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A Novel Vicinal Lesion Obtained from the Oxidative Photosensitization of TpdG: Characterization and Mechanistic Aspects

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A new type of vicinal base lesion was isolated from the photosensitization of TpdG in aerated aqueous solution. One- and two-dimensional NMR measurements were used together with mass spectrometry to accurately characterize the new adduct. Chemical detection of guanidine provided additional structural information on the base moiety at the 3′-OH terminal end. Altogether the experiments results were indicative of the occurrence of a covalent bonding between the pyrimidine ring on the 5′-OH terminal end and the imidazole ring on the 3′-OH terminal end through a methylene bridge. Photosensitization studies of TpdG, thymidine, and 2′-deoxyguanosine in the presence of either benzophenone, menadione, or riboflavin associated with isotopic labeling experiments using enriched oxygen and water provided relevant information on the mechanism of formation of the adduct. The results of these experiments clearly demonstrated that the initial event leading to the formation of the lesion is the abstraction of a hydrogen atom from the methyl group of the thymine base moiety of TpdG. This is followed by the addition of the methyl-centered radical to the C-4 atom of the guanine ring which gives rise to the vicinal lesion after reaction with molecular oxygen and subsequent rearrangement.

Introduction

It is now very well documented that exposure of cells to electromagnetic radiation has deleterious effects *(1)*. DNA is a major cellular target for the biological effects of UV light. The formation of a wide array of UV photoproducts is known to interfere with cellular processes such as DNA replication *(2, 3)* and transcription *(4)*. The type of photochemical modifications depends on the wavelength of the incident radiation. At wavelengths shorter than 330 nm, DNA directly absorbs energy *(5)* and the bulk of the generated photoproducts involves the pyrimidine bases *(6−8)*. At wavelengths in the UVA range (330−400 nm), the primary photochemical target is the guanine base *(9)*. The latter reactions occur through the intermediary of either endogenous or exogenous photosensitizers which, in mostly a triplet state, generate oxidation products according to two main competitive mechanisms. Type I mechanism involves either initial electron or hydrogen abstraction by the excited photosensitizer from the substrate. Type II mechanism is accounted for by the generation of singlet oxygen ($^1O_2$) as the result of energy transfer from the excited photosensitizer to oxygen. Subsequently $^1O_2$ reacts with the substrate leading to the formation of oxidation products. These two different mechanisms give rise, at least partly, to different sets of photoproducts.

Many efforts have been devoted during the past decade to elucidate the mechanism of photosensitization of nucleosides *(10−12)* and short oligonucleotides *(13−15)* used as DNA models. Emphasis has been placed on the isolation and characterization of the final photooxidation products. Recently, efforts have been devoted to the characterization of a radiation-induced tandem lesion involving the formamido remnant as a modification of thymine on one hand and the 8-oxo-7,8-dihydroguanine as a modification of the guanine base on the other hand *(16)*. This particuliar lesion was found to be generated by X-radiolysis of aerated aqueous solution of di- and 4-mers *(16, 17)*. Covalent bond formation between two vicinal bases was also shown to occur upon X-irradiation of oligomers in oxygen-free aqueous solutions *(18, 19)*. Vicinal lesions are interesting structural modifications because they are expected to represent a real challenge for repair enzymes. Moreover, elucidation of the mechanism involved in the chemical formation of vicinal lesions could be a first step in the understanding of the generation of bulky DNA base oxidation products *(20)*.

We report here the description of a new adduct derived from TpdG upon 350 nm sensitization in neutral aerated aqueous solution. The adduct is produced in sufficient amounts to allow its accurate characterization and a mechanistic study. The use of ^1H and ^{13}C NMR spectroscopies provided evidence for a covalent linkage between the methyl of thymine and the C-4 position of the 2′-deoxyguanosine through a methylene bridge (Fig-

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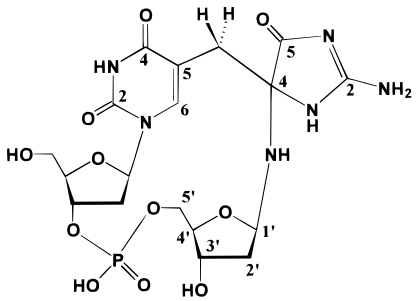

Figure 1. Structure of the adduct ⟨TpdG⟩. The numbers used for each position are indicated.

ure 1). Relevant mechanistic information on the initial steps of the formation of the adduct was gained from photosensitization studies. In particular, the latter investigations are strongly indicative of the occurrence of a radical centered on the methyl group of the thymidine moiety in the initial step of the formation of the adduct.

Materials and Methods

Chemicals. 2′-Deoxyguanosine and thymidine were purchased from Pharma-Waldhof (Düsseldorf, Germany). Benzophenone (diphenyl ketone) was obtained from Merck (Darmstadt, Germany). Menadione (2-methyl-1,4-naphthoquinone) was from Sigma (St Louis, MO), and riboflavin (7,8-dimethyl-10-(D-*ribo*-2,3,4,5-tetrahydroxypentyl)isoalloxazine), from BDH chemicals (Poole, U.K.). Water was purified on a Milli-Q system (Millipore, Milford, MA). D_2O, $H_2{}^{18}O$ (50.1%), and $^{18}O_2$ (97.54%) were obtained from Euriso-Top (Saclay, France). Thymidylyl-(3′→5′)-2′-deoxyguanosine (TpdG) was synthesized using a phosphotriester method *(21, 22)*. Phosphorylation of thymidine was carried out according to Chattopadhyaya and Reese *(23)* whereas the condensation of isobutyrylated 2′-deoxyguanosine was achieved using the method developed by Miyoshi and Takura *(24)*.

HPLC Purification and Analysis. The HPLC apparatus consisted of a L-7100 pump (Merck, Darmstadt, Germany) equipped with a Rheodyne injector (Cotati, CA). The detection for the preparative separations was provided by a LKB Bromma UV detector (Knauer, Berlin, Germany) connected to a Linear 1200 plotter (Barnstead-Thermolyne, Dubuque, IA). A Waters 990 photodiode array detector (Millipore, Milford, MA) associated to a Waters 990/600E system controller was used for the detection of the analytical separations. The fluorescent detection was provided by a fluorescence spectrophotometer F1000 (Hitachi, Tokyo, Japan). Four different chromatographic systems were used as follows.

System 1: Home-packed semipreparative column (300 × 7.5 mm); stationary phase, octadecylsilyl silica gel 10 μm, Nucleosil 100-10 C18 (Macherey-Nagel, Düren, Germany); mobile phase, gradient of 25 mM ammonium formate aqueous solution (A) and methanol (B), 100% solution A for 5 min followed by a linear gradient to 80/20 (A/B, v/v) over 50 min; flow rate, 3 mL/min; detection wavelength, 260 nm.

System 2: Interchrom HC18-25F column (250 × 4.6 mm) (Interchim, Montluçon, France); stationary phase, octadecylsilyl silica gel 5 μm, Hypersil 5μ C18; mobile phase, isocratic eluent of 25 mM ammonium formate aqueous solution; flow rate, 1 mL/min; detection wavelength, 260 nm.

System 3: Interchrom HC18-25F column (250 × 4.6 mm) (Interchim, Montluçon, France); stationary phase, octadecylsilyl silica gel 5 μm, Hypersil 5μ C18; mobile phase, gradient of 25 mM ammonium formate aqueous solution (A) and methanol (B), 100% solution of A for 5 min followed by a linear gradient to 80/20 (A/B, v/v) over 50 min; flow rate, 1 mL/min; detection wavelength, 260 nm.

System 4: Interchrom HC18-25F column (250 × 4.6 mm) (Interchim, Montluçon, France); stationary phase, octadecylsilyl silica gel 5 μm, Hypersil 5μ C18; mobile phase, linear gradient of 25 mM ammonium formate aqueous solution (A) and methanol (B), from 5% of B to 10% over 10 min; flow rate, 1 mL/min; fluorescence detection, excitation wavelength, 355 nm; emission wavelength, 405 nm.

UV Spectroscopy. The UV absorption spectra were obtained in pure water on a HP8452 spectrophotometer (Hewlett-Packard, Palo Alto, CA) controlled by a HP89531A data processing system (Hewlett-Packard).

Mass Spectrometry. Electrospray ionization (ESI+)[1] mass spectra were obtained in the positive mode on a VG Platform (Fisons, Manchester, U.K.). The capillary, HV lens, cone, and skimmer offset tensions were set at 3.81 kV, 0.64 kV, 48, and 6 V, respectively. Samples to be analyzed were dissolved in 50/50 (v/v) acetonitrile/water that contained 0.2% of formic acid *(25)*. MS/MS spectrum was obtained on a Quattro II spectrometer (Micromass, Altrincham, U.K.) equipped with an electrospray ionization source. The sample was dissolved in a mixture of 70/30 (v/v) water/acetonitrile that contained ammonium formate at a concentration of 7 mM (pH 6.2) prior to be analyzed in the negative mode.

NMR Spectroscopy. Samples were freeze-dried twice in D_2O (99.8% D) prior to dissolution in 99.96% D D_2O. ^{1}H NMR spectra were recorded in the Fourier transform mode at 500.62 and 600.13 MHz using a Unity 500 Varian (Palo Alto, CA) and a AMX 600 Bruker (Wissembourg, France) spectrometer, respectively. ^{13}C NMR spectra (DEPT 135°) were recorded in the Fourier transform mode on AM 300 and AM 400 Bruker instruments. Totally correlated scalar homonuclear ^{1}H−^{1}H experiments (TOCSY) were carried out on a Unity 500 Varian. The mixing delays used to correlate the protons were 20, 50, 80, 100, and 150 ms. Homonuclear ^{1}H−^{1}H (COSY), one-bond (HMQC), and long-range (HMBC) ^{1}H−^{13}C scalar correlated 2D NMR experiments were carried out on the Unity 500 Varian spectrometer. Two different HMBC experiments were performed with delays of 100 and 200 ms, respectively. Nuclear Overhauser effects were observed through a two-dimensional NOE experiment. The mixing delay was 450 ms. The chemical shifts and the coupling constants were checked by simulating the spectra using the PERCH program (University of Kuopio, Kuopio, Finland). The chemical shifts of the proton spectra are expressed in ppm. The HOD peak was used as a secondary reference, set at 4.9 ppm with respect to sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ (TSP). The chemical shifts of the carbon spectra were determined with respect to tetramethylsilane (TMS) used as the external reference. 1D- and 2D-spectra were recorded at 300 K. Theoretical determination of ^{13}C NMR chemical shifts was carried out using the CNMR-DB software (Advanced Chemistry Developments, Toronto, Canada).

Isolation and Characterization of ⟨TpdG⟩. A 1 mM TpdG solution (60 mL) that contained a photosensitizer (benzophenone, menadione, or riboflavin) was exposed at room temperature to the near-UV light emitted by the 350 nm lamps (16 × 15 W) of a Rayonet RPR-100 photochemical reactor (Southern New England Ultraviolet Co., Hamden, MA). The TpdG solution was saturated with either benzophenone or riboflavin. Menadione was used at a concentration of 0.1 mM. The

[1] Abbreviations: TpdG, thymidylyl-(3′→5′)-2′-deoxyguanosine; ⟨TpdG⟩, 3′-thymidylic acid, α-[2-amino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-4,5-dihydro-5-oxo-1*H*-imidazol-4-yl]-, intramolecular 3′→5′ ester; TSP, sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$; TMS, tetramethylsilane; ESI+/MS, positive electrospray ionization coupled to mass spectrometry with quadripolar detection; COSY, correlated spectrosocopy (homonuclear ^{1}H−^{1}H correlations); NOESY, nuclear Overhauser enhancement spectrosocopy (two-dimensional NOE experiment); DEPT, distortionless enhancement by polarization transfer (^{13}C NMR with selective responses of methyl, methylene, and methine carbons); HMQC, heteronuclear multiquanta correlation (one-bond ^{13}C−^{1}H scalar correlated 2D NMR experiment); HMBC, heteronuclear multibond correlation (long-range ^{13}C−^{1}H scalar correlated 2D NMR experiment).

Table 1. Proton Chemical Shifts (δ in ppm Referenced to TSP) of TpdG and ⟨TpdG⟩ in D_2O (300 K)

	TpdG		⟨TpdG⟩	
	Tp	pdG	⟨Tp	pdG⟩
1′	6.19	6.32	6.48	5.97
2′	1.85	2.94	2.86	2.51
2″	2.39	2.63	2.83	2.44
3′	4.75	4.87	4.90	4.71
4′	4.18	4.30	4.12	4.23
5′	3.78	4.18	4.00	4.14
5″	3.78	4.18	3.88	4.12
6/8	7.54	8.16	7.40	
Me/5-CH_2	1.97		3.57, 3.07	

Table 2. Proton Coupling Constants (J in Hz) of TpdG and ⟨TpdG⟩

	TpdG		⟨TpdG⟩	
	Tp	pdG	⟨Tp	pdG⟩
1′2′	8.9	6.9	3.8	8.7
1′2″	5.6	6.6	6.7	5.5
2′2″	−14.0	−13.9	−14.5	−13.3
2′3′	5.9	6.6	8.3	5.9
2″3′	2.1	4.2	3.8	2.7
3′4′	2.1	3.7	3.8	2.8
4′5′	4.2	2.8	2.4	5.4
4′5″	4.2	2.8	3.7	2.5
5′5″	−12.8	−11.8	−12.7	−11.6
5-CH_2			−14.6	

Table 3. Carbon Chemical Shifts (δ in ppm Referenced to TMS) of ⟨TpdG⟩ in D_2O (300 K)

	⟨TpdG⟩	
	⟨Tp	pdG⟩
1′	82.9	83.6
2′	36.3	38.9
3′	71.1	70.8
4′	83.8	85.2
5′	59.1	64.2
2	151.9	n.d.
4	164.1	93.2
5	106.7	181.0
6	140.7	
5-CH_2	32.6	

irradiations were carried out under continuous air bubbling in order to maintain the solution saturated with oxygen. A 30% degradation of TpdG was reached after 5 h of irradiation with benzophenone, 10 min with riboflavin, or 30 min with menadione. To isolate stable degradation products, the irradiation solutions were maintained 6 days at 40 °C prior to being freeze-dried. The irradiated solutions of TpdG were analyzed with the HPLC system 3. The dry residues were solubilized in 2 mL of water prior to a first round of HPLC isolation of ⟨TpdG⟩ (system 1). Under these chromatographic conditions, the capacity factor k' ($k' = (t - t_0)/t_0$) of ⟨TpdG⟩ is 2.2. Then, the ⟨TpdG⟩ purity was improved by a second HPLC purification using system 2. The adduct ⟨TpdG⟩ was characterized by UV, MS, and NMR analyses. UV: $\lambda_{max} = 210$ nm (major), $\lambda_{max} = 250$ nm (minor), $\lambda_{min} = 240$ nm, $\lambda_{min} = 266$ nm (shoulder). MS {ESI+, *m/z* (relative intensity, [ionic species])}: 533 (100, [M + H]), 555 (49, [M + Na]), 577 (19, [M + 2Na − H]). MS/MS {ESI−, *m/z* (relative intensity, [ionic species])}: 531 (100, [M − H]), 220 (20, [M − sugar moieties − H_2O]). NMR: data are listed in Tables 1−3.

Similar analyses were made for TpdG. k': 15.0 (system 1). UV: $\lambda_{max} = 252$ nm, $\lambda_{min} = 240$ nm. MS {ESI+, *m/z* (relative intensity, [ionic species])}: 572 (100, [M + H]), 594 (23, [M + Na]).

Laser Irradiation of TpdG. The 266 nm ps laser irradiation of an aqueous solution of TpdG was performed on an apparatus whose features are given elsewhere *(26)*. The absorbance of the solution of TpdG was 0.5 OD (2 mL). Several 40 μL aliquots of the solution were submitted to one laser pulse irradiation of 20 mJ. All irradiated aliquots were pooled together prior HPLC analysis on system 2.

Photosensitization of Thymidine and 2′-Deoxyguanosine. UVA photosensitizations of thymidine and 2′-deoxyguanosine by riboflavin, benzophenone, and menadione were performed as described for TpdG. Menadione-mediated photosensitization of 2′-deoxyguanosine was carried out during 5 h. To reach 30% degradation, thymidine was photosensitized for periods ranging from 20 min for riboflavin up to 6 h for benzophenone with 4 h for menadione. Photosensitized samples were freeze-dried overnight prior to HPLC separation. Analyses of irradiated solutions of thymidine and 2′-deoxyguanosine were achieved using HPLC system 3.

Chemical Detection of Guanidine. The method designed for the measurement of guanidine *(27)* is an adaptation of the protocol initially described for a postcolumn derivatization assay *(28)*. Typically, 50 μL of the sample was placed in a polypropylene vial. Sodium hydroxide (1 M, 50 μL) was then added. After homogenization, the solution was maintained for 30 min at 70 °C in a water bath and subsequently cooled to room temperature. A solution of 1,2-naphthoquinone-4-sulfonic acid (10 μL; 8 mg/mL) was added and the resulting solution incubated for 15 min in a 37 °C water bath. After being cooled to room temperature, the sample was neutralized by addition of 50 μL of 1 M HCl. The formation of the resulting aromatic fluorescent derivative ($k' = 1.7$) was monitored using HPLC system 4.

Stable Isotopic Labeling of ⟨TpdG⟩. Photosensitization of TpdG by benzophenone was performed in either $H_2{}^{18}O$ or in the presence of $^{18}O_2$. The irradiation of TpdG in $H_2{}^{18}O$ (50.1%) was carried out in 1 mL of water under conditions previously described (vide supra). The photosensitization of TpdG under $^{18}O_2$ (97.54%) bubbling was performed as follows. A solution of 1 mM TpdG (10 mL) that contained benzophenone was placed in a airtight vial. The air was first removed from the solution by bubbling argon (Air Produts, Paris, France) in the vial. The deaerated solution was then saturated with a stream of $^{18}O_2$ which was maintained in the vial at a low flow rate during 30 min. In both cases, ⟨TpdG⟩ was isolated using HPLC systems 1 and 2. The mass measurements were performed by ESI+/MS. MS {ESI+, *m/z* (relative intensity, [ionic species])}: $H_2{}^{18}O$ experiment, 533 (100, [M + H]), 555 (17, [M + Na]); $^{18}O_2$ experiment, 535 (100, [M + H]), 557 (23, [M + Na]). The isotopic enrichment of ⟨TpdG⟩ with $^{18}O_2$ was 100%.

Acidic Hydrolysis of ⟨TpdG⟩. The hydrolysis of ⟨TpdG⟩ was performed with hydrogen fluoride stabilized in pyridine *(29, 30)*. Typically, the sample to be hydrolyzed was evaporated to dryness in a polypropylene vial. Then, 150 μL of hydrogen fluoride stabilized in pyridine was added to the vial. The sample was maintained at 37 °C during 90 min. The neutralization of the sample was carried out under vigorous stirring in a glass tube that contained 300 mg of calcium carbonate suspended in 1 mL of water. When the pH reached the value of 7, the suspension was centrifuged and the liquid phase was collected. The solid fraction was washed by 500 μL of water with subsequent stirring and centrifugation. The new liquid phase was collected and pooled with the first one. The resulting solution (1.5 mL) was freeze-dried overnight. Finally, the dry sample was dissolved in water prior to HPLC purification using system 2. The main product (95% of the UV-absorbing products) ($k' = 1.4$) was collected and analyzed by ESI+/MS and NMR. ESI+ {*m/z* (relative intensity, [ionic species])}: 239 (100, [M + H]); 221 (58, [M + H + H_2O]); 210 (40, [M + H − CO]). ^{1}H NMR [δ (ppm)]: 7.63 (s, 1H, H-6); 3.24 (d, 1H, methylenic-H, $J = -14.7$ Hz); 3.10 (d, 1H, methylenic-H, $J = -14.7$ Hz).

Results

Isolation and Characterization of the Photoproduct. UVA photosensitization of TpdG in aerated aqueous solution gave rise to a complex mixture of photoproducts

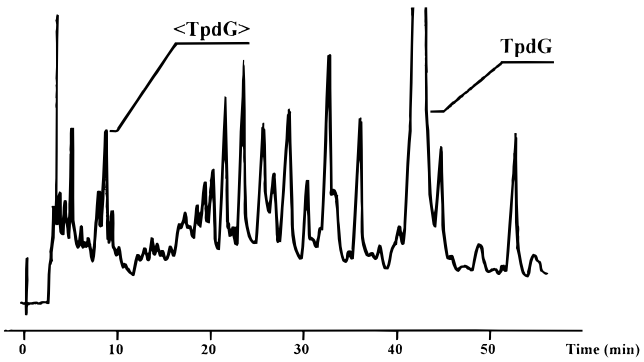

Figure 2. HPLC profile of a 1 mM photosensitized solution of TpG on a octadecylsilyl silica gel column (UV detection at 260 nm).

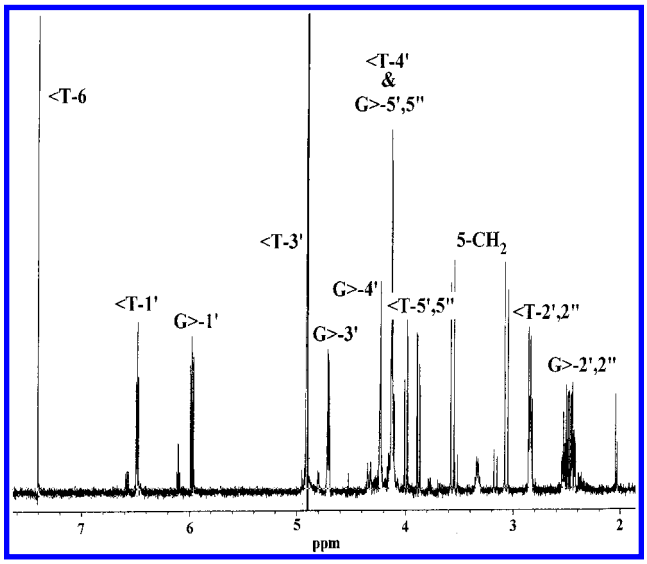

Figure 3. ^{1}H NMR 500 MHz spectrum of ⟨TpdG⟩ (D_2O, 300 K). The chemical shifts are relative to TSP.

which was resolved by HPLC analysis (Figure 2). Most of the oxidative decomposition products exhibited an undamaged thymine residue as referred from ^{1}H NMR measurement (data not shown). However, the ^{1}H NMR spectrum of the photoproduct corresponding to the peak eluting with a capacity factor of 2.2 (retention time of 8.8 min in Figure 2) exhibits neither the thymine characteristic signals of H-6 and $-CH_3$ nor the H-8 signal of the guanine base moiety (Figure 3). The latter photoproduct (⟨TpdG⟩) was isolated on a semipreparative reversed phase column. Its purity was further increased (>95%) after a second round of separation on an analytical reversed-phase column. It was found that ⟨TpdG⟩ is stable in aqueous solution (pH 7) at 40 °C over a period of at least 6 days.

The unambiguous assignment of the two sets of 2-deoxyribose protons was accomplished on the basis of TOCSY measurements. Selective irradiation of each of the two anomeric protons (δ = 6.48 and 5.98 ppm) allowed the assignment of the set of protons belonging to the related 2-deoxyribose moiety. The attribution was further confirmed by a COSY experiment. The assignment of the respective resonances of the H-2′ and H-2″ protons in each 2-deoxyribose was inferred from 2D-NOE analysis. The correlation map exhibits a cross-peak between the H-3′ signal at δ = 4.71 ppm and the signal at δ = 2.51 ppm, one of the two C-2 multiplet protons of the 3′-OH terminal end. A correlation is also observed between the H-1′ signal at δ = 5.97 ppm and the resonance signal at δ = 2.44 ppm. These observations strongly suggest that the H-2′ and H-2″ are resonating at δ = 2.51 and δ = 2.44 ppm, respectivley. For the 5′-end sugar, a similar line of reasoning was applied considering the dipolar interactions involving the pyrimidine H-6 on one hand and the 2′-methylenic signals at δ = 2.86 and 2.83 ppm on the other hand. It may be conclued that the H-2′ is resonating at δ = 2.86 ppm whereas the signal at δ = 2.83 ppm is assigned as H-2″.

The specific assignment of the two 2-deoxyribose moieties at either the 3′- or 5′-end was inferred from the values of the chemical shifts of the H-3′ and H-5′,H-5″ signals. The resonance of the H-3′ on the 5′-end together with those of H-5′,H-5″ on the 3′-end are downfield shifted as the result of the electronic-withdrawing properties of the phosphate group. Altogether, these results provided also evidence that none of the two 2-deoxyribose moieties was altered. Further support for the lack of modifications within the 2-deoxyribose moieties was provided by the similarity in the ^{13}C chemical shifts of the DEPT spectrum of the related carbons with those of TpdG.

Inspection of the chemical shifts of both protons and carbons of the two bases strongly suggests that the dinucleoside monophosphate photoproduct is modified on both the guanine (loss of the H-8 signal at δ = 8.04 ppm) and the thymine moieties (loss of the methyl signal at δ = 1.84 ppm). The long-range ^{1}H−^{13}C correlated (HMBC) spectrum showed a cross-peak between the anomeric proton of the 5′-end sugar (δ = 6.48 ppm) and the thymine C-6 at δ = 140.7 ppm. Moreover, the one-bond ^{1}H−^{13}C correlated (HMQC) spectrum exhibits a cross-peak between the C-6 at δ = 140.7 ppm and the proton resonance at δ = 7.40 ppm. These results confirm that the signal at δ = 7.40 ppm is accounted for by the H-6 proton of the pyrimidine base and not by a downfield shifted H-8 of the guanine moiety. The assignment of the other quaternary carbons of the former thymine ring moiety was inferred from the HMBC analysis. Several correlations observed in the latter 2D measurements provided evidence for a lack of modification of the pyrimidine ring. Indeed, the C-2, C-4, and C-5 pyrimidine signals resonating at δ = 151.9, 164.1, and 106.7 ppm, respectively are similar to those of thymine, 5-(hydroperoxymethyl)uracil, and 5-(hydroxymethyl)uracil *(31, 32)*. Furthermore, the UV spectrum of the adduct exhibits an absorption band centered at 250 nm which is strongly indicative of the aromatic feature of the pyrimidine base.

The ^{1}H NMR spectrum of the adduct exhibits two doublets at δ = 3.07 and 3.57 ppm, respectively. The strong value of the coupling constant between the two protons (J = −14.6 Hz) is strongly indicative of the occurrence of a *geminal* coupling. This received confirmation from the observation of a cross-peak between the two latter signals (δ = 3.07 and 3.57 ppm) in the COSY spectrum. Additional relevant structural information was provided by the DEPT spectrum which exhibits five $-CH_2-$ type carbons instead of four in the TpdG spectrum. Four of them were assigned to the C-2′ and C-5′ of the two 2-deoxyribose moieties. The fifth $-CH_2-$ signal resonance at δ = 32.6 ppm exhibits, in the HMQC map, two correlation peaks with the proton signals at δ = 3.07 and 3.57 ppm, respectively. These results clearly

demonstrate that the pyrimidine base of ⟨TpdG⟩ is substituted by a methylene group.

The HMBC spectrum exhibits low-intensity correlation peaks between each of the methylenic protons at $\delta = 3.07$ and 3.57 ppm and both C-4 at $\delta = 164.1$ ppm and C-6 at $\delta = 140.7$ ppm. This can be rationalized in terms of the occurrence of a ^3J coupling between the methylene protons on one hand and the C-4 and C-6 carbons of the pyrimidine ring on the other hand. The heteronuclear long-range 2D map also exhibits a correlation between the protons at $\delta = 3.07$ and 3.57 ppm on one hand and the C-5 at $\delta = 106.7$ ppm on the other hand. This can be rationalized in terms of a ^2J scalar coupling between the latter protons and the carbon C-5 of the former thymine base moiety. This is confirmed by the high intensity of the cross-peaks between the methylenic protons at $\delta = 3.07$ and 3.57 ppm and the C-5 signal which suggests a ^2J coupling. Moreover, the HMBC map exhibits two cross-peaks between the protons at $\delta = 3.07$ and 3.57 ppm on one hand and a quaternary carbon at $\delta = 93.2$ ppm on the other hand. The high intensity of the latter correlation peaks strongly suggests a ^2J coupling between the methylenic protons and the carbon at $\delta = 93.2$ ppm. Furthermore, the observed correlation peak in the HMBC map between the proton at $\delta = 3.57$ ppm and a quaternary carbon at $\delta = 181.0$ ppm is indicative of the occurrence of a $^3J(^{13}C,^1H)$ coupling. Altogether, this demonstrates that the methylene group is covalently linked to a carbon atom of the remnant of the guanine base moiety. In addition, the low magnitude of the trans coupling constant $J_{1'2'}$ on the 5′-end sugar strongly suggests that ⟨TpdG⟩ has a rigid constrained structure *(33−37)*.

The lack of a correlation peak between the methylenic proton at $\delta = 3.07$ ppm and the carbon at $\delta = 181.0$ ppm can be rationalized in terms of the presence of an atom in the α-position of the methylene group. Indeed, two dihedral angles can be defined between each of the methylenic protons and the carbon at $\delta = 181.0$ ppm. The value of one of them is close to 180°, corresponding to the ^3J coupling between the methylenic proton at $\delta = 3.57$ ppm and the carbon at $\delta = 181.0$ ppm. The value of the second dihedral angle is close to 90°; it corresponds to a low ^3J coupling between the proton at $\delta = 3.07$ ppm and the carbon at $\delta = 181.0$ ppm *(38−43)*. This additional atom involved in the dihedral angle was assigned to the carbon resonating at $\delta = 93.2$ ppm. The chemical shift of the latter carbon can be rationalized in terms of a sp^3 carbon in α position to the carbon at $\delta = 32.6$ ppm This was confirmed by the observed downfield shift of the carbon of the methylene group resonance in the DEPT spectrum ($\delta = 32.6$ ppm) compared to the chemical shift value of the methyl of the thymine base moiety ($\Delta\delta = +20.9$ ppm). Moreover, the evidence for a rigid constrained structure of the adduct (vide supra) and the presence of unmodified sugars indicate that the N-glycosidic bonds of ⟨TpdG⟩ are not cleaved.

Since NMR studies provided strong support for the integrity of both the sugar moieties and the pyrimidine base, 420 amu of the molecular mass of ⟨TpdG⟩ could easily be explained ($C_{15}H_{21}N_2O_{10}P$). Moreover, evidence has been provided for the presence of two quaternary carbons (^{13}C NMR: $\delta = 181.0$ and 93.2 ppm) and the implication of N-9 in the N-glycosidic bond on the 3′-end. These elements indicate that 38 amu (24 from the carbon and 14 from the nitrogen) can be added to the previous calculated molecular weight, leading to the following formula: $C_{17}H_{21}N_3O_{10}P$ (MW = 458). In addition, the chemical detection of guanidine allows to add 56 extra amu to the partial mass, suggesting the following partial formula: $C_{18}H_{22}N_6O_{11}P$ (MW = 514). Altogether, 18 additional amu are necessary to reach the molecular mass of *m/z* 532. This can be accounted for by the presence of one oxygen and two hydrogens. The presence of an oxygen atom is strongly suggested by the chemical shift of the carbon resonating at $\delta = 181.0$ ppm, corresponding to a carbonyl. In addition, the experiment with isotopically enriched oxygen ($^{18}O_2$) unambiguously demonstrated the incorporation of an oxygen atom in ⟨TpdG⟩. Altogether, these results provide an elemental composition of $C_3N_4H_4O$ for the modified guanine ring (Figure 1).

The occurrence of a *pseudo*-lactam derivative is confirmed by the loss of a CO fragment in the ESI+ spectrum *(44−46)* of the base moiety of the adduct obtained by acidic hydrolysis of ⟨TpdG⟩. The structure of ⟨TpdG⟩ received further confirmation from the computer prediction of the carbon chemical shifts. A good agreement was observed for the carbons at $\delta = 32.6$, 93.2, and 181.0 ppm. Interestingly, the calculated chemical shifts for the three latter carbons are respectively 31 ± 8, 88 ± 3, and 172 ± 6 ppm. In addition, the high stability (6 days in water at 40 °C or in hydrogen fluoride stabilized in pyridine) of the compound strongly suggests that the guanidine group is stabilized in a cyclic structure. Indeed, exocyclic guanidine is unstable in water *(37)*. It can be added that the compound released by mild acidic hydrolysis of ⟨TpdG⟩ exhibits ^1H NMR and ESI+/MS features in agreement with the structure proposed for the aglycone moiety of ⟨TpdG⟩.

It should be emphasized that the proposed structure exhibits an asymmetric carbon. A second diastereoisomer is thus expected to be found. As a matter of fact, benzophenone-mediated photosensitization (but not menadione) of TpdG provided a minor product coeluting with ⟨TpdG⟩ which exhibits similar ^1H NMR features. Two anomeric protons at $\delta = 6.58$ and 6.10 ppm, respectively, were observed. Moreover, one of them ($\delta = 6.58$ ppm) exhibited a low coupling constant ($J = 5.4$ Hz) which could correspond to the trans coupling between the H-1′ and H-2′ protons. This low value strongly suggests a constrained rigid structure of the molecule. The resonance signal of a H-3′ proton with unusual couplings is also observed at $\delta = 4.80$ ppm. In addition, the spectrum also exhibits two methylenic protons resonating at $\delta = 3.53$ and 3.17 ppm with a strong *geminal* coupling constant ($J = -14.8$ Hz) and a singlet at $\delta = 7.73$ ppm. This minor adduct can be assumed to be the other diastereoisomer of ⟨TpdG⟩.

Conformational Analyses. The 5′-CH_2OH terminal exocyclic group is submitted to a rapid interconversion between three staggered rotamers: *gauche−gauche (gg)*, *trans−gauche (tg)*, and *gauche−trans (gt) (47)*. An empirical expression allows the estimation of the ratio of the *gg* population: $gg\ (\%) = 100 \times [(13.7 - J_{4'5'} + J_{4'5''})/9.7]$ *(48)*. The relative importance of the *gg* conformer for ⟨TpdG⟩ was estimated to be 78%. Such a high value is not compatible with a typical syn orientation of the pyrimidine ring which is expected to lead to a destabilization of the *gg* rotamer. It should be added that the occurrence of a methylene bridge between the pyrimidine and imidazole rings of ⟨TpdG⟩ would rule out the pos-

Table 4. Effect of Photoexcited Menadione (MQ), Benzophenone (Bz), and Riboflavin (Rb) on TpdG, Thymidine, and 2′-Deoxyguanosine: Degradation of Each Base Involved (Top) and Formation of Mechanistically Relevant Degradation Products (Bottom)

	MQ	Bz	Rb
thymidine	yes	yes	no
2′-deoxyguanosine	no	yes	yes
dT → HMdU	yes	yes	no
dG → dZ	no	yes	yes
TpdG → ⟨TpdG⟩	yes	yes	no

sibility to have an anti orientation for the pyrimidine ring. Then, the oxygen of the C-2 carbonyl group of the pyrimidine ring is likely to lie between C-2′ and C-3′. This may be inferred from the significant downfield shift effect on the H-2′ ($\Delta\delta = +1.01$ ppm) and the H-2″ ($\Delta\delta = +0.44$ ppm) with respect to TpdG. This is likely to be due to the deshielding cone of the C-2 carbonyl of the pyrimidine ring. This effect can also be observed on the H-3′ proton whose chemical shift is downfield shifted ($\Delta\delta = +0.15$ ppm) with respect to the corresponding signal of TpdG. The high values of the cis coupling constant $J_{1'2''}$ and $J_{2'3'}$ as well as the low value of the trans coupling constant $J_{1'2'}$ are indicative of a lack of significant packering of the 5′-end sugar. On the other hand, the high value of the trans coupling constant $J_{1'2'}$ as well as the low values of the $J_{2''3'}$ and $J_{3'4'}$ in the 3′-end sugar strongly suggest a C-2′*endo*−C-3′*exo* conformation of the sugar *(49, 50)*.

Photosensitization and Irradiation of Thymidine, 2′-deoxyguanosine, and TpdG. Photooxidative sensitizations of TpdG were carried out with several photodynamic agents including benzophenone, menadione, and riboflavin in order to gain insights into the mechansim of formation of ⟨TpdG⟩. The vicinal lesion was formed in significant amounts only when benzophenone and menadione were used as the photosensitizers. In contrast, photoexcited riboflavin was not able to induce any detectable amount of ⟨TpdG⟩ (Table 4). The benzophenone- or menadione-mediated photosensitization formation of ⟨TpdG⟩ was about $^1/_8$ of the yield of thymidylyl-(3′→5′)-2,2-diamino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-5(2*H*)-oxazolone, the major degradation product from the photosensitization of TpdG. The study of the formation of ⟨TpdG⟩ as a function of the UVA dose showed a linear increase ($r^2 = 0.991$, 11 measurements) for a degradation of TpdG ranging between 0 and 40%. In addition, degradation products from the photosensitization of thymidine by benzophenone and menadione were analyzed. Photosensitization of thymidine by benzophenone gave rise to several nonpolar compounds on the HPLC profile. Two of them were identified as 5-(hydroxymethyl)-2′-deoxyuridine ($k' = 5.5$) *(51)* and 5-formyl-2′-deoxyuridine ($k' = 6.4$) *(10)*. In contrast, the photosensitization of 2′-deoxyguanosine by menadione did not lead to any degradation. The laser irradiation of TpdG provided evidence for the formation of the adduct ⟨TpdG⟩ with a significant yield. It could be added that the formation yield of ⟨TpdG⟩ was approximately $^1/_6$ of thymidylyl-(3′→5′)-2,2-diamino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-5(2*H*)-oxazolone which was the major degradation product of the laser irradiation of TpdG.

Discussion

Photosensitization of TpdG to UVA by either menadione or benzophenone led to the formation of an adduct involving the two base moieties. The structure of the compound was determined by extensive UV, NMR, and MS analyses.

The comparative study of the photosensitization of 2′-deoxyguanosine, thymidine, and TpdG by various sensitizers provided relevant mechanistic information. While, it is well documented that 2′-deoxyguanosine is degradated by photoexcited benzophenone and riboflavin *(12, 14, 52)*, menadione-mediated photosensitization does not induce any detectable degradation of 2′-deoxyguanosine (Table 4). These results strongly suggest that the guanine moiety of TpdG is not the initial target involved in the formation of ⟨TpdG⟩, since the latter photoproduct is generated upon menadione photosensitization.

In contrast, riboflavin-mediated photosensitization induces neither thymidine degradation nor ⟨TpdG⟩ formation. This is in good agreement with previous works which demonstrate that photoexcited riboflavin induces mainly degradation of guanine residues in DNA *(53, 54)*. In addition, photosensitization of thymidine to UVA by benzophenone gives rise to the same oxidation products than with photoexcited menadione *(10, 55)*.

Nevertheless, the comparison of the amount of 5-(hydroxymethyl)-2′-deoxyuridine and 5-formyl-2′-deoxyuridine, on one hand, and the amount of the four diastereoisomers of 5,6-dihydroxy-5,6-dihydrothymidine, on the other hand, produced upon photosensitization of thymidine by either benzophenone or menadione showed the ability for benzophenone to generate the neutral radical on the methyl group of thymidine. Indeed, the ratio between the combined yield of 5-(hydroxymethyl)-2′-deoxyuridine and 5-formyl-2′-dexoyuridine together and the yield of 5,6-dihydroxy-5,6-dihydrothymidine was higher with benzophenone than menadione.

The formation of the methyl-centered radical can thus be proposed to be the initial step of the formation of ⟨TpdG⟩. This could account for the results of the γ radiolysis of aerated aqueous solution of TpdG (data not shown) which clearly showed that ⟨TpdG⟩ was not generated, at least, in a detectable amount. This may be accounted for by the fact that the formation of the 5-methylene radical by the hydroxyl radicals arising from the water radiolysis occurred in a very low yield.

The formation of a similar yield of ⟨TpdG⟩ by either benzophenone or menadione is related to their specificity in their photosensitizing features. There is a competition between the thymine and guanine bases of TpdG for the photoexcited reaction of benzophenone. With menadione, the photosensitizer mostly reacts on the thymine moiety of TpdG. Then, the high similarity in the amount of generated ⟨TpdG⟩ could be summed up as the following: the high competition for the reaction of benzophenone on each base of TpdG is largely compensated by the high selectivity of the mechanism involved in the sensitization of the thymine base moiety. The high selectivity of the action of menadione on the thymine base moiety of TpdG is largely compensated by the competition between the two mechanistic paths from the radical cation of thymine.

The high formation yield of the adduct suggests that the vicinal lesion is a primary product i.e. formed from a single initial radical event. This is confirmed by the study of the formation of ⟨TpdG⟩ as a function of the UVA

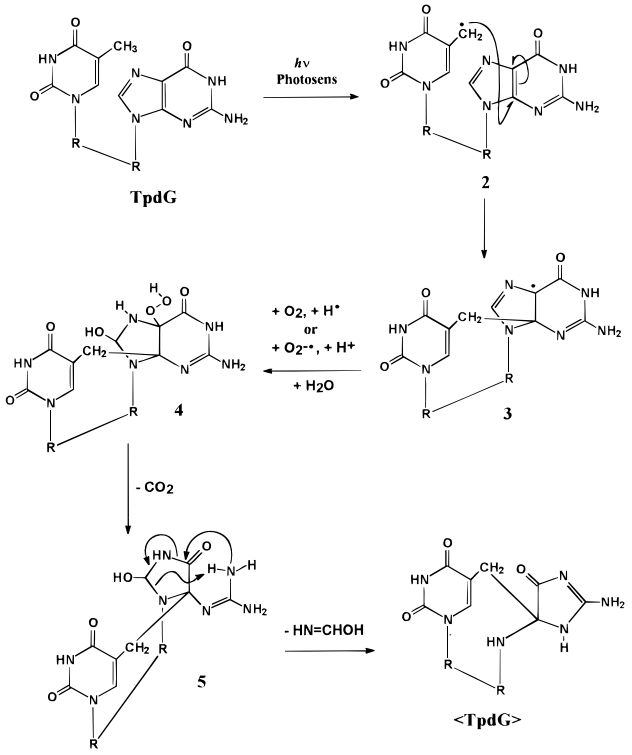

Figure 4. Suggested mechanism for the photosensitized formation of the ⟨TpdG⟩ adduct.

dose which presents a linear increase in the amount of ⟨TpdG⟩ and the laser irradiation of TpdG which gives rise to ⟨TpdG⟩.

From all these considerations, the following mechanism for the formation of ⟨TpdG⟩ may be proposed (Figure 4). The photosensitization of TpdG in the presence of either menadione or benzophenone leads to the formation of the neutral radical centered on the methyl position of thymine moiety **2**. Biphotonic ionization of the thymine moiety, followed by fast deprotonation of the excited pyrimidine radical cation, is also likely to generate the radical **2** upon exposition of TpdG to ps-UV-laser irradiation *(56)*. In a subsequent step, the methyl radical of thymine would add to the C-4 position of the guanine moiety leading to the formation of the radical intermediate **3**. Then, molecular oxygen is likely to add to the C-5 position of the guanine base. Then, hydration of the N-7−C-8 unsaturated bond can occur, giving rise to the transient species **4** as proposed for the formation of imidazolone *(14)*. Conversion of the hydroperoxide **4** into its corresponding intermediate **5** would lead to the opening of the C-5−C-6 bond of the pyrimidine ring of the guanine moiety through the loss of CO_2. In a subsequent step, the nucleophilic attack of an amino group of the guanidine residue on the carbonyl would lead to the formation of the photoproduct ⟨TpdG⟩. This mechanism is in agreement with the isotopic labeling experiments. Interestingly, the $^{18}O_2$ labeling demonstrated the incorporation of one oxygen atom (from **3** to **4**) in ⟨TpdG⟩. In contrast, no oxygen was incorporated from water (no mass enrichment of ⟨TpdG⟩ was observed in the case of the photosensitization of TpdG in $H_2{}^{18}O$).

The possibility that the formation of ⟨TpdG⟩ would be induced by an intramolecular rearrangement of the methyl radical of thymine on the C-8 position of guanine base moiety could be ruled out. Indeed, structural considerations indicate that if the latter reaction occurred, the guanidine residue would be linked to N-9 through the C-4 carbon of guanine. Therefore, in such a structure, the former high value of the chemical shift of the carbon at $\delta = 181.0$ ppm shows that the C-4 of the guanine base would be implicated in a carbonyl group. In this case, the high intensity of the cross-peak observed in the HMBC map between the methylenic proton at $\delta = 3.57$ ppm on one hand and the carbon at $\delta = 181.0$ ppm on the other hand would be explained in terms of a very unlikely ^5J coupling involving an heteroatom.

The mechanism of formation of ⟨TpdG⟩ involves the addition of the methyl-centered radical of thymine to the C-4−C-5 double bond. The reactivity of the neutral radical centered on the methyl group of thymidine with aromatic compounds is a likely process, even in the presence of oxygen. Similar type of intramolecular reaction has been already observed, like the formation of 5′,6 cyclic adduct of 2′-deoxyuridine *(57)*. The formation of ⟨TpdG⟩ shows that the methyl-centered radical of thymine can add to the C-4 position of guanine. This is an additional possibility to the addition to the C-8 position reported by Box and co-workers *(18, 19)*. In that respect, methyl-centered radical of thymine reacts in a way similar to HO• which can also add to either the C-4 or C-8 position of guanine.

The isolation of ⟨TpdG⟩ is an additional example of vicinal oxidative lesion *(16, 58)*. Viewing the highly modified structure of the latter adduct, work is needed to establish its biological properties in terms of repair and mutagenicity.

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