Photoinduced Hydroxylation of Deoxyguanosine in DNA by Pterins: Sequence Specificity and Mechanism†

Kimiko Ito‡ and Shosuke Kawanishi*,§

Department of Public Health, Graduate School of Medicine, Kyoto University, Kyoto 606, Japan, and Department of Hygiene, Mie University School of Medicine, Tsu, Mie 514, Japan

Received August 13, 1996; Revised Manuscript Received December 9, 1996⊗

ABSTRACT: Pterin-sensitized DNA photodamage has been characterized by a DNA sequencing technique. Exposure of double-stranded DNA to 365-nm light in the presence of pterin, 6-carboxypterin, biopterin, neopterin, and folic acid produced sequence-specific DNA lesions, whereas photoinduced DNA lesions were not observed in the presence of xanthopterin or isoxanthopterin. The DNA photodamage induced by these pterin derivatives occurred specifically at the guanine located 5′ to guanine. High-pressure liquid chromatography (HPLC) analysis revealed that the pterin-sensitized DNA photodamage was predominantly due to the formation of 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG). The DNA photodamage with pterin was not enhanced in D_2O, suggesting that $^1O_2$ is not the main active species. Electron spin resonance (ESR) spin destruction experiments demonstrated that the photoexcited pterins reacted specifically with dGMP to produce pterin anion radicals. In addition, the reactivities of the photoexcited pterin derivatives with dGMP were found to correlate well with their efficiencies of DNA photodamage induction. These results indicate that the photoexcited pterins specifically oxidize deoxyguanosine in DNA to produce 8-oxo-dG through an electron transfer reaction. With denatured single-stranded DNA, the extent of pterin-sensitized photodamage was decreased and the damage occurred at most guanine residues without specificity for those located 5′ to guanine. The mechanism of pterin-induced sequence-specific guanine photodamage could be explained on the basis of a recent theoretical study [Sugiyama, H., & Saito, I. (1996) *J. Am. Chem. Soc. 118*, 7063−7068] concerning the ionization potentials of stacked dinucleotide base pairs. Sepiapterin, a model compound for the dihydropterins, induced similar sequence-specific photolesions in double-stranded DNA. However, DNA photodamage by sepiapterin was more extensive in the presence of Cu(II), and the sites of the photolesions were different from those induced in the absence of Cu(II). These data may provide a basis for the elucidation of the molecular mechanism of solar UV carcinogenesis.

The mechanism of DNA damage caused by solar UV light distinctly differs with UV wavelength (Cadet *et al*., 1986; Coohill *et al*., 1987). Wavelengths shorter than about 320 nm directly photoactivate the DNA molecule to generate mainly pyrimidime photoproducts, while UVA (320−380 nm) wavelengths can indirectly damage the DNA *via* photosensitized reactions. This indirect action of UV light is mediated by a non-DNA molecule through type I (electron transfer) and type II (energy transfer) processes (Foote, 1991). As is well-known, the solar UV at the surface of the earth is largely in the range of UVA. UVB (290−320 nm) radiation is a small portion. Studies with laboratory animals have shown that UVB is generally most effective in inducing skin cancer (International Agency for Research on Cancer, 1992). UVB light can be absorbed by DNA and produces two major types of base lesions, i.e., cyclobutane pyrimidine dimers and pyrimidine(6-4)pyrimidone photoadducts. It has been well-recognized that the two photoproducts are relevant to UV carcinogenesis (Ananthaswamy & Pierceall, 1990). However, recent studies have demonstrated that UVA wavelengths are also mutagenic and carcinogenic (Tyrrell & Keyse, 1990; Setlow *et al*., 1993). It has been shown that the action spectra for mutation induction and tumorigenesis do not coincide with the DNA absorption spectrum in the UVA region (Suzuki *et al*., 1981; Jones *et al*., 1987). These data suggest that DNA damage *via* indirect photosensitization reactions may be partly responsible for the natural sunlight-induced skin cancer (Coohill *et al*., 1987). Therefore, it is considered necessary to clarify the precise mechanisms of DNA damage *via* photosensitized reactions.

Recently, mutations in *ras* oncogenes and in the *p53* tumor suppressor gene have been found in human skin cancers located at sun-exposed body sites (van der Schroeff *et al*., 1990; Pierceall *et al*., 1991; Brash *et al*., 1991). Since these genetic changes in tumor DNA might reflect the chemical nature of the DNA photoproduct, comparison of the sunlight-induced mutation spectrum with the DNA photodamage profiles obtained in cell-free systems can potentially help in elucidating the molecular mechanism of UV carcinogenesis. For this reason, it is appropriate to perform detailed studies of the DNA damage profile within a defined DNA sequence induced by solar UV wavelengths through photosensitization pathways.

One of the oxidized DNA products, 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG),[1] has attracted much attention in relation to carcinogenesis, since its presence in DNA causes GC to TA transversions (Shibutani *et al*., 1991; Cheng *et al*., 1992). In previous work, we have demonstrated that the exposure of double-stranded DNA to UVA or UVB in

† This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

* Author to whom correspondence should be addressed.
‡ Kyoto University.
§ Mie University School of Medicine.

⊗ Abstract published in *Advance ACS Abstracts,* January 15, 1997.

S0006-2960(96)02016-8 CCC: $14.00

the presence of riboflavin produces 8-oxo-dG through an electron transfer mechanism (type I) (Ito *et al*., 1993). These findings allow us to assume that other endogenous molecules are also able to generate 8-oxo-dG through the type I mechanism upon exposure to solar UV.

Pterins, the 2-amino-4-hydroxypteridine derivatives, occur in conjugated or free form in a wide range of biological systems. They are involved in the metabolism of various biomolecules (Nichol *et al*., 1985). The presence of biopterin in human skin, as well as its accumulation in patients with dispigmentation disorder, has been recently demonstrated (Schallreuter *et al*., 1994). In addition, it has been shown that DNA photolyases, which catalyze photosensitized cleavage of pyrimidine dimers, utilize a pterin derivative as the light-gathering chromophore (Sancar & Sancar, 1988; Johnson *et al*., 1988). Thus, this study was carried out to examine DNA photodamage induced in the presence of pterins to improve understanding of the mechanism of sunlight-induced skin cancers.

