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Characterization of the Deoxyguanosine−Lysine Cross-Link of Methylglyoxal

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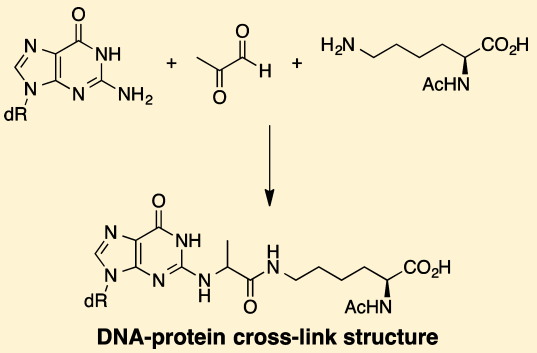
Supporting Information

ABSTRACT: Methylglyoxal is a mutagenic bis-electrophile that is produced endogenously from carbohydrate precursors. Methylglyoxal has been reported to induce DNA−protein cross-links (DPCs) in vitro and in cultured cells. Previous work suggests that these cross-links are formed between guanine and either lysine or cysteine side chains. However, the chemical nature of the methylglyoxal induced DPC have not been determined. We have examined the reaction of methylglyoxal, deoxyguanosine (dGuo), and N^{α}-acetyllysine (AcLys) and determined the structure of the cross-link to be the N^2-ethyl-1-carboxamide with the lysine side chain amino group (**1**). The cross-link was identified by mass spectrometry and the structure confirmed by comparison to a synthetic sample. Further, the cross-link between methylglyoxal, dGuo, and a peptide (AcAVAGKAGAR) was also characterized. The mechanism of cross-link formation is likely to involve an Amadori rearrangement.



INTRODUCTION

Methylglyoxal (pyruvaldehyde or 2-oxo-propanal) is a widely dispersed, reactive bis-electrophile found in food and beverages, cigarette smoke, and automobile exhaust.[1,2] The major endogenous source of methylglyoxal is from the enzymatic and nonenzymatic breakdown of carbohydrates;[3,4] it is also a product of lipid peroxidation and threonine catabolism.[5] Methylglyoxal covalently modifies both DNA and proteins,[6] forming protein adducts with arginine (MG-H1, MG-H2, and MG-H3), and lysine (N^{ω}-CE-Lys) side chains and DNA adducts with guanine and adenine bases (Figure 1).[7,8] Interestingly, the MG-H3 adduct can undergo 2-electron oxidation to MG-I3, which hydrolyzes back to Arg and methylglyoxal.[9] A related adduct of glyoxal (ethane-1,2-dione) with a cysteine side chain (*S*-CM-Cys) has also been characterized,[10] and methylglyoxal has been shown to inactivate a cysteine protease suggesting the formation of the corresponding *S*-carboxyethyl-Cys adduct.[11,12] Protein and DNA adducts of glyoxal, methylglyoxal, and related species are collectively referred to as advanced glycation end-products (AGEs). AGEs have been implicated in the pathologies of many diseases, including cancer. Elevated levels of AGEs are observed in diabetics, and there is a long established epidemiological link between diabetes and higher risk of certain cancers (liver and pancreatic).[13] Glyoxal and methylglyoxal are mutagenic in the bacteria and mammalian cells.[14−16]

Since methylglyoxal and glyoxal are bis-electrophiles, they react with dGuo to form an initial 1,N^2-cyclic dGuo adduct (MG-dGuo and GX-dGuo); these adducts form reversibly but can rearrange to stable N^2-(1-carboxyethyl)-dGuo (CE-dGuo) and N^2-(1-carboxymethyl)-dGuo (CM-dGuo), respectively.[17,18] The related N^6-(1-carboxyethyl)-dAdo (CE-dAdo) adduct has also been characterized.[7] The CE-dGuo adduct has been identified from calf thymus DNA and cultured cells treated with methylglyoxal, and in background levels of human melanoma

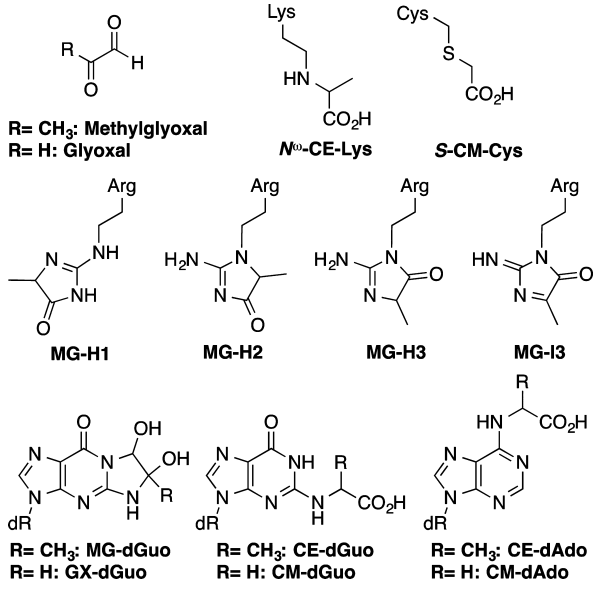


Figure 1. Protein and DNA adducts of methylglyoxal and glyoxal.

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cells and in plasmid DNA isolated from *Escherichia coli*.[7,8,19−22] CE-dGuo has been observed in the urine of healthy humans, and increased levels were measured from tissue samples from diabetic and uremic patients.[23,24] As such, significant attention has been dedicated to the CE-dGuo adduct lesion.

The bifunctional nature of glyoxal and methylglyoxal can also lead to the formation of DNA−DNA, protein−protein, and DNA−protein cross-links (DPCs). Some cross-links between amino acid side chains have been structurally characterized.[25−28] DNA−protein cross-links have been observed when Chinese hamster ovary, human skin, or human endothelial (ECV304) cells were exposed to methylglyoxal.[29−31] Additionally, cross-links between the dGuo adduct of glyoxal and other DNA bases have been characterized and observed from the DNA of exposed cells.[32−34]

Methylglyoxal was shown to cause a stable DNA−protein cross-link with the exonuclease deficient Klenow fragment (Kf^-) of *E. coli* DNA polymerase I.[35] The level of the cross-link increased by ∼50% if $NaBH_4$ was added, suggesting that the cross-linking mechanism involved a Schiff base intermediate. The cross-linking was most efficient if the single-stranded region of the template strand consisted of a run of dGuo's, indicating that dGuo was involved in the cross-link. The reaction of dGuo, methylglyoxal, and N^{α}-acetyl-derivatives of several amino acids (Gly, Ala, Lys, Arg, His, Cys, Trp, Asn, and Asp) was examined, and cross-links between N^{α}-acetyl-Lys (AcLys) and N^{α}-acetyl-Cys were reported.[35] However, the chemical nature of the cross-links was not determined. On the basis of the mechanism of formation of CE-dGuo and related protein adducts of methylglyoxal (vide infra), we hypothesized that the structure of the dGuo, AcLys, and methylglyoxal cross-link to be that shown in Figure 2. The determination of this structure is reported herein.

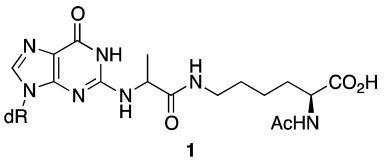

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Figure 2. Structure of the dGuo, methylglyoxal, and N^{α}-AcLys cross-link **1**.

