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Independent Generation of 5-(2′-Deoxycytidinyl)methyl Radical and the Formation of a Novel Cross-Link Lesion between 5-Methylcytosine and Guanine

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Abstract: Reactive oxygen species (ROS) can damage DNA. Although a number of single nucleobase lesions induced by ROS have been structurally characterized, only a few intrastrand cross-link lesions have been identified and characterized, and all of them involve adjacent thymine and guanine or adenine. In mammalian cells, the cytosines at CpG sites are methylated. On the basis of the similar reactivity of 5-methylcytosine and thymine toward hydroxyl radical and the similar orientation of adjacent thymine guanine (TG) and 5-methylcytosine guanine (mCG) in B-DNA, we predict that the cross-link lesion, which was identified in TG and has a covalent bond formed between the 5-methyl carbon atom of T and the C8 carbon atom of G, should also form at mCG site. Here, we report for the first time the independent generation of 5-(2′-deoxycytidinyl)methyl radical, and our results demonstrate that this radical can give rise to the predicted novel intrastrand cross-link lesion in dinucleoside monophosphates d(mCG) and d(GmC). Furthermore, we show that the cross-link lesion can also form in d(mCG) from γ irradiation under anaerobic conditions.

Introduction

Reactive oxygen species (ROS) are generated by either aerobic metabolism or exogenous oxidizing agents.[1] ROS can damage nucleic acids and a multitude of damaged products involving a single nucleobase are known.[2] Those adducts may contribute to a number of pathological conditions including cancer,[2] neurodegeneration,[3] and natural processes of aging.[1]

In mammalian cells, the cytosines at CpG sites are methylated. Although CpGs are under-represented by 5-fold of their expected frequency in mammalian DNA, methylated CpGs are mutational hotspots and the most common mutations observed are C to T transition mutations.[4] In addition to single-nucleobase lesions, several intrastrand cross-link lesions formed at adjacent TG and GT sites have been structurally characterized.[5−14] However, no cross-link lesion between mC and G has been reported.

It is known that, upon γ irradiation, the three major sites for hydroxyl radical attack on both thymine and mC are the C5, C6, and the 5-methyl carbon atoms, and the yields for the hydroxyl radical attack at these three different carbon atoms are similar for the two nucleobases.[15] Furthermore, in B-DNA, the distance between the methyl carbon atom of thymine and C8 carbon atom of the vicinal guanine is 6.36 Å, whereas the corresponding distance in mCG is 5.51 Å.[16] On the basis of the similar reactivity between mC and T toward hydroxyl radical and the similar orientation of the adjacent pyrimidine-purine nucleobases at mCG and TG sites in B-DNA, we predict that a similar cross-link lesion should form between the 5-methyl carbon atom of mC and the C8 carbon atom of its 3′ adjacent guanine. In addition, Pfeifer and co-workers[17] recently observed mCG → TT tandem double mutation in nucleotide excision repair (NER)-deficient XP-A cells, indicating that cross-link lesion might form at mCG site.

To address the mechanism of ROS-induced nucleic acid damage, Greenberg,[18−24] Giese,[25−28] and Cadet et al.[5,6] reported

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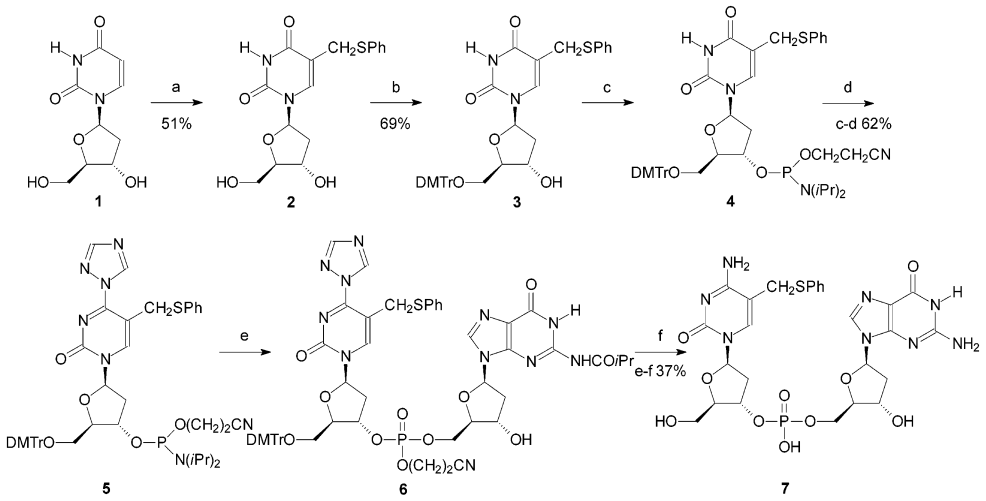
Scheme 1



Reagents: (a) (i) $(CH_2O)_n$/TEA/H_2O/60 °C. (ii) $(CH_3CO)_2O$, pyridine. (iii) PhSH/TEA/DMF/70 °C. (iv) NH_3/MeOH. (b) DMTrCl/DMAP/TEA/pyridine. (c) $NC(CH_2)_2OP(Cl)N(iPr)_2$/DIEA/CH_2Cl_2. (d) $POCl_3$/1,2,4-triazole/TEA/CH_3CN. (e) (i) N^2-isobutyryl-dG/tetrazole/DMF/CH_3CN. (ii) I_2/THF/H_2O/pyridine. (f) (i) 1% TFA/CH_2Cl_2. (ii) 29% NH_4OH.

the synthesis and characterization of a number of photolabile precursors of radical intermediates involved in radiation-induced nucleic acid damage. One of the precursors, 5-(phenylthiomethyl)-2′-deoxyuridine, which yields a 5-methyl radical of thymine upon 254-nm irradiation, can give rise to a known cross-link lesion where the 5-methyl carbon atom of thymine and the C8 carbon atom of an adjacent guanine or adenine are covalently bonded.[5,6] This and earlier work by Box et al.[11] demonstrated that the intrastrand cross-link lesion can initiate from a single radical event. Therefore, we reason that a similar photolabile radical precursor of mC may facilitate us to obtain the predicted cross-link lesion between mC and guanine.

Here, in this paper, we report for the first time the independent generation of the 5-(2′-deoxycytidinyl)methyl radical and the formation of a novel intrastrand cross-link lesion between the 5-methyl carbon atom of 5-methylcytosine and the C8 carbon atom of the adjacent guanine in dinucleoside monophosphates.

