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Characterization of Lysine−Guanine Cross-Links upon One-Electron Oxidation of a Guanine-Containing Oligonucleotide in the Presence of a Trilysine Peptide

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Abstract: Formation of DNA−protein cross-links involving the initial formation of a guanine radical cation was investigated. For this purpose, riboflavin-mediated photosensitization of a TGT oligonucleotide in aerated aqueous solution in the presence of the KKK tripeptide was performed. We have shown that the nucleophilic addition of the ϵ-amino group of the central lysine residue of KKK to the C8 atom of either the guanine radical cation or its deprotonated form gives rise to the efficient formation of a $N\epsilon$-(guanin-8-yl)-lysine cross-link. Interestingly, the time course of formation of the above-mentioned cross-link was found to be not linear with the time of irradiation, and its formation rapidly reached a plateau. This is explained by secondary decomposition of the initially generated cross-link which could be further oxidized more efficiently than starting TGT oligonucleotide. One-electron oxidation of the initially generated cross-link was found to produce mainly two diastereomeric cross-links exhibiting a spiroimino-trilysine-dihydantoin structure as inferred from enzymatic digestion, CD, UV, NMR and mass spectrometry measurements. In addition, other minor cross-links, for which formation was favored at acidic pH, were assigned as lysine−guanine adducts in which the modified guanine base exhibits a guanidino-trilysine-iminohydantoin structure. A proposed mechanism for the formation of the different detected oligonucleotide−peptide cross-links is given. The high yield of formation of the detected cross-links strongly suggests that a DNA−protein cross-link involving a lysine residue linked to the C8 position of guanine could be generated in cellular systems if a lysine is located in the close vicinity of a guanine radical cation. KEYWORDS: oxidatively generated DNA damage, photosensitization, guanine radical cation, DNA-protein cross-links.

Introduction

It is now generally accepted that oxidatively generated DNA lesions are implicated in lethality, mutagenesis, and carcinogenesis.[1] The spectrum of oxidatively damaged DNA includes base modifications, abasic sites and DNA strand breaks. Another possible consequence of cellular oxidation reactions is the production of covalent adducts between DNA and protein, the so-called DNA−protein cross-links (DPCs).[2] DNA in cells is closely surrounded by a variety of proteins that range in function from structural organization of the genome to the control of cellular processes. It has been shown that different physical agents including high-intensity UV laser pulses,[3] ionizing radiation,[4] and photosensitizers[5] together with several chemicals[6] can induce formation of DPCs.[7] However, there is still an almost complete lack of information on the chemical structure of DPCs as well as on their mechanism of formation. One exception concerns detailed investigations performed by Dizdaroglu et al. who have characterized several radiation-induced thymine−amino acids cross-links[8−10] in oxygen-free aqueous solutions.

Recently, several experimental approaches have been used to generate DPCs following selective oxidation of guanines in double-stranded DNA. For example, Stemp and co-workers[11,12] have investigated DPCs formation using the flash−quench technique after one-electron oxidation of the guanine bases in DNA. For this purpose the DNA bound intercalator Ru(1,10-phenanthroline)$_2$dipyridophenazine^{+3} was used in the presence of histones or other proteins. Besides forming DPCs and other

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lesions, the guanine radical is a strong oxidant that can also participate in hole transfer reactions toward intercalated amino acids. Thus, oxidation of Tyr in Lys-Tyr-Lys peptides by guanine radicals results in a tyrosine-thymine cross-link presumably via the transient formation of a phenoxyl radical. Interestingly, Burrows' group has demonstrated that one-electron oxidation of 8-oxo-7,8-dihydroguanine (8-oxoGua), the major lesion formed by oxidation of guanine within double-stranded DNA, by Ir(IV) led to the formation of a specific cross-linking reaction between 8-oxoGua and lysine 142 of the DNA repair protein MutY.[13] A similar observation was recently made using a *Escherichia coli* single-stranded binding protein as the probe.[14] In both cases, the authors proposed that the cross-linking reaction occurs between C5 of 8-oxoGua and a nucleophilic group of amino acid side chains. It may be added that complete characterization of DPCs failed due to the instability of the latter adducts and the possible occurrence of multiple oxidative events within the protein. However, it may be noted that the same research group succeeded in identifying the nature and the mechanism of formation of single-stranded DNA−spermine adducts following one-electron oxidation of 8-oxoGua.[15]

Guanine, which possesses the lowest ionization potential among DNA components,[16] is the preferential DNA target for one-electron oxidation. The guanine radical cation can undergo hydration giving rise to 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine,[17] while the deprotonated neutral guanine radical has been suggested to be the precursor of 2-amino-5-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-4*H*-imidazol-4-one (imidazolone nucleoside) and its hydration decomposition product, 2,2-diamino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-5(2*H*)-oxazolone[18] (oxazolone nucleoside). Imidazolone and oxazolone were also found to be generated upon one-electron oxidation of 8-oxodGuo,[19−21] although in a lower yield with respect to spiroiminodihydantoin and guanidinohydantoin (or iminoallantoin) nucleosides.[22,23] It was found that for free 2′-deoxyguanosine (dGuo), nucleophilic attack of the 5′-hydroxyl group of the 2-deoxyribose moiety onto the guanine radical at C8 gives rise to a cyclonucleoside[24] in a competitive pathway with the formation of imidazolone, produced by nucleophilic addition of a water molecule.[18] Methanol adducts[25] were also shown to be generated when the reaction was carried out in the presence of MeOH. Moreover, one-electron oxidation of either 5′-amino-2′,5′-dideoxyguanosine[26] or 5′-lysine-2′,5′-dideoxyguanosine[27] was found to lead to the formation of cyclonucleosides through the initial nucleophilic addition of an amino group onto the guanine radical. These nucleophilic reactions that take place at the C8 position of the guanine base represent relevant model systems of DPCs generation, mimicking the potential addition of a free nucleophilic residue of amino acid side chains onto a guanine base upon one-electron oxidation of DNA.