EXPERIMENTAL PROCEDURES

Reaction of dGuo, AcLys, and Methylglyoxal. dGuo·H_2O (5 mM) and AcLys (5 mM) were mixed in sodium phosphate buffer (100 mM, pH 7.4) or sodium carbonate−bicarbonate buffer (100 mM, pH 9.0) containing 1 mM EDTA. The mixture was vortexed until both reagents dissolved. Methylglyoxal (20 mM, 5 mM, 1.25 mM, or 0.5 mM) was then added, and the reaction was incubated at 37 °C with continuous shaking for 7 days. At the end of the incubation time, the samples were filtered through a 0.45 μm Millipore cartridge, and aliquots of the reaction mixture were analyzed by HPLC-UV (gradient II) with the remainder of the samples stored at −70 °C pending UPLC-ESI-MS/MS analysis in negative ion SRM mode (see Supporting Information for details of the MS analysis). Negative ion SRM mode was shown to be more sensitive for the analysis of the CE-dGuo adducts.[7]

The above reaction was also performed in which methylglyoxal was added in 20 equal portions over 2.5 days. The methylglyoxal portions (0.25 mM) were added every 2 h (8 additions on the first and second days and 4 on the third day) so that the final concentration was 5 mM. The reaction was incubated at 37 °C for an additional 10 h and analyzed as above.

The experiments described above were performed and analyzed in triplicate. As a control, the cross-linking reaction was repeated in which each of the components was omitted. In these cases, cross-link **1** was not observed upon MS analysis.

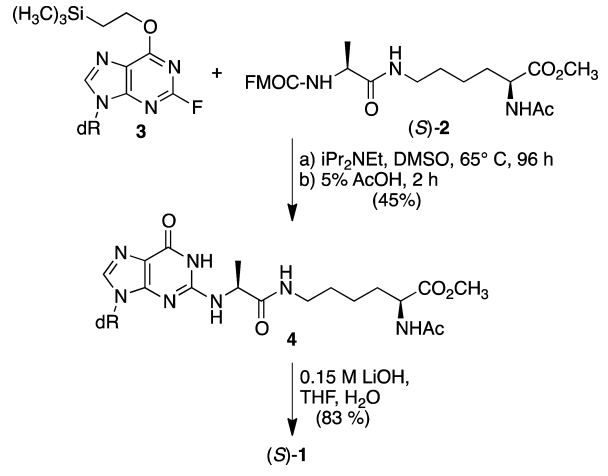
Quantitation of the Cross-Link 1. Aliquots of the reaction of dGuo, AcLys, and methylglyoxal were diluted by 5-fold (v/v) with H_2O. A portion of this sample (10 μL) was mixed with a solution of [$^{13}C_3{}^{15}N_1$]-**1** (10 ng, 10 μL). A portion of this mixture (10 μL) was then subjected to UPLC-ESI-MS/MS analysis performed in negative SRM mode using UPLC gradient **I** by monitoring the transition corresponding to a major fragment ion observed upon CID fragmentation of cross-link **1** in an LTQ mass spectrometer (transitions of m/z 508 → 392 for cross-link **1** and m/z 512 →396 for [$^{13}C_3{}^{15}N_1$]-**1**). The quantitation of cross-link **1** using UPLC-ESI-MS/MS was repeated twice.

Reaction of dGuo, AcAVAGKAGAR, and Methylglyoxal. dGuo·H_2O (5 mM) and the synthetic AcAVAGKAGAR peptide (5 mM) were mixed in sodium phosphate (100 mM, pH 7.4) buffer containing 1 mM EDTA. The mixture was vortexed until both reagents were dissolved. Methylglyoxal (5 mM) was then added and the mixture incubated at 37 °C with continuous shaking for 48 h. At the end of the incubation, the sample was filtered through a 0.45 μm Millipore cartridge and stored at −70 °C pending UPLC-ESI-MS/MS analysis in positive SRM mode (see Supporting Information for details of the MS analysis). The experiments described above were performed and analyzed in triplicate. As a control, the reaction was repeated in which each of the components was omitted. No cross-link was observed by MS analysis if the peptide, dGuo, or methylglyoxal was omitted.

RESULTS

Synthesis of the dGuo-Methylglyoxal-N^{α}-AcLys Cross-Link (1). The chemical synthesis of the putative cross-link is outlined in Scheme 1 (see Supporting Information for experimental procedures). FMOC-(*S*)-Ala was coupled to the side chain amino group of the AcLys methyl ester in 78% yield (HBTU, iPr_2NEt, DMF). The FMOC group was removed using diisopropylethylamine in DMSO and reacted in situ with O^6-(2-(trimethylsilyl)ethyl)-2-fluoro-2′-deoxyinosine (**3**) under conditions previously employed by Tretyakova and co-workers.[36,37] The O^6-protecting group was removed by brief treatment with acetic acid at ambient temperature, and the modified nucleotide (**4**) was obtained in 45% yield after purification by HPLC. Hydrolysis with LiOH afforded the desired cross-link (*S*)-**1** in 83% yield after purification by solid phase extraction. The cross-linking reaction was anticipated to

Scheme 1. Synthesis of Cross-Link (1) Standards



afford a mixture of stereoisomers at the position adjacent to N^2. Thus, (*R*)-**1** was synthesized starting from FMOC-(*R*)-Ala by the same sequence. The NMR chemical shifts of the 2′-protons, which are in the typical range for deoxyguanosine, and the deoxyribose coupling constants suggest that the nucleoside exists predominantly in the *anti* glycosidic conformation with the deoxyribose largely (~63%) in the 2′-endo conformation.[38−40]

The mass spectrum of cross-link **1** (*m/z* 508.2 $[M - H]^{1-}$) and its fragmentation in negative ion mode is shown in Figure 3B. The initial characteristic loss of the deoxyribose unit (−116 Da, 508 → 392) is followed by decarboxylation of the lysine portion (−44, 392 → 349). Interestingly, the amide bond involving the lysine side chain readily fragmented (−145, 349 → 204) at low collision energy (10 eV). The mass spectra of both diastereomers were identical. An authentic standard of the CE-dGuo adduct was also synthesized from the reaction of dGuo and glyceraldehyde.[41] The mass spectral fragmentation of CE-dGuo in negative ion mode involved decarboxylation (−44, 338 → 294) followed by neutral loss of the deoxyribose (−116, 294 → 178) (see Figure S17 of the Supporting Information), which is consistent with a previous analysis.[7,8] Cross-link **1** and CE-dGuo adducts were also characterized by 1D- and 2D-^{1}H NMR spectroscopy (see Figure S8−S13 of the Supporting Information).