Results and Discussion

1. Synthesis of Phosphoramidite Building Block of 4-(1,2,4-Triazol-1-yl)-5-(phenylthiomethyl)-2′-deoxyuridine. Because the 5-methyl radical of mC is expected to give rise to a cross-link lesion where the 5-methyl carbon atom of mC and the C8 carbon atom of the adjacent G are covalently bonded, we decided to first synthesize a photolabile precursor for this radical. A phosphoramidite building block containing a photolabile precursor of the 5-methyl radical of thymidine has been reported by Romieu and co-workers[5] and Anderson et al.[24] using phenylthio photochemistry and Norrish type I photocleavage, respectively.

We adopted the phenylthio photochemistry and employed a nucleoside conversion[29] step to prepare the desired phosphoramidite building block **5** from building block **4** (Scheme 1). Compound **4** was prepared according to that reported by Cadet and co-workers[5,6] with some modifications to improve the yield. The key modification was in step (a), where we added more paraformaldehyde during the course of the reaction followed by addition of triethylamine to keep the reaction solution basic, which afforded a 51% yield for step a, compared to a 13% yield reported in the previous study.[5]

Under standard nucleobase deprotection conditions, i.e., incubation with 30% ammonium hydroxide at 55 °C for 12 h, the phenylthio moiety fully decomposed.[5] We found that the phenylthio moiety is stable upon prolonged treatment with 30% ammonium hydroxide at room temperature. Such treatment for 48 h is sufficient for the removal of standard nucleobase protecting groups. Therefore, we employed standard nucleobase protection for the synthesis of dinucleoside monophosphates containing 5-(phenylthiomethyl)-2′-deoxycytidine. Furthermore, the base deprotection with ammonium hydroxide also converts the 1,2,4-triazol-1-yl group to an amino group,[30] which gives us dinucleoside monophosphates with the desired 5-(phenylthiomethyl)-2′-deoxycytidine.

With building block **5**, we synthesized dinucleoside monophosphate d($mC^{SPh}G$) (**7**), where mC^{SPh} is 5-(phenylthiomethyl)-2′-deoxycytidine, using solution-phase phosphoramidite chemistry (Scheme 1).[31] We also prepared d(GmC^{SPh}) (**12**) using solution-phase chemistry starting from compound **3** (Scheme 2).

2. Demonstration of the Formation of the Intrastrand Cross-Link Lesions from d($mC^{SPh}G$) and d(GmC^{SPh}). We then irradiated d($mC^{SPh}G$) and d(GmC^{SPh}) with 254-nm UV

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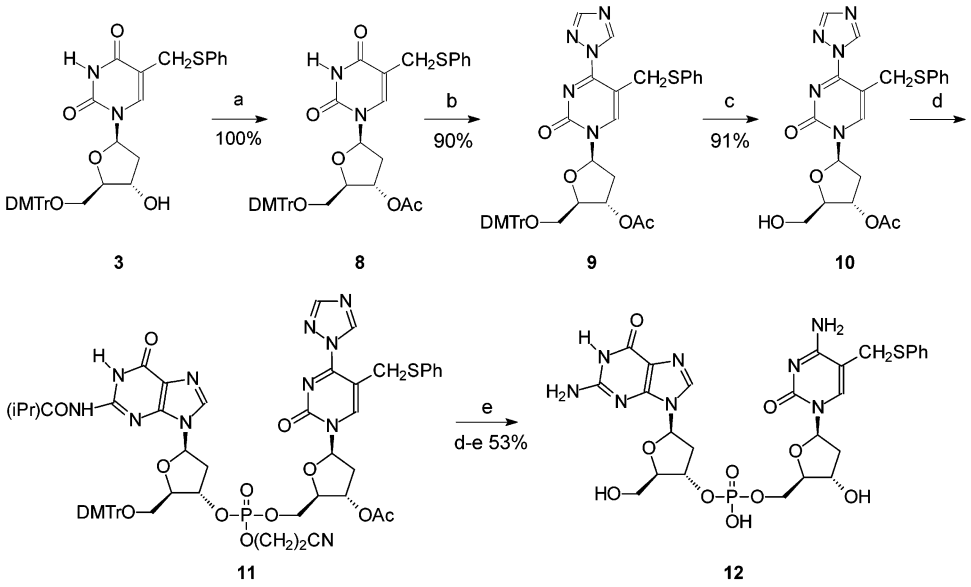
Scheme 2



Reagents: (a) Ac_2O/pyridine. (b) $POCl_3$/1,2,4-triazole/TEA/CH_3CN. (c) 80% AcOH. (d) (i) dG-CE phosphoramidite/DMF/CH_3CN (ii) I_2/THF/H_2O/pyridine. (e) (i) 80% AcOH. (ii) 29% NH_4OH.

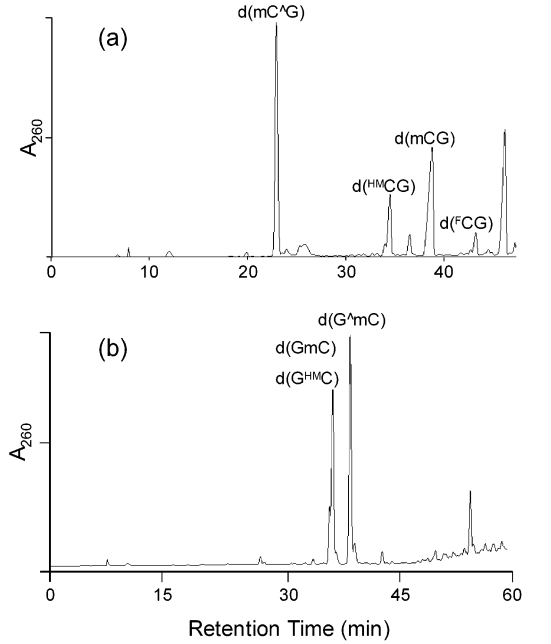


Figure 1. HPLC traces for the separation of the 254-nm irradiation mixtures of d(mC^{SPh}G) (a) and d(GmC^{SPh}) (b).