MATERIALS AND METHODS

Materials. Pterin, biopterin, neopterin, isoxanthopterin, and 4-oxo-TEMPO were obtained from Aldrich Chemical Co., Milwaukee, WI. L-Sepiapterin was from Alexis Corp., and 6-carboxypterin, folic acid, and xanthopterin were from Nacalai Tesque Co., Kyoto, Japan. [γ-^{32}P]ATP (6000 Ci/mmol) was supplied by New England Nuclear, Boston, MA. Calf intestine alkaline phosphatase (3000 units/mg) was obtained from Boehringer Mannheim GmbH, Mannheim, Germany. Calf thymus DNA was from Sigma Chemical Co., St. Louis, MO. Nuclease P_1 (400 units/mg) was from Yamasa Shoyu Co., Chiba, Japan. D_2O (99.95%) was obtained from Commissariat à l'Énergie Atomique, France.

Preparation of ^{32}P 5′-End-Labeled DNA Fragments. DNA fragments were prepared from plasmid pbcNI which carries a 6.6-kilobase *Bam*HI chromosomal DNA segment containing human c-Ha-*ras*-1 protooncogene (Yamamoto *et al*., 1989). A singly labeled 261-base-pair fragment (*Ava*I* 1645−*Xba*I 1905), a 341-base-pair fragment (*Xba*I 1906−*Ava*I* 2246), and a 337-base-pair fragment (*Pst*I 2345−*Ava*I* 2681) were obtained as previously described (Yamamoto *et al*., 1989). The asterisk indicates ^{32}P labeling, and nucleotide numbering starts with the *Bam*HI site (Capon *et al*., 1983).

UV Radiation in the Presence of Pterins. The standard reaction mixture, in a microtube (1.5-mL Eppendorf), contained 0.1 mM pterin, *ca*. 0.1 μM/base [^{32}P]DNA fragment, and 2 μM/base sonicated calf thymus DNA in 100 μL of 10 mM sodium phosphate buffer at pH 7.9. Irradiation was performed using a transilluminator (UVP, Model NTL-33) equipped with six 15-W black-light lamps which have an emission peak at approximately 365 nm: a long-wave filter was used to reduce the light intensity at wavelengths shorter than 300 nm negligibly small as compared with that of the 365-nm line. The fluence rate at 365 nm was measured using a UVX radiometer (UVP). The reaction mixture was protected from direct sunlight. After irradiation, the DNA fragments were heated at 90 °C in 1 M piperidine for 20 min and treated as previously described (Maxam & Gilbert, 1980; Yamamoto *et al*., 1989). Denatured DNA fragments were prepared by heating double-stranded DNA fragments at 90 °C for 5 min, followed by quick chilling. Where indicated, D_2O was used in place of H_2O. The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam−Gilbert procedure (Maxam & Gilbert, 1980) using a DNA sequencing system (LKB2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL) was used for the measurement of the relative amounts of oligonucleotides from treated DNA fragments.

Measurement of 8-Oxo-dG. The content of 8-oxo-dG was determined by the modified method of Kasai *et al*. (1987). Calf thymus DNA (50 μM/base) was irradiated with 365-nm light in the presence of 50 μM pterin in 100 μL of 4 mM phosphate buffer (pH 7.9). After irradiation, the DNA was separated by ethanol precipitation, dissolved in 20 mM acetate buffer (pH 5.0), and digested to deoxynucleosides by incubation first with 8 units of nuclease P1 at 37 °C for 30 min and then with 1.3 units of calf intestine alkaline phosphatase at 37 °C for 1 h in 0.1 M Tris-HCl buffer (pH 7.5). The resulting deoxynucleoside mixture was analyzed by HPLC-ECD as described previously (Ito *et al*., 1993).

Analysis of the Reactivity of Photoexcited Pterins by ESR Spin Destruction Method. The reactivity of photoexcited pterins with mononucleotides was investigated by the ESR spin destruction method (Moan, 1980; Reszka & Sealy, 1984). The standard reaction solutions (50 μL) contained 20 mM sodium phosphate buffer (pH 7.9), 0.1 mM pterin, and 5 μM 4-oxo-TEMPO. The samples, in a microtube (1.5-mL Eppendorf), were irradiated with 365-nm light placed at 15 cm. For ESR measurements, aliquots of the solutions were taken in calibrated capillaries (Clay Adams, 50 μL) before irradiation or after different irradiation times. ESR spectra were measured at room temperature using a JES-FE-3XG (JEOL, Tokyo, Japan) spectrometer with 100-kHz field modulation. The magnetic fields were calculated by the splitting of Mn^{2+} in MgO ($\Delta H_{3-4} = 86.9$ G). Spectra were recorded with a microwave power of 1 mW and a modulation amplitude of 1.0 G.

Fluorescence Spectra Measurements. The fluorescence spectra of pterin (1 μM) were measured with and without addition of sonicated calf thymus DNA in 20 mM phosphate buffer at room temperature using a Hitachi MPF-4 spectrofluorophotometer. The excitation wavelength was set at 353 nm and the emission spectra were measured in the 430−500-nm range. The DNA was added at a concentration range of 0.08−1 mM/base.

RESULTS

DNA Cleavage by 365-nm Radiation in the Presence of Various Pterins. The extent of damage induced in singly 5′-end-labeled DNA fragments by 365-nm light with several pterin derivatives was investigated by gel electrophoretic analysis. The six unconjugated pterins, i.e., pterin, 6-carboxypterin, biopterin, neopterin, xanthopterin, and isoxanthopterin, and one conjugated pterin, i.e., folic acid, were chosen in the present study to compare their DNA-damaging potentials in the presence of UV light. Figures 1−3 show

[1] Abbreviations: 8-oxo-dG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; HPLC, high-pressure liquid chromatography; HPLC-ECD, electrochemical detector coupled to high-pressure liquid chromatography; ESR, electron spin resonance; $^1O_2$, singlet oxygen; 4-oxo-TEMPO, 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl.

