Reaction of Singlet Oxygen with 2′-Deoxyguanosine and DNA. Isolation and Characterization of the Main Oxidation Products

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The reaction of singlet molecular oxygen with 2′-deoxyguanosine and DNA was studied. Emphasis was placed on the identification and characterization of the main methylene blue mediated type II (singlet oxygen) oxidation products of 2′-deoxyguanosine and its corresponding 3′,5′-di-*O*-acetylated derivative. Two major oxidation products of 2′-deoxyguanosine were isolated and characterized by mass spectrometry analysis and extensive ^1H and ^{13}C NMR measurements as the two 4*R** and 4*S** diastereomers of 4,8-dihydro-4-hydroxy-8-oxo-2′-deoxyguanosine. The addition of $^1O_2$ was also found to occur to the base moiety of the corresponding 3′,5′-di-*O*-acetylated derivative. Methylene blue mediated photosensitization of 2′-deoxyguanosine led also to the production of 7,8-dihydro-8-oxo-2′-deoxyguanosine, but in a relatively lower yield with respect to the two above diastereomers. The participation of singlet oxygen in the mechanism of formation of these oxidation products was confirmed. A reasonable mechanism involving the transient formation of an unstable endoperoxide produced through a Diels-Alder 1,4-cycloaddition of singlet oxygen to the purine ring is suggested. Quantitative analysis allowed us to demonstrate that the two diastereomers of 4,8-dihydro-4-hydroxy-8-oxo-2′-deoxyguanosine are the main singlet oxygen oxidation products of the guanine moiety within nucleosides, whereas 7,8-dihydro-8-oxoguanine was found to be the major $^1O_2$ oxidation product of guanine in double-stranded DNA.

Introduction

The genotoxicity and the mutagenicity of $^1O_2$, as the result of DNA oxidation, have been determined (for recent reviews see refs 1–3). A major source of $^1O_2$ is photosensitization reactions (*4–6*). Generation of $^1O_2$ through energy transfer from an excited triplet-state sensitizer to ground-state molecular oxygen is defined as a type II photosensitization mechanism (*7, 8*). Tyrrell *et al.* have suggested the involvement of singlet oxygen in the induction of the human heme oxygenase gene and in the inactivation of cultured human fibroblasts by UVA irradiation (*9*). Bezman *et al.* (*10*) have also shown the participation of $^1O_2$ in the inactivation of *Escherichia coli* by rose bengal photosensitization. Singlet oxygen was also found to be able to induce the loss of biological activity of bacteria (*10*) and plasmids (*11*). It is important to note that $^1O_2$ may also be produced by other chemical and biochemical systems. Among others, we may cite the decomposition of dioxetanes (*12*), the dismutation of superoxide radical (*13*), and also enzymatic processes involving oxidases (*14, 15*). Because of its relatively long lifetime, $^1O_2$ may diffuse toward potential targets and reacts like a strong electrophile with biomolecules that contain regions of high electron density. Therefore, cellular targets may include DNA (*2*), ribosomes (*16*), mitochondria (*17*), and cellular membranes (*18*). Among the nucleosides, $^1O_2$ has been shown to react preferentially with 2′-deoxyguanosine (dGuo)[1] (*19–21*), leading to complex mixtures of oxidation products which were only partly characterized (*22, 23*). Exposure of DNA to $^1O_2$ led to base damage and strand breaks, both of which specifically occur at guanine residues (for reviews see refs 24–26). 7,8-Dihydro-8-oxo-2′-deoxyguanosine (8-oxodGuo) is the only $^1O_2$ mediated guanine modification that has been detected in DNA (*27, 28*). Several assays, including HPLC–electrochemical detection (HPLC-EC) (*29*), ^{32}P-postlabeling (*30*), and also gas chromatography–mass spectrometry (GC/MS) (*31, 32*), are now available to monitor the formation of 8-oxodGuo, either as the nucleoside or as the free base, in both isolated and cellular DNA. In addition, a monoclonal antibody column is now available for the prepurification by affinity chromatography of 8-oxodGuo in urine (*33*).

In the present work we report the results of the study on the type II (singlet oxygen) photosensitization reaction of 2′-deoxyguanosine and DNA. The main objective of the work was to provide a detailed characterization of the singlet oxygen mediated oxidation products of dGuo (**1**) and its corresponding 3′,5′-di-*O*-acetylated derivative (**1a**), whose structures were described in preliminary communications (*34, 35*).

Experimental Procedures

Chemicals. Caution: *Because of its toxicity, hydrogen fluoride (Aldrich Chemie, Milwaukee, WI) should be handled with care, and reactions have to be carried out in nonglass flasks such as polypropylene tubes (Eppendorf R).* Methylene blue and dGuo were purchased from Sigma Chemical Co. (St. Louis, MO)

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[1] Abbreviations: 4-OH-8-oxodGuo, 4,8-dihydro-4-hydroxy-8-oxo-2′-deoxyguanosine; 4-OH-8-oxoGua, 4,8-dihydro-4-hydroxy-8-oxoguanine; 8-oxodGuo, 7,8-dihydro-8-oxo-2′-deoxyguanosine; 8-oxoGua, 7,8-dihydro-8-oxoguanine; dGuo, 2′-deoxyguanosine; di-O-Ac-dGuo, 3′,5′-di-*O*-acetyl-2′-deoxyguanosine; FAB/MS, fast atom bombardment mass spectrometry; HF/Pyr, hydrogen fluoride stabilized in pyridine; HPLC-EC, high performance liquid chromatography–electrochemical detection; TSP, 3-trimethylsilyl(2,2,3,3-$^2H_4$)propionate.

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and were used without further purification. 7,8-Dihydro-8-oxoguanine (8-oxoGua) was obtained from Chemical Dynamics (South Plainfield, NJ). HPLC grade acetonitrile and ammonium formate were obtained from Carlo Erba (Farmitalia Carlo Erba, Milan, Italy) and Kodak (Eastman Kodak Co., Rochester, NY) respectively. Disulfonated phthalocyanine complexed with zinc which was a gift of Dr. J. E. van Lier (University of Sherbrooke, Sherbrooke, Québec) was prepared as described by Langlois *et al.* (*36*). Irradiation solutions containing Fe^{2+} were prepared by appropriate dilution of $FeSO_4,(NH_4)_2SO_4,6H_2O$ purchased from Prolabo (Paris, France) to a 0.2 mM final concentration. A clean source of singlet oxygen was provided by the thermal decomposition of the endoperoxide of 3,3′-(1,4-naphthylidene)-dipropionate (*37*). $^{18}O_2$ was obtained from Eurisotop (Paris, France).

Irradiation System. The visible light was generated by a 100 W tungsten lamp, filtered with a 590 nm cutoff filter no. 23A (Eastman Kodak Co., Rochester, NY). During photolysis, the solution, placed at a distance of 20 cm from the irradiation source, was kept saturated with oxygen by continuous air bubbling and maintained at room temperature by circulating water. For isotopic labeling experiments, oxygen was removed from the irradiation solution under vacuum, and $^{18}O_2$ was introduced prior to irradiation in a close flask.