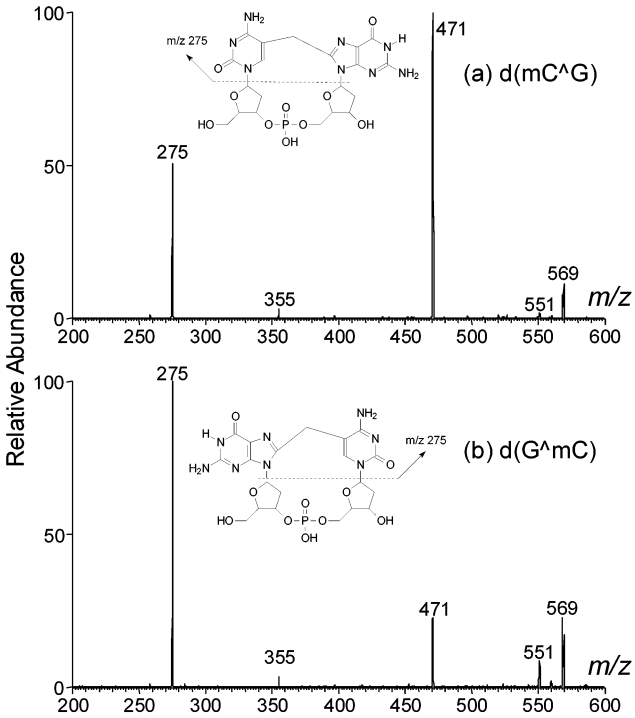


Figure 2. Product-ion spectra of the ESI-produced $[M + H]^+$ ions of d(mC^G) (a) and d(G^mC) (b).

light, and indeed the cross-link lesion, in which the 5-methyl carbon atom of mC and the C8 carbon atom of the adjacent guanine are cross-linked, forms from both dinucleoside monophosphates. The 22.9-min fraction from the HPLC of the irradiation mixture of d(mC^{SPh}G) is the anticipated cross-link product [labeled as d(mC^G) in Figure 1].

ESI−MS and tandem MS (MS/MS) indicate the presence of the cross-link lesion. The cross-link lesion has a molecular weight that is 2 amu less than that of the unmodified d(mCG), which is what we observed in the positive-ion ESI−MS (ion of *m*/*z* 569, data not shown). Product-ion spectrum of the $[M + H]^+$ ion of d(mC^G) showed the formation of an abundant fragment ion of *m*/*z* 275, which is attributed to the protonated ion of the cross-linked nucleobase moiety (Figure 2a). In addition, we observed an ion corresponding to the loss of a 2-deoxyribose (ion of *m*/*z* 471). The assignments of the fragment ions are supported by exact mass measurements on a Fourier transform ion cyclotron resonance mass spectrometer (Table 1). Similar fragment ions have also been observed for the cross-link products between T and G or A.[5,6] In contrast to the product-ion spectrum of d(mCG) (see the Supporting Information), which shows a facile neutral loss of 5-methylcytosine, we did not observe that loss in the product-ion spectrum of d(mC^G). The result is again in accord with that the 5-methylcytosine is being cross-linked. ESI−MS/MS of the $[M + H]^+$ ion of d(G^mC) gave similar results (Figure 2b).

Table 1. Results for Exact Mass Measurement of Fragment Ions Observed in the Product-Ion Spectra of the $[M + H]^+$ Ions of d(mC^G) and d(G^mC)

ion identities	calculated mass	measured mass	deviation (ppm)
	d(G^mC)		
H_2O loss	551.1404	551.1410	1.1
		551.1407	0.5
		551.1408	0.7
2-deoxyribose loss	471.1142	471.1140	−0.4
		471.1134	−1.7
		471.1137	−1.1
[nucleobase + H]$^+$	275.1005	275.1000	−1.8
		275.1000	−1.8
		275.1000	−1.8
	d(mC^G)		
2-deoxyribose loss	471.1142	471.1137	−1.1
		471.1142	0.0
		471.1145	0.6
[nucleobase + H]$^+$	275.1005	275.1004	−0.4
		275.1002	−1.1
		275.1004	−0.4

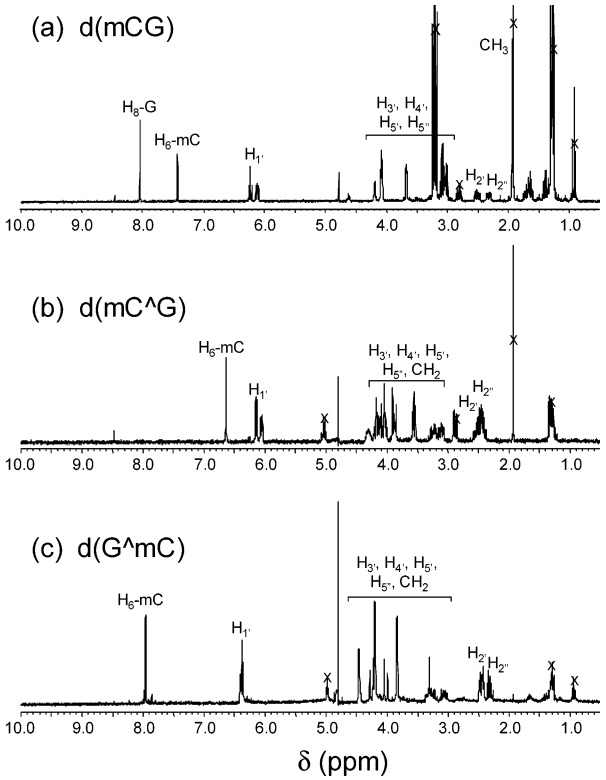


Figure 3. ^{1}H NMR spectra of d(mCG) (a), d(mC^G) (b), and d(G^mC) (c). D_2O was used as solvent, and impurity peaks were marked with a "X".

^{1}H NMR spectra of d(mC^G) and d(G^mC) further demonstrate the presence of the cross-link lesion. Most strikingly, the ^{1}H NMR spectra of both d(mC^G) and d(G^mC) showed the absence of the H8 proton of guanine (Figure 3b&c) compared to that of unmodified d(mCG) (Figure 3a). In addition, the 5-methylene protons in d(mC^G) and d(G^mC) occur as two doublets [δ 3.86, 4.07 for d(mC^G) and δ 4.03, 4.26 for d(G^mC)], whereas the methyl proton resonance shows as a singlet in d(mCG). The NMR results are consistent with the proposed structure and with previous results for the similar cross-link lesion formed between T and G.[5]

In addition to the novel cross-link lesion, we observed the formation of other products initiated from the 5-methyl radical

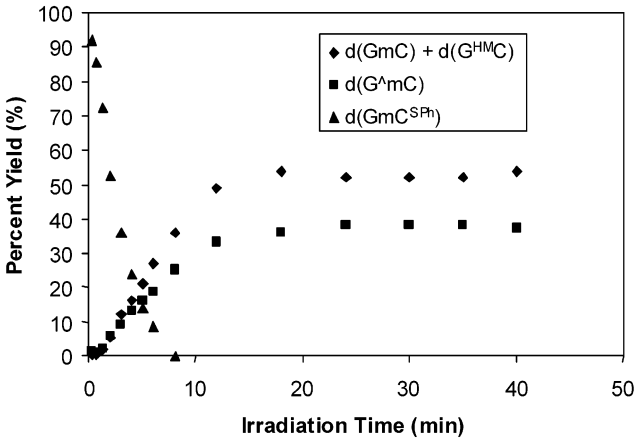


Figure 4. Time-dependent study of 254-nm irradiation of d(GmC^{SPh}), which shows the %yield of d(G^mC), the combined %yield of d(G^{HM}C) and d(GmC), and the decay of starting material d(GX) as a function of time. The experiment was done by taking aliquots out at different irradiation times, and the aliquots were dried and subjected to HPLC analysis. The yield was based on the peak areas in the HPLC traces.