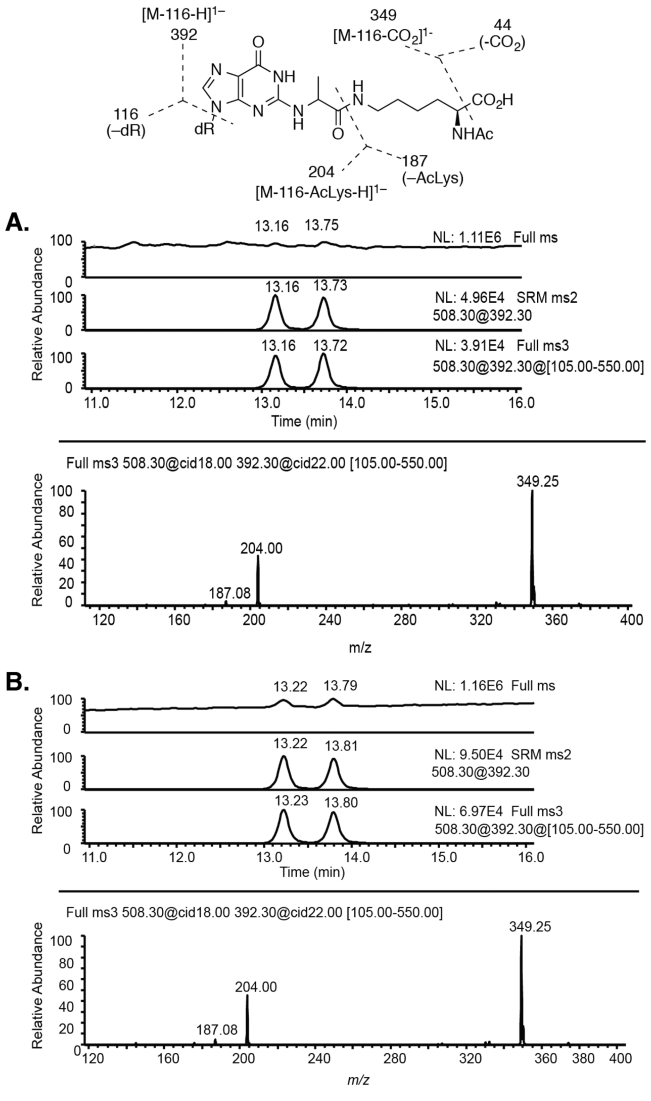


Figure 3. UPLC chromatogram of the full scan mode (105 − 1100 Da), reconstructed ion chromatograms of SRM scan mode and MS^3 fragment ions, and MS^3 product ion spectrum of the dGuo-methylglyoxal-AcLys cross-link (**1**). (A) From the reaction of dGuo, AcLys, and methylglyoxal (1:1:4). (B) Authentic cross-link standards of **1**.

Identification and Quantitation of the Cross-Link (1) from the Reaction of dGuo, Ac-Lys, and Methylglyoxal. The cross-linking reaction was initially investigated using a 4-fold molar excess of methylglyoxal in phosphate buffer (100 mM, pH 7.4) at 37 °C for 7 days. HPLC analysis of the reaction showed three predominant products (Figure 4A) having retention times (t_r) between ~16 and 19 min. UPLC-MS analysis (Figure 4B) of the reaction revealed that the major products had masses consistent with 2:1 methylglyoxal-dGuo adducts (*m/z* 410 $[M - H]^{1-}$). Minor products, which coeluted with the 2:1 adducts, had masses consistent with a 1:1 adduct between methylglyoxal and dGuo (*m/z* 338 $[M - H]^{1-}$) and are likely to be the diastereomeric 1,N^2-cyclic adducts (MG-dGuo) and CE-dGuo. Under the conditions of the reaction, the cross-links were not detectable by HPLC (Figure 4A, insert) but could be readily identified by UPLC-ESI-MS^n (Figure 4B). With standards in hand, we identified the diastereomeric cross-links of interest (Figure 3A), and their mass spectra were identical to those of the standard (Figure 3B).

Structures of the 2:1 dGuo adducts of methylglyoxal and glyoxal have been proposed previously (**5**−**7**, Figure 5).[7,42,43] The formation of the proposed 2:1 adducts are anticipated to be reversible and therefore expected to equilibrate with dGuo or the 1:1 adduct after isolation;[7] this was in fact shown to be the case for the 2:1 glyoxal-dGuo adduct (**7**).[43] The 2:1 methylglyoxal adducts we observed were stable, which would seemingly rule out the previously proposed structures. Our data for one of the 2:1 methylglyoxal-dGuo adducts (t_r 16.6 min, Figure 4C) suggested **8** (stereochemistry unknown) as the structure, and details of the NMR and mass spectrometric analyses will be reported separately. The proposed structure is related to the previously characterized 2:1 methylglyoxal-arginine adduct **9**.[44,45] We assume that the remaining 2:1 adducts are diastereomers of **8** (t_r 17.1 and 17.9 min, Figure 4C), although it is possible that other isomeric species are present and that their distribution is dependent upon the reaction conditions.

An isotopically labeled cross-link standard was synthesized starting from [$^{13}C_3{}^{15}N_1$]-FMOC-(*S*)-Ala in order to quantitate the cross-links by stable isotope dilution mass spectrometry.[46] A calibration curve was developed by plotting the ratio of the cross-link (*S*)-**1** (0.25 − 100 ng)/[$^{13}C_3{}^{15}N_1$]-(*S*)-**1** (50 ng) versus the integrated ratio of (*S*)-**1** (*m/z* 508 → 392)/[$^{13}C_3{}^{15}N_1$]-(*S*)-**1** (*m/z* 512 → 396). The calibration curve was linear over the concentration range examined (r^2 = 0.998, Figure S18 and S19 of the Supporting Information).

The yield of the cross-link when methylglyoxal was in 4-fold excess was 0.06% at pH 7.4 (100 mM phosphate buffer, 37 °C) based on the initial dGuo concentration. Consistent with the previous work of Murata-Kamiya and Kamiya,[35] the yield of cross-linking was higher at pH 9.0 (0.3%), which may reflect the protonation state of the side chain amino group of AcLys. We hypothesized that formation of the stable 2:1 methylglyoxal-dGuo adduct is much more favorable than the cross-linking chemistry, thereby suppressing cross-link formation. The

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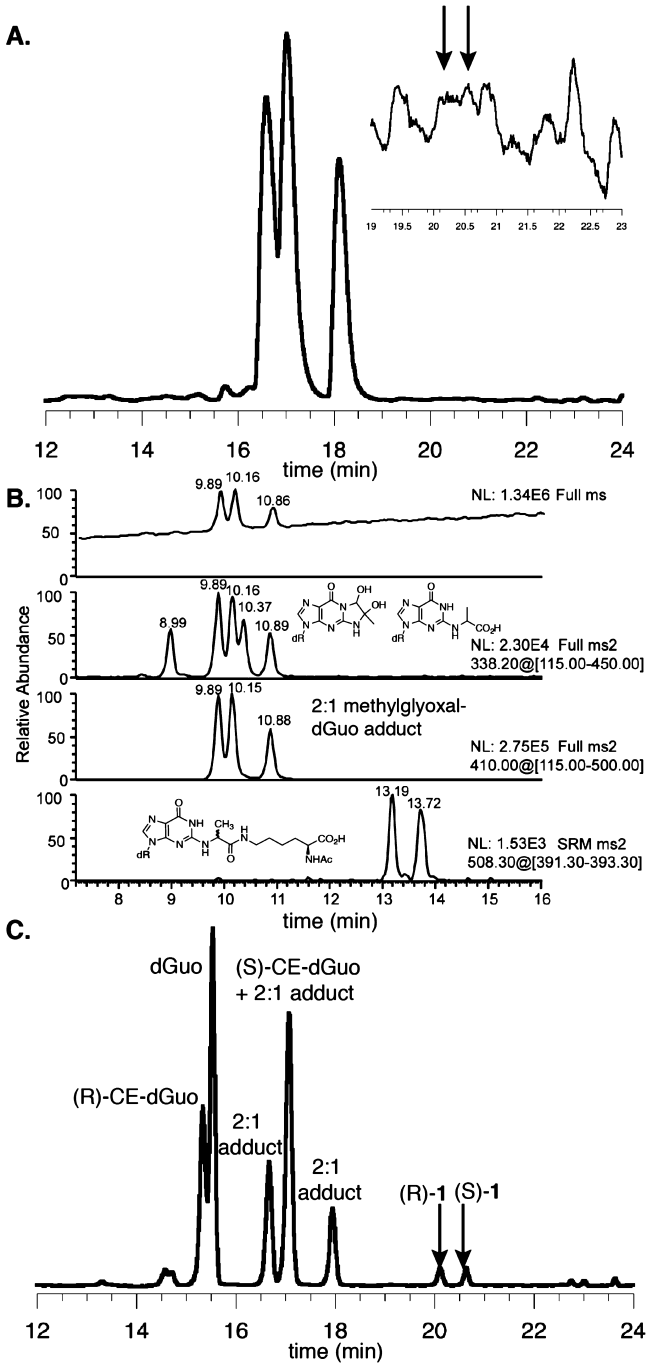