Spectrometer Analysis. UV absorption spectra were recorded in H_2O with a spectrophotometer DU 8B (Beckman, Fillerton, CA). Circular dischroism spectra (for H_2O solution) were obtained by using a Model III Roussel-Jouan dichrograph (Jobin-Yvon, Paris, France). 400.13 MHz ^1H NMR spectra were recorded on a Bruker AM-400 spectrometer at 300 K either in 400 μL of 99.8% ^2H_2O (Merck, Darmstad, Germany) or in 400 μL of $(C^2H_3)_2SO$ (Merck, Darmstad, Germany). 3-(Trimethylsilyl)(2,2,3,3-$^2H_4$)propionate (TSP) was used as the internal standard (TSP = 0.00) in ^2H_2O, whereas tetramethylsilane (TMS) was utilized for the calibration of the spectra in $(C^2H_3)_2SO$. Assignments were checked by homonuclear double- or triple-resonance experiments. H2′ and H2″ proton signals were further assigned on the basis of coupling constant arguments (*38*, *39*). In order to obtain accurate coupling constants, the spectra were simulated using the Bruker PANIC program. 50.13 MHz ^{13}C NMR spectra were obtained in $(C^2H_3)_2SO$ at 300 K with a Bruker AM-200 spectrometer. Long range heteronuclear scalar correlations were established by using the "INVDR2LP. AU" Bruker program on the AM-400 spectrometer. Fast atom bombardment mass spectra (FAB/MS) (thioglycerol matrix, 35-keV Cs atoms) were obtained in the positive ion mode on a VG ZAB 2-EQ instrument (Fisons-V. G., Manchester, U.K.).

HPLC Analysis. Several HPLC apparatus were used for both analytical and preparative separations. Large scale purification of synthesized di-O-Ac-dGuo (**1a**) was achieved by using a Model 500 Waters HPLC preparative pumping system (Millipore, Mildford, MA) equipped with a differential refractometer and a Prep/Pack 500 (500 × 50 mm i.d.) octadecylsilyl silica gel column (Millipore). The second system, used for semipreparative and analytical purifications of nucleoside photooxidation products, consisted of two Model 302 Gilson pumps (Middleton, WI) equipped with a Sil-9A Shimadzu automatic injector (Touzart & Matignon, Paris, France), a dynamic mixer Model 811 (Gilson), and a L-4000 spectrophotometer (Hitachi, Tokyo, Japan). The pumps were connected to an Apple II microcomputer that controled the eluent composition and the flow rate, usually set at 1 and 2 mL/min for analytical and semipreparative separations, respectively. HPLC elution profiles were recorded and the peaks of interest were integrated by using a Model 621 Data Master (Gilson) interfaced with the microcomputer through the HPLC system manager software Model 704 (Gilson). Quantitation was achieved by using an external calibration procedure. The HPLC-EC detection system consisted of a LKB pump Model 2150 (Pharmacia LKB Biotechnology, Uppsala, Sweden) equipped with a Waters automatic injector Wisp 710B (Millipore) and a Model 111B UV detector (Gilson). The electrochemical detection was accomplished by amperometry using a Model LC-4B/LC-17A(T) apparatus (Bioanalytical Systems, West Lafayette, IN) set at 0.5 nA. The amperometric detection of 8-oxoGua and its related nucleoside was achieved by using two glassy-carbon electrodes in parallel that were set at a potential of +650 mV (*40*).

Analytical reverse phase columns (250 × 4.6 mm i.d.) for electrochemical detection were home-packed with 10 μm Nucleosil octadecylsilyl silica gel (Macherey Nagel, Düren, Germany). The analytical (250 × 4.6 mm i.d.) and semipreparative (250 × 6.2 mm i.d.) amino substituted silica gel (mean particle 5 μm) Hypersil NH_2 columns were purchased from Interchim (Montluçon, France).

For dose curve response studies, aliquots (200 μL) were periodically removed from the irradiated solutions, in order to monitor the formation of photoproducts by HPLC. For this purpose, 20 μL were injected onto the reverse phase column and 8-oxodGuo (**4**) was quantified by using the HPLC-EC assay, as previously described by Berger *et al.* (*40*). The formation of 4*R** and 4*S** diastereomers **2** and **3** was monitored by introducing 50 μL of the photolyzed solution of **1** onto the analytical NH_2 column, using a mixture of acetonitrile and 25 mM ammonium formate (80:20) as the mobile phase at a flow rate of 1 mL/min (*41*). The 4*R** and 4*S** diastereomers **2** and **3** were detected at 230 nm and quantified by external calibration.

Chemical Synthesis. 8-OxodGuo (**4**) was prepared by hydrogenation of 8-(benzoyloxy)-2′-deoxyguanosine as previously described by Lin *et al.* (*42*). Di-O-Ac-dGuo (**1a**) was synthesized by acetylation of 2′-deoxyguanosine (**1**). Typically, 2.67 g (10 mM) of dGuo (**1**) was dissolved in 17.5 mL of tetraethylammonium hydroxide (Merck, Darmstad, Germany). After evaporation of the solution to dryness, the resulting salt was dried by evaporation of 3 × 30 mL of anhydrous pyridine (Sigma). Then, the dry nucleoside was dissolved in a mixture of 50 mL of anhydride acetic (Sigma) and 175 mL of anhydrous pyridine. The solution was stirred in the dark for 16 h. After completion of the reaction, the excess of anhydride acetic was destroyed by the addition of 50 mL of ethanol. Then, di-O-Ac-dGuo (**1a**) was purified, after lyophilization, by preparative liquid chromatography, using a mobile phase constituted of 40% of methanol in water, the flow rate being 100 mL/min. The collected fraction (capacity factor: $k' = 4.0$) gave 2.2 g of di-O-Ac-dGuo (**1a**) (yield 60%). ^1H NMR (200.13 MHz, ^2H_2O, TSP) δ 2.12 (s, 3H, CH_3-CO), 2.23 (s, 3H, CH_3CO), 3.06 (m, 1H, H-2′), 2.79 (m, 1H, H-2″), 4.42 (m, 2H, H-5′ H-5″), 4.55 (m, 1H, H-4′), 5.59 (m, 1H, H-3′), 6.40 (t, 1H, H-1′), 8.07 (s, 1H, H-8). ^{13}C NMR (50.3 MHz, $(C^2H_3)_2SO$, TMS) δ 20.5 (CH_3CO), 20.7 (CH_3CO), 35.6 (C-2′), 63.7 (C-5′), 74.6 (C-3′), 81.6 (C-4′), 82.8 (C-1′), 116.9 (C-5), 135.4 (C-8), 151.2 (C-4), 153.8 (C-2), 157.0 (C-6), 169.9 (CH_3CO), 170.1 (CH_3CO).

4,8-Dihydro-4-hydroxy-8-oxoguanine (4-OH-8-oxoGua) was quantitatively obtained by chemical hydrolysis of the corresponding nucleosides (4-OH-8-oxodGuo) using hydrogen fluoride stabilized in pyridine (HF/Pyr) as previously described by Polverelli *et al.* (*43*) with the following modifications. Typically, 1 mg of 4-OH-8-oxodGuo (**2** or **3**) was treated with 50 μL of HF/Pyr at 37 °C during 30 min. Then, the residual HF was neutralized by addition of 1 mL of an aqueous solution containing 80 mg of calcium carbonate (Sigma). A vigorous agitation was maintained until neutral pH was obtained. The resulting insoluble salts (CaF_2) and the excess of calcium carbonate salts were eliminated by filtration through a Millex-GS 0.22 μm filter (Millipore). Then, pyridine was removed by repeated lyophilization, and the final residue was analyzed by HPLC. 4-OH-8-oxoGua was separated by HPLC using an analytical NH_2 silica gel column and a mixture of acetonitrile and 25 mM ammonium formate (80:20) as the mobile phase, the flow rate being 1 mL/min. The collected fraction ($k' = 3.1$), as detected by UV absorption at 230 nm, was lyophilized twice in order to eliminate ammonium formate prior to ^{13}C NMR and mass spectrometry analysis. FAB/MS: m/z (relative intensity) (positive mode) 206 (16, $[M + Na]^+$), 184 (100, $[M + H]^+$). ^{13}C NMR (50.3 MHz, $(C^2H_3)_2SO$, TMS) δ 78.9 (C-4), 156.2 (C-8), 170.8, 172.1, 181.5.