of mC. In Figure 1, the fractions eluting at 34.5, 38.7, and 43.2 min are the dinucleoside monophosphates with the mC moiety being oxidized to 5-(hydroxymethyl)cytosine [d(^{HM}CG)], 5-methylcytosine [d(mCG)], and 5-formylcytosine [d(^{F}CG)], respectively. Similarly, we observed the formation of d(GmC) and d(G^{HM}C) upon the 254-nm irradiation of d(GmC^{SPh}). The formation of d(^{HM}CG), d(^{F}CG), and d(G^{HM}C) indicates that there might be residual amount of oxygen present in the irradiation solution.[32] If that is the case and the amount of molecular oxygen is not in large excess, we would expect to see that the formation of these oxidation products and cross-link lesion both increase with time at short irradiation time. However, these oxidation products are expected to reach a plateau sooner than the cross-link lesion. Our time-dependent irradiation experiment with d(GmC^{SPh}) (Figure 4) demonstrates that the yield of d(G^mC) and the overall yield of d(G^{HM}C) and d(GmC) reach the plateau at a similar time. The above results were obtained with continuous argon bubbling during the 254-nm irradiation. We did similar experiment except that the solution was not bubbled with argon during the irradiation, and it turned out that the two yields again reach the plateau at a similar time (data not shown). Therefore, it remains unclear about the reason for the formation of the oxidation products where the 5-methyl group is oxidized to 5-formyl or 5-hydroxymethyl group.

By using the peak areas and assuming that the extinction coefficients at 260 nm are similar for different products, we estimated that the yield for the formation of d(mC^G) is 21.4%, which is lower than that for the formation of d(G^mC) from d(GmC^{SPh}) (37.4%, HPLC trace shown in Figure 1b) under similar irradiation conditions. This result is consistent with the distances between the methyl carbon atom of mC and the C8 carbon atom of the adjacent G in these two dinucleoside monophosphates, which, in standard B-DNA geometry, are 3.95 and 5.51 Å for adjacent GmC and mCG, respectively.[16] Although a higher cross-linking yield was obtained when G is the 5′ nucleobase, the difference between those two is not as drastic as that reported for the similar cross-link lesions between T and purines.[6] The different observation with adjacent mCG/

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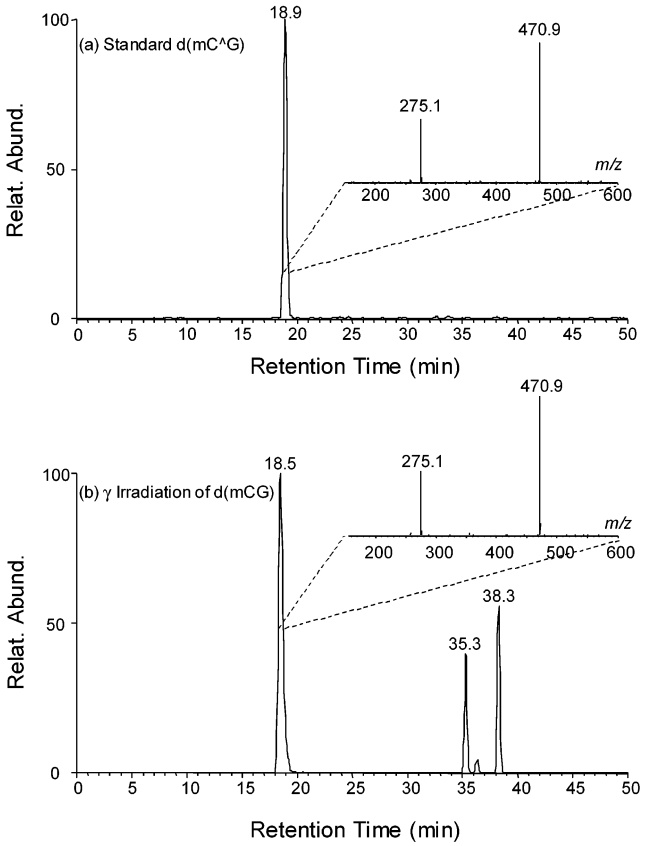

Figure 5. LC−MS/MS analysis of 125-pmol authentic d(mC^G) (a) and 40-nmol γ irradiation mixture of d(mCG) under anaerobic conditions (b). The mass spectrometer was set to monitor the fragmentation of the ion of *m*/*z* 569. Shown in the insets are the MS/MS for the ion of *m*/*z* 569.

GmC and TG/GT might be due to different sequences studied. Here, we studied dinucleoside monophosphates, whereas 3-mer and 15-mer oligodeoxynucleotides were investigated in the previous study.[6]

3. Demonstration of the Formation of the Cross-Link Lesion in d(mCG) Induced by γ Irradiation under Anaerobic Conditions. Next we confirmed, by LC−MS/MS experiment, that γ irradiation of d(mCG) in the absence of oxygen can also give rise to the cross-link lesion. In this experiment, the MS/MS was set to monitor the fragmentation of the *m*/*z* 569 ion, which is the protonated ion of the cross-link lesion d(mC^G) (*vide supra*), in the positive-ion mode. The total-ion current of the γ irradiation mixture (Figure 5b) showed a peak eluting at a very similar time as that of the standard cross-link lesion d(mC^G) (Figure 5a). Furthermore, MS/MS of the 18.5-min peak for the γ irradiation sample gives identical fragmentation as that of the authentic standard (MS/MS are shown as insets in the Figure 5). In addition to the cross-link lesions, we observed other two fractions eluting at 35.3 and 38.3 min, which are very likely the dinucleoside monophosphates with the 2′-deoxyguanosine being oxidized to two stereoisomers of the 5′,8-cyclo-2′-deoxyguanosine,[33] which have the same molecular weight as the d(mC^G). Furthermore, MS/MS of the two cyclo adducts showed that the most abundant fragment ions are due to the loss of the unmodified mC and 5-methyl-2′-deoxycytidine (Figure 6).