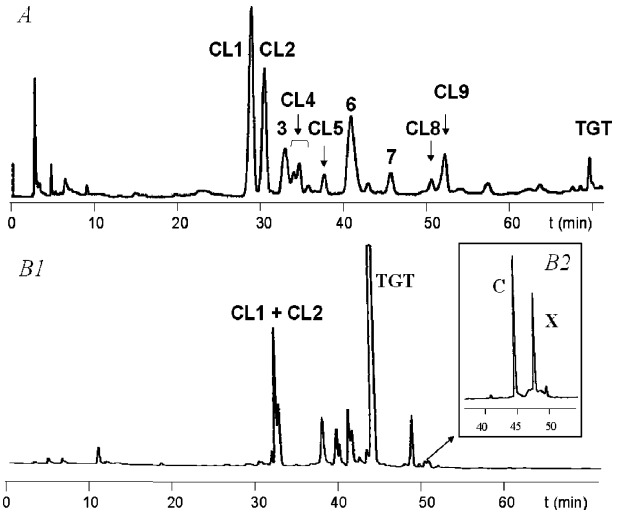

Figure 1. Reversed-phase HPLC analysis of products generated upon one-electron oxidation of TGT oligonucleotide in aerated aqueous solution in the presence of KKK peptide (see Experimental Section for details).

The previously reported studies strongly suggest that lysine residues (K) could be suitable amino acid candidates to be involved in the formation of DPCs if they are closely located to a guanine radical cation. Lysine is of particular interest because of its relatively large abundance in histones, one of the main protein components of eukaryotic chromatin. One major difficulty in the actual identification of DPCs when DNA−protein complexes are irradiated in aqueous solution deals with the competitive oxidation of amino acid residues such as tryptophan and tyrosine that have lower oxidation potentials than guanine. Therefore, the choice of a suitable model system appears critical in such studies. In the present work, we have investigated the putative formation of guanine-lysine cross-links upon riboflavin-mediated one-electron photooxidation of TGT oligonucleotide in aerated aqueous solution in the presence of KKK tripeptide. Emphasis was placed on the search and characterization of potential oligonucleotide−peptide cross-links together with the delineation of their mechanism of formation. We report here the isolation of cross-links arising from the addition of the ϵ-amino group of lysine to the C8 of the guanine radical. The major cross-links were characterized by mass spectrometry and ^{1}H NMR analyses. Mechanistic insights were gained from isotopic labeling experiments that involved HPLC-ESI-MS/MS measurements. A detailed mechanism of formation of the primary and secondary generated cross-links observed between TGT and KKK is proposed.

Results and Discussion

Following photosensitization of TGT in the presence of KKK tripeptide, the main modified DNA oligonucleotides were separated by reversed-phase HPLC as shown in Figure 1. The irradiation time was first chosen to allow an almost complete

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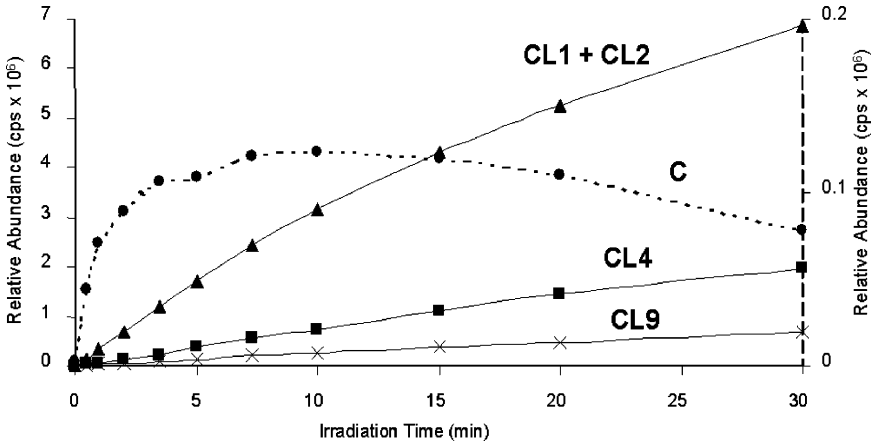
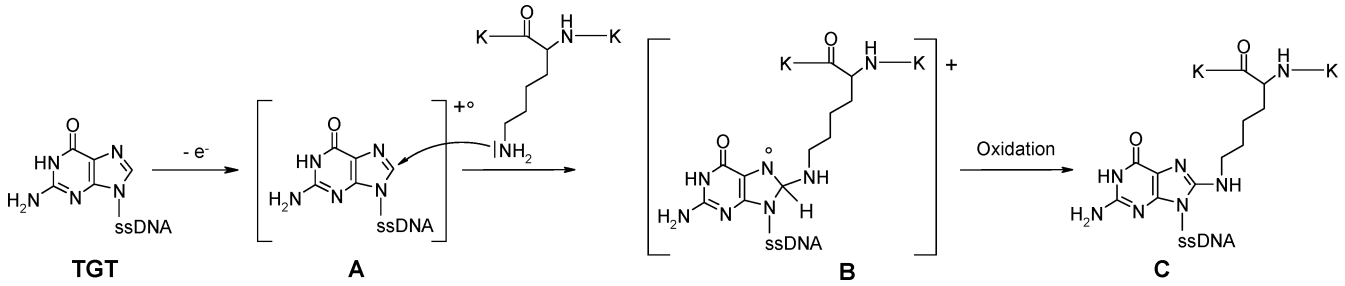


Figure 2. Time course formation of cross-links **C** (●) (dashed curve and enlarged right scale), cross-links **CL1** + **CL2** (▲), **CL4** (■), and **CL9** (×) (full curves and left scale) as a function of the irradiation time. Cross-links have been detected by HPLC-ESI-MS/MS (see Experimental Section for details).