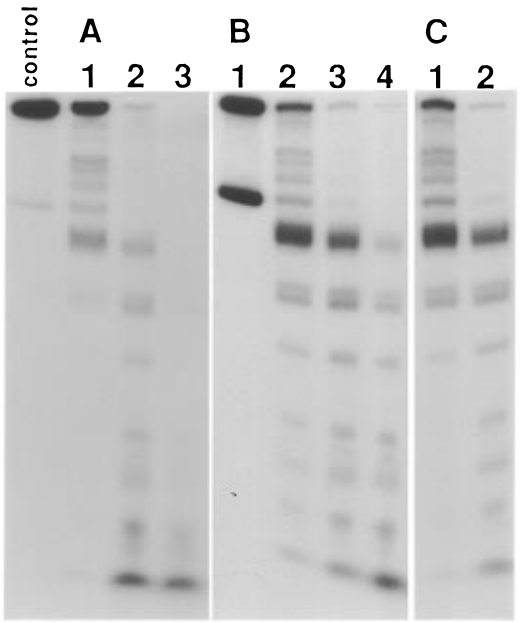

FIGURE 1: Autoradiogram of ^{32}P-labeled DNA fragments irradiated at 365 nm in the presence of pterin. The sample solution contained ^{32}P 5′-end-labeled 337-base-pair fragment (*Pst*I 2345−*Ava*I* 2681), sonicated calf thymus DNA (2 μM/base) and pterin in 100 μL of 10 mM sodium phosphate buffer (pH 7.9). Following exposure to 365-nm light, the DNA fragments were treated with 1 M piperidine at 90 °C for 20 min and subjected to electrophoresis on an 8 M urea-containing 8% polyacrylamide gel. (A) The DNA was exposed to light at 10 J/cm^2. Lane 1, 0.05 mM pterin; lane 2, 0.1 mM pterin; lane 3, 0.2 mM pterin. (B) Pterin (0.1 mM) was added. Lane 1, 0 J/cm^2; lane 2, 2 J/cm^2; lane 3, 5 J/cm^2; lane 4, 10 J/cm^2. (C) The DNA was exposed to light at 5 J/cm^2 in the presence of 0.1 mM pterin in D_2O (lane 1) or H_2O (lane 2) buffer.

the autoradiograms of double-stranded DNA fragments exposed to these pterins and 365-nm light. UV irradiation in the presence of pterin induced DNA photodegradation in a concentration- (Figure 1A) and UV dose- (Figure 1B) dependent manner. The pterin-induced DNA photolesions were not enhanced in D_2O (Figure 1C). As shown in Figure 2, 6-carboxypterin (lane 2), biopterin (lane 3), neopterin (lane 4), and folic acid (lane 7) also induced DNA damage in the presence of 365-nm light. In contrast, little or no DNA damage was observed with xanthopterin (lane 5) or isoxanthopterin (lane 6) under the present conditions. The efficiency of DNA damage induction by 365-nm light decreased in the following order: pterin $\approx$ 6-carboxypterin $>$ biopterin $\approx$ neopterin $\gg$ folic acid. The pterin-induced DNA photolesions were enhanced upon piperidine treatment (data not shown), suggesting that base alteration and/or liberation occurred. None of these pterins induced DNA damage without UV radiation. No oligonucleotide was observed with UV radiation alone (data not shown). Interestingly, when the double-stranded DNA fragments were denatured to single-stranded DNA by heating at 90 °C for 5 min, followed by rapid cooling, pterin-induced DNA photolesions were decreased (Figure 3).

Sequence Specificity of DNA Cleavage by UV Radiation with Pterins. ^{32}P 5′-end-labeled DNA fragments irradiated at 365 nm in the presence of pterins, and subsequently treated with piperidine, were electrophoresed and the autoradiograms obtained were scanned with a laser densitometer (Figures 4 and 5). The cleavage sites were determined with reference to the cleavage sites produced by Maxam−Gilbert sequencing reactions (Maxam & Gilbert, 1980). With double-

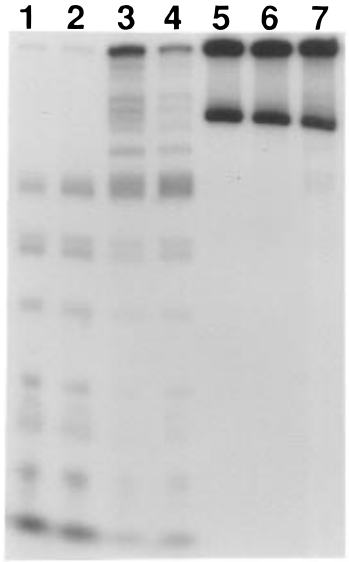

FIGURE 2: Autoradiogram of ^{32}P-labeled DNA fragments irradiated at 365 nm in the presence of various pterin derivatives. The ^{32}P 5′-end-labeled 337-base-pair fragment (*Pst*I 2345−*Ava*I* 2681) was exposed to 10 J/cm^2 of 365-nm light in the presence of 0.1 mM pterin (lane 1), 0.1 mM carboxypterin (lane 2), 0.1 mM biopterin (lane 3), 0.1 mM neopterin (lane 4), 0.2 mM xanthopterin (lane 5), 0.2 mM isoxanthopterin (lane 6), or 0.4 mM folic acid (lane 7) in 100 μL of 10 mM sodium phosphate buffer (pH 7.9) containing 2 μM/base sonicated calf thymus DNA. After irradiation, the DNA fragments were treated as described in the Figure 1 legend.