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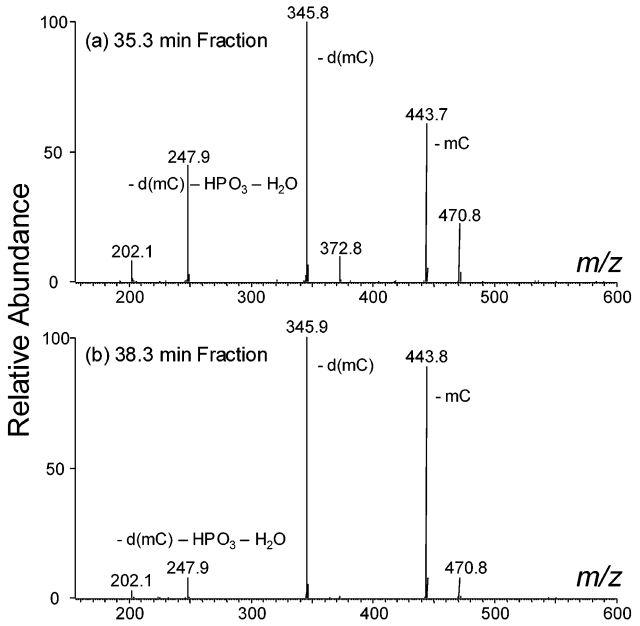

Figure 6. Product-ion spectra of the *m*/*z* 569 ion for the fractions with retention times of 35.3 min (a) and 38.3 min (b) from the γ irradiation mixture.

Conclusions

The 5-(2′-deoxycytidinyl)methyl radical can form from 5-methyl-2′-deoxycytidine upon either γ irradiation,[15] during which the hydroxyl radical can attack the 5-methyl carbon atom of 5-methylcytosine via hydrogen abstraction, or one-electron photooxidation with 2-methyl-1,4-naphthoquinone as a photosensitizer.[32] The resulting cation radical from the latter process can deprotonate to give the 5-methyl radical.[32] Here, we demonstrate that the 5-(2′-deoxycytidinyl)methyl radical can be specifically generated by 254-nm irradiation of 5-(phenylthiomethyl)-2′-deoxycytidine in dinucleoside monophosphates d(mC^{SPh}G) and d(GmC^{SPh}). Moreover, the resultant radical can attack the C8 carbon atom of the adjacent guanine to yield a novel cross-link lesion where the 5-methyl carbon atom of mC and the C8 carbon atom of guanine are covalently bonded. In addition, we demonstrated for the first time that the cross-link lesion can form in dinucleoside monophosphate d(mCG) via γ irradiation under anaerobic conditions.

To our knowledge, this is the first report about the formation of a cross-link lesion between 5-methylcytosine and guanine. Next, we will quantify the formation of the novel cross-link lesions in isolated and cellular DNA and investigate the biological implications of this type of cross-link lesion.

Materials and Methods

All chemicals, unless otherwise specified, were from Sigma-Aldrich (St. Louis, MO). The reagents for solid-phase DNA synthesis were purchased from Glen Research (Sterling, VA). All other solvents, silica gel, and TLC plates were obtained from EM Science (Gibbstown, NJ).

Mass Spectrometry. ESI−MS and MS/MS experiments were carried out on an LCQ Deca XP ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). An equal-volume solvent mixture of acetonitrile and water was used as solvent for electrospray, and a 2-μL aliquot of a ~5 μM sample solution was injected in each run. The spray voltage was 3.4 kV, and the capillary temperature was maintained at 200 °C.

An IonSpec HiResESI external ion source FTICR mass spectrometer (IonSpec Co., Lake Forest, CA) equipped with a 4.7 T unshielded superconducting magnet was used for exact mass measurements. The instrument was equipped with an Analytica (Branford, CT) electrospray ion source. Samples were dissolved in CH_3CN/H_2O (1/1, v/v) solution at a concentration of 5 μM and infused by a syringe pump at a flow rate of 1 μL/min. The source end plate was kept at 3.5 and 3.0 kV for experiments in positive- and negative-ion modes, respectively. Ions were accumulated in a hexapole for 1000−1500 ms and transported through a quadrupole ion guide to the analyzer cell by standard pulse sequences.

Exact mass measurement of the molecular ions were done by using the $[M + 2H]^{2+}$ and $[M + H]^+$ ions of a peptide PRCGVPDVA and the $[M - H]^-$ and $[M - Ade]^-$ ions of ApA as internal standards in the positive- and negative-ion modes, respectively. All spectra were acquired in the broadband mode and, with the internal standards, the mass accuracy for all mass measurements reported in this work was within 5 ppm for the monoisotopic ion peaks.

For exact mass measurements in MS/MS, the $[M + H]^+$ ions of the analyte of interest and 2′-deoxyguanosine were first isolated in the ICR cell by ejection of all other species. Sustained off-resonance irradiation/collisionally activated dissociation (SORI-CAD) of the analyte ions were performed for 1000 ms by a r.f. burst with an amplitude of 1.2 to 2.5 V depending on the analytes (i.e., at a frequency around 1000 Hz from the cyclotron frequency of the isolated ions). Ions were excited as a result of multiple collisions with pulsed nitrogen gas. A 400-ms pulse of nitrogen was introduced at the beginning of the irradiation period (the maximum pressure read-out on the ion gauge was 3×10^{-6} Torr). After a delay of approximately 2 s, the resulting fragment ions were accelerated for detection by a r.f. sweep excitation waveform (100 V p−p). The image current was amplified, digitized at an acquisition rate of 2 MHz with 2048 k data points, and Fourier transformed to yield a mass spectrum.

NMR Measurements. All NMR spectra were recorded on a Varian Unity Inova 300 MHz instrument (Palo Alto, CA). The residual proton signal of the solvent serves as internal reference.

UVC Irradiation. An aqueous solution of dinucleoside monophosphate $d(mC^{SPh}G)$ or $d(GmC^{SPh})$ with AU_{260} of 0.4 in a quartz tube was degassed by argon bubbling for 30 min and irradiated for 15−20 min with 254-nm light generated by a Rayonet photochemical reactor that is equipped with 16 light tubes (The Southern New England Ultraviolet Company, CT). The irradiation was done under continuous argon bubbling. The resulting solution was then lyophilized to dryness.