Photosensitization of 2′-Deoxyguanosine. One hundred milliliters of an aqueous (either water Milli-Q, pH 6, or ^2H_2O

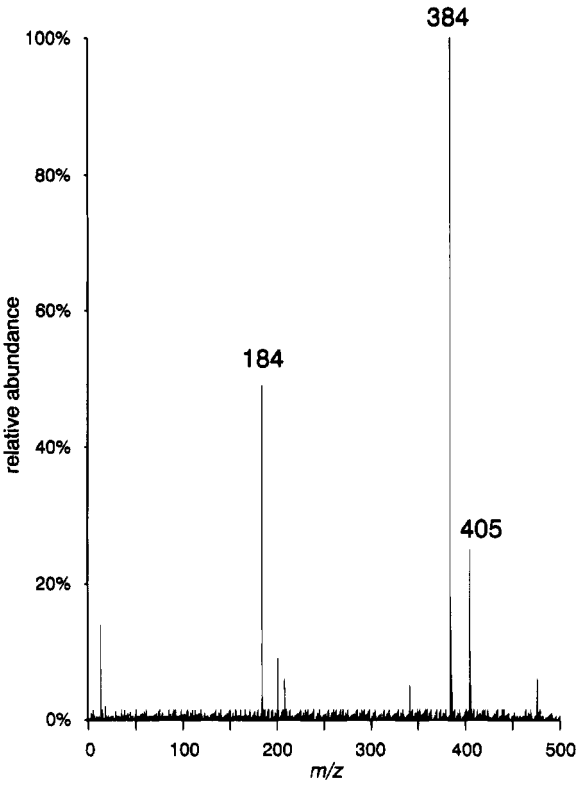

Figure 1. FAB/MS (positive ion mode) spectrum of photooxidation product **2a**.

purchased from Merck) 1 mM solution of either dGuo (**1**) or its corresponding di-*O*-acetylated derivative **1a**, containing 0.5 mM methylene blue, was irradiated for 1 h at room temperature in a large flask (15 cm diameter). Photosensitization with the zinc complexed phthalocyanine was performed using sufficient sensitizer to give an optical density absorbance of 2 at the maximum absorption of the dye (*44*). After irradiation, the solution was evaporated to dryness, and the resulting residue was dissolved in 5 mL of H_2O. Then, the solution was centrifuged at 12000*g* in order to eliminate the excess of the starting unmodified nucleoside. The supernatant was evaporated to dryness, and the residue was dissolved in a minimum of HPLC buffer prior to the injection onto the NH_2 semipreparative HPLC column.

Nucleoside Photooxidation Products. (4*R)-4,8-Dihydro-4-hydroxy-8-oxo-3′,5′-di-*O*-acetyl-2′-deoxyguanosine (2a).** The separation of di-O-Ac-dGuo photoproducts was achieved on a NH_2 silica gel column under isocratic conditions using a mixture of acetonitrile and 25 mM ammonium formate (85:15) as previously described by Ravanat *et al.* (*41*). Under these conditions, the unmodified nucleoside **1a** was found to be eluted more rapidly ($k' = 1.5$) than the main type I ($k' = 2.0$) and the two predominant type II ($k' = 3.1$, $k' = 3.3$) photooxidation products. Lyophilization of the combined fractions ($k' = 3.1$) gave 3.8 mg (yield 10%) of oxidation product **2a**. The FAB/MS spectrum (positive ion mode) is shown in Figure 1. FAB/MS: *m*/*z* (relative intensity) (positive mode) 405 (25, $[M + Na]^+$), 384 (100, $[M + Na]^+$), 184 (50, $[B + H]^+$). The 400.13 MHz ^1H NMR spectroscopic parameters, including chemical shifts and coupling constants of **2a** in ^2H_2O, are listed in Table 1. The chemical shifts of **2a** in $(C^2H_3)_2SO$ are listed in Table 2. The 50.3 MHz ^{13}C NMR chemical shifts of **2a** in $(C^2H_3)_2SO$ are listed in Table 3.

(4*S)-4,8-Dihydro-4-hydroxy-8-oxo-3′,5′-di-*O*-acetyl-2′-deoxyguanosine (3a).** Lyophilization of the combined fractions ($k' = 3.3$) gave 3.8 mg of **3a** (yield 10%). The FAB/MS spectrum of the compound was found to be similar to that of photoproduct **2a**. FAB/MS: *m*/*z* (relative intensity) (positive mode) 405 (25, $[M + Na]^+$), 384 (100, $[M + Na]^+$), 184 (50, $[B + H]^+$). The 400.13 MHz ^1H NMR spectroscopic parameters, including chemical shifts of **3a** in ^2H_2O and $(C^2H_3)_2SO$, are

Table 1. 400.13 MHz ^1H NMR Features of 2′-Deoxyguanosine (1) and the Main Singlet Oxygen Oxidation Product 2, 3, 2a, and 3a in ^2H_2O

protons	1	2	3	2a	3a
1′	6.30	5.87	5.90	5.90	5.90
2′	2.79	2.55	2.34	2.60	2.41
2″	2.52	2.30	2.21	2.40	2.32
3′	4.64	4.34	4.42	5.23	5.30
4′	4.14	3.94	3.98	4.28	4.29
5′	3.82	3.70	3.74	4.18	4.26
5″	3.77	3.68	3.69	4.15	4.28
8	7.99				
CH_3CO				2.18, 2.20	2.18, 2.19
$J_{1'-2'}$	7.4	7.6	8.7	8.8	9.2
$J_{1'-2''}$	6.4	6.7	6.3	5.9	5.8
$J_{2'-2''}$	−14.1	−13.9	−13.8	−14.2	−14.0
$J_{2'-3'}$	6.1	6.6	6.2	6.5	5.8
$J_{2''-3'}$	3.6	4.0	3.1	2.4	2.2
$J_{3'-4'}$	2.6	3.9	3.0	2.4	2.0
$J_{4'-5'}$	3.7	4.9	4.8	3.8	3.5
$J_{4'-5''}$	4.7	6.4	6.0	6.2	6.1
$J_{5'-5''}$	−12.6	−12.0	−12.0	−11.7	−12.7

Table 2. Chemical Shifts of the Exchangeable Protons of 3′,5′-Di-*O*-acetyl-2′-deoxyguanosine (1a) and Photoproducts 2a and 3a in $(C^2H_3)_2SO$

protons	1a	2a	3a
1′	6.24	5.08	5.46
2′	2.56	2.75	2.30
2″	2.03	2.09	1.97
3′	5.40	5.12	5.05
4′	nd[a]	4.15	nd
5′	nd	nd	nd
5″	nd	nd	nd
8	8.03		
CH_3	2.14	2.03	2.03
CH_3	2.18	2.03	2.03
NH	10.87	11.57	11.51
C2-NH_2	6.65	8.59	8.50
C4-OH		8.26	8.20

[a] nd = not determined due to the overlapping between signals of protons 4′, 5′, and 5″.

Table 3. 50.3 MHz ^{13}C NMR Chemical Shifts of 2′-Deoxyguanosine (1) and of the Main Singlet Oxygen Oxidation Products 2, 3, 2a, 3a, and 4 in $(C^2H_3)_2SO$

carbons	1	2	3	2a	3a	4
1′	83.2	82.6	81.1	80.3	79.6	
2′	40.0	36.3	35.0	33.7	32.6	
3′	71.2	70.8	70.6	74.0	74.0	
4′	88.0	86.4	86.0	82.7	81.3	
5′	62.1	62.0	62.1	63.6	63.8	
4	151.4	80.2	79.2	80.5	79.5	78.9
8	136.1	156.0	155.7	155.3	156.0	156.2
	117.0 (**5**)	169.9	169.3	169.2	169.5	170.8
	157.5 (**6**)	172.2	171.7	172.3	171.7	172.1
	154.1 (**2**)	180.3	180.8	180.0	180.5	181.5
CH_3CO				20.6	20.6	
CH_3CO				170.2	170.2	

reported in Tables 1 and 2, respectively. The 50.3 MHz ^{13}C NMR chemical shifts of **3a** are listed in Table 3.