Scheme 1. Proposed Mechanism for the Formation of Cross-Link **C** upon Riboflavin-Mediated One-Electron Oxidation of TGT in the Presence of KKK (ssDNA Means Single-Stranded DNA)



decomposition of starting oligonucleotide TGT. Some of the decomposition products were assigned as modified oligonucleotides without any KKK cross-links since their formation was also observed upon riboflavin-mediated photosensitization of TGT in the absence of KKK (peaks **3**, **6**, and **7**, Figure 1A). These products, as inferred from mass spectrometry analysis, were assigned as oligonucleotides that exhibited loss or modification of a guanine residue, including an abasic site (**6**), imidazolone residue (**7**), and its hydrolyzed decomposition product, namely oxazolone (**3**). When riboflavin-mediated decomposition of TGT was performed in the presence of 10 equiv of KKK, additional products were detected. These new photoproducts that were shown to be oligonucleotide−peptide cross-links according to their molecular weight represent about 70% of the overall decomposition products.

Experimental evidence, as discussed below, strongly suggests that the major products named **CL1** and **CL2** (Figure 1A) are two diastereomeric products of TGT covalently cross-linked to KKK through the guanine moiety exhibiting a spiroiminotrilysine-dihydantoin structure. According to our experimental data it could not be completely ruled out that **CL1** and **CL2** arose from the secondary oxidation of T-8-oxoGua-T generated during irradiation and that the peptide is linked to the C5 position of the guanine base. In fact, HPLC-ESI-MS/MS analysis indicated that **CL1** and **CL2** are not generated upon riboflavin-mediated photooxidation of T-8-oxoGua-T in the presence of 10 equiv of KKK. However, several oligonucleotide−peptide cross-links were found to be generated from T-8-oxoGua-T, in agreement with Burrows' work.[13−15] Then, it was postulated that **CL1** and **CL2** could be secondary oxidation products of an initially generated *N*ϵ-(guanin-8-yl)-lysine cross-link (structure **C**, Scheme 1) as described by Johnson et al. for C8-arylamine adducts of guanine.[28,29] The initial formation of a C8 adduct is expected from the results obtained using model systems that have highlighted the efficient addition of several nucleophiles to the C8 position of either the guanine radical cation or its deprotonated neutral radical. Therefore, attempts were made, taking advantage of the sensitivity and specificity of the HPLC-ESI-MS/MS analytical tool to search for the formation of **C** and to determine the time course of its generation, together with that of **CL1** and **CL2**, following one-electron oxidation of TGT in the presence of KKK. In agreement with the postulated mechanism depicted in Scheme 1, a product with a molecular weight corresponding to cross-link **C** was detected. Its yield of formation rapidly reached a plateau with the time of irradiation (Figure 2) and represents, at best, 5% of that of **CL1** and **CL2**. The nonlinear formation of **C** could be attributed to its efficient secondary decomposition into **CL1** and **CL2** as a result of one-electron oxidation of the C-8 substituted guanine moiety similarly to 8-oxoGua[20] and 8-arylamine guanine derivatives.[28,29]

To confirm our hypothesis, **C** was isolated by HPLC purification (Figure 1B), and riboflavin-mediated photosensitization of **C** in an aerated aqueous solution was found to lead almost exclusively to **CL1** and **CL2**. Further information was obtained following its enzymatic digestion leading to the cross-linked nucleoside **Cd**. It should be noted that, unlike **CL1** and **CL2** (vide infra), **C** was efficiently digested to the corresponding nucleoside **Cd** by the combined use of nuclease P1 and acid phosphatase. The UV absorption spectrum of **Cd** exhibits two

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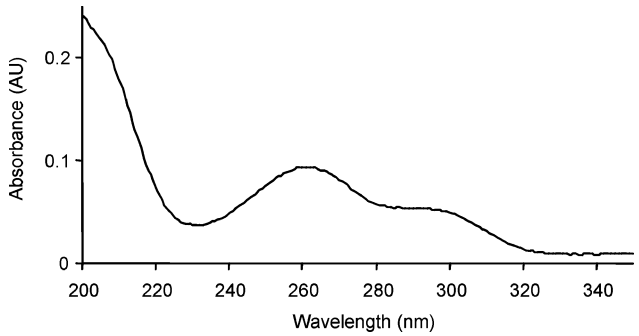

Figure 3. UV absorption spectrum of *N*ϵ-(2-deoxyguanosin-8-yl)trilysine **Cd** in water.

maxima of absorbance at 260 and 295 nm (Figure 3) similar to 8-aminoguanine[30] and 8-oxoGua. Moreover, the high-resolution mass spectrum of **Cd** is consistent with the molecular formula for *N*ϵ-(2′-deoxyguanosin-8-yl)-trilysine (Table 1). To demonstrate that the trilysine residue is linked to the C8 of guanine, a TGT oligonucleotide containing a deuterium atom at the C8 position of guanine was prepared (Figure S1[31]). After riboflavin-mediated photosensitization of such an oligonucleotide in the presence of 10 equiv of KKK, the reaction mixture was analyzed by HPLC-ESI-MS/MS. The loss of the deuterium atom in **C** (and also in **CL1** and **CL2**) confirms the nucleophilic addition of the peptide to the C8 position of guanine.

