone oxidation, rather than DNA oxidation.[16] They detected protein-derived N-centered lysine radicals and showed that these radicals can react with pyrimidine bases to yield covalent adducts. Furthermore, there are several examples which conclude that radical formation associated with amino acid residues results in covalent bond formation between nucleobases and proteins.[17−20] Kelley et al. also observed that upon exposure of proteins to $^1O_2$, peroxides generated from amino acid oxidation ultimately led to DNA strand scission.[21,22]

A third alternative for DPC formation involving radical−radical coupling (Figure 1, path C) might be possible if either the protein radical or the nucleobase radical is sufficiently long-lived. However, this would require oxidation events on both the protein and the nucleic acid components to occur in close proximity as well as within a relatively short time frame.

Of the four nucleobases, G has the lowest reduction potential and is the most susceptible to oxidative stress. As a major terminal product of the oxidation of G and OG, spiroiminodihydantoin (Sp) has been characterized in our laboratory and others under a wide range of reaction conditions.[23−35] In the case of singlet oxygen oxidation of G, we postulated that Sp is formed by the nucleophilic addition of water to C5 of a two-electron oxidized form of OG (OG^{ox} in Scheme 1).[36] Nucleophilic trapping by a protein nucleophile, such as the ϵ-amino

(13) Hickerson, R. P.; Chepanoske, C. L.; Williams, S. D.; David, S. S.; Burrows, C. J. *J. Am. Chem. Soc.* **1999**, *121*, 9901−9902.
(14) Johansen, M. E.; Muller, J. G.; Xu, X.; Burrows, C. J. *Biochemistry* **2005**, *44*, 5660−5671.
(15) Steenken, S.; Jovanovic, S. *J. Am. Chem. Soc.* **1997**, *119*, 617−618.
(16) Hawkins, C. L.; Pattison, D. I.; Davies, M. J. *Biochem. J.* **2002**, *365*, 605−615.
(17) Luxford, C.; Dean, R. T.; Davies, M. J. *Biogerontology* **2002**, *3*, 95−102.
(18) Luxford, C.; Dean, R. T.; Davies, M. J. *Chem. Res. Toxicol.* **2000**, *13*, 665−672.
(19) Luxford, C.; Morin, B.; Dean, R. T.; Davies, M. J. *Biochem. J.* **1999**, *344*, 125−134.
(20) Distel, L.; Distel, B.; Schussler, H. *Radiat. Phys. Chem.* **2002**, *65*, 141−149.
(21) Prestwich, E. G.; Roy, M. D.; Rego, J.; Kelley, S. O. *Chem. Biol.* **2005**, *12*, 695−701.
(22) Mahon, K. P.; Ortiz-Meoz, R. F.; Prestwich, E. G.; Kelley, S. O. *Chem. Commun.* **2003**, *15*, 1956−1957.
(23) Luo, W.; Muller, J. G.; Rachlin, E. M.; Burrows, C. J. *Org. Lett.* **2000**, *2*, 613−617.
(24) Neeley, W. L.; Essigmann, J. M. *Chem. Res. Toxicol.* **2006**, *19*, 491−505.
(25) Pratviel, G.; Meunier, B. *Chem. Eur. J.* **2006**, *12*, 6018−6030.
(26) Gimisis, T.; Cismas, C. *Eur. J. Org. Chem.* **2006**, *6*, 1351−1378.
(27) Misiaszek, R.; Uvaydov, Y.; Crean, C.; Geacintov, N. E.; Shafirovich, V. *J. Biol. Chem.* **2005**, *280*, 6293−6300.
(28) Misiaszek, R.; Crean, C.; Geacintov, N.; Shafirovich, V. *J. Am. Chem. Soc.* **2005**, *127*, 2191−2200.
(29) Jia, L.; Shafirovich, V.; Shapiro, R.; Geacintov, N. E.; Broyde, S. *Biochemistry* **2005**, *44*, 6043−6051.
(30) Joffe, A.; Geacintov, N. E.; Shafirovich, V. *Chem. Res. Toxicol.* **2003**, *16*, 1528−1538.
(31) Niles, J. C.; Wishnok, J. S.; Tannenbaum, S. R. *Chem. Res. Toxicol.* **2004**, *17*, 1510−1519.
(32) Niles, J. C.; Wishnok, J. S.; Tannenbaum, S. R. *Org. Lett.* **2001**, *3*, 963−966.
(33) Sugden, K. D.; Campo, C. K.; Martin, B. D. *Chem. Res. Toxicol.* **2001**, *14*, 1315−1322.
(34) Martinez, G. R.; Medeiros, M. H. G.; Ravanat, J.-L.; Cadet, J.; Di Mascio, P. *Biol. Chem.* **2002**, *383*, 607−617.
(35) Cadet, J.; Ravanat, J.-L.; Martinez, G. R.; Medeiros, M. H. G.; Mascio, P. D. *Photochem. Photobiol.* **2006**, *82*, 1219−1225.
(36) 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*, 13926−13927.

Scheme 2. Lysine Radical-Mediated Guanine Oxidation



group of lysine, would generate the analogous spirocycle with lysine appended at the original C5 position of the purine (5-Lys-Sp in Scheme 1). In contrast, oxidation of G by a one-electron mechanism generates a radical species with unpaired electron density at both C5 and C8. Furthermore, the redox potentials of lysine and tyrosine fall below that of guanine, bringing in the possibility that protein radicals add to closed-shell guanine at either C5 or C8.