(4*R)-4,8-Dihydro-4-hydroxy-8-oxo-2′-deoxyguanosine (2).** The main photooxidation products of dGuo (**1**) were separated on a NH_2 semipreparative HPLC column under isocratic condition using a mixture of acetonitrile and 25 mM ammonium formate (80:20) at a flow rate of 2.0 mL/min. Under these conditions, it should be noted that methylene blue was found to be eluted in the void volume of the column (*41*). The oxidized nucleosides were eluted in the same order as the corresponding 3′,5′-di-*O*-acetylated derivatives. Lyophilization of the combined fractions ($k' = 4.0$) gave 3 mg of **2** (yield 10%). FAB/MS: *m*/*z* (relative intensity) (positive mode) 344 (10, $[M + 2Na]^+$), 322 (25, $[M + Na]^+$), 300 (12, $[M + H]^+$). The 400.13 MHz ^1H NMR spectroscopic parameters including chemical shifts and coupling

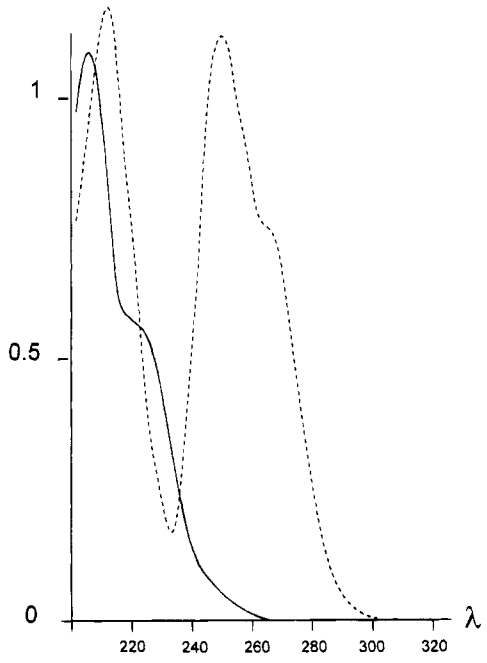

Figure 2. Ultraviolet absorption spectra of dGuo (**1**) (dashed line) and photooxidation product **2** (solid line).

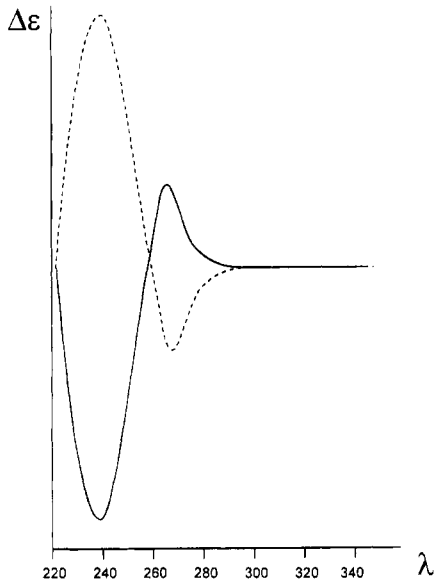

Figure 3. Circular dichroism spectra of photooxidation products **2** (solid line) and **3** (dashed line) in H_2O.

constants of 4-OH-8-oxodGuo (**2**) obtained in ^2H_2O are listed in Table 1. The 50.3 MHz ^{13}C NMR chemical shifts of **2** in $(C^2H_3)_2$-SO are reported in Table 3. The UV absorption spectrum of the 4*R** diastereomer of 4-OH-8-oxodGuo (**2**) is shown in Figure 2. The spectrum exhibits a shoulder at 230 nm; the molecular absorption coefficient at this wavelength was determined to be 5240 mol^{-1} cm^{-1} L^{-1} for both photoproducts **2** and **3**. The circular dichroism spectrum of **2** is shown in Figure 3.

(4S*)-4,8-Dihydro-4-hydroxy-8-oxo-2′-deoxyguanosine (3). Lyophilization of the combined fractions ($k' = 4.5$) gave 3 mg of homogeneous **3** (yield 10%). FAB/MS: *m*/*z* (relative intensity) (positive mode) 344 (10, $[M + 2Na]^+$), 322 (25, $[M + Na]^+$), 300 (12, $[M + H]^+$). The 400.13 MHz ^1H NMR spectroscopic parameters and the 50.3 MHz ^{13}C NMR chemical shifts are listed in Tables 1 and 3, respectively. The circular dichroism spectrum of **3** is shown in Figure 3.

7,8-Dihydro-8-oxo-2′-deoxyguanosine (4). 7,8-Dihydro-8-oxo-2′-deoxyguanosine (**4**) was purified on a semipreparative home-packed C_{18} reverse phase column using a mixture (93:7) of water and acetonitrile as the mobile phase. The flow rate was 2 mL/min, and the detection was achieved by UV monitoring at 290 nm. The product contained in the fraction ($k' = 3.2$) was purified twice on the HPLC system in order to remove the starting 2′-deoxyguanosine (**1**) that is eluted very closely ($k' = 3.0$) to **4**. The product was characterized as 7,8-dihydro-8-oxo-2′-deoxyguanosine (**4**) by comparison of its ^1H and ^{13}C NMR spectroscopic features with those of the authentic sample previously described by Lin *et al.* (*42*).

DNA Photosensitization. Calf thymus DNA (500 μg) (Boeringher, Mannheim, Germany), dialyzed against water during 30 min by using a VM 0.05 μm Milipore filter, was dissolved in 5 mL of Milli-Q water that contained 50 μg of methylene blue. After stirring in the dark for 1 h, the solution, saturated with oxygen by continuous air bubbling, was irradiated during 30 min in a 2.5 cm round-bottom glass flask. After irradiation, the DNA solution was reduced to about 200 μL in a Speed Vac concentrator (Savant Instrument, Farmingdale, NY). In order to eliminate the photosensitizer, the DNA was twice precipitated using 0.1 volume of 3 M (pH = 5.3) ammonium acetate and 2.5 volumes of ethanol. Then, the DNA pellet was dried by lyophilization prior to acidic hydrolysis.

HF/Pyr Hydrolysis of DNA. Photosensitized DNA (500 μg) was dissolved in 50 μL of HF/Pyr. In a subsequent step, the hydrolysis was performed during 30 min at 37 °C. Then, the residual HF was neutralized by addition of 1 mL of an aqueous solution containing 80 mg of calcium carbonate. A vigorous agitation was maintained until neutral pH was obtained. The resulting insoluble salts (CaF_2) and the excess of calcium carbonate salts were eliminated by filtration through a Millex-GS 0.22 μm filter. Then, pyridine was removed by repeated lyophilization, and the final residue was dissolved in 100 μL of water prior to HPLC analysis for the detection of the oxidation products of guanine. Ten microliters of the latter solution was used for the detection of 8-oxoGua by amperometry (*40*). Detection of 4-OH-8-oxoGua was performed in two steps. The residual 90 μL was injected onto a C_{18} reverse phase column which was subsequently eluted by using 10 mM ammonium formate as the mobile phase in order to eliminate the unmodified nucleobases. 4-OH-8-oxoGua which was rapidly eluted was collected in a large fraction, $k' < 1$, with a recovery of almost 100%. After repeated lyophilization, the content of the collected fraction was analyzed by HPLC under isocratic conditions on an analytical NH_2 column with a mobile phase constituted of a mixture of acetonitrile and 25 mM ammonium formate (80:20), the flow rate being 1 mL/min. Quantitation was achieved by UV measurement at 230 nm using the synthetic 4-OH-8-oxoGua ($k' = 3.1$) as the external standard.