Figure 4. (A) HPLC chromatogram for the reaction of dGuo, AcLys, and methylglyoxal in a 1:1:4 ratio (pH 7.4). (B) UPLC chromatogram of full scan mode (105 − 1100 Da), reconstructed UPLC-ESI -MS/MS and SRM (−116 Da) ion chromatograms from the reaction of dGuo, AcLys, and methylglyoxal (1:1:4). (C) HPLC chromatogram from the cross-linking reaction with a slow addition of methylglyoxal.

reaction was re-examined with equimolar ratio of the three components, and with methylglyoxal as the limiting reagent. The 1:1 and 2:1 adducts were still the predominant adducts under these conditions. However, the yield of the cross-link with equimolar concentrations of three components was 1.1% at pH 7.4, which is nearly 20 times higher than when methylglyoxal was used in 4-fold excess. The yield of the cross-link further increased to 3.6% and 7.1% when methylglyoxal was the limiting reagent (0.25 and 0.1 equiv respectively) based

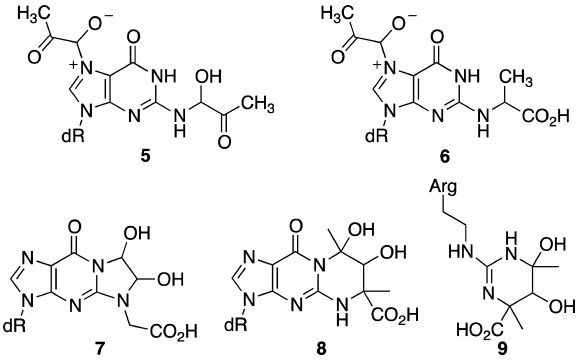

Figure 5. Proposed structures of the 2:1 adducts of methylglyoxal or glyoxal with dGuo.

on the initial methylglyoxal concentration. Isolated yields of 5.3% and 4.9% were realized for the (*S*)- and (*R*)-cross-links when one equivalent of methylglyoxal was added slowly (0.05 equiv every 2 h) over time. An HPLC trace of this reaction is shown in Figure 4C.

Cross-Link Formation from the Reaction of dGuo, Methylglyoxal, and a Model Peptide. Equimolar ratios of methylglyoxal, dGuo, and AcAVAGKAGAR peptide (m/z 842.5 $[M + H]^{1+}$ and 421.8 $[M + 2H]^{2+}$) were reacted in pH 7.4 phosphate buffer for 48 h at 37 °C. Mass spectrometric analysis of the reaction in positive ion mode is shown in Figure 6A (also see Figure S20 of the Supporting Information for the reconstructed ion chromatogram). The product observed at m/z 1163.5 $[M + H]^{1+}$ and 582.2 $[M + 2H]^{2+}$ is consistent with the peptide-methylglyoxal-dGuo cross-link (**10**); additionally, the ion with m/z of 524.3 is consistent with the $[M + 2H]^{2+}$ ion of the cross-link following the neutral loss of the deoxyribose in source (m/z 582.2 → 524.3). The MS^2 spectrum of the m/z 524.3 ion is shown in Figure 6B (collision energy 35 eV), and Table 1 lists the theoretical b- and y-fragment ions for the peptide cross-link involving the lysine side chain at position 5. The most prominent ion in the MS^2 spectrum is at m/z 421.8 (Figure 6B); this mass corresponds to the $[M + 2H]^{2+}$ ion of the starting peptide and results from fragmentation of the amide bond involving the lysine side chain. This amide bond readily fragmented in the MS^2 spectrum of the dGuo-methylglyoxal-AcLys (**1**) at low collision energy. However, b_2−b_9 and y_2−y_9 fragment ions were clearly observed consistent with a Gua-methylglyoxal cross-link at the Lys residue (Figure 6B and Table 1) and strongly supports the structural assignment.

Minor products (Figures 6A and 7) were observed that had masses consistent with the cross-link plus an additional mol of methylglyoxal (**11**, m/z 1235.4 $[M + H]^{1+}$ and 618.1 $[M + 2H]^{2+}$) and the cross-link plus an additional mol of methylglyoxal and loss of H_2O (**12**, m/z 1217.2 $[M + H]^{1+}$ and 609.1 $[M + 2H]^{2+}$) (Figures S22 and S23 of the Supporting Information). Fragmentation of the product ions suggested that in addition to the lysine cross-link, the C-terminal arginine residue was also modified. The latter is consistent with the cross-link and the MG-H1, MG-H2, or MG-H3 arginine adduct (**12**); although only the MG-H1 adduct is shown, it is likely that a mixture of these isomeric adducts are present. The product with m/z 1235.4 $[M + H]^{+1}$ and 618.1 $[M + 2H]^{2+}$ is likely to be the cyclic vicinal diol that results from the addition of methylglyoxal to arginine and is a probable precursor of MG-H1, MG-H2, and MG-H3 modifications (**11**).[47,48] The MG-