HPLC. The HPLC separation was performed on a system composed of a Hitachi L-6200A pump (Hitachi Ltd, Tokyo, Japan), a SSI 500 variable wavelength UV Detector (Scientific System Inc., State College, PA), and a Peak Simple Chromatography Data System (SRI Instruments Inc., Las Vegas, NV). A 4.6 × 250 mm Apollo C18 column (5 μm in particle size and 300 Å in pore size, Alltech Associates Inc., Deerfield, IL) was used for the separation. A solution of 50 mM TEAA (solution A) and a mixture of 50 mM TEAA and acetonitrile (70/30, v/v) (solution B) were used as the mobile phases. A gradient of 40-min 0−30% solution B, 5-min 30−100% B, and 5-min 100−0% B was used for the separation of the 254-nm irradiation products of $d(mC^{SPh}G)$ and $d(GmC^{SPh})$. Under this condition, d(GmC) and $d(G^{HM}C)$ were not well resolved. Therefore, the fraction containing the two components was further separated by using a gradient of 5-min 0−15% B, 15-min 15−28% B, and 2-min 28−0% B, and the column was heated to 45 °C. In all separations, the flow rate was 0.8 mL/min, and the fractions were monitored at 260 nm. The HPLC condition for LC−MS experiments was different and described separately in a latter section.

γ-Irradiation of d(mCG) and LC−MS Identification of d(mC^G) from the Irradiation Mixture. A 3-mL solution of d(mCG) (300 nmol) in a 50-mL round-bottom flask was degassed by three freeze−pump−thaw cycles, filled with argon, and exposed to a Mark I ^{137}Cs Irradiator (JL Shepherd and Associates, San Fernando, CA) at a dose rate of 2.4 Gy/min for 3 h (total dose: ∼430 Gy). The resulting solution was dried and subjected directly to LC−MS analysis.

A 2*250 mm Microsorb C18 column with a particle size of 5 μm and a pore size of 100 Å (Varian, Walnut Creek, CA) was used for separation, and a 50-min gradient of 0−28% acetonitrile in 10 mM ammonium acetate was employed. The flow rate was 100 μL/min, and the effluent was coupled to the LCQ Deca XP ion-trap mass spectrometer without splitting. The mass spectrometer was set up to monitor the fragmentation of the ion of *m*/*z* 569 in the positive-ion mode. Approximately 40-nmol irradiation mixture was injected in each run. As a control, standard d(mC^G) (125 pmol) was also injected to the column in a separate LC−MS/MS run with identical experimental setup as for the γ-irradiation mixture.

Synthesis and Characterization of Compounds. Preparation of 2. We followed the procedures described by Ueda et al.[34] with some modifications. Paraformaldehyde (3.1 g) and 2′-deoxyuridine (**1**) (5.25 g, 23 mmol) were dissolved in 0.5 M triethylamine (TEA) in H_2O (80 mL) and the solution was stirred at 60 °C. Additional paraformaldehyde in two portions (4.5 g, 3.2 g) was added after 1 d and 2 d, immediately after which TEA (1 mL) and H_2O (10 mL) were also added to keep the solution basic. After 4 d, the solution was concentrated. The residue was coevaporated three times with benzene and once with pyridine, and dried under vacuum overnight. The final residue was dissolved in pyridine (60 mL), to which acetic anhydride (21 mL) was added. The solution was stirred at room-temperature overnight. After adding a small volume of methanol (5 mL), the solution was concentrated *in vacuo*. The residue was then partitioned between $CHCl_3$ and H_2O, and the organic layer was washed sequentially with saturated $NaHCO_3$, saturated NaCl, and H_2O. The dried residue was dissolved in DMF (10 mL), to which PhSH (2.6 mL, 25.3 mmol) and TEA (5 mL) were added, and the mixture was stirred overnight at 70 °C. The whole mixture was partitioned between EtOAc and H_2O, and the organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed *in vacuo*. The latter dried residue was dissolved in methanolic ammonia (50 mL) and kept at room-temperature overnight. The solvent was then removed and the residue was dissolved in CH_3OH, to which silica gel was added. The gel was dried and placed on top of a silica gel column, which was eluted with $CH_3OH/CHCl_3$ (1/5, v/v). Appropriate fractions were pooled and solvent evaporated to leave compound **2** as a white foam (4.08 g, 51%). ESI-MS: *m*/*z* 350.9 $[M + H]^+$, 373.1 $[M + Na]^+$; 349.1 $[M - H]^-$. ^1H NMR (DMSO-d_6): δ 11.47 (s, 1H, NH), 7.69 (s, 1H, H-6), 7.35 (m, 5H, Ar−H of PhS), 6.12 (t, $J_{H1'-H2'} = 6.2$ Hz, $J_{H1'-H2''} = 7.2$ Hz, 1H, H-1′), 5.23 (d, $J_{OH-H3'} = 4.6$ Hz, 1H, 3′-OH), 5.00 (t, $J_{OH-H5'} = J_{OH-H5''} = 5.1$ Hz, 1H, 5′-OH), 4.16 (m, 1H, H-3′), 3.82 (s, 2H, CH_2−SPh), 3.75 (m, 1H, H-4′), 3.51 (m, 2H, H-5′ and H-5″), 2.00 (m, 1H, H-2′), 1.82 (m, 1H, H-2″).

Preparation of 3. Compound **2** (2.26 g, 6.46 mmol) was dissolved in dry pyridine (15 mL) and the resulting solution was evaporated to dryness. The procedure was repeated twice. The resulting residue was again dissolved in dry pyridine (40 mL), to which 4,4′-dimethoxytrityl chloride (DMTrCl) (2.63 g, 7.76 mmol) and 4-(dimethylamino)pyridine (DMAP) (40 mg, 0.33 mmol) were quickly added, followed by slow addition of TEA (1.5 mL, 10.76 mmol). The reaction mixture was stirred at room temperature for 3 h. After the reaction was complete as monitored by TLC (95/5, $CHCl_3/CH_3OH$, v/v), the solution was cooled to 5 °C, and a small volume of methanol (5 mL) was added. After 10 min, the mixture was evaporated to dryness *in vacuo*. The resulting residue was purified by silica gel chromatography with a step gradient of methanol (0−5%) in $CHCl_3$/TEA (99/1, v/v). Appropriate fractions were pooled and concentrated to dryness to give compound **3** as a white foam (2.91 g, 69%). ESI−MS: *m*/*z* 652.7 $[M + H]^+$, 675.1 $[M + Na]^+$; *m*/*z* 651.2 $[M - H]^-$. ^1H NMR (DMSO-d_6): δ 11.53 (s, 1H, NH), 7.44−6.82 (m, 19H, H-6 and aromatic H), 6.10 (t, $J_{H1'-H2'} = 6.2$

(34) Suzuki, Y.; Matsuda, A.; Ueda, T. *Chem. Pharm. Bull.* **1987**, *35*, 1085−1092.