Results

Isolation of the Singlet Oxygen Oxidation Photoproducts of 2′-Deoxyguanosine. The separation of the methylene blue mediated photooxidation products of dGuo (**1**) was achieved on a silica gel NH_2 column as previously described by Ravanat *et al.* (*41*). The methylene blue photosensitization reaction of **1** was found to give rise to the formation of four main stable photoproducts. Under the HPLC analytical conditions, the 4*R** and 4*S** diastereomers of 4-OH-8-oxodGuo (**2** and **3**) are well separated from the starting 2′-deoxyguanosine (**1**) and 2,2-diamino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-5-(2*H*)-oxazolone (**5**), one of the main stable type I photooxidation products (*41*). The other type I photoproduct, (2*S*)-2,5′-anhydro-1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-guanidinylidene-2-hydroxy-4-oxoimidazolidine (**6**) (*45*), coelutes with starting 2′-deoxyguanosine under these conditions. The formation of **5**, whose characterization is reported elsewhere (*46*), involves the transient formation of a purine radical cation (*46*, *47*). The purity of each of the two diastereomers of 4-OH-8-oxodGuo was greater than 95% as determined by ^1H

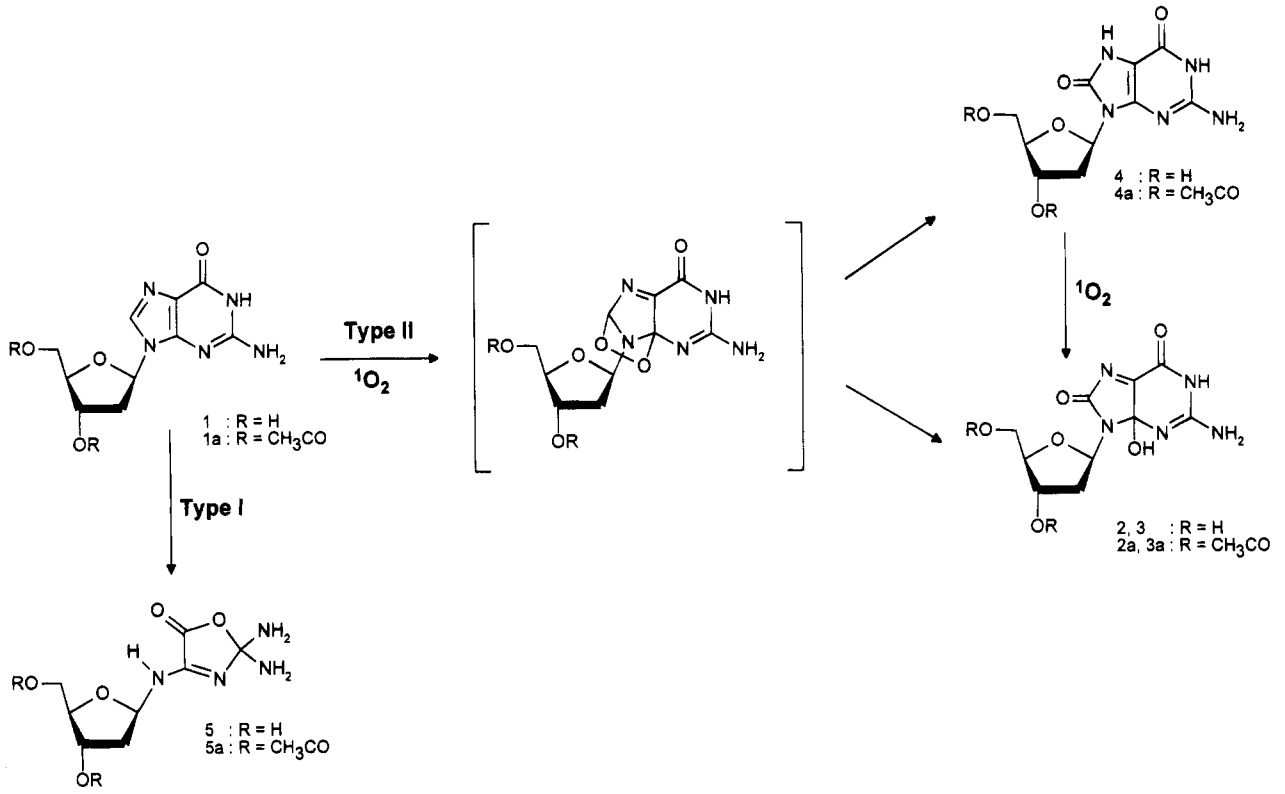

Figure 4. Mechanism of formation of the main type II oxidation products of dGuo (**1**) and 3′,5′-di-*O*-acetyl-2′-deoxyguanosine (**1a**) in neutral aqueous solution.

NMR spectroscopy. It is important to note that the separation of the photoproducts of 3′,5′-di-*O*-acetyl-2′-deoxyguanosine (**1a**) was achieved under similar chromatographic conditions by using a mixture of acetonitrile and ammonium formate (85:15). Again, three main products, **1a**, **2a**, and **3a**, were detected, corresponding to the 3′,5′-di-*O*-acetylated derivatives of the unprotected nucleosides **1**, **2**, and **3**, respectively. Methylene blue was found to have no interaction with the amino silica gel column and was eluted in the void volume of the HPLC column.

In addition, it should be noted that another singlet oxygen oxidation product of **1** was found to be generated in a relatively lower yield as compared to the two diastereomers **2** and **3**. This compound, which was characterized as 8-oxodGuo (**4**), is not separated from **1** under the analytical HPLC conditions used.

Structure Assignment. The two main methylene blue photooxidation products (**2** and **3**) arising from type II photosensitization reaction (*vide infra*) of dGuo (**1**) as well as the corresponding 3′,5′-di-*O*-acetylated derivatives **2a** and **3a** were unambiguously assigned on the basis of FAB/MS mass spectrometry analysis and extensive ^1H and ^{13}C NMR measurements (the structure of the 4*R** and 4*S** diastereomers of 3′,5′-di-*O*-acetyl-4,8-dihydro-4-hydroxy-8-oxo-2′-deoxyguanosine is illustrated in Figure 4).

Mass Spectrometry. The FAB/MS spectrum of photoproduct **2a** (Figure 1) shows a notable pseudomolecular ion at *m*/*z* 384 ($[M + H]^+$) together with a quasi-molecular ion at *m*/*z* 405 ($[M + Na]^+$). This is indicative of the gain in mass of 32 amu from the starting nucleoside **1a**, corresponding to the incorporation of two oxygen atoms. In addition, the presence of a fragment at *m*/*z* 184 ($[B + H]^+$) indicates that the incorporation had occurred within the base moiety. Additional structural information was inferred from the mass spectra of the nonacetylated nucleosides **2** and **3**. The mass spectrum of (**2**) (data not shown) shows a notable pseudomolecular ion at *m*/*z* 299 together with a quasi-molecular ion at *m*/*z* 322 ($[M + Na]^+$). The fragment at *m*/*z* 184 ($[B + H]^+$) is also detectable. These results are indicative of the molecular formula of $C_{10}H_{13}N_5O_6$ for the nucleosides **2** and **3** and $C_5H_4N_5O_3$ for the corresponding base moiety. Further confirmation was provided by the FAB/MS spectra features of the corresponding base (4-OH-8-oxoGua) obtained after acidic hydrolysis of **2** or **3** which exhibit a pseudomolecular ion and a quasi-molecular ion ($[M + Na]^+$) at *m*/*z* 184 and 206, respectively (*vide infra*).