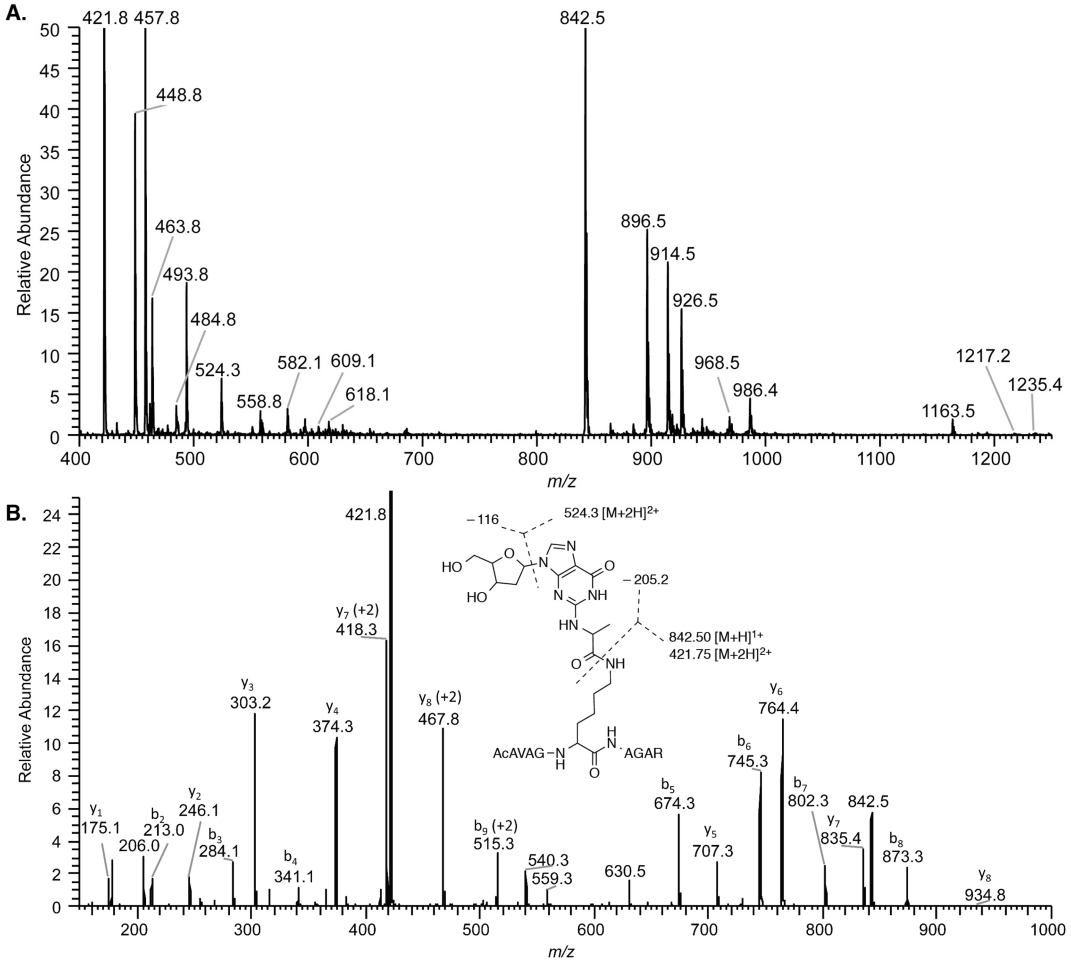

Figure 6. (A) Full-scan spectrum from the reaction of dGuo, methylglyoxal, and the AcAVAGKAGAR peptide in 100 mM, pH 7.4 phosphate buffer. (B) Collision-induced dissociation (CID) mass spectrum of the *m/z* 524.3 $[M + 2H]^{2+}$. This ion results from the in source, neutral loss of the deoxyribose (see Table 1 for theoretical b- and y-ion masses).

Table 1. Theoretical b- and y-Fragment Ion Masses for the dGuo-Methylglyoxal-Lysine Cross-Link of the AcAVAGKAGAR Peptide[a]

	b-ions	y-ions	
1	114.1	1047.5	9
2	**213.1**	**934.4**	8
		467.3 $[M + 2H]^{2+}$	
3	**284.2**	**835.4**	7
		418.2 $[M + 2H]^{2+}$	
4	**341.2**	**764.3**	6
5	**674.3**	**707.3**	5
6	**745.3**	**374.2**	4
7	**802.3**	**303.1**	3
8	**873.4**	**246.1**	2
9	1029.5	**175.1**	1
	515.3 $[M + 2H]^{2+}$		

[a]Observed ions are in bold.

H1, MG-H2, and MG-H3 arginine adducts (**14**) were also observed at *m/z* 896.5 and 448.8 for the singly and doubly charged ions, respectively (Figure S25 of the Supporting Information), as well the diol precursor (**13**) at *m/z* 914.6 and 457.8 (Figure S24 of the Supporting Information). We note that the CE-Lys adduct of the peptide would also be consistent with *m/z* 914.6 $[M + H]^{1+}$ and 457.8 $[M + 2H]^{2+}$; fragmentation of the *m/z* 914.6 revealed a nearly complete b- and y-ion series. The b_5−b_8 and y_1−y_4 fragment ions for the CE-Lys and Arg adducts are predicted to differ, and the observed fragment ions are clearly consistent with the Arg-adduct (**13**). The *m/z* 986.3 and 493.8 ions are consistent with the singly and doubly charge peptide plus 2 mols of methylglyoxal (+144). Fragmentation of this ion resulted in dehydration to *m/z* 484.7. Further fragmentation was consistent with the MG-H1−3 modifications of Arg and the CE-Lys adduct (**15**), which undergoes decarboxylation (−44) (Figure S26 of the Supporting Information). A reasonably abundant product with *m/z* 926.5 and 463.8 for the singly and doubly charged ions is also observed. A complete y-ion series was observed upon CID consistent with the addition of 84 Da to the Arg. (Figure S27 of the Supporting Information). The structure of this adduct is unknown.

Formation of the CE-dGuo Adduct and Lysine Cross-Link (1). Proposed mechanisms for the formation of the CE-dGuo adduct from MG-dGuo are shown in Scheme 2.[7,17,43,49] The mechanism could potentially proceed through the ring closed MG-dGuo or through a ring-opened N^2-dGuo adduct (**16**), in which the aldehyde is hydrated. Dehydration of the carbinolamine involving the N^2-atom would provide intermediate **17** or **19**. In the case of the open-chain pathway, **17** could undergo a 1,2-hydride shift (pinacol rearrangement) to