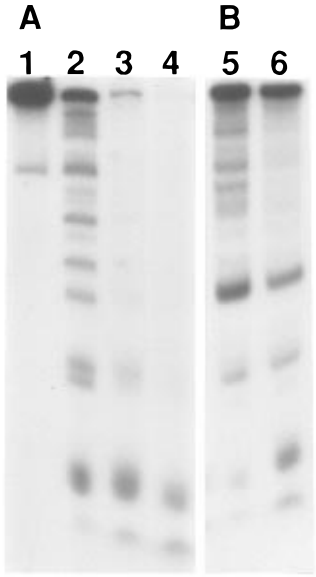

FIGURE 3: Comparison of DNA damage induced by pterin in single- and double-stranded DNA. The ^{32}P 5′-end-labeled 337-base-pair fragment (*Pst*I 2345−*Ava*I* 2681) was exposed to 365-nm UV light in the presence of 0.2 mM pterin in 100 μL of 10 mM sodium phosphate buffer (pH 7.9) containing 2 μM/base sonicated calf thymus DNA. After irradiation, the DNA fragments were treated as described in the legend to Figure 1. For the experiment with denatured single-stranded DNA (B), the 5′-end-labeled DNA fragment was treated at 90 °C for 5 min and quickly chilled before the addition of pterin. (A) Lane 1, 0 J/cm^2; lane 2, 2 J/cm^2; lane 3, 5 J/cm^2; lane 4, 10 J/cm^2. (B) Lane 5, 5 J/cm^2; lane 6, 10 J/cm^2.

stranded DNA, pterin induced cleavage specifically at the 5′ site of 5′-GG-3′ sequences (Figure 4A). Although very weak cleavage at the 5′ site of 5′-GA-3′ sequences occurred, no cleavage was observed at other sequences, including single guanine residues. Similar results were also observed with 6-carboxypterin (Figure 4B), neopterin (Figure 4C),

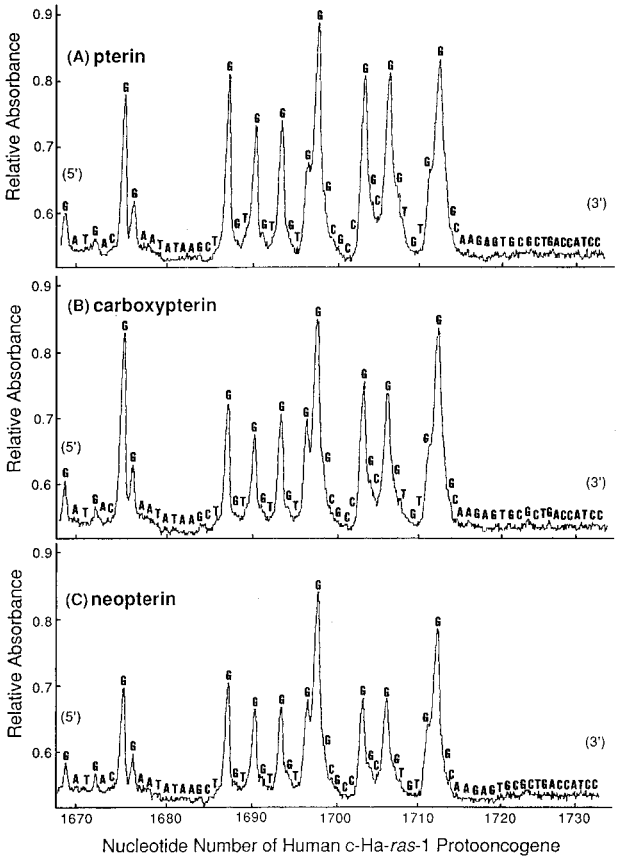

FIGURE 4: Site specificity of DNA cleavage induced by 365-nm radiation in the presence of pterins. The ^{32}P 5′-end-labeled 261-base-pair fragment (*Ava*I* 1645−*Xba*I 1905) was exposed to 365-nm light with pterin (A), carboxypterin (B), or neopterin (C) in 100 μL of 10 mM sodium phosphate buffer (pH 7.9) containing 2 μM/base sonicated calf thymus DNA. After piperidine treatment, the DNA fragments were electrophoresed on an 8 M urea-containing 8% polyacrylamide gel using a DNA-sequencing system and the autoradiogram was obtained by exposing X-ray film to the gel. The relative amounts of oligonucleotides produced were measured by scanning the autoradiogram with a laser densitometer. The piperidine-labile sites of the treated DNA were determined by direct comparison of the positions with those produced by the chemical reactions of the Maxam−Gilbert procedure (Maxam & Gilbert 1980). The nucleotide number of the human c-Ha-*ras*-1 proto-oncogene starting with the *Bam*HI site is given on the horizontal axis (Capon *et al*., 1983). (A) 0.1 mM pterin, 5 J/cm^2; (B) 0.1 mM carboxypterin, 5 J/cm^2; (C) 0.2 mM neopterin, 10 J/cm^2.

biopterin, and folic acid (data not shown). On the other hand, when denatured single-stranded DNA was irradiated at 365 nm in the presence of pterin, the cleavage occurred at most guanine residues (Figure 5).

Effect of DNA Binders on Pterin-Induced DNA Photodamage. The extent of the pterin-induced DNA photodamage was examined in the presence of various DNA binders. Both divalent cations ($MgCl_2$) and polycations (spermine), which bind nonspecifically to DNA as counterions, decreased pterin-sensitized photodamage (data not shown). These results indicate that the DNA damage was caused by photoactivated pterin generated in the very close proximity of DNA. In addition, distamycin A and berenil, minor-groove binders with a preference for AT regions, decreased pterin-sensitized photodamage (Figure 6). Since binding of these agents in the minor groove of DNA can produce alterations in the DNA structure, this may imply that the DNA double-helical structure is important for the induction of sequence-specific DNA damage by pterin.