To confirm the proposed mechanism, experiments were performed to determine the structure of **CL1** and **CL2** cross-links arising from secondary oxidation of **C**. **CL1** and **CL2** exhibit a molecular weight of 1291 g/mol, 14 mass units higher than the sum of TGT and KKK. Two successive losses of 322 amu that could be attributed to the loss of one and two thymidine monophosphate residues were observed in the collision-induced fragmentation mass spectra of **CL1** and **CL2** (in either positive or negative ionization modes). In addition, when mass spectra were recorded in the positive ionization mode (Figure 4), the intense fragment detected at *m*/*z* 568.2 was attributed to the peptide linked to the modified guanine moiety $[BH_2]^+$. NMR assignment of all the nonwater exchangeable protons in **CL1** and **CL2** was established by combining ^{1}H (Figure S2[31]), 2D-NOESY, and 2D-TOCSY NMR analyses. ^{1}H NMR spectra of **CL1** and **CL2** clearly indicate the lack of the signal resonance corresponding to the aromatic H8 proton of guanine, in agreement with the spiroimino-trilysine-dihydantoin structure proposed in Scheme 2. Moreover, the nonequivalent chemical shifts of the two ϵ-H of the central lysine of KKK in the ^{1}H NMR spectrum of **CL1** strongly suggest that the tripeptide is covalently linked to the modified guanine base by the ϵ-amino group of its central lysine.

Further information was obtained from enzymatic digestion of the oligonucleotide moiety of **CL1** and **CL2**. First, partial hydrolysis by either nuclease P1 or 5′-exonuclease indicates that, in agreement with the data obtained from collision-induced dissociation mass spectra and ^{1}H NMR analysis of **CL1** and **CL2**, neither 5′-T nor 3′-T is modified in both cross-links. It is worth noting that the efficiency of enzymatic hydrolysis of **CL1** and **CL2** was significantly reduced compared to that of TGT, as already observed for spiroiminodihydantoin containing oligonucleotides.[32,33] In addition, **CL1** could be fully digested to the corresponding nucleoside **CL1d** by the combined use of nuclease P1, 5′-exonuclease, and either alkaline or acid phosphatase, whereas, for **CL2**, complete hydrolysis of the 5′-phosphate group of the 2-deoxyribose moiety bound to the modified guanine base in the corresponding nucleotide **CL2mp** was unsuccessful.

Further relevant experimental supports for the structure of **CL1** and **CL2** were gained from complementary spectroscopic measurements performed on nucleoside **CL1d** and nucleotide **CL2mp**. The exact mass determination of **CL1d** and **CL2mp** is in agreement with the respective molecular formulas of $C_{28}H_{49}N_{11}O_9$ and $C_{28}H_{50}N_{11}O_{12}P$ expected from the trilysine-substituted spiroiminodihydantoin cross-links (Table 1). In addition, the measured mass of the intense fragment could be attributed to the protonated base $[BH_2]^+$ (Table 1). Furthermore, **CL1d** and **CL2mp** exhibit almost opposite CD spectra strongly suggestive of a diastereomeric relationship between the two adducts (Figure 5). Finally, the UV absorption spectra of the two cross-linked nucleoside **CL1d** and nucleotide **CL2mp**, that present a lack of absorption around 260 nm (Figure S3[31]), are similar to those of spiroiminodihydantoin adducts formed by one-electron oxidation of 2′,3′,5′-triacetoxy-8-oxo-7,8-dihydroguanosine in the presence of nucleophilic 1,4-diaminobutane.[15]

One-electron oxidation of **C** was found to generate other minor products that could correspond (Scheme 2) to guanidino-trilysine-iminohydantoin cross-links **CL4** (and/or their isomers analogous to iminoallantoin adducts) and *N*,*N*ϵ-(guanin-5,8-yl)-trilysine cross-links **CL9** (Scheme 2), as inferred from their molecular masses determined by HPLC-ESI-MS/MS. These products were also detected following one-electron oxidation of TGT in the presence of KKK (Figure 1), although in lower amounts compared to **CL1** and **CL2**. For **CL4**, the presence of two photoproducts with similar masses and fragmentation pattern was detected. However, HPLC separation of the two modified oligonucleotides was not possible. **CL4** cross-links were enzymatically hydrolyzed into two nucleosides **CL4d** that exhibit different HPLC retention times and were found to be in a dynamic equilibrium. The high-resolution mass spectrum of **CL4d** (Table 1) confirms the elemental composition proposed for the guanidino-trilysine-iminohydantoin structure (Scheme 2). As observed for the secondary oxidation of 8-oxodGuo,[19] the yield of formation of **CL4** was found to be significantly increased when the photosensitization reaction of **C** was performed under acidic conditions (Figure S4[31]), at the apparent expense of **CL1** and **CL2** photoproducts.

Concerning **CL5** (Figure 1), the photoproduct exhibits a molecular weight identical to that of **CL1** and **CL2**. **CL5** was also detected upon riboflavin-mediated one-electron oxidation of T-8-oxoGua-T in the presence of KKK. Such information strongly suggests that **CL5** is a KKK adduct to T-8-oxoGua-T with the peptide likely tethered to the C5 position of 8-oxoGua, as observed by Burrows' group.[15] Therefore, a minor reaction is observed upon one-electron oxidation of TGT in the presence of KKK, involving the initial formation of T-8-oxoGua-T that could be further oxidized to generate cross-links, including **CL5**.