Hz, $J_{H1'-H2''}$ = 6.7 Hz, 1H, H-1′), 5.35 (d, $J_{OH-H3'}$ = 4.1 Hz, 1H, 3′-OH), 4.14 (m, 1H, H-3′), 3.86 (m, 1H, H-4′), 3.74 (s, 6H, O−CH_3), 3.63 (d, J_{Ha-Hb} = 13.3 Hz, 1H, CH_{2a}-SPh), 3.50 (d, J_{Hb-Ha} = 13.3 Hz, 1H, CH_{2b}-SPh), 3.14 (m, 2H, H-5′ and H-5″), 2.10 (m, 1H, H-2′), 1.96 (m, 1H, H-2″).

Preparation of 4. Compound **3** (1.26 g, 1.93 mmol) was dissolved in dry CH_2Cl_2 (15 mL) and the resulting solution was evaporated to dryness. The operation was repeated twice. The resulting residue was dissolved in dry CH_2Cl_2 (14 mL) and kept under an argon atmosphere. Dry diisopropylethylamine (DIEA) (0.84 mL, 4.83 mmol) was then added, followed by dropwise addition of 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.65 mL, 2.91 mmol). The reaction mixture was stirred for 15 min at room temperature, then another portion of DIEA (0.84 mL) was added, and the reaction mixture was stirred for another 15 min. TLC (toluene/EtOAc, 1/1, v/v) check found two diastereomers (Rf = 0.65 and 0.54). Workup was done by cooling the reaction mixture in an ice bath followed by addition of CH_3OH (3 mL). The solution was then quickly extracted with EtOAc (70 mL). The EtOAc layer was washed twice with saturated $NaHCO_3$ (30 mL) and once with saturated NaCl (30 mL). The organic layer was dried with anhydrous Na_2SO_4, and solvent was evaporated off to give **4** in white foam. ESI−MS: *m*/*z* 852.9 $[M + H]^+$, 875.3 $[M + Na]^+$.

Preparation of 5. Compound **5** was synthesized from compound **4** by nucleoside conversion using Reese Reagent [29,30,35]. 1,2,4-triazole (3.4 g, 49 mmol) was suspended in dry acetonitrile (70 mL) which was cooled in an ice bath, then $POCl_3$ (0.8 mL, 8.75 mmol) was slowly added with rapid stirring. TEA (9 mL, 64.5 mmol) was then added dropwise and the suspension was stirred for 30 min. Compound **4** was dissolved in dry acetonitrile (20 mL) and added over 20 min to the solution, and the solution was continuously stirred for another 90 min. The reaction was stopped by adding saturated $NaHCO_3$ solution (100 mL) and extracted with CH_2Cl_2 (200 mL). The organic layer was washed sequentially with saturated $NaHCO_3$ (50 mL) and brine (50 mL), dried with Na_2SO_4, and evaporated under reduced pressure to a small volume. The resulting residue was loaded onto a silica gel column and eluted with EtOAc:CH_2Cl_2:TEA (7:5:1, v/v), and appropriate fractions were pooled and solvent evaporated under reduced pressure to give a white foam (1.1 g, overall yield for the above two steps 62%). ESI−MS: *m*/*z* 904.1 $[M + H]^+$, *m*/*z* 926.3 $[M + Na]^+$. ^{1}H NMR (DMSO-d_6) (Figure S1): δ 9.36 (s, 1H, H-triazolyl), 8.41 (s, 1H, H-triazolyl), 8.14 (s, 1H, H-6), 7.40−6.83 (m, 18H, Ar−H of DMTr and PhS), 6.03 (t, $J_{H1'-H2'}$ = 5.6 Hz, $J_{H1'-H2''}$ = 6.2 Hz, 1H, H-1′), 4.44 (m, 1H, H-3′), 4.39 (d, J_{Ha-Hb} = 13.3 Hz, 1H, CH_{2a}-SPh), 4.16 (m, 1H, H-4′), 3.92 (d, J_{Hb-Ha} = 13.3 Hz, 1H, CH_{2b}-SPh), 3.73 (s, 6H, O−CH_3), 3.68−3.51 (m, 4H, CH_2−OP and CH-*i*Pr), 3.41−3.24 (m, 2H, H-5′), 2.67 (t, $J_{CH2-CH2CN}$ = 5.6 Hz, 2H, CH_2CN), 2.45−2.18 (m, 2H, H-2′ and H-2″), 1.22−1.09 (m, 12H, CH_3-*i*Pr). ^{31}P NMR (DMSO-d_6): δ 151.61, 152.15.

Synthesis of d(mC^{SPh}G) (7, Scheme 1). Dinucleoside monophosphates were synthesized using the procedures described by Cadet and co-workers.[5] N^2-isobutyryl-2′-deoxyguanosine was synthesized following a transient protection procedure developed by Ti and co-workers.[36] Compound **5** (505 mg, 0.61 mmol) was dissolved in dry CH_3CN (27 mL), then N^2-isobutyryl-2′-deoxyguanosine (308 mg, 0.91 mmol) in 6 mL DMF was added followed by 0.45 M tetrazole in CH_3CN (6.7 mL). The resulting solution was stirred at room temperature for 25 min, and the volume of the reaction mixture was reduced to 15 mL. A 0.1-M iodine solution in THF/H_2O/pyridine (78/20/2, v/v) was added until the dark brown color did not dissipate, and the reaction mixture was stirred for another 50 min. The reaction was then quenched by addition of 1 M $Na_2S_2O_3$ (5 mL) and $CHCl_3$ (50 mL). The organic layer was washed with water, dried with anhydrous Na_2SO_4, and the solvent was evaporated off under reduced pressure to give a yellow oil (compound **6**). ESI−MS: *m*/*z* 1178.1 $[M + Na]^+$.

Detritylation was done by dissolving **6** in 1% TFA solution in CH_2Cl_2. The solution was stirred for 30 min, and the solvent was removed by evaporation under reduced pressure. The residue was purified by silica gel chromatography using a step gradient of CH_3OH (0−10%) in CH_2Cl_2. Appropriate fractions were combined and solvent was evaporated off under vacuum to give a glass (detritylation product). ESI−MS: *m*/*z* 854.0 $[M + H]^+$, 876.1 $[M + Na]^+$.