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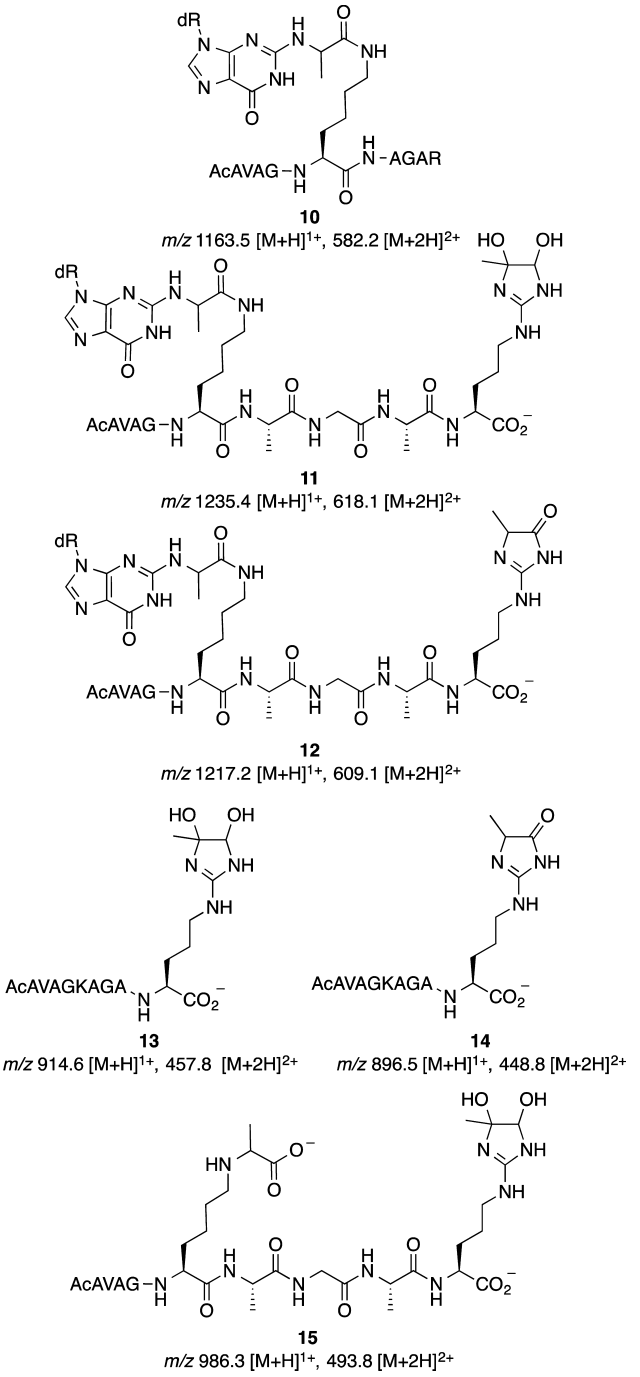
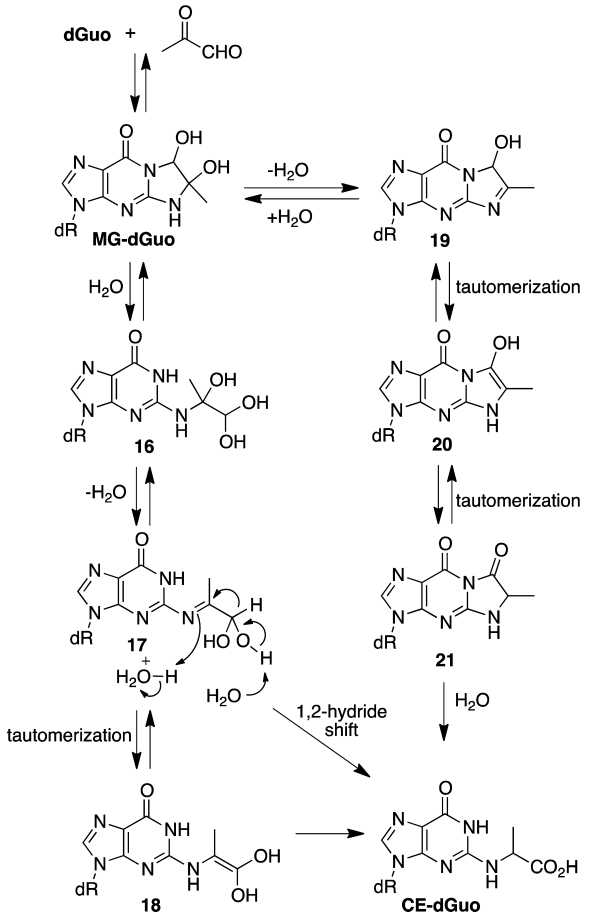


Figure 7. Products identified by the mass spectrometric analysis of the reaction of dGuo, methylglyoxal, and the AcAVAGKAGAR peptide in 100 mM, pH 7.4 phosphate buffer.

the CE-dGuo adduct. Alternatively, imine **17** could undergo tautomerization to the enamine **18**, which is the enol form of the CE-dGuo adduct (Amadori rearrangement). An analogous mechanism can be envisioned from the ring-closed imine **19**. Amadori rearrangement leads to imide **21** through **20** or a 1,2-hydride of **19** gives **21** directly (not shown). Hydrolysis of imide **21** results in CE-dGuo.

The proton adjacent to N^2 will be derived from the solvent in the Amadori pathway, whereas it is derived from the aldehyde proton of methylglyoxal in the 1,2-hydride shift mechanism. Therefore, conducting the adduction reaction in deuterated buffer can differentiate the Amadori versus 1,2-hydride shift

Scheme 2. Mechanism of CE-dGuo Formation



pathways. Methylglyoxal and dGuo were incubated at 37 °C in deuterated phosphate buffer (100 mM, pH 7.4) for 7 days. To enhance the yield of the CE-dGuo adducts, one equivalent of methylglyoxal was added in equal portions over 5 days. The formation of the 2:1 adduct was lower under these conditions compared to reactions when equimolar or excess methylgyoxal was added at once.

The later eluting CE-dGuo isomer, assigned as the (*S*)-isomer based on previous work,[50] coeluted with one of the 2:1 methylglyoxal-dGuo adducts under the chromatographic conditions used. Therefore, (*R*)-CE-dGuo was isolated for NMR analysis. An expanded region of the spectrum is shown in Figure 8A, and the full spectrum is shown in Figure S14 of the Supporting Information. The analysis showed that the proton adjacent to the N^2-atom and carboxylate group, which normally appears as a quartet at δ 4.22 ppm, was not observed from the product obtained in deuterated buffer, and the adjacent methyl group at δ 1.36 ppm, which was normally a doublet, was now a singlet. The proton spectrum was integrated relative to the integration of the anomeric proton (δ 6.19 ppm). This showed that the H8 had exchanged by ~60%,[51] while the methyl group had ~25% deuterium incorporation. The latter probably occurred as methylglyoxal, rather than at the CE-dGuo adduct stage. No exchange of the proton adjacent to the N^2-atom and carboxylate group was observed when a (*R*)-CE-dGuo standard was incubated in deutrated buffer for 7 days at 37 °C, indicating that the deuterium atom was incorporated as part of the mechanism of CE-dGuo formation from dGuo and methylglyoxal. The results are consistent with the Amadori rearrange-

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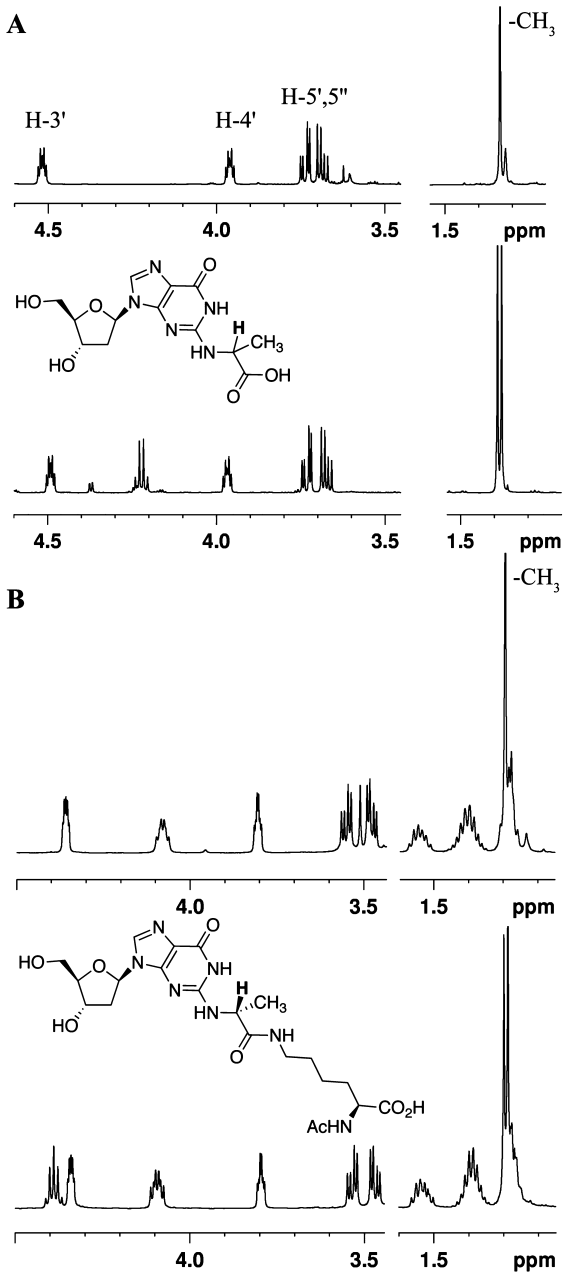