The obtained results allowed us to propose a coherent mechanism for the formation of the detected oligonucleotide−

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Table 1. Exact Mass Determination (Electrospray Ionization) of Digested Cross-Links **CL1d**, **CL2mp**, **CL4d**, and **Cd** and of Their Major Fragmentation Products Assigned as the Protonated Cross-Linked Bases

	cross-linked nucleos(t)ide $[M + H]^+$ or $[M + Na]^+$		cross-linked base fragment $[BH_2]^+$	
cross-link	elemental composition	measured mass[a]	elemental composition	measured mass[a]
CL1d	$C_{28}H_{50}N_{11}O_9$	684.3760 (−0.0027)	$C_{23}H_{42}N_{11}O_6$	568.3280 (−0.0034)
CL2mp	$C_{28}H_{51}N_{11}O_{12}P$	764.3440 (−0.0011)	$C_{23}H_{42}N_{11}O_6$	568.3310 (−0.0004)
CL4d	$C_{27}H_{52}N_{11}O_8$	658.3980 (−0.0015)	$C_{22}H_{44}N_{11}O_5$	n.d.
Cd	$C_{28}H_{49}N_{11}O_8Na$	690.3640 (−0.0018)	$C_{23}H_{42}N_{11}O_5$	552.3360 (−0.0005)

[a] Exact masses are given in atomic mass units, and the deviation, written in parentheses, corresponds to the difference between the measured and the calculated masses. n.d. means not determined.

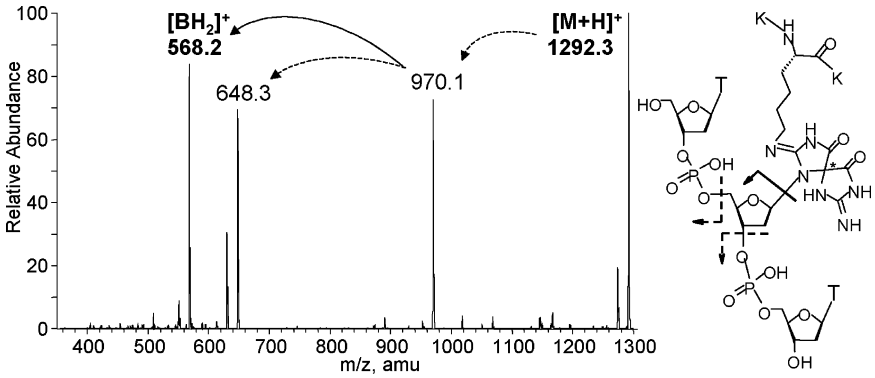
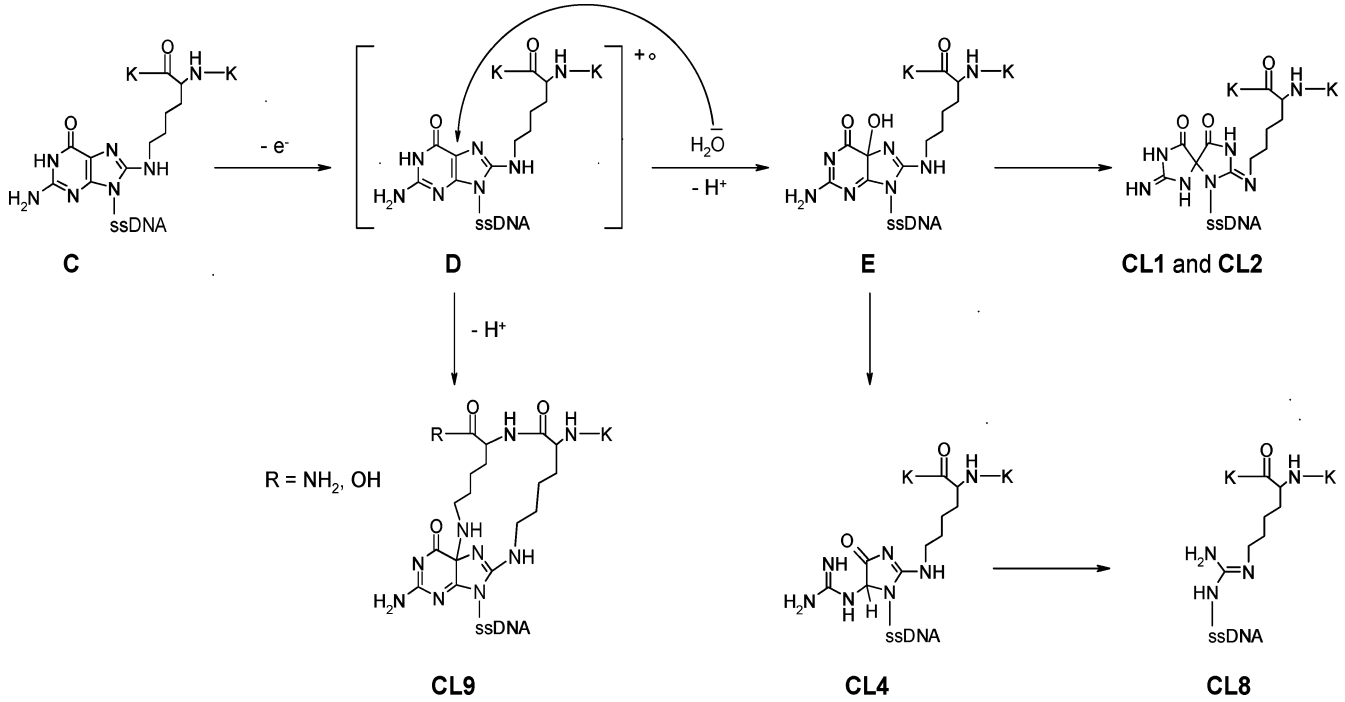


Figure 4. Collision-induced dissociation mass spectrum (electrospray ionization, positive mode) of the pseudomolecular ion $m/z = 1292.3$ of **CL2**. $[BH_2]^+$ ion corresponds to the protonated base moiety of the cross-linked **CL2**.