After detritylation, the compound was further deprotected under a mild condition using aqueous ammonium hydroxide (29%) for 60 h at room-temperature instead of using standard base deprotection conditions (29% ammonium hydroxide, 55 °C, 12 h). Under the mild condition, no degradation of the phenylthio moiety was observed. The final product was obtained by lyophilization. Overall yield (steps e−f in Scheme 1) of compound **7** (153 mg, 37%). HRMS (ESI−FTICR) calcd 679.1700 $[M + H]^+$, found 679.1683. ^{1}H NMR (D_2O) (Figure S2): δ 8.06 (s, 1H), 7.30 (m, 5H), 7.00 (s, 1H), 6.29 (t, J = 6.7 Hz, 1H), 6.00 (t, J = 6.6, 7.2 Hz, 1H), 4.83 (m, 1H), 4.48 (m, 1H), 4.22 (m, 1H), 4.09 (m, 2H), 3.98 (m, 1H), 3.83 (s, 2H), 3.46 (d, J = 4.1 Hz, 2H), 2.54 (m, 2H), 2.25 (m, 2H).

Preparation of 8 (Scheme 2). Compound **3** (1.58 g, 2.42 mmol) was dried twice by coevaporation with dry pyridine and dissolved in dry pyridine (40 mL). After stirring at room temperature for 10 min, acetic anhydride (0.5 mL, 5.29 mmol) was added. Reaction was kept at room temperature and stirred for overnight. The workup was done by evaporating the solvent off under reduced pressure, and the resulting residue was coevaporated with toluene (20 mL) for three times. After drying under vacuum, the product was obtained as a white/light yellow foam almost quantitatively. ESI−MS: *m*/*z* 717.1 $[M + Na]^+$; 693.1 $[M - H]^-$.

Preparation of 9. Compound **9** was synthesized from compound **8** by a nucleoside conversion procedure using Reese Reagent.[30] 1,2,4-triazole (3.2 g, 46 mmol) was suspended in dry acetonitrile (75 mL) in an ice bath, to which $POCl_3$ (0.9 mL) was slowly added with rapid stirring. Then TEA (7.5 mL) was added slowly and the suspension was stirred for another 30 min. Compound **8** (1.67 g, 2.41 mmol) in dry acetonitrile (10 mL) was added slowly and the solution was stirred for 80 min. The reaction was terminated by pouring the mixture into cold saturated $NaHCO_3$ solution (80 mL), and the resulting solution was extracted with CH_2Cl_2 (250 mL). The organic layer was washed with brine (100 mL) and dried with anhydrous Na_2SO_4. The solvent was evaporated off under reduced pressure to afford **9** in a white foam (1.62 g, 90%). ESI−MS: *m*/*z* 768.1 $[M + Na]^+$, 784.13 $[M + K]^+$. ^{1}H NMR (DMSO-d_6): δ 9.40 (s, 1H, H-triazolyl), 8.43 (s, 1H, H-triazolyl), 8.12 (s, 1H, H-6), 7.40−6.85 (m, 18H, Ar−H of DMTr and PhS), 6.06 (t, $J_{H1'-H2'}$ = 6.2 Hz, $J_{H1'-H2''}$ = 6.7 Hz, 1H, H-1′); 5.21 (m, 1H, H-3′), 4.43 (d, J_{Ha-Hb} = 13.3 Hz, 1H, CH_{2a}-SPh), 4.20 (m, 1H, H-4′), 4.05 (d, J_{Hb-Ha} = 13.3 Hz, 1H, CH_{2b}-SPh), 3.71 (s, 6H, O−CH_3), 3.24 (m, 2H, H-5′ and H-5″), 2.28 (m, 2H, H-2′ and H-2″), 2.05 (s, 3H, C(O)CH_3).

Preparation of 10. Compound **9** (1.52 g, 2.04 mmol) was detritylated with 80% acetic acid for 3 h and neutralized with saturated $NaHCO_3$ solution. The solution was then extracted with $CHCl_3$, and the organic layer was dried with anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure, and the residue was purified on silica gel column using 5% CH_3OH in EtOAc. Appropriate fractions were combined and solvent evaporated under reduced pressure. Compound **10** was obtained as a white foam (0.82 g, 91%). ESI−MS: *m*/*z* 443.9 $[M + H]^+$, 466.1 $[M + Na]^+$, 482.1 $[M + K]^+$; 442.1 $[M - H]^-$. ^{1}H NMR (DMSO-d_6) (Figure S3): δ 9.39 (s, 1H, H-triazolyl), 8.45 (s, 1H, H-triazolyl), 8.43 (s, 1H, H-6), 7.29 (m, 5H, Ar−H of PhS), 6.08 (t, $J_{H1'-H2'}$ = 7.7 Hz, $J_{H1'-H2''}$ = 6.2 Hz, 1H, H-1′), 5.27 (t, $J_{OH-H5'}$ = 5.1 Hz, $J_{OH-H5''}$ = 4.6 Hz, 1H, 5′-OH), 5.20 (m, 1H, H-3′), 4.54 (d, J_{Ha-Hb} = 13.8 Hz, 1H, CH_{2a}-SPh), 4.46 (d, J_{Hb-Ha} = 13.8 Hz,

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1H, CH_{2b}-SPh), 4.15 (m, 1H, H-4′), 3.63 (m, 2H, H-5′ and H-5″), 2.46 (m, 1H, H-2′), 2.02 (m, 1H, H-2″), 2.09 (s, 3H, $C(O)CH_3$).

Preparation of d(GmC^{SPh}) (12, Scheme 2). Compound **11** was prepared using the same procedure as for the preparation of compound **6**, which was obtained as a foam. ESI-MS: *m*/*z* 1198.0 $[M + H]^+$, 1220.3 $[M + Na]^+$; 1195.9 $[M - H]^-$.

Compound **11** was detritylated with 80% acetic acid for 3 h at room temperature, then deprotected in a similar way to give **12** as white powder (95 mg, overall yield for steps d−e in Scheme 2: 53%). HRMS (ESI−FTICR) calcd 679.1700 $[M + H]^+$, found 679.1682. ^{1}H NMR (D_2O) (Figure S4): δ 7.97 (s, 1H), 7.36 (m, 5H), 7.19 (s, 1H), 6.19 (t, $J = 6.2$ Hz, 1H), 6.10 (t, $J = 5.1$, 6.7 Hz, 1H), 4.89 (m, 1H), 4.27 (m, 1H), 4.17 (m, 2H), 4.02 (m, 2H), 3.78 (d, $J_{Ha-Hb} = 14.3$ Hz, 1H), 3.66 (d, $J_{Hb-Ha} = 14.3$ Hz, 1H), 2.74 (m, 2H), 2.25 (m, 1H), 1.75 (m, 1H).

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Supporting Information Available: ^{1}H NMR spectra of synthetic compounds, and ^{1}H NMR and mass spectrometric characterization of the 254-nm irradiation products of d(mC^{SPh}G) and d(GmC^{SPh}). This material is available free of charge via the Internet at http://pubs.acs.org.

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