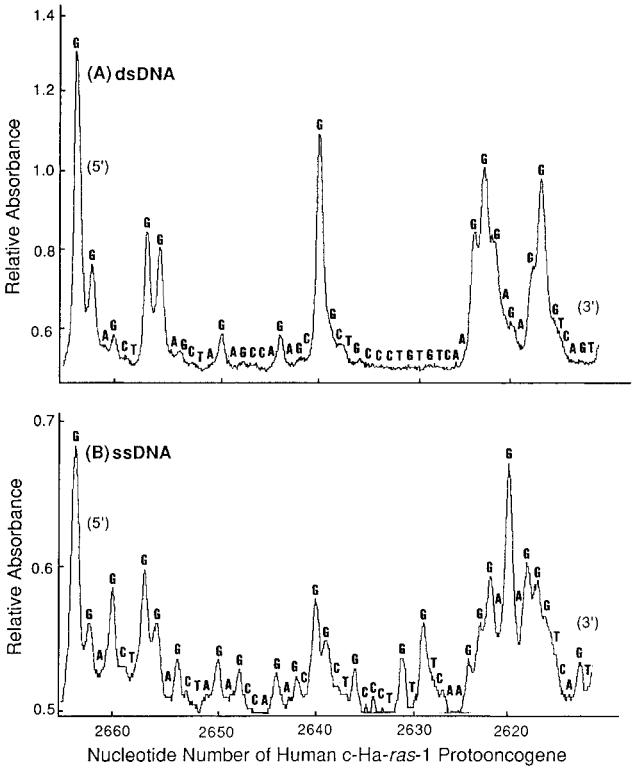

FIGURE 5: Comparison of cleavage sites in single- and double-stranded DNA induced by pterin plus 365-nm light. The ^{32}P 5′-end-labeled 337-base-pair fragment (*Pst*I 2345−*Ava*I* 2681) was exposed to 5 J/cm^2 of UV light in the presence of 0.1 mM pterin (A, double-stranded DNA) or 0.2 mM pterin (B, denatured single-stranded DNA) in 100 μL of 10 mM sodium phosphate buffer (pH 7.9) containing 2 μM/base sonicated calf thymus DNA. For the experiment with denatured single-stranded DNA (B), the 5′-end-labeled DNA fragment was treated at 90 °C for 5 min and quickly chilled before the addition of the sensitizer. After the piperidine treatment, the DNA fragments were analyzed as described in the Figure 4 legend.

Formation of 8-Oxo-dG in Calf Thymus DNA by UV Radiation with Pterin. Production of 8-oxo-dG in DNA following exposure to pterin and 365-nm UV light was measured as a function of pterin concentration and also as a function of radiation dose (Figure 7). The amount of 8-oxo-dG produced in double-stranded DNA was about 4 times more than that produced in denatured DNA. In D_2O, 8-oxo-dG formation decreased, using both double-stranded and denatured single-stranded DNA. The yields of 8-oxo-dG were roughly from 20% to 70% of the total guanine modifications under the conditions employed; the ratios were found to decrease with the UV dose and the pterin concentration.

Reactivity of Photoexcited Pterins with dGMP. We studied the reactivity of photoexcited pterin with mononucleotides by the spin destruction method in which 4-oxo-TEMPO, a stable nitroxide radical, is used as an indirect probe for monitoring the yields of radicals. Figure 8 shows that marked loss of the nitroxide radical occurred during 365-nm UV irradiation of pterin in the presence of dGMP, whereas a decrease of nitroxide radical did not occur in the absence of dGMP. The addition of dAMP, dCMP, or dTMP caused only a small decrease of the radical (data not shown). In contrast, loss of the nitroxide radical did not occur when xanthopterin was exposed to UV light with and without dGMP (Figure 8). Neopterin and folic acid also induced a loss of nitroxide radical during 365-nm UV irradiation in

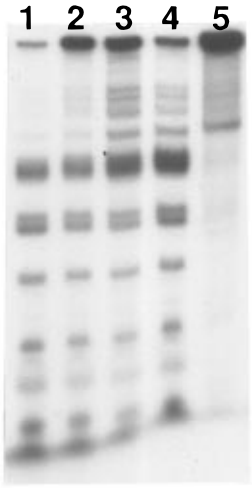

FIGURE 6: Effect of DNA binders on pterin-induced DNA photodamage. The ^{32}P 5′-end-labeled 337-base-pair fragment (*Pst*I 2345−*Ava*I* 2681) was exposed to 5 J/cm^2 of 365-nm light in the presence of 0.1 mM pterin in 100 μL of 10 mM sodium phosphate buffer (pH 7.9) containing 2 μM/base sonicated calf thymus DNA and a DNA binder. After irradiation, followed by the piperidine treatment, the DNA fragments were analyzed by the method described in the legend to Figure 1. Lane 1, without a DNA binder; lane 2, 0.5 μM distamycin A; lane 3, 2 μM distamycin A; lane 4, 0.05 μM berenil; lane 5, 0.5 μM berenil.

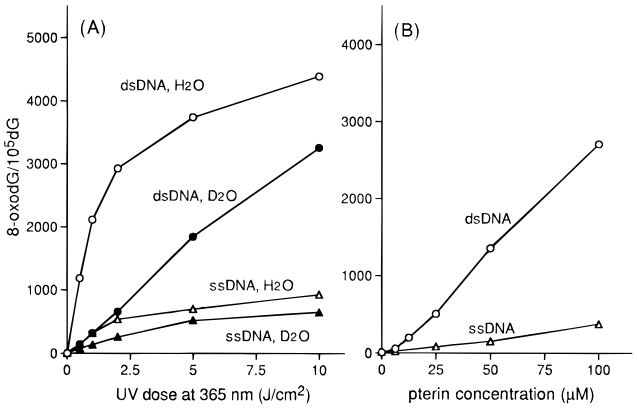

FIGURE 7: Formation of 8-oxo-dG in calf thymus DNA irradiated at 365 nm in the presence of pterin. Calf thymus DNA (50 μM/base) was exposed to 365-nm light in the presence of pterin in 100 μL of 4 mM sodium phosphate buffer (pH 7.9). Where indicated, D_2O was used in place of H_2O (closed symbols). For the experiment with denatured single-stranded DNA (△), the DNA was treated at 90 °C for 5 min and quickly chilled before the addition of the sensitizer. After irradiation, the DNA was subjected to enzyme digestion and analyzed by HPLC-ECD as descried under Materials and Methods . (A) The pterin concentration was 0.05 mM; (B) irradiation dose was 0.5 J/cm^2.

the presence of dGMP (Figure 8). The reactivities of the photoexcited pterins with dGMP decreased in the following order: pterin > neopterin > folic acid. This shows good correlation with their efficiency of DNA photodamage induction.