Scheme 2. Proposed Mechanism for the One-Electron Oxidation of Initially Generated Cross-Link **C**[a]



[a] Decomposition of **C** leads to the main formation of spiroimino-trilysine-dihydantoin diastereomers **CL1** and **CL2** and, to a lesser extent, to the guanidino-trilysine-iminohydantoin diastereomers **CL4**, which may further decompose into **CL8**. Formation of **CL9** could be also explained by an intramolecular nucleophilic addition of an amino group onto the C5 atom of guanine.

peptide cross-links. In the first step, (Scheme 1) the guanine radical cation **A**, or rather its deprotonated form that should predominate at neutral pH ($pK_a = 3.9$),[16] is generated by riboflavin-mediated one-electron oxidation of guanine. Subsequent KKK ϵ-amino group nucleophilic attack at the C8 position of the guanine radical **A** leads to a transient guanyl radical **B** that through oxidation, similar to the chemical reaction of 8-hydroxyguanyl radical, is able to give rise to a diamagnetic C8-substituted guanine compound. This was assigned as the oligonucleotide−peptide cross-link **C** exhibiting a *N*ϵ-(guanin-8-yl)-trilysine structure. Subsequently, a secondary oxidation reaction could take place to generate the related radical cation **D** which could be trapped by a water molecule at C5 giving **E** (Scheme 2). Unstable *N*ϵ-(5-hydroxyguanin-8-yl)-trilysine cross-

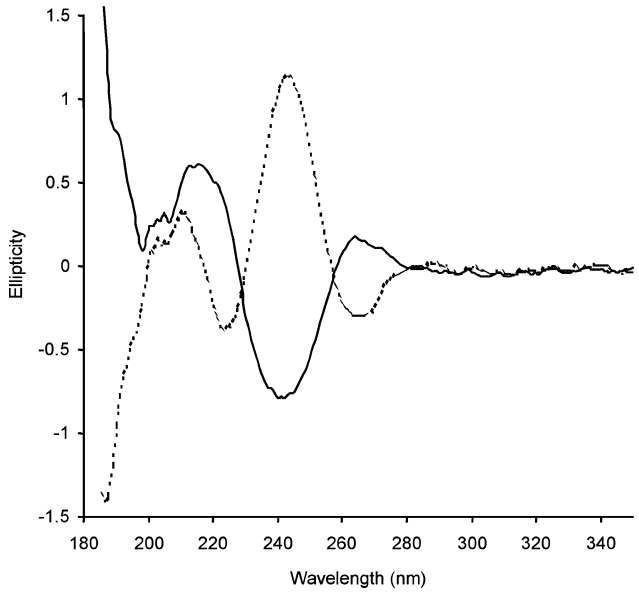


Figure 5. CD spectra of the two diastereomeric digested cross-links **CL1d** (full curve) and **CL2mp** (dashed curve) in water.

link **E** ultimately rearranges into two pairs of diastereomers: the spiroimino-trilysine-dihydantoin **CL1** and **CL2** and, to a lesser extent, into guanidino-trilysine-iminohydantoin **CL4**. In agreement with that proposal, a photosensitization reaction performed in $H_2{}^{18}O$ has shown that any of **CL1**, **CL2**, and **CL4** is able to incorporate one oxygen atom from water (Figure S5[31]) and not from $^{18}O_2$. Such a mechanism of formation is similar to that proposed for the one-electron oxidation of 8-oxoGua[19] and also the formation of 8-oxo-7,8-dihydroguanine-polyamine adducts.[14] Therefore, the 5-hydroxyguanine structure of **E** has been proposed by analogy with the transient formation of 5-hydroxy-8-oxodGuo[34] involved in the one-electron oxidation pathway of 8-oxodGuo, and it is supported by the pH dependent formation of the final **CL1**, **CL2**, and **CL4** cross-links (Figure S4[31]). Moreover, we have observed during HPLC purification of **CL4d** nucleosides an isomerization indicative of the postulated equilibrium between guanidinohydantoin and iminoallantoin species.[19] In the same way, Johnson et al. have described that aerobic oxidation of 8-arylamine derivatives of 2′-deoxyguanosine or a related single-stranded oligomer generated mainly a pair of spiroiminodihydantoin adducts together with a mixture of isomers analogous to guanidinohydantoin and/or iminoallantoin adducts.[28] The latter adducts were found to be converted into a guanidine adduct which was subject to further decomposition under alkaline conditions leading to an abasic site. Interestingly, the molecular weight of the minor cross-link **CL8** detected in the present work (Figure 1) could correspond to such a guanidine adduct with one of the amino groups of the guanidine belonging to the trilysine peptide (Scheme 2); it is likely that **CL8** derives from **CL4** since their formation was found to be concomitant.

A mechanism of formation of **CL9**, which exhibits a molecular weight two mass units lower than **C**, can also be proposed. Thus, initially produced radical cation **D** that reacts with a water molecule to generate intermediate **E** could also undergo a favored intramolecular addition at C5 by one of the free amino groups of the linked trilysine residue, generating *N*,*N*ϵ-(guanin-5,8-yl)-trilysine adducts (structure **CL9** in Scheme 2). In agreement with such a proposal, the formation of **CL9** was also found to be preponderant at basic pH when the peptide amino groups are not protonated and therefore more nucleophilic.

In conclusion, in the present work we have shown the efficient formation in aerated aqueous solutions of oligonucleotide−peptide cross-links following one-electron oxidation of the guanine moiety of a short single-stranded oligonucleotide in the presence of a trilysine peptide. Under the present experimental conditions, the formation of cross-links between TGT and KKK is very efficient since the DNA−peptide adducts represent about 70% of all TGT decomposition products. This could be explained by an electrostatic interaction between the two phosphate groups of TGT and the amino groups of lysine that are supposed to be protonated at neutral pH. Such a complex formation may favor the positioning of the free central lysine residue in the close vicinity of the guanine base. This could explain the predominant formation of cross-links that competes with the generation of TGT decomposition products through addition of either water or the superoxide radical[35] to the oxidizing guanine radical initially produced. In that respect, it is interesting to note that **CL1** and **CL2** are still the major photooxidation products when the reaction is performed with only 1 equiv of KKK.