Figure 8. NMR analysis of the reaction of dGuo and methylglyoxal (A) and dGuo, AcLys, and methylglyoxal (B) in deuterated buffer (top) versus standards (bottom).

ment mechanism but do not distinguish between the ring-opened or ring-closed pathways. We hypothesize that formation of cross-link **1** proceeds through a related mechanism (vide infra), and similar results were obtained when AcLys, dGuo, and methylglyoxal were reacted in deuterated buffer. An apparent quintet at δ 4.37 ppm was not observed for the methine proton adjacent to the N^2-atom of cross-link (*R*)-**1** and the methyl group at δ 1.30 ppm appeared as a singlet instead of a doublet (Figure 8B and S15 of the Supporting Information).

DISCUSSION

DPCs are intermediates of natural DNA processing or result through exposure to environmental, occupational, or endogenous toxicants, as well as chemotherapeutic agents.[52−54] DPCs are formed by numerous mechanisms, and one of the major challenges to studying them is their structural heterogeneity. DPCs can result from oxidative damage to DNA[55−57] or through reaction of the protein with oxidation products such as oxanine,[58−61] ribonolactone[62,63] lesions, or further oxidation of 8-oxoguanine.[64−66] Such cross-links involve direct reaction with amino acid side chains and DNA. DPCs are also formed with bifunctional electrophiles, which can react with a nucleophilic site on both the DNA and amino acid side chain. Formaldehyde,[67] dihaloalkanes,[68,69] butadiene diepoxide,[70,71] and some heavy metals[72] are examples of environmental/occupational agents that form DPCs. DPCs have also been characterized from chemotherapeutic agents such as nitrogen mustards[73] and cisplatin.[74] DPCs can form reversibly with abasic sites[75−77] or enal adducts[78−82] of dGuo via Schiff base formation with a lysine side chain or the N-terminal amino group; such DPCs have been reductively trapped.

Comparative mutagenesis between glyoxal and methylglyoxal in African green monkey kidney (COS-7) cells using the *supF* reporter gene showed dramatically different mutagenic spectra. While glyoxal resulted largely in base-pair substitutions (mainly G → T transversions), multiple base deletions were the predominant mutation observed for methylglyoxal.[14,16,83] Interestingly, exposure of human skin cells to methylglyoxal and glyoxal showed that methylglyoxal was significantly more likely to form cross-links, while glyoxal induced DNA strand breaks.[84] In one study, DPC formation was reported to be ∼10-fold higher with methylglyoxal than glyoxal in vitro.[35] It is tempting to attribute the higher level of deletion mutations observed in the methylglyoxal mammalian mutagenesis studies to DPCs.

The *supF* containing DNA vector was treated with glyoxal or methylglyoxal then immediately transfected into the cells for these initial mutagenesis experiments; the nature and distribution of the DNA adducts were not determined. In a more recent study, a DNA vector was analyzed by mass spectrometry after modification by methylglyoxal, and the MG-dGuo adduct accounted for less than 1% that of the diastereomeric CE-dGuo adducts, and the 2:1 adducts were not detected.[22] The major mutation observed after transfection into human fibroblast cells was G → T transversions, with deletions accounting for less than 10% of the observed mutation frequency.

Oligonucleotides containing CE-dGuo have been synthesized in a sequence specific and stereospecific manner,[50,85] allowing for replication studies in vitro and intact cells. DNA polymerases prefer to insert dCTP opposite a template CE-dGuo resulting in error-free or nonmutagenic bypass. However, when replications errors occur purines are most commonly misinserted opposite the CE-dGuo lesions leading to G → T and G → C transversions. CE-dGuo was weakly mutagenic in *E. coli* resulting in low levels of G → T transversions with a mutagenic frequency of <0.5%; the mutagenicity increases to 1.1 and 2.3% for the *R*- and *S*-isomers in a pol IV deficient background, suggesting that this polymerase participates in the replication bypass of the CE-dGuo adducts in vivo. Interestingly, the CE-dGuo adducts were not mutagenic when replicated in wild-type murine embryonic fibroblast cells.[86] However, G → A transitions and G → T transversions were observed in a pol κ deficient background suggesting a role for this polymerase in the error-free bypass of the CE-dGuo adducts in mammalian cells. Pol κ and pol IV are both part of the DinB family of DNA polymerases.

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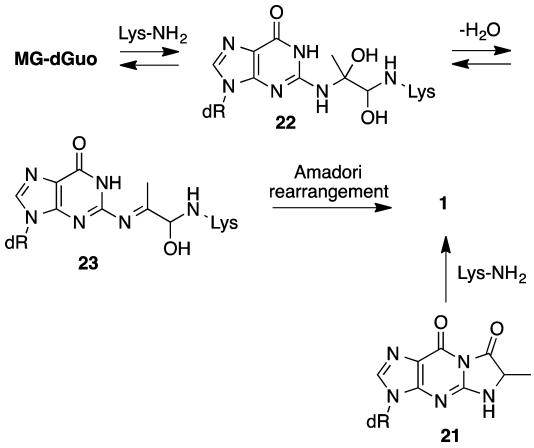
Scheme 3. Mechanism of Cross-Link (1) Formation



Altogether, the most recent random and site-specific mutagenesis data suggests that CE-dGuo is weakly mutagenic and not likely to be the source of the deletion mutations observed in early experiments. It is possible that residual MG-dGuo reacts with cellular proteins after transfection and that the resulting DPCs give rise to deletions. Model studies with amino acids suggested that lysine and cysteine, but not arginine, formed cross-links with the MG-dGuo adduct.[35]

Two mechanisms for CE-dGuo formation have been proposed involving either a series of tautomerizations (Amadori rearrangement) or a 1,2-hydride shift (pinacol rearrangement) as the key step (Scheme 2).[7,17,43,49] The mechanism can occur through a ring-opened N^2-adduct or the ring-closed 1,N^2-exocyclic adduct. Labeling studies are consistent with the Amadori rearrangement mechanism. We hypothesized that the mechanism of lysine cross-link formation is related to the mechanism of CE-dGuo formation. Nucleophilic attack of the side chain amino group of lysine forming a carbinolamine intermediate (**22**, Scheme 3) rather than hydration of the open-chain aldehyde, followed by dehydration and Amadori rearrangement affords cross-link **1** with a stable amide linkage. Alternatively, the nucleophilic opening of the cyclic imide **21** by lysine also leads to cross-link **1**. Although the labeling studies cannot distinguish between the ring-opened or ring-closed pathways, it was previously noted that the yield of cross-link improved in the presence of $NaBH_4$.[35] This observation is more consistent with the ring-opened pathway since further dehydration of **23** would provide a bis-imine intermediate that could be reduced to form a stable cross-link.