Photoinduced DNA Cleavage with Sepiapterin. The DNA damage induced by 365-nm irradiation in the presence of sepiapterin, a model compound for the dihydropterins, was investigated by DNA sequencing analysis. Figure 9A shows that sepiapterin induced DNA damage upon UV irradiation. The cleavage sites after piperidine treatment were at the 5′ site of 5′-GG-3′ sequences (Figure 10A). This pattern was similar to those induced by the fully oxidized form of pterins. However, when Cu(II) was added, the extent of DNA

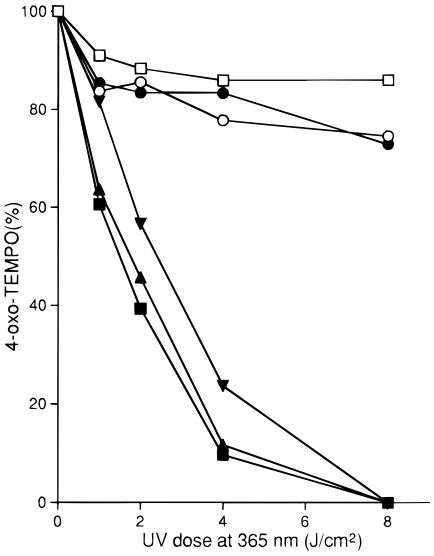

FIGURE 8: Reactivity of photoexcited pterins with dGMP. The sample contained 5 μM 4-oxo-TEMPO and 0.1 mM pterin (■), neopterin (▲), folic acid (▼), or xanthopterin (●) in the presence (closed symbols) and the absence (open symbols) of 2.5 mM dGMP in 50 μL of 20 mM sodium phosphate buffer at pH 7.9. The samples were irradiated with the indicated doses of 365-nm light. After irradiation, each solution was taken in a calibrated capillary, and ESR spectra were measured at room temperature as described under Materials and Methods.

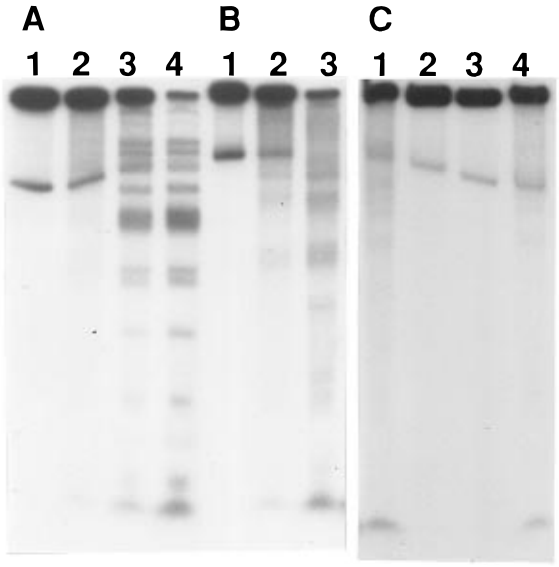

FIGURE 9: Sepiapterin-induced DNA photodamage in the presence of and the absence of Cu(II). The sample solution contained ^{32}P 5′-end-labeled 337-base-pair fragment (*Pst*I 2345−*Ava*I* 2681), 2 μM/base sonicated calf thymus DNA, and 0.1 mM sepiapterin in 100 μL of 10 mM sodium phosphate buffer (pH 7.9). After the addition (B, C) or without the addition (A) of 20 μM Cu(II), the sample was exposed to the indicated dose of 365-nm light, treated with 1 M piperidine, and subjected to electrophoresis on an 8 M urea-containing 8% polyacrylamide gel. (A) Lane 1, 0 J/cm^2; lane 2, 2 J/cm^2; lane 3 5 J/cm^2; lane 4, 10 J/cm^2; (B) lane 1, 0 J/cm^2; lane 2, 1 J/cm^2; lane 3, 2 J/cm^2; (C) lane 1, 2 J/cm^2; lane 2, 2 J/cm^2 + 50 μM bathocuproine; lane 3, 2 J/cm^2 + 15 units of catalase; lane 4, 2 J/cm^2 + 15 units of heat-denatured catalase.

photodamage by sepiapterin was more extensive than that in the absence of Cu(II) (Figure 9B). The DNA photolesions with sepiapterin plus Cu(II) were not at the 5′ site of 5′-GG-3′ (Figure 10B). The preferential piperidine-labile sites were simiar to those induced with Cu(II) plus H_2O_2 (Yamamoto & Kawanishi, 1989; Ito *et al.*, 1992). In addition,