Under the present experimental conditions, most of the final identified cross-links are generated by a secondary oxidation reaction of the initially formed *N*ϵ-(guanin-8-yl)-lysine adduct. It is obvious that, as observed for 8-oxodGuo, such a secondary oxidation reaction is likely to be at best a minor process in cells and that the first generated C8-amino acid substituted guanine cross-link **C** is the potential adduct that could be generated in cells. The high yield of the adduct formation strongly suggests that nucleophilic amino acids, such as lysine of DNA binding proteins, located in the DNA major groove may induce cross-links upon one-electron oxidation of cellular DNA. Interestingly, preliminary work indicates that such a reaction could also take place in double-stranded DNA (not shown), and additional work is in progress to search for the formation of such DNA−protein cross-links within cells.

Experimental Section

Products. Acetonitrile (HPLC grade) and acetic acid were purchased from SDS (Valdonne, France). Ammonium acetate and formate, trifluoroacetic and formic acid, trilysine peptide, Nuclease P1, acid phosphatase I (from sweet potatoes), and phosphodiesterase I were from Sigma Co. (St Louis, MO). Riboflavin was obtained from BDH Biochemicals (Poole, U.K.). Alkaline phosphatase was obtained from Roche Diagnostic (Mannheim, Germany). Enriched $^{18}O_2$ and $H_2{}^{18}O$ (isotopic purity > 95%) were from Eurisotop (Paris, France).

Oligonucleotide Synthesis. TGT and T-8-oxoGua-T oligodeoxyribonucleotides were synthesized by standard phosphoramidite chemistry using an Applied Biosystems Inc. 392 DNA synthesizer (Toronto, Canada). The T-8-oxoGua-T oligonucleotide was prepared using a commercially available phosphoramidite monomer of 8-oxodGuo (Glen Research, Sterling, VA). Oligonucleotides were deprotected in a concentrated ammonia solution for 16 h at 55 °C. For T-8-oxoGua-T deprotection, the ammonia solution contained 0.25 M β-mercaptoethanol

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to prevent further oxidation of 8-oxoGua. Oligonucleotides were purified by a reversed-phase HPLC C_{18} column, 5 μm, 250 mm × 7.0 mm (Interchim, Montluçon, France) with acetonitrile and ammonium formate buffer (pH 6.4).

Synthesis of TGT Decomposition Products. An aqueous solution containing riboflavin (0.04 mM), TGT (0.1 mM), and KKK (1 mM) was irradiated at room temperature for 30 min with a 500 W halogen lamp placed at a distance of 20 cm. Air was continuously bubbled into the solution during the irradiation to keep the solution saturated with oxygen. Then, decomposition products were purified by reversed-phase HPLC on an Uptisphere-ODB (5 μm, 250 mm × 4.6 mm) column (Interchim, Montluçon, France). The elution was achieved at a flow rate of 1 mL/min in the gradient mode, and the detection of the products was performed with a UV−visible spectrophotometer set at 260 nm. Elution was achieved using a gradient of a 50/50 water/acetonitrile solution (from 5 to 10% in 60 min and from 10 to 20% in 10 min) in 5 mM ammonium acetate buffer (pH 4), the column being maintained at 40 °C (Figure 1, panel A). For $H_2{}^{18}O$ labeling experiments, the irradiated solution was prepared as described above and then lyophilized to dryness. The resulting dry products were dissolved in $H_2{}^{18}O$ before irradiation. For the experiment performed using enriched $^{18}O_2$, the oxygen contained in the aqueous solution was removed by successive freezing and thawing steps under vacuum. The procedure was repeated at least 3 times, and then the solution was saturated with $^{18}O_2$ to restore atmospheric pressure, before being irradiated. After irradiation, the reaction mixture was analyzed by HPLC/ESI-MS/MS (vide infra).

Purification of C. To obtain a maximum amount of cross-link **C**, the solution containing TGT (0.1 mM), KKK (1 mM), and riboflavin (0.04 mM) was irradiated for only 10 min. Two successive HPLC separations were necessary to purify **C**. Separations were performed using a gradient of acetonitrile (from 0 to 15% in 60 min, column temperature 28 °C), first in 5 mM ammonium formate buffer, pH 6.4 (Figure 1, panel B1), and then the fraction containing cross-link **C** was further purified in 5 mM ammonium acetate buffer pH 4 using the same gradient (Figure 1, panel B2).

Time Course of Formation Studies. To assess the level of formation of the different products with respect to the irradiation time, aliquots were withdrawn from the irradiated solution as a function of time and directly placed into darkness to stop the reaction. The samples were kept frozen until being analyzed by HPLC-ESI-MS/MS.

Mass Spectrometry. Electrospray ionization (ESI)-MS measurements and collision induced dissociation mass spectrometry analysis of the HPLC purified irradiation products were carried out on an LC-Q ion-trap model mass spectrometer from Thermo-Finnigan (San Jose, CA). Typically, for the analysis in the negative ionization mode, products were dissolved in a mixture of acetonitrile and water (50/50, v/v) that contained 1% of triethylamine, whereas, in the positive ionization mode, the samples were dissolved in a water/methanol mixture (50/50, v/v).