^1H NMR Spectroscopy. Inspection of the ^1H NMR data (Table 1) shows that the sugar moiety of the photooxidation products **2** and **3** and their di-*O*-acetylated derivatives **2a** and **3a** is intact. Important structural information is provided by the lack of the H8 signal, in the products **2** and **3** as well as **2a** and **3a**. This indicates that one of the two incorporated oxygen atoms is likely to be attached to the C8 carbon of the guanine moiety of the oxidation products. It should be added that the ^1H NMR features are similar for compounds **2** and **3** (and also for **2a** and **3a**). The relative high magnitude value for the *trans* coupling constant $J_{1'-2'}$ and the low value of the two other *trans* coupling constants $J_{2''-3'}$ and $J_{3'-4'}$ suggest that the two nucleosides adopt preferentially a C2′ *endo* puckered conformation (*48*). In addition, the downfield shift of the NMR signal of the H2′ proton, together with the upfield shift of the C2′ signal, with respect to those of **1**, is indicative of a preferential *syn* conformation (*49*). In addition, a significant destabilization of the *gauche-gauche* rotameric population may be inferred from the high value of the sum of $J_{4'-5'}$ and $J_{4'-5''}$ coupling constants.

The ^1H NMR spectra of **2a** and **3a** in $(C^2H_3)_2SO$ exhibit three additional signals which may be easily chemically exchanged by addition of D_2O (Table 2). Two of them correspond to the resonance signal of the exocyclic amino group and a N(H) respectively. The third exchangeable signal in the NMR spectrum of **2a** and **3a** is likely to be assign to the proton of a hydroxyl group at position 4. The exchangeable protons were also observed in the two corresponding nonacetylated photoproducts **2** and **3**.

^{13}C NMR Spectroscopy. The assignment of the osidic carbons (Table 3) was unambiguously achieved on the basis of heteronuclear decoupling experiments. The chemical shifts of the five osidic carbons of nucleosides **2** and **3** and of the seven osidic carbons of the corresponding 3′,5′-di-*O*-acetylated derivatives **2a** and **3a** are reported in Table 3. It should be noted that no important shift was observed for the resonance signals by comparison with those of the osidic carbons of dGuo (**1**) with the exception of the C2′ signal. Comparison of the chemical shifts of the base moiety of **2** and **3** with those of **1** indicates a notable upfield shift for the resonance signal of C4 carbon (Table 3). Such a large upfield shift indicates a change in the carbon hybridization from a sp^2 to a sp^3 structure, due to the addition of an oxygen molecule to the base moiety.

Long range scalar correlations between anomeric proton and carbons of the base moiety were used in order to assign the C4 and C8 carbons. The related long range heteronuclear scalar correlations of photoproducts **2** and dGuo (**1**) are shown in Figure 5. A correlation between the anomeric proton and two carbons of the purine ring of **1** was observed (Figure 5, part A). These carbons were found to be the C8 and C4 carbons in agreement with available data from the literature (*50, 51*). This indicates that a three bond scalar correlation between H1′ and the C4 and C8 guanine carbons is present. Similar correlations were observed for photoproduct **2** (Figure 5, part B). Therefore, these observations allowed us to assign the C4 and C8 carbons of **2** (Table 3). A similar result was obtained for the other diastereomer **3**, providing confirmation that the hydroxyl group is attached to the C4 carbon in both photoproducts **2** and **3**.

UV Absorption Spectrum. The UV absorption spectra of **1** and **2** in H_2O are shown in Figure 2. Confirmation of the sp^2 to sp^3 change in the hybridation of the C4 carbon upon addition of $^1O_2$ is provided by the decrease in the aromaticity of the nucleoside with the loss of absorption around 260 nm. It is interesting to note that the UV absorption spectrum of the photooxidation product **2** exhibits a shoulder around 230 nm. The molecular absorption coefficient at this wavelength was determined experimentally to be 5240 mol^{-1} cm^{-1} L^{-1}. It should be added that the other diastereomeric photoproduct **3**, as well as the corresponding 3′,5′-di-*O*-acetylated derivatives **2a** and **3a**, exhibits similar UV absorption features.

Circular Dichroism. The two nucleosides **2** and **3** exhibit quite similar but opposite n–π^* transitions with two maxima at 235 and 260 nm, respectively (Figure 3). This strongly suggests a diastereomeric relationship between **2** and **3**.

Altogether, the above spectroscopic data may be rationalized in terms of a 4,8-dihydro-4-hydroxy-8-oxoguanine structure for the aglycon of **2** and **3** as well as for **2a** and **3a**.

Characterization of 4,8-Dihydro-4-hydroxy-8-oxoguanine (4-OH-8-oxoGua). Chemical hydrolysis of the two photoproducts **2** and **3** (or the corresponding acetylated derivatives **2a** and **3a**) was carried out by using hydrogen fluoride stabilized in pyridine (HF/Pyr). This allowed the quantitative release of the two 4*R** and 4*S** enantiomers of 4-OH-8-oxoGua which were purified by HPLC separation.

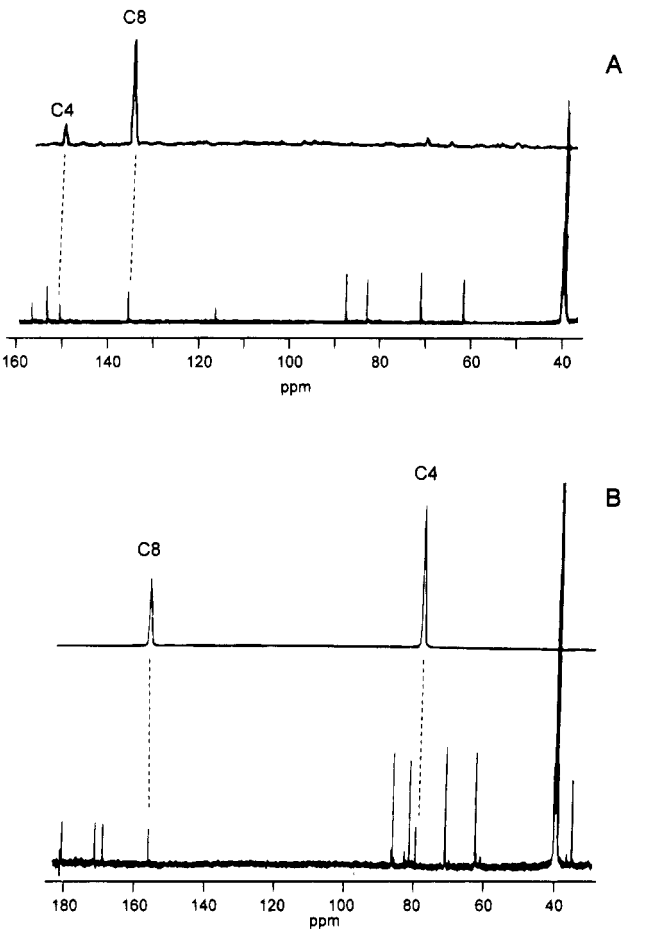

Figure 5. Long range scalar correlations between the anomeric proton and C4 and C8 carbons of the purine moiety of **1** (part A) and **2** (part B), respectively. The bottom of each part represents the ^{13}C NMR spectrum of **1** (part A) and **2** (part B), and the top shows the long range correlation between the anomeric proton and the carbons of the corresponding nucleoside.

FAB Mass Spectrometry. The FAB/MS spectrum of 4-OH-8-oxoGua obtained in the positive mode shows a pseudomolecular ion at *m/z* 184 $[M + H]^+$ and a quasi-molecular ion at *m/z* 206 $[M + Na]^+$. This could be rationalized in terms of a molecular weight of 183, corresponding to the elemental composition of $C_5H_5N_5O_3$, confirming the presence of two additional atoms of oxygen within the guanine moiety.