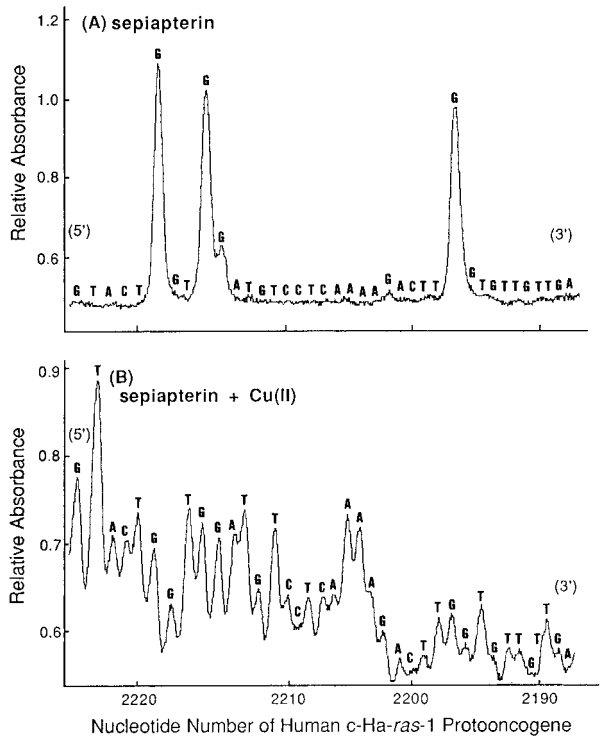

FIGURE 10: Site specificity of sepiapterin-mediated DNA photocleavage in the presence and the absence of Cu(II). The ^{32}P 5′-end-labeled 341-base-pair fragment (*Xba*I 1906−*Ava*I* 2246) was exposed to 365-nm light in the presence of 0.1 mM sepiapterin at 10 J/cm^2 (A) or 0.1 mM sepiapterin plus 20 μM Cu(II) at 2 J/cm^2 (B) in 100 μL of 10 mM sodium phosphate buffer (pH 7.9) containing 2 μM/base sonicated calf thymus DNA. After piperidine treatment, the DNA fragments were analyzed as described in the legend to Figure 4.

catalase and bathocuproione, a chelating agent of Cu(I), inhibited the sepiapterin plus Cu(II)-induced DNA photodamage (Figure 9C).

Fluorescence Spectra of Pterin with DNA. The fluorescence emission spectrum of 1 μM pterin was not affected by the addition of DNA under the conditions we employed (data not shown), suggesting that pterin has little binding affinity to DNA.

DISCUSSION

In the present study, we have demonstrated that the exposure of double-stranded DNA to 365-nm light in the presence of various pterin derivatives, i.e., pterin, 6-carboxypterin, biopterin, neopterin, and folic acid, followed by hot piperidine treatment, produces sequence-specific cleavage at the 5′ site of 5′-GG-3′ sequences. The photoinduced DNA lesions were not observed in the presence of xanthopterin or isoxanthopterin. The efficiency of inducing DNA damage with UV light decreased in the following order: pterin ≈ 6-carboxypterin > biopterin ≈ neopterin ≫ folic acid. It is not likely that the observed differences are due to differences in their absorbance characteristics, since xanthopterin and isoxanthopterin, both of which have significant absorbance at 365 nm, do not induce DNA damage by photosensitization. DNA cleavage was significantly increased by piperidine treatment, suggesting that base alteration was induced. In addition, HPLC analysis revealed that pterin plus 365-nm light increased the 8-oxo-dG content of double-stranded DNA. The estimated ratio of 8-oxo-dG yield to total guanine modification reached as high as approximately 70% under

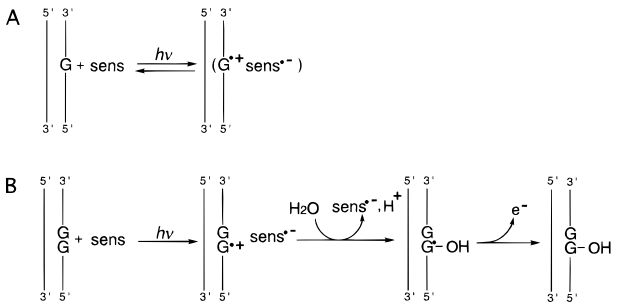

FIGURE 11: Possible mechanism of 8-oxo-dG formation at the 5′ site of 5′-GG-3′ sequences in DNA by UV radiation in the presence of a sensitizer (sens).

our experimental conditions. It has been reported that 8-oxo-dG lesions in DNA are piperidine-labile (Chung *et al*., 1992). Therefore, it is reasonable to assume that pterins induce hydroxylation at the C-8 of guanine located 5′ to guanine with exposure to 365-nm light.

In order to clarify the photosensitization mechanism, we examined the effect of D_2O, in which the lifetime of $^1O_2$ is 10 times or more longer than in H_2O (Foote, 1979). Under the conditions we employed, an enhancing effect of D_2O was not observed in either DNA sequence-specific cleavage or 8-oxo-dG formation. These results suggest that $^1O_2$ plays only a little, if any, role in pterin-sensitized DNA damage. D_2O actually reduced the photodamage; the reason for this reduction remains unclear at present. By ESR spectroscopy, the reactivity of photoexcited pterins with mononucleotides was examined. The method used is based on the reaction between 4-oxo-TEMPO, a stable nitroxide radical, and reducing radicals, pterin anion radicals in the present study, leading to a decrease in the nitroxide ESR signal amplitude. The present results suggest that photoexcited pterins react preferentially with dGMP to produce a pterin anion radical and a guanine cation radical. The reactivity of the photoexcited pterin derivatives with dGMP decreased in the following order: pterin > neopterin > folic acid. This is similar to the order in which the pterin derivatives induce DNA photodamage. The small discrepancy between the reactivity of folic acid with dGMP and the extent of folic acid-induced photodamage in isolated DNA may be due to steric hindrance caused by the bulky side chains of folic acid when in close proximity to the DNA double helix. This view is consistent with the finding that pterin-induced DNA photodamage was reduced in the presence of magnesium or spermine, which bind nonspecifically to DNA as counterions. Therefore, it is possible that pterin-sensitized sequence-specific DNA photodamage occurs predominantly through an electron transfer reaction. It has been suggested that the guanine cation radical generated by photosensitization or by γ-irradiation may react with water to give the C-8 OH adduct radical (Symons, 1987; Boiteux *et al*., 1992). In addition, Kasai *et al*. (1992) have demonstrated by an isotopic experiment that the guanine cation radical formed in DNA by photoinduced electron transfer predominantly undergoes a hydration reaction, leading to the formation of the C-8 OH adduct radical. Formation of 8-oxo-dG involves the one-electron oxidation of the C-8 OH adduct radical of guanine (Steenken, 1989). Therefore, it is possible to assume that, as illustrated in Figure 11B, photoexcited pterins react with the consecutive guanine residues within DNA to produce a guanine cation radical, which subsequently undergoes a