Both diastereoisomers of the proposed cross-link (**1**) were synthesized according to Scheme 1 and used as authentic standards. Additionally, an isotopically labeled standard, [$^{13}C_3{}^{15}N_1$]-(*S*)-**1**, was synthesized starting from [$^{13}C_3{}^{15}N_1$]-FMOC-(*S*)-Ala, which was used to quantitated cross-link **1** by stable isotope dilution mass spectrometry. Initial model cross-linking reactions were examined with dGuo, AcLys, and excess methylglyoxal. Large excesses of the electrophile are typically used for such model reactions owing to the low nucleophilicity of the nucleoside bases. These conditions gave vanishing low yields of the cross-link with the 2:1 adducts being highly favored. We hypothesized that the favorable formation of the 2:1 adducts, which are of questionable biological relevance, were greatly out competing the cross-linking reaction and reasoned that lower concentrations of methylglyoxal might make cross-link formation more competitive. Consistent with this hypothesis, the yield of the cross-link significantly improved through lowering the methylglyoxal concentration. The methylglyoxal concentration in cells is likely to be much lower than that in in vitro model systems. Our results suggest that the cross-linking chemistry may be more efficient under cellular conditions than might be predicted based on model systems that use a large excess of the electrophile.

The reaction of equimolar concentrations of dGuo, methylglyoxal, and a model peptide, AcAVAGKAGAR, was examined by mass spectrometry. The most abundant products (Figure 6A) were identified as the arginine adducts of methylglyoxal (**13** and **14**) in addition to modification of both the arginine and lysine residues (**15**). Products with masses consistent with the predicted methylglyoxal cross-link between the lysine side chain and dGuo were also observed (**10**−**12**). The cross-link products, which also include products in which the Arg is also modified, appear to be in lower abundance than the simple peptide adducts. The CID spectra are consistent with cross-linking to the lysine side chain. The major fragment ion results from neutral loss of the dGuo-methylglyoxal unit to the original peptide. This fragmentation suggests a possible method for detection of the cross-link from proteomic analysis of cells or tissue samples. The cross-linked tryptic peptide could be identified by monitoring for the neutral loss the dGuo-methylglyoxal mass (−321 Da) after digestion of the DNA portion of the DPC to a single nucleoside or loss of Gua-methylglyoxal base (−205 Da) after deglycosylation of the DNA similar to that in Figure 6B.[79] The mass of the resulting peptide fragment would represent a tryptic peptide with a missed cleavage site since the lysine involved in cross-link formation is unlikely to be a substrate for trypsin.

Reactive electrophiles other than methylglyoxal are formed from the degradation of carbohydrates, which can also modify proteins and DNA. It was observed that amines can accelerate the modification of DNA and in some cases be incorporated into the product. For example, the reaction of guanosine with glucose in the presence of *n*-propylamine resulted in amide cross-links related to **1** (Figure 9).[87] Analogous products were observed when D-ribose was used. The work provided the first insight into the structure of DPCs derived from Maillard products. Subsequently, cross-link **1** was observed from the reaction of Ac-Lys, dGuo, and either dihydroxyacetone or glyceraldehyde.[88] It is possible that methylglyoxal is the reactive bis-electrophile in these examples or that perhaps other electrophilic Maillard products are capable of forming the

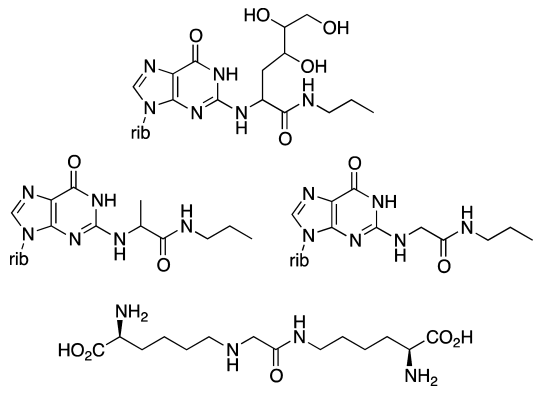


Figure 9. Model cross-links formed by methylglyoxal and related 1,2-dicarbonyls.

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same cross-link. A related glyoxal cross-link between Lys side chains has also been characterized in vitro from the reaction of glyoxal with bovine serum albumin (BSA) and human lens proteins.[25] Interestingly, the yield of this cross-link for the in vitro reactions increased at lower glyoxal concentrations.

CONCLUSIONS

The reaction of dGuo, methylglyoxal, and either N^{α}-AcLys or a Lys-containing peptide has been examined as a model for DPC formation and analysis. The structure of the stable cross-link between dGuo, N^{α}-AcLys, and methylglyoxal (**1**) was hypothesized based on the mechanism of CE-dGuo formation (Schemes 2 and 3). The structure was confirmed by chemical synthesis and mass spectrometry. Importantly, we observed that cross-link formation increased at lower methylglyoxal concentrations. Cross-link formation was also characterized on a model peptide. The amide linkage between the lysine side chain and the methylglyoxal-dGuo readily fragmented during MS analysis at low collision energy. The neutral loss of this portion to give the original peptide can potentially be monitored as a means to identify cellular proteins involved in DPCs by methylglyoxal.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures for the synthesis of **1**; parameters and details of analytical procedures; and ^{1}H, ^{13}C NMR, and LC-ESI-MS^{n} spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

DEDICATION

We dedicate this paper to the memory of Edward Keith Hawkins (1964−2011).

ABBREVIATIONS

MG-H1, N^{5}-(4,5-dihydro-4-methyl-5-oxo-1*H*-imidazol-2-yl)-L-ornithine; MG-H2, α,2-diamino-4,5-dihydro-5-methyl-4-oxo-1*H*-imidazole-1-pentanoic acid; MG-H3, α,2-diamino-4,5-dihydro-4-methyl-5-oxo-1*H*-imidazole-1-pentanoic acid; CE-Lys, N^{6}-(1-carboxyethyl)-L-lysine; AGE, advanced glycation end-products; MG-dGuo, 3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,4,6,7-tetrahydro-6,7-dihydroxy-6-methyl-9*H*-imidazo[1,2-*a*]-purin-9-one; GX-dGuo, 3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,4,6,7-tetrahydro-6,7-dihydroxy-9*H*-imidazo[1,2-*a*]purin-9-one; CE-dGuo, *N*-[9-(2-deoxy-β-D-*erythro*-pentofuranosyl)-6,9-dihydro-6-oxo-1*H*-purin-2-yl]-alanine; CM-dGuo, *N*-[9-(2-deoxy-β-D-*erythro*-pentofuranosyl)-6,9-dihydro-6-oxo-1*H*-purin-2-yl]-glycine; DPC, DNA−protein cross-link; AcLys, N^{2}-acetyllysine; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium-*N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)-uronium hexafluorophosphate; FMOC, fluorenylmethyloxycarbonyl; UPLC, ultra performance liquid chromatography; SRM, selected reaction monitoring

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