Because of this dichotomy of reaction sites, principally C5 vs C8, as well as the diversity of initial oxidation substrates, we hypothesized that there would be a distribution of spirocyclic adducts dependent upon the oxidation pathway. In the present work, we have explored the cross-link formation between guanine and lysine in a series of oxidation systems. To prevent reactions from the α-amino group, it and the carboxylate terminus were protected as N^{α}-acetyllysine methyl ester (Lys) for reactions with guanosine. By employing a simple system that includes only a single nucleoside, deoxyguanosine, and a single amino acid, Lys, we could completely characterize product distributions under a variety of oxidation conditions. We then compare these reactions to those of single- and double-stranded oligodeoxynucleotides with lysine.

Results

Cross-linking Studies between dGuo and Lys. In nucleoside experiments, 1.5 mM dGuo was allowed to react with 15 mM N^{α}-acetyllysine methyl ester (Lys) in the presence of various oxidants, which yielded covalent adducts between the ϵ-amino group of lysine and the C5 or C8 position of guanine. These overall 4-electron oxidation processes included a ring-contracting rearrangement yielding the spirocyclic adducts 5-Lys-Sp, 8-Lys-Sp, and 5,8-diLys-Sp (Schemes 1 and 2). The structural assignments of these adducts were based on 2D NMR spectra and MS fragmentation studies of the collision-induced free base (see Supporting Information) that are consistent with the previously reported MS/MS for Sp[23] as well as with the characterization of lysine-containing peptide adducts reported by Ravanat and co-workers.[12] The type II photosensitizers Rose Bengal (RB) and methylene blue (MB) are known to produce $^1O_2$, and their photochemical reactions resulted in a Lys adduct at the C5 position of Sp, supporting a [4+2] cycloaddition pathway (Scheme 1).[36,37] The relative product yields for singlet oxygen-mediated adduct formation are summarized in Figure 2, columns 1 and 2.

(37) Kang, P.; Foote, C. S. *J. Am. Chem. Soc.* **2002**, *124*, 4865−4873.

In sharp contrast, the type I photosensitizers benzophenone (BP) and riboflavin (Rf) led to the formation of 8-Lys-Sp as the major adduct, whereas the iridium(IV) complex Na_2IrCl_6 and sulfate radicals (generated from photolysis of $K_2S_2O_8$) initiated sequential one-electron oxidation producing both 5-Lys and 8-Lys adducts. In addition, small amounts of the 5,8-diLys adduct were produced with all oxidant systems investigated (except $^1O_2$) due to the stoichiometric excess of Lys used in these studies.

Cross-linking Studies between d(ATGC) and Lys. In order to gain further insight for adduct formation, studies were conducted with a tetramer d(ATGC) and Lys in the presence of RB or Rf. Adducts were analyzed after digestion and HPLC separation. For RB-mediated $^1O_2$ oxidation in the absence of Lys, more than 90% of the guanine residues were converted to Sp, in which water served as the nucleophile. In the presence of Lys as a competing nucleophile, increasing amounts of 5-Lys-Sp were produced (Figure 3, columns 1−3), fully consistent with the nucleoside studies. However, when Rf was the photocatalyst for oxidation, the overall yield was low in the absence of Lys; only 11% of d(ATGC) was converted to d(ATSpC). In the presence of increasing concentrations of Lys (15 and 75 mM), the yield of guanine-modified products

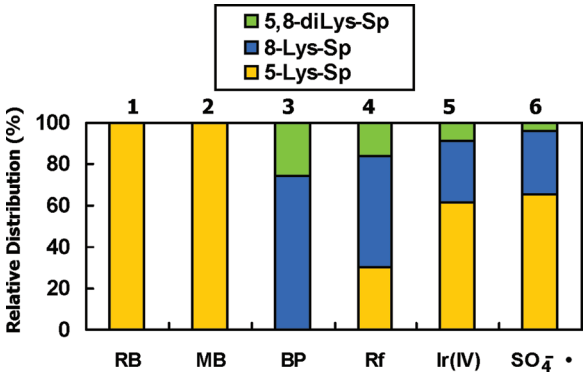


Figure 2. Relative product distributions of Lys-Sp adducts under different oxidation conditions using Rose Bengal (RB), methylene blue (MB), benzophenone (BP), riboflavin (Rf), Na_2IrCl_6 (IrIV), or $K_2S_2O_8$. (1) [RB] = 150 μM, tungsten lamp (300 W), 3 h. (2) [MB] = 150 μM, tungsten lamp (300 W), 3 h. (3) [BP] = ~20μM, 365 nm UV lamp, 20h. (4) [Rf] = 150 μM, tungsten lamp (300 W), 3 h. (5) [Ir(IV)] = 10 mM, 0.5 h. (6) [$SO_4^{-\bullet}$] = 40 mM, 254 nm UV lamp, 6 h. In all reactions: [dGuo] = 1.5 mM; [Lys] =15 mM; pH = 7.5. 95% of dGuo was oxidized in the presence of RB, MB, Rf, and Ir, 30% of dGuo underwent reaction in the reactions with $K_2S_2O_8$, and 10% of dG was reacted in the reactions with BP. The relative distributions are based on the formation of Sp-like adducts between Lys and dGuo.[38]

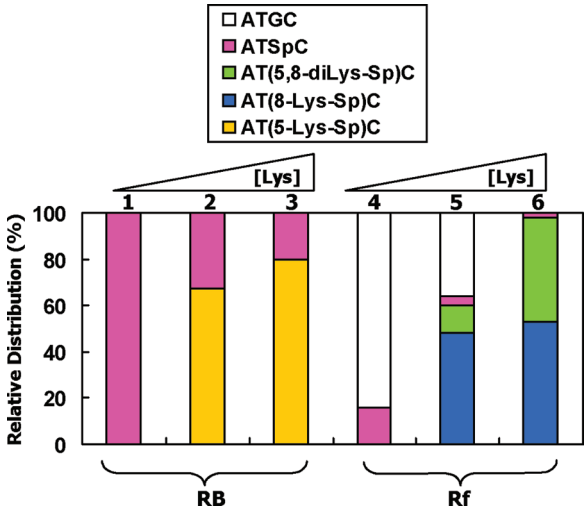


Figure 3. Relative distributions of the adducts of ATGC and Lys. (1) [RB] = 150 μM, no Lys. (2) [RB] = 150 μM, [Lys] = 15 mM. (3) [RB] = 150 μM, [Lys] = 75 mM. (4) [Rf] = 150 μM, no Lys. (5) [Rf] = 150 μM, [Lys] = 15 mM. (6) [Rf] = 150 μM, [Lys] = 75 mM. For all reactions, [dATGC] = 50 μM, pH = 7.5, tungsten lamp (300 W), 3 h.

increased dramatically: 48−53% of G residues were converted to 8-Lys-Sp, and 12−45% were converted to 5,8-diLys-Sp (Figure 3, columns 4−6). These results suggest that Lys participates in the first step of the type I photooxidation process forming a reactive intermediate, likely an aminyl radical, that leads to C8-adduct formation (Scheme 2).

For Rf photooxidations, the product distributions differed for the tetranucleotide vs nucleoside. No 5-Lys-Sp was produced from oxidation of d(ATGC), whereas 5-Lys-Sp was a significant product when dGuo was oxidized by Rf in the presence of Lys. One possible explanation for this result is that, although G is harder to oxidize than Lys, small amounts of oxidized dGuo may be produced (Figure 1, Pathway A) due to the relatively higher concentration of guanine in the monomer experiment (1.5 mM dGuo) compared to the tetranucleotide (50 μM). Consistent with type I photochemical oxidations, $IrCl_6^{2-}$ ($E_{1/2}$ = 0.9 V vs NHE) reactions produced both 5-Lys-Sp and 8-Lys-Sp in the presence of dGuo, apparently because it could oxidize both Lys and G and proceed through both the Lys oxidation pathway (Figure 1, path B and Scheme 2) and the G oxidation pathway (Figure 1, path A). Significantly, equimolar amounts of dA, dT, and dC were detected, and there was no evidence that any base other than G underwent oxidation or adduct formation.[39]

Cross-linking Studies between Duplex DNA and Lys. Adduct formation was also explored in Ir^{IV}-initiated reactions with two single-stranded DNA oligomers, 5′-TCATGGGTCG-TCGGTATA-3′ (**1**) and 5′-TATACCGACGACCCATGA-3′ (**2**), and a double-stranded DNA duplex containing these two oligodeoxynucleotides, with the goal of understanding how accessibility to C5 and C8 might change upon formation of the duplex. Chemical oxidation using the outer-sphere oxidant Na_2IrCl_6 was chosen because the reactivity of some oxidants,

(38) Although other guanine oxidation products such as Sp, imidazolone (Iz), and oxazolone (Oz) may also be produced, these highly polar products were not measurable by this HPLC assay because they would elute with the solvent front. By comparison to the study of d(ATGC) shown in Figure 2, it can be estimated that these products, if present, are less than 10% of the total.

(39) Interestingly, the parallel pathway to guanidinohydantion-lysine adducts was not observed under these conditions, although it was a minor product when an 18-mer oligonucleotide was studied.[14]

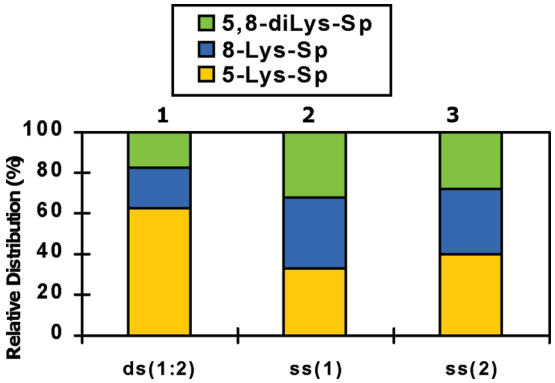


Figure 4. Relative distributions of the Ir^{IV}-mediated adducts of guanine and Lys in ssDNA and dsDNA analyzed after nuclease digestion. (1) [ds**1**·**2**] = 0.05 mM, [Lys] = 75 mM, [Ir] = 2.5 mM. (2)[1] = 0.05 mM, [Lys] = 75 mM, [Ir] = 1.25 mM. (3) [**2**] = 0.05 mM, [Lys] = 75 mM, [Ir] = 1.25 mM. In all reactions, pH = 7.5.