hydration reaction to yield the C-8 OH adduct radical, and ultimately 8-oxo-dG, at the 5′ side. Similar conclusions were reached in our earlier studies with riboflavin (Ito *et al*., 1993). On the basis of these studies, it is proposed here that some chemical classes of photosensitizers induce hydroxylation at the C-8 of guanine located 5′ to guanine upon exposure to 365-nm light by a mechanism similar to that shown in Figure 11B. It should be noted that the C-8 OH adduct radical of guanine can also be converted into a formamidopyrimidine, involving ring opening of the C-8 OH adduct radical and one-electron reduction (Steenken, 1989).

A possible mechanism for the sequence-specific hydroxylation of deoxyguanosine in DNA could be explained as follows. The present ESR experiments showed that the photoexcited pterin derivatives reacted with monoguanine, which contrasted with the results of the DNA sequencing experiments where DNA damage by these pterins occurred at the 5′ site of contiguous guanines but not at isolated guanines. These results suggest that the specific binding of pterins to polyguanine is not necessary for electron transfer from guanine to the photoexcited pterins. In addition, the fluorescence of pterin was not quenched by the addition of DNA, suggesting that the association of pterin to DNA by intercalation or strong binding in a groove of the DNA helix is unlikely. Therefore, it may be considered that the absence of photodamage at isolated guanines is due to the high rate of back electron transfer between radical ion pairs consisting of a single guanine residue and a sensitizer (Figure 11A). Recently, Sugiyama and Saito (1996) performed molecular orbital calculations on stacked dinucleotide base pairs and demonstrated that the stacking of two guanine bases in B form DNA significantly lowers the ionization potential; the GG sequence was shown to have the lowest ionization potential among the seven guanine-containing base pairs studied. These workers have also reported that electron-loss centers are localized on the 5′-G of 5′-GG-3′. These theoretical results could explain the sequence specificity of the pterin-sensitized guanine damage at the 5′-GG-3′ sequence by an electron transfer mechanism. In this study, we observed that single-stranded DNA was less damaged than double-stranded DNA and that the cleavage induced in single-stranded DNA was at guanines, but not specifically at the 5′ site of 5′-GG-3′ sequences. Also, pterin-induced photodamage in double-stranded DNA was reduced in the presence of minor-groove binders (distamycin A and berenil), which can locally alter the DNA structure. These observations also suggest that the sequence-specific DNA photodamage by pterins was due to the stacking interaction of consecutive guanines in the DNA duplex.

Sepiapterin, a model compound of the dihydropterins, was also found to induce sequence-selective DNA photolesions similar to those observed with the fully oxidized pterins. In the sepiapterin-induced DNA photolesions, however, the addition of Cu(II) produced a significant increase in DNA damage that showed little preference for contiguous guanines. The preferential piperidine-labile sites were similar to those induced with Cu(II) plus H_2O_2 (Yamamoto & Kawanishi, 1989). The inhibitory effects of catalase and bathocuproione, a chelating agent for Cu(I), on the DNA photolesions with sepiapterin plus Cu(II) also indicate the involvement of H_2O_2 and Cu(I) in the sepiapterin-induced DNA damage. It is, therefore, speculated that photoexcited sepiapterin reacted with oxygen to form the superoxide anion (Foote, 1991), which subsequently autodismutated to H_2O_2, and active species generated from the reaction of H_2O_2 with Cu(I) caused the DNA photodamage (Yamamoto & Kawanishi, 1989).

Although various attempts have been made to elucidate the photosensitization effects of solar UV on cellular DNA, the nature of the DNA photoproducts, as well as their role in the carcinogenic process, still remains unclear. It has been shown that single- and double-strand breaks and DNA−protein cross-links are formed in cells exposed to UVA through mainly oxygen-involving mechanisms (Peak *et al*., 1987; Kochevar & Dunn, 1990; Cadet *et al*., 1992). In addition, Hattori-Nakakuki *et al*. (1994) have recently reported an increase in 8-oxo-dG in the epidermis of hairless mice upon exposure to near-UV light. Pflaum *et al*. (1994) have also reported that DNA damage induced in mammalian cells by visible light consists predominantly of oxidized bases sensitive to formamidopyrimidine DNA glycosylase, particularly 8-oxo-dG. In the present study, we have demonstrated that the 5′-G of 5′-GG-3′ sequences is a hotspot for the formation of 8-oxo-dG by various structurally unrelated compounds through an electron transfer reaction. These results suggest that 8-oxo-dG may play a role in the induction of skin cancers. The extent to which 8-oxo-dG formation is involved in human carcinogenicity may be greatly reduced, since this modification is expected to be excised efficiently in mammalian cells. However, if the repair system is modulated, then 8-oxo-dG formation through electron transfer by various endogeneous and exogenous compounds could have important implications regarding sunlight-induced skin cancers. It is known that a variety of endogenous and exogenous chromophores can be activated with exposure to solar UV radiation (Cadet *et al*., 1992; Kochevar & Dunn, 1990). Therefore, it is likely that the final DNA photoproducts generated during sunlight exposure would vary considerably among individuals, not only due to the efficiencies of the cellular defense systems, such as the levels of repair enzymes, but also due to the differences in the type and amount of chromophore present in human skin cells.

REFERENCES

Ananthaswamy, H. N., & Pierceall, W. E. (1990) *Photochem. Photobiol. 52*, 1119−1136.

Brash, D. E., Rudolph, J. A., Simon, J. A., Lin, A., Makenna, G. J., Baden, H. P., Halperin, A. J., & Ponten, J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10124−10128.

Boiteux, S., Gajewski, E