^{13}C NMR. The ^{13}C NMR features of 4-OH-8-oxoGua (Table 3) indicate the presence of 5 carbon resonances, as expected from the results obtained with the related nucleosides **2a**, **3a**, **2**, and **3**. The chemical shift values are similar to those of the base moiety of the nucleosides **2** and **3**. Therefore, the downfield shift signal of 4-OH-8-oxoGua (78.9 ppm) and the signal at 156.2 ppm were assigned to the C4 and C8 carbons, respectively, by comparison with the data obtained for **2** and **3** (Table 3).

Taken together, all the spectrometric data can be rationalized in terms of a 4,8-dihydro-4-hydroxy-8-oxo-2′-deoxyguanosine structure for photoproducts **2** and **3** (Figure 4) which are the two 4*R** and 4*S** diastereomers. The photoproducts **2a** and **3a** correspond to the 3′,5′-di-

Table 4. Relative Abundance of the Major Ions Observed in the FAB/MS Spectra (Positive Ion Mode) of Photoproduct 2a (MW = 383) and 8-OxodGuo (4) (MW = 283) Obtained after Methylene Blue Photosensitization in the Presence of $^{18}O_2$

	m/z	rel abundance
2a		
$[M + H]^+$	384	75
	386	100
	388	40
$[M + Na]^+$	406	50
	408	68
	410	26
8-oxodGuo (**4**)		
$[M + H]^+$	284	45
	286	100
$[B + H]^+$	170	80
	168	40

O-acetylated derivatives of **2** and **3**, respectively, whereas product **4** may be assigned as 4,8-dihydro-4-hydroxy-8-oxoguanine.

Mechanism of Formation. Oxygen Incorporation. In order to establish the origin of the two additional oxygen atoms in the main photooxidation products **2a** and **3a**, di-O-Ac-dGuo (**1a**) was exposed to photoexcited methylene blue in an aqueous solution enriched with ^{18}O isotopically labeled oxygen. The incorporation of ^{18}O in **2a** was determined by FAB/MS (positive ion mode) analysis (Table 4) after HPLC separation of each of the two diastereomers. The presence of the pseudomolecular ion $[M + H]^+$ at $m/z = 388$ and $m/z = 386$, as well as the ion $[M + Na]^+$ at $m/z = 410$ and $m/z = 408$, indicates that the two oxygen atoms incorporated in the photoproducts come from molecular oxygen and not from water. It should be noted that the presence of the pseudomolecular ion $[M + H]^+$ at $m/z = 386$ and $[M + Na]^+$ at $m/z = 406$, respectively, indicates that the enrichment of the irradiated solution in $^{18}O_2$ was not quantitative but approximately 60%, due to air contamination. In this respect, it is interesting to note that a similar yield of incorporation of ^{18}O was observed for the main stable type I photooxidation product (*46*).

A similar experiment was carried out in order to determine the origin of the additional oxygen atom in 8-oxodGuo (**4**), the other $^1O_2$ oxidation product of **1**. The FAB/MS (positive ion mode) spectrum of **4** obtained under these conditions showed the presence of a pseudomolecular ions $[M + H]^+$ at $m/z = 286$ and a fragment $[B + H]^+$ at $m/z = 170$ (Table 4). This indicates that the C8 oxygen atom in **4** comes from molecular oxygen.

Singlet Oxygen Participation. The methylene blue photosensitized oxidation of dGuo in aqueous aerated solution may proceed either through a radical (type I) and/or a singlet oxygen (type II) mediated mechanism (*8*). In order to establish the origin of the main photoproducts, different experimental approaches were used:

(a) Enhancing Isotopic Effect of ^2H_2O. The enhancing effect of ^2H_2O was used in order to establish the participation of singlet oxygen in the mechanism of formation of photoproducts **2**, **3**, and **4**. The formation of 8-oxodGuo (**4**) and 4-OH-8-oxodGuo (**2** + **3**) with respect to increasing periods of irradiation in H_2O and ^2H_2O solution is illustrated in Figure 6. Results indicate that the formation of 4-OH-8-oxodGuo is linear with the time of exposure of **1** to visible light. In addition, an enhancing effect was observed when the irradiation was carried out in heavy water (Figure 6, lower part). This confirms the participation of singlet oxygen in the mechanism of formation of such photoproducts. In contrast, no heavy water enhancing effect was observed for the formation of **5**, whose formation involves a type I mechanism (*46*). It has to be noted that the formation of **4** is not linear with the time of irradiation (Figure 6, upper part); a plateau is reached at approximately 1% of the original amount of dGuo. The plateau is reached more rapidly in ^2H_2O than in H_2O solution, indicating that singlet oxygen is implicated in the mechanism of formation of **4**. As we have already reported previously (*44*), a steady-state level in the production of **4** is obtained as its formation is balanced by a degradation reaction. This may be explained by the fact that 8-oxodGuo (**4**) is a better substrate for singlet oxygen than dGuo (**1**). Moreover, phthalocyanine, a photosensitizer that predominantly produces type II (*44*) mediated photosensitization reaction of **4**, was found to generate 4-OH-8-oxodGuo (**2** and **3**) as the main $^1O_2$ oxidation products, as inferred from HPLC and ^1H NMR analysis of the irradiated solution.

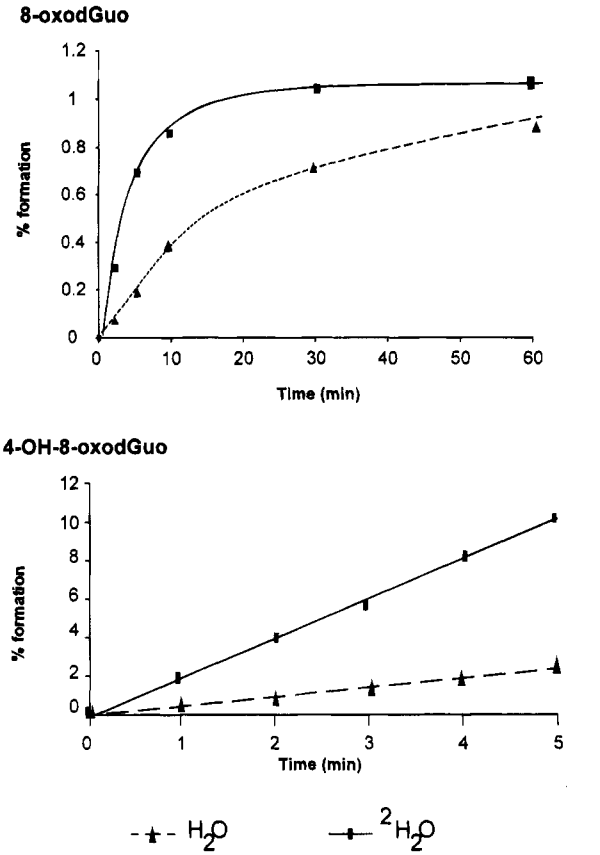

Figure 6. Formation of photooxidation products 4-OH-8-oxodGuo (sum of **2** and **3**) (lower part) and 8-oxodGuo (**4**) (upper part) with respect to increasing periods of methylene blue mediated photosensitization of dGuo (**1**) in H_2O (dashed line) or ^2H_2O (solid line).

(b) The Use of Selected Sensitizers. The use of selected sensitizers, such as riboflavin and benzophenone, which predominantly act through a type I photosensitization reaction, was found to generate **5** as the main photooxidation product. In contrast, a relatively low level, when detectable, of photoproducts **2**, **3**, and **4** (4-OH-8-oxodGuo and 8-oxodGuo) was observed. On the other hand, the use of phthalocyanines and naphthalocyanines, well-known type II photosensitizers, was found to give rise to the predominant formation of the nucleosides **2**, **3**, and **4** with a very low yield of **5** (*44*).