High-resolution mass spectra were acquired on a Micromass QToF-2 instrument (Micromass, Manchester, UK). The electrospray (needle) voltage was set at 3 kV, and the temperature of the ion source block, at 120 °C. The cone gas (nitrogen) was set to a flow rate of 50 L/min, and the desolvation gas to 300 L/min at a temperature of 140 °C. Data acquisition and data evaluation were performed using the Micromass MassLynx 4.0 software, SP4. Samples were dissolved in water/methanol/acetic acid (50/50/1, v/v/v), and survey mass spectra were acquired by directly infusing the solution into the electrospray ion source of the instrument at a flow rate of 5 μL/min. Accurate mass measurements were then realized by mixing an aliquot of the sample with a calibration solution (consisting of 1 mg/mL NaI and 0.05 mg/mL CsI in water/isopropanol, 60/40, v/v) and infused as above. About 10 scans were acquired over the mass range m/z 100−1400, with the cone voltage alternating between 25 and 50 V (1 min total acquisition time at ca. 1 scan/s, resolution about 10000 fwhm); exact mass data were then obtained using a suitable lockmass from the calibrant.[36] The elemental composition of the resulting data was calculated using a software that was developed in-house.

On-line HPLC-ESI-MS/MS measurements were carried out using a 1100 HPLC system (Agilent, Massy, France), equipped with a thermostated autosampler, a binary HPLC pumping system, an oven, and a UV detector. Separations were performed using a reversed-phase Uptisphere-ODB (3 μm, 0.2 cm × 15 cm) column from Interchim (Montluçon, France). The elution was achieved at a flow rate of 0.2 mL/min in the gradient mode, the column being maintained at 28 °C. The proportion of acetonitrile in 2 mM ammonium formate (pH 6.5), starting from 0%, reached the 15% value within 30 min for the measurement of TGT decomposition products including cross-links. At the output of the column, the eluent circulated first through a UV detector set at 260 nm. Then, after addition of MeOH (0.1 mL/min), the eluent was directed onto a API3000 tandem mass spectrometer (Applied Biosystems) through a "Turbospray" electrospray source (Sciex, Thornil, Canada) as described in detail elsewhere.[37,38] The negative ionization mode was chosen to improve the sensitivity of detection using two different acquisition modes. First, for the detection of TGT decomposition products, the API3000 instrument was used in the precursor ion scan mode of the ion at $m/z = 321$. Such a fragment that corresponds to a thymidine-monophosphate residue was found to be a major fragment of TGT and was also found to be common to all the TGT decomposition products including TGT-KKK cross-links. The masses scanned by the first quadrupole ranged from m/z 500 to m/z 1500, the scan time being 2 s, and the collision energy used (55 eV) was that optimized for TGT. Thereafter, to increase the sensitivity of detection, the multiple reactions monitoring (MRM) mode was chosen and two specific transitions $(M - H)^- \rightarrow 321$ and $(M - 2H)^{2-} \rightarrow 321$ were used for the detection of the products. The transitions used were as follows: 1290.4 → 321 and 645.2 → 321 for both **CL1** and **CL2**, 1264.4 → 321 and 632.2 → 321 for **CL4**, 1274.4 → 321 and 637.2 → 321 for **C**, 1272.4 → 321 and 636.2 → 321 for **CL9**, and 1167.5 → 321 and 583.3 → 321 for **CL8**.

Oligonucleotide Enzymatic Digestion. Concentrations of peptide cross-linked oligonucleotides **CL1**, **CL2**, and **CL4** were estimated using an absorption coefficient being twice that of thymine at 260 nm, whereas concentration of **C** was assessed using the molecular extinction coefficient of TGT. To about 8 nmol of **CL1** or **CL2** or **CL4** was added 40 U of nuclease P1 with 10 μL of buffer P1 10× (200 mM succinic acid, 100 mM $CaCl_2$, pH 6). The resulting solution was incubated for 48 h at 37 °C. Thereafter, 10 μL of alkaline phosphatase buffer 10× (500 mM tris, 1 mM EDTA, pH 8) was added together with 0.03 U of phosphodiesterase I and 5 U of alkaline phosphatase. The samples were then incubated at 37 °C for 48 h. After enzymatic digestion, the digested cross-links were purified by HPLC using a Hypercarb column, 5 μm, 100 mm × 3 mm (Thermo Electron corporation, Bellefonte, PA) with the following conditions: flow rate = 0.5 mL/min; column temperature = 35 °C; UV detection at 240 nm; elution using a gradient of acetonitrile (from 0 to 50% in 60 min) in 0.1% trifluoroacetic acid aqueous solution. Enzymatic digestion of **CL1** and **CL4** was found to liberate the corresponding nucleosides **CL1d** and **CL4d** together with Thd, whereas, for **CL2**, enzymatic digestion gave rise to the related nucleotide **CL2mp**. Under the HPLC conditions used, Thd elutes at 36.7 min, **CL1d**, at 15.3 min, and **CL2mp**, at 17.7 min, whereas two isomers eluting at 16.4 and 18.2 min were detected for **CL4d**.

Digestion of **C** (about 10 μM, 100 μL) was performed using 8 U of nuclease P1 with 10 μL of buffer P1 10× and 1 U of acid phosphatase. The resulting solution was incubated for 24 h at 37 °C. After enzymatic digestion, the resulting cross-link **Cd** was purified by reversed-phase

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HPLC (Interchim C_{18} Uptisphere column, 5 μm, 250 mm $\times$ 4.6 mm) with the following conditions: flow rate = 1 mL/min; column temperature = 28 °C; UV detection at 260 nm; elution using a gradient of a 50/50 water/acetonitrile solution (from 0 to 30% in 60 min) in 2 mM ammonium formate (pH 3). Under these conditions nucleoside **Cd** eluted at 17.2 min, whereas the retention time of Thd was 26.8 min.

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Supporting Information Available: ^{1}H NMR spectra of **CL1** and **CL2**, experimental procedure for deuteration of TGT, pH dependent formation of **CL1** + **CL2**, **CL9** and **CL4**, UV spectra of **CL4d**, **CL1d**, and **CL2mp** and mass spectra of **CL1**+**CL2**, **CL4**, **CL9**, and **C** obtained upon one-electron oxidation of TGT in $H_2{}^{18}O$. This material is available free of charge via the Internet at http://pubs.acs.org.

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