notably $^1O_2$, is extremely low with duplex DNA.[40] The reaction mixtures were digested by P1 nuclease, snake venom phosphodiesterase, and alkaline phosphatase, and then analyzed by HPLC. Although both ssDNA and dsDNA yielded 5-Lys-Sp and 8-Lys-Sp adducts, a marked increase in formation of the 5-Lys-Sp was observed in dsDNA (Figure 4), which may be a measure of the greater accessibility of C5 compared to C8 in the major groove of duplex DNA. Reactions of dsDNA and ssDNA oligomers were also conducted with Rf photocatalysis and analyzed by HPLC after enzyme digestion. Both the 8-Lys-Sp and the 5,8-diLys-Sp adducts were detected in yields comparable to those formed from Ir^{IV} oxidation (data not shown). However, consistent with the data of Figure 3, the yield of the 5-Lys-Sp from Rf was too low to estimate, and a comparison of any change between ssDNA and dsDNA could not be made.

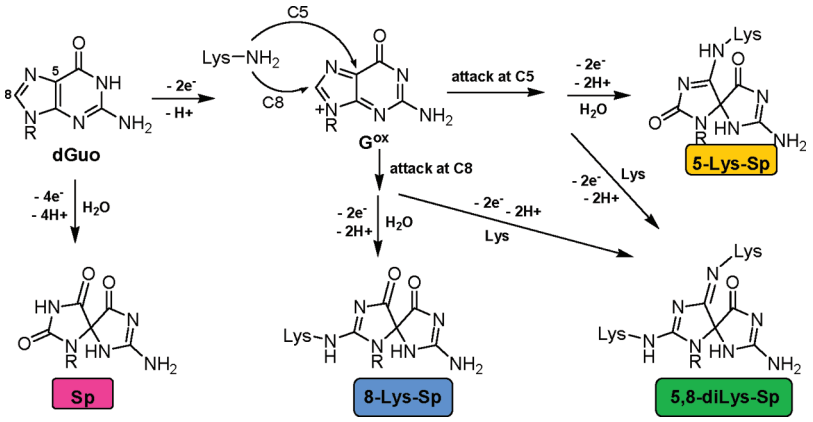
Discussion

Two previous studies in our laboratory implicated lysine side chains and 8-oxo-7,8-dihydroguanosine as key partners in oxidative DNA−protein cross-linking.[13,14] When OG is present in a DNA−protein complex, it is clearly the most readily oxidized site, and its further oxidation by any oxidant leads to an electron-deficient intermediate that can be trapped at C5 of the purine by nucleophiles such as the ϵ-amino group of lysine in competition with water. When OG is absent, it is less clear that a nucleobase will be the initial site of oxidation; instead, it will depend upon the nature of the oxidant and its mechanism of action. In the present studies, singlet oxygen oxidation of either the mononucleoside dGuo or oligonucleotides containing G led exclusively to lysine adduct formation at C5 of the purine. This result is fully consistent with the previously proposed mechanism[36] in which singlet oxygen first introduces an oxygen atom at C8 via cycloaddition, followed by attack of a nucleophile at C5 of the quinonoid OG^{ox} intermediate (Scheme 1). Subsequent rearrangement yields the final stable adduct, 5-Lys-Sp. Thus, oxidation initiated by $^1O_2$ leads exclusively to the lysine adduct at C5 of the purine via initial oxidation of DNA rather than lysine.

In contrast, one-electron oxidants will select the most readily oxidized partner according to one-electron reduction potentials.

(40) Hickerson, R. P.; Prat, F.; Muller, J. G.; Foote, C. S.; Burrows, C. J. *J. Am. Chem. Soc.* **1999**, *121*, 9423−9428.

Scheme 3. Two-Electron Oxidation of G in the Presence of Lys



In general, primary amines have a lower redox potential (1.1 V vs NHE, pH 10)[41] than G (1.3 V vs NHE, pH 7)[15] and are therefore more readily oxidized. Protonation inhibits the oxidation process, and lysine or Lys residues in proteins would be predominantly protonated under the reaction conditions used here (pH 7.5). Nevertheless, transient deprotonation of Lys, present in 10-fold excess in these experiments, could lead to the generation of the ϵ-aminyl radical (or aminium radical cation). For example, oxidation of histones with HOCl led to a 50:1 preference for oxidation of lysine and histidine compared to DNA bases, and the lysine aminyl radical was shown to react with the 5,6 double bond of pyrimidine bases to form covalent adducts.[16] Therefore, it was anticipated that oxidation of a DNA−protein complex by Type I photooxidants (Rf or BP) or by one-electron oxidants (Ir^{IV} or sulfate radical) would lead predominantly to protein-centered oxidation.

Hydroxyl radicals are also known to attack guanines at C8 to form 8-oxoG.[42] Recent work by Manderville and co-workers demonstrated that phenoxyl radicals reacted at the C8 site of G and led to various C8-G adducts, further indicating that C8 is the target site of radical attack.[43−46] Additionally, heterocyclic aromatic amines are known to covalently modify DNA to give the C8-G adduct with a covalent bond being formed between N and C8 of G upon biological activation,[47] although the mechanism in this case is thought to involve aryl nitrenium ions. In our studies of Rf-mediated Lys adduct formation with d(ATGC), DNA hydrolysis of the reaction adducts yielded equivalent amounts of dA, dT, and dC, indicating that Lys radical reacted exclusively with guanine. Taken together, these data suggest that Lys oxidation leads to an initial formation of an 8-Lys-G adduct through attack of an aminyl radical (or aminium radical cation) at C8 of G (Scheme 2). Primary aminyl radicals are extremely reactive toward addition to π bonds.[48−50] Coupled with the numerous examples of preferential radical attack at the C8 position of G, it is not surprising that oxidation centered on Lys leads primarily to adduct formation at C8.

The initially formed adduct 8-Lys-G is not observed; such a species would be expected to have a redox potential in the range of or lower than OG, the best precedence for this being the recently measured potentials of C8-G arylamine adducts that display $E_{1/2}$ values about 100 mV lower than OG.[51] Under the reactions conditions employed here that optimize adduct formation, i.e., excess oxidant and Lys, the initial 8-Lys-G adduct is likely to undergo a second two-electron oxidation to the analog of OG^{ox}, adding an H_2O or second Lys as a nucleophile at C5 (Scheme 2). The isolated products are then 8-Lys-Sp or 5,8-diLys-Sp after rearrangement.

Nucleoside studies with cationic Lys were conducted with higher concentrations of neutral 2′-deoxyguanosine compared to polyanionic tetranucleotide. These conditions optimize adduct formation and facilitate isolation and characterization. However, the higher concentrations used in monomer studies may also explain the mixture of isomers obtained when more potent oxidants were employed, particularly sulfate radical. This oxidant with a reduction potential of ~2.5 V can readily oxidize either dGuo or Lys leading to a more complex mixture of products.

Another explanation for the mixture of 5-Lys-Sp and 8-Lys-Sp adducts observed with Ir^{IV} and sulfate radical oxidation is two-electron oxidation of G to the quinonoid species G^{ox} (Scheme 3). Although OG formation is normally ascribed to H_2O attack at C8 of a guanine radical cation, a second one-electron oxidation of G to G^{ox} would explain the dual reaction sites of C5 and C8 toward nucleophiles. This mechanism has been discussed in some detail by Pratviel and co-workers for two-electron oxidants, and might be operating here under conditions of higher concentrations of one-electron oxidants.[52] Interestingly, literature examples show that some reaction conditions favor oxidation at C5 of G without oxidation of the C8 position, notably certain transition metal complexes that lead to a hydantoin that is not C8 oxidized,[25,53,54] as well as pathways

(41) Koppang, M. D.; Witek, M.; Blau, J.; Swain, G. M. *Anal. Chem.* **1999**, *71*, 1188−1195.
(42) Burrows, C. J.; Muller, J. G. *Chem. Rev.* **1998**, *98*, 1109−1151.
(43) Dai, J.; Wright, M. W.; Manderville, R. A. *J. Am. Chem. Soc.* **2003**, *125*, 3716−3717.
(44) Dai, J.; Wright, M. W.; Manderville, R. A. *Chem. Res. Toxicol.* **2003**, *16*, 817−821.
(45) Faucet, V.; Pfohl-Leszkowicz, A.; Dai, J.; Castegnaro, M.; Manderville, R. A. *Chem. Res. Toxicol.* **2004**, *17*, 1289−1296.
(46) Manderville, R. A. *Can. J. Chem.* **2005**, *83*, 1261−1267.
(47) Elmquist, C. E.; Stover, J. S.; Wang, Z.; Rizzo, C. J. *J. Am. Chem. Soc.* **2004**, *126*, 11189−11201.
(48) Newcomb, M.; Weber, K. A. *J. Org. Chem.* **1991**, *56*, 1309−1313.
(49) Guindon, Y.; Guerin, B.; Landry, S. R. *Org. Lett.* **2001**, *3*, 2293−2296.
(50) Benati, L.; Bencivenni, G.; Leardini, R.; Nanni, D.; Minozzi, M.; Spagnolo, P.; Scialpi, R.; Zanardi, G. *Org. Lett.* **2006**, *8*, 2499−2502.
(51) Stover, J. S.; Ciobanu, M.; Cliffel, D. E.; Rizzo, C. J. *J. Am. Chem. Soc.* **2007**, *129*, 2074−2081.
(52) Kupan, A.; Sauliere, A.; Broussy, S. C.; Seguy, S.; Pratviel, G.; Meunier, B. *ChemBioChem* **2006**, *7*, 125−133.
(53) Ye, W.; Sangaiah, R.; Degen, D. E.; Gold, A.; Jayaraj, K.; Koshlap, K. M.; Boysen, G.; Williams, J.; Tomer, K. B.; Ball, L. M. *Chem. Res. Toxicol.* **2006**, *19*, 506−510.
(54) Li, L.; Karlin, K. D.; Rokita, S. E. *J. Am. Chem. Soc.* **2005**, *127*, 520−521.

leading to imidazolone/oxazolone products.[55] Formation of the ring-opened guanidinohydantoin (Gh) analog is disfavored under these slightly basic conditions (pH = 7.5) and in the absence of stacking interactions, as in nucleoside (2′-deoxyguanosine) or tetranucleotide (d(ATGC)) studies.[23] Therefore, it was not surprising that no Gh-like adducts were detected.

The oxidation of reaction mixtures containing Lys plus double-stranded vs single-stranded DNA showed a small but noticeable difference in the position of the lysine adduct when Ir^{IV} was used as the oxidant. The relative amount of 5-Lys-Sp formation increased in dsDNA compared to C8, suggesting that Lys had better access to the purine's C5 in the major groove. The comparable study with $^1O_2$ oxidation could not be conducted because its reaction mechanism is a cycloaddition with guanine and consequently double-stranded DNA has very low reactivity. Any small amounts of oxidation products from duplex DNA + $^1O_2$ could be due to dynamic breathing of the duplex giving locally melted structures and would not necessarily reflect the intrinsic reactivity of the duplex. Oxidation of G by $^1O_2$ in a duplex structure is anticipated to yield some amount of guanidinohydantoin,[56] but its detection via enzyme digestion and HPLC analysis have been problematic. We cannot rule out the formation of Gh-like adducts in dsDNA in addition to the Sp structures reported herein.

Conclusions

DNA−protein cross-links are observed from cells under oxidative stress, including those exposed to carcinogenic metal ions such as nickel and chromium.[33,57−63] DPCs are potent blocks to transcription and replication and must be repaired for cell survival.[64,65] Their mechanisms of repair likely depend upon the types of covalent cross-links being formed, and therefore, it is important to understand the chemical structures of nucleobase-amino acid adducts as well as the conditions of oxidative stress that produce them. DPCs are excellent substrates for the nucleotide excision repair pathway (NER),[65,66] although some models propose that proteolytic digestion first produces a DNA−peptide cross-link before NER.[67−70] It is not known if subtle differences in the position of the cross-link, C5 vs C8 adduction to G for example, would undergo differential rates of repair, but such studies could now be envisioned.

DPC formation between guanine and lysine has been investigated in this study because guanine oxidation is implicated in most forms of oxidative DNA damage. Furthermore, the abundance of lysine residues in DNA-binding proteins as well as its susceptibility to oxidation makes lysine one of the key amino acids in formation of DPCs. Our efforts focused on an investigation of the spirodihydantoin-lysine adducts as stable products emanating from a range of oxidation systems with nucleosides, single-stranded and duplex DNA, and these were characterized by MS/MS, NMR, and UV. To date, only the parent spiroiminodihydantoin lesion has been detected in cells exposed to chromate;[71] the present studies should aid in the detection of amino acid, peptide or protein adducts of Sp.

We propose here the mechanisms for the formation of 5-Lys-Sp, 8-Lys-Sp and 5,8-diLys-Sp adducts under these oxidation conditions. Lysine oxidation leads to an 8-Lys-G adduct, and its facile further oxidation produced 8-Lys-Sp and 5,8-diLys-Sp since lysine serves as a competitive nucleophile with water. In contrast, $^1O_2$ oxidation of dGuo generates 5-Lys-Sp exclusively because of its initial cycloaddition reaction with guanine; lysine then enters as a nucleophile at a later stage. The pattern is complicated for certain one-electron oxidants that react with dGuo to yield both 5-Lys-Sp and 8-Lys-Sp in the presence of Lys.

Riboflavin photooxidation is commonly employed to mimic one-electron oxidation processes in the cell generating the guanine radical cation and subsequent DNA damage products.[72,73] A significant observation in the present study is the fact that the extent of Rf-mediated guanine modification in the tetramer d(ATGC) was greatly enhanced in the presence of lysine. This is ascribed to preferential oxidation of Lys generating a highly reactive aminium radical cation that can add to C8 of G. This observation underscores the concept that cellular DNA, which exists mainly in the form of lysine-rich DNA−protein complexes, may in fact be more likely to undergo attack from lysine radicals than from direct oxidation and electron loss at guanine.

Overall, these studies strongly suggest that amino acid radical cation formation leads to Lys adducts at the C8 position of G (path B in Figure 1), whereas nucleobase oxidation produces adducts at both C-5 and C-8 depending upon the oxidant: singlet oxygen leads exclusively to 5-Lys-Sp (path A in Figure 1), whereas the C5:C8 ratio for other oxidants depends on the concentration and the accessibility of these site in single or double-stranded DNA. In any case, it is clear that the precise structure of DNA−protein cross-links can serve as an indication of the type of oxidant leading to these lesions, and thus, DPC structure may serve as a signature of different types of oxidative stress in the cell. Furthermore, the benefit of polyamines in protecting against oxidative damage to DNA[74,75] may be mitigated by the reactivity of aminium radicals toward guanine bases.

(55) Cadet, J.; Berger, M.; Buchko, G. W.; Joshi, P. C.; Raoul, S.; Ravanat, J.-L. *J. Am. Chem. Soc.* **1994**, *116*, 7403−7404.
(56) Luo, W.; Muller, J. G.; Rachlin, E. M.; Burrows, C. J. *Chem. Res. Toxicol.* **2001**, *14*, 927−938.
(57) Zhitkovich, A.; Voitkun, V.; Kluz, T.; Costa, M. *Environ. Health Perspect.* **1998**, *106*, 969−974.
(58) Zhang, Q.; Kluz, T.; Salnikow, K.; Costa, M. *Biol. Trace Elem. Res.* **2002**, *86*, 11−22.
(59) Costa, M.; Zhitkovich, A.; Harris, M.; Paustenbach, D.; Gargas, M. *J. Toxicol. Environ. Health* **1997**, *50*, 433−449.
(60) Taioli, E.; Zhitkovich, A.; Kinney, P.; Udasin, I.; Toniolo, P.; Costa, M. *Biol. Trace Elem. Res.* **1995**, *50*, 175−180.
(61) Zhitkovich, A.; Lukanova, A.; Popov, T.; Taioli, E.; Cohen, H.; Costa, M.; Toniolo, P. *Biomarkers* **1996**, *1*, 86−93.
(62) Borges, K. M.; Wetterhahn, K. E. *Carcinogenesis* **1989**, *10*, 2165−2168.
(63) Tsapakos, M. J.; Hampton, T. H.; Wetterhahn, K. E. *Cancer Res.* **1983**, *43*, 5662−5667.
(64) Reardon, J. T.; Cheng, Y.; Sancar, A. *Cell Cycle* **2006**, *5*, 1366−1370.
(65) Minko, I. G.; Kurtz, A. J.; Croteau, D. L.; VanHouten, B.; Harris, T. M.; Lloyd, R. S. *Biochemistry* **2005**, *44*, 3000−3009.
(66) Minko, I. G.; Zou, Y.; Lloyd, R. S. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 1905−1909.
(67) Desai, S. D.; Zhang, H.; Rodriguez-Bauman, A.; Yang, J.-M.; Wu, X.; Gounder, M. K.; Rubin, E. H.; Liu, L. F. *Mol. Cell. Biol.* **2003**, *23*, 2341−2350.
(68) Mao, Y.; Desai, S. D.; Ting, C.-Y.; Hwang, J.; Liu, L. F. *J. Biol. Chem.* **2001**, *276*, 40652−40658.
(69) Quievryn, G.; Zhitkovich, A. *Carcinogenesis* **2000**, *21*, 1573−1580.
(70) Baker, D. J.; Wuenschell, G.; Xia, L.; Termini, J.; Bates, S. E.; Riggs, A. D.; O'Connor, T. R. *J. Biol. Chem.* **2007**, *282*, 22592−22604.
(71) Hailer, M. K.; Slade, P. G.; Martin, B. D.; Sugden, K. D. *Chem. Res. Toxicol.* **2005**, *18*, 1378−1383.
(72) Luo, W.; Muller, J. G.; Burrows, C. J. *Org. Lett.* **2001**, *3*, 2801−2804.
(73) Ravanat, J.-L.; Remaud, G.; Cadet, J. *Arch. Biochem. Biophys.* **2000**, *374*, 188−127.
(74) Newton, G. L.; Ly, A.; Tran, N. Q.; Ward, J. F.; Milligan, J. R. *Int. J. Radiat. Biol.* **2004**, *80*, 643−651.
(75) Ly, A.; Bullick, S.; Won, J.-H.; Milligan, J. R. *Int. J. Radiat. Biol.* **2006**, *82*, 421−433.

Experimental Section

Materials. N^{α}-acetyllysine methyl ester (Lys), 2′-deoxyguanosine (dGuo), Rose Bengal (RB), benzophenone (BP), $K_2S_2O_8$, methylene blue (MB), riboflavin (Rf) and Na_2IrCl_6 were used as purchased from commercial suppliers. The oligodeoxynucleotides 5′- d(ATGC)-3′ (ATGC), 5′-TCA TGG GTC GTC GGT ATA-3′ (**1**), and 5′-TAT ACC GAC GAC CCA TGA-3′ (**2**) were obtained from the DNA/Peptide Core Facility (University of Utah). Nuclease P1 was obtained from U.S. Biological (Swampscott, MA), snake venom phosphodiesterase was from USB Co. (Cleveland, OH), and alkaline phosphatase was from Promega (Madison, WI).

Cross-linking Studies with Lys and 2′-Deoxyguanosine (dGuo) in the Presence of Rose Bengal, Methylene Blue, or Riboflavin. A 3-mM concentration of 2′-deoxyguanosine and 30 mM Lys were prepared in 250 μL 75 mM phosphate buffer (pH = 7.5). A 250-μL portion of Rose Bengal, methylene blue, or riboflavin dissolved in 75 mM NaP_i (pH = 7.5) was then added to a final concentration of 150 μM. The reaction mixture was then incubated at 37 °C in a water bath and irradiated with a 300 W tungsten lamp at a distance of 20 cm for 3 h (6.4×10^3 kJm^{-2}). The photosensitizers were removed using a NAP-25 column, and the eluant was then analyzed by HPLC using a Synergi 4u Polar-RP (Phenomenex) column (Solvent A: 0.1% TFA in H_2O; Solvent B: methanol. The first 15-min period was isocratic at 12% B, followed by a linear gradient to 40% B over 25 min. This was maintained for 5 min, and then raised to 65% B over a 15-min period. The flow rate was 1 mL/min and UV detector was set at 220 nm.).

Cross-linking Studies with Lys and dGuo in the Presence of Benzophenone. A 3-mM concentration of 2′-deoxyguanosine and 30 mM Lys were prepared in 250 μL 75 mM phosphate buffer (pH = 7.5). A 250-μL portion of benzophenone solution (~40 μM) in 75 mM NaP_i (pH = 7.5) was then added. The reaction mixture was irradiated with a 365 nm UV lamp (115V, 0.16A) at a distance of 4 cm for 20 h (1.4×10^3 kJm^{-2}) at room temperature. The reaction mixture was then analyzed by RP−HPLC as described above.

Cross-linking Studies with Lys and dGuo in the Presence of Ir-(IV). A 3-mM concentration of 2′-deoxyguanosine and 30 mM Lys were prepared in 100 μL 75 mM phosphate buffer (pH = 7.5); another 100 μL 500 mM NaP_i buffer (pH = 7.5) and 10 μL 200 mM Na_2IrCl_6 were then added. After incubation at 37 °C for 30 min, the reaction mixture was quenched with 20 μL 100 mM EDTA, and then analyzed by RP−HPLC as described above.

Cross-linking Studies with Lys and dGuo in the Presence of $SO_4^{-\bullet}$. A 3-mM concentration of 2′-deoxyguanosine and 30 mM Lys were prepared in 250 μL 75 mM phosphate buffer (pH = 7.5). Another 200 μL 500 mM NaP_i buffer (pH = 7.5) and 50 μL 200 mM $K_2S_2O_8$ were then added. The reaction mixture was irradiated with a 254 nm UV lamp (115V, 0.16A) at a distance of 4 cm for 6 h (2.4×10^2 kJm^{-2}) at room temperature, and then analyzed by RP−HPLC as described above.

Cross-linking Studies with Lys and ATGC in the Presence of Rose Bengal or Riboflavin. A 25-nmol portion of ATGC and 0, 30, or 150 mM Lys were prepared in 250 μL 75 mM phosphate buffer (pH = 7.5); 250 μL Rose Bengal or riboflavin dissolved in 75 mM NaP_i (pH = 7.5) was then added to a final concentration of 150 μM photosensitizer. The reaction mixture was then incubated at 37 °C in a water bath and irradiated with a 300 W tungsten lamp at a distance of 20 cm for 3 h (6.4×10^3 kJm^{-2}). Rose Bengal or riboflavin was removed using a NAP-25 column and the eluant was then analyzed by AE-HPLC using a DNAPac PA-100 (DIONEX) 4 × 250 mm analytical column (Solvent A: 10% of acetonitrile in water; Solvent B: 1.5M Sodium acetate, 10% of acetonitrile in water, pH 7.0. A linear gradient from 1 to 12% B over 20 min was followed by a second gradient to 100% B over the next 30 min. The flow rate was 1 mL/min with UV detection at 260 nm).

Cross-linking Studies with Lys and 1, 2 or dsDNA in the Presence of Ir(IV). Double-stranded DNA was prepared by heating equivalent moles of **1** (5′-TCATGGGTCGTCGGTATA-3′) and **2** (5′-TATAC-CGACGACCCATGA-3′) at 90 °C for 3 min, followed by cooling down to room-temperature slowly. A 10-nmol dsDNA, **1** or **2**, was incubated with 75 mM Lys at 37 °C for 30 min (200 μL, 300 mM NaP_i, pH = 7.5). The reaction was initiated by adding 20, 10, or 10 μL 25 mM Na_2IrCl_6, respectively, and was quenched with 5, 2.5, or 2.5 μL of 100 mM EDTA, respectively, after 30 min. The reaction mixtures were then dialyzed against distilled water. The desalted samples were dried under vacuum and hydrolyzed by 5 u of nuclease P1 in 33 mM NaOAc and 2 mM $Zn(OAc)_2$ (pH = 5.3) for 12 h and then by 5 u of snake venom phosphodiesterase and 10 u of alkaline phosphatase in 200 mM Tris·HCl and 10 mM $MgCl_2$ (pH = 8.0) for another 12 h. The resulting mixtures were then loaded on HPLC using a Synergi 4u Polar-RP (Phenomenex) column. (Solvent A: 0.1% TFA in H_2O; Solvent B: methanol. The solvent was isochratic at 0.5% B for 15 min, followed by a linear gradient to 20% B in 25 min, and then another linear gradient to 65% B over 20 min. The flow rate was 1 mL/min and UV detector was set at 220 nm.)

ESI−MS/MS Analysis of the Various Adducts of Deoxyguanosine and N^{α}-Acetyllysine methyl ester (Lys). The nucleoside samples were analyzed by positive ion electrospray ionization (ESI) on a Micromass Quattro II tandem mass spectrometer equipped with Zspray API source. Samples were dissolved in acetonitrile and water (1:1) and introduced via infusion at a flow rate of 7 μL/min. The source and desolvation temperatures were 80 and 120 °C, respectively. The capillary voltage was set to 3.25 kV, the sampling cone voltage was set to 45 V, and the extractor cone was set to 3 V. The collision energy was set to 18 eV. Argon, used as a gas collision for CID experiments, was adjusted to a pressure of 1.7×10^{-4} mBar. The mass for the nucleoside base was set in the first scanning analyzer (MS-1), and the precursor ion was subjected to CID in the static quadrupole. The resulting spectra of the products were recorded by scanning the second scanning analyzer (MS-2) between 50 and 600 Da. The scan duration and interscan delay were 3.0 and 0.1 s, respectively. The instrument was operated and the data were accumulated with Micromass Masslynx software (version 3.5).

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Supporting Information Available: HPLC traces of each dGuo reaction, MS/MS and UV spectra of 5-Lys-Sp, 8-Lys-Sp, and 5,8-diLys-Sp, HPLC of ATGC reaction with Rose Bengal or riboflavin, HMBC NMR spectrum of 5-Lys-Sp. This material is available free of charge via the Internet at http://pubs.acs.org.

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