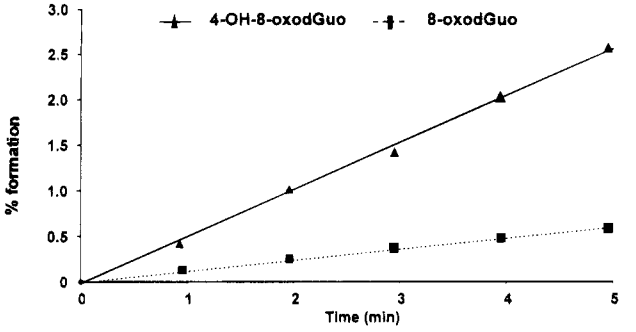

Figure 7. Formation of photooxidation products 4-OH-8-oxodGuo (**2**, **3**) (dashed line) and 8-oxodGuo (**4**) (solid line) versus increasing period of methylene blue mediated photosensitization of dGuo (**1**).

Table 5. Yield[a] of Type II Photooxidation Products in Methylene Blue Mediated Photosensitization of Double-Stranded Calf Thymus DNA in Aqueous Aerated Solution

	8-oxodGuo	4-OH-8-oxodGuo
control	2 (*10*)	<1 (*4*)
photosensitized	1500 (*5700*)	3.5 (*15*)

[a] In ng per 500 μg of DNA (and in *modified nucleosides per 10^6 DNA bases*). Irradiation time = 30 min.

(c) Chemical Singlet Oxygen Production. The thermal dissociation of the endoperoxide of 3,3′-(1,4-naphthylidene)dipropionate (*37*) was used in order to provide a clean source of singlet oxygen. HPLC and ^{1}H NMR analysis of the mixture of endoperoxide mediated decomposition products of dGuo (**1**) was found to contain the nucleosides **2**, **3**, and **4** as the main oxidation products.

(d) Effects of a Reducing Agent. The methylene blue mediated photosensitization of dGuo in aqueous solution containing 0.2 M $FeSO_4$ was found to produce **4** as the main oxidation product, with a concomitant decrease in the formation of photoproducts **2** and **3**.

Quantitative Analysis. Quantitative analysis were carried out in order to establish the yield of formation of the main two classes of type II photoproducts of dGuo (**1**). Formation of the two diastereomers of 4-OH-8-oxodGuo (sum **2** + **3**) and 8-oxodGuo (**4**) versus time of exposure of a 1 mM dGuo solution containing methylene blue to visible light is reported in Figure 7. Short irradiation times were chosen in order to minimize the secondary reaction of singlet oxygen with **4**. Comparison between the slope of the two curves indicates that the two diastereomers **2** and **3** are the main singlet oxygen oxidation products of 2′-deoxyguanosine. The overall yield of formation of the two diastereomers 4-OH-8-oxodGuo (**2** + **3**) was determined to be 7-fold higher than those of 8-oxodGuo (**4**).

DNA Photosensitization. Attempts were made in order to detect the main singlet oxygen oxidation products within methylene blue mediated photosensitization of DNA. The yield of the formation of the two classes of singlet oxygen photoproducts in DNA is given in Table 5. It should be noted that the two diastereomers of 4-OH-8-oxoGua are only produced in a minor yield since their level of formation was found to be close to the limit of detection of the HPLC-UV assay, estimated to be 1 ng per 500μg of DNA. Therefore, it may be concluded that the main singlet oxygen oxidation product of the guanine residues within double-stranded DNA is **4**, whereas 4-OH-8-oxoGua, the predominant $^1O_2$ oxidation product of the guanine moiety of **1**, is formed at a very low level (less than 1%).

Discussion

The two 4*R** and 4*S** diastereomers of 4-OH-8-oxodGuo (**2** and **3**) are the main singlet oxygen mediated oxidation products of dGuo. 8-OxodGuo (**4**) is also produced, through $^1O_2$ reaction but in an about 7-fold lower yield than the two diastereomers **2** and **3**. A reasonable mechanism for the formation of the oxidation products **2** and **3** involves the initial formation of a transient endoperoxide through a Diels–Alder 1,4-cycloaddition of $^1O_2$ to the purine ring. In a subsequent step, the unstable endoperoxides decompose to give rise to the formation of the two diastereomers **2** and **3**. It should be pointed out that the endoperoxides could not be isolated at room temperature; however, using low-temperature photooxygenation conditions and 2′,3′,5′-*O*-(*tert*-butyldimethylsilyl)-8-methylguanosine as the substrate, Sheu and Foote (*52*) have recently characterized such intermediates by ^{1}H NMR analysis. It is also important to note that the two diastereomers **2** and **3** are produced in relatively similar yields. This indicates that the molecular addition of oxygen could occur on both sides of the base without any pronounced stereospecificity.

The other oxidation product, 8-oxodGuo (**4**), is produced under methylene blue photosensitization but in a relatively low yield (ratio 1/7) compared to photoproducts **2** and **3** (Figure 7). It is also important to note that **4** was found to react efficiently with singlet oxygen. As a consequence, formation of the latter oxidized nucleoside is not linear with the time of irradiation of **1**. In addition, it is interesting to note that the two diastereomers of 4-OH-8-oxodGuo (**2**, **3**) are among the main singlet oxygen secondary oxidation products of 8-oxodGuo (Figure 4).

It is also important to note that, under the conditions used in the present study, no other predominant products, including cyanuric acid nucleoside (*34*), were observed. This may be explained by the alkaline conditions of irradiation which were used in earliest studies (*22*, 34). Such conditions probably affect the reaction of singlet oxygen with dGuo and also the nature of the oxidation products formed.

A reasonable mechanism for the formation of **4** may involve a competitive reduction reaction of the initial unstable endoperoxide (*24*) through a not yet identified process. Such a hypothesis is inferred from the observation that the presence of a reducing agent such Fe^{2+} in the photooxidation reaction leads to the predominant formation of 8-oxoGua at the expense of the formation of the two diastereomers **2** and **3**. In addition, the formation of **4** arises from the addition of an oxygen atom coming from air and not from water as inferred form the $^{18}O_2$ experiment (Table 4). Interestingly, the ratio of formation of (**2** + **3**)/**4** is very low in double-stranded DNA upon exposure to singlet oxygen (Table 5). In contrast, the two diastereomers **2** and **3** were found to be the major guanine singlet oxygen oxidation products when the nucleoside was acetylated (photoproduct **2a**, **3a**) or when incorporated in a short oligonucleotide (*53*). It is interesting to note that van der Akker *et al.* (*54*) have recently shown the formation of one-G deletions as a consequence of singlet oxygen induced damage to single-stranded DNA. The nature of the singlet oxygen oxidation product

responsible for such a mutation was not identified. However, one possibility to be further explored would be that the two diastereomers **2** and **3** could be responsible for such a mutation since 8-oxoGua did not induce any base deletion but rather G → T transversions (*55*). Work is in progress in our laboratory to determine how reducing agents and DNA structure may affect the chemical reactions of the unstable endoperoxides.

Meanwhile, a GC/MS assay has been developed (*56*) for monitoring the formation of diastereomers **2** and **3** in both isolated and cellular DNA. It is important to note that the two diastereomers of 4-OH-8-oxodGuo **2** and **3** can be considered as biological markers of singlet oxygen formation since these two compounds are not produced under other oxidation conditions, including γ radiolysis (OH° formation) or type I photooxidation reactions. In addition, detection of the 2′-deoxyguanosine oxidation products **2** and **3** may be used as a tool in order to determine the ability of a dye to produce singlet oxygen via a type II photosensitization reaction (*44*, *57*). On the other hand, it is worth mentioning that 8-oxoGua cannot be considered as a potential marker of the presence of singlet oxygen since this modified nucleoside is also produced through the reaction of hydroxyl radicals (*29*). In addition, it has been reported recently that 8-oxodGuo is also produced by a type I riboflavin mediated photooxidation reaction involving initial formation of a guanine radical cation within double-stranded DNA (*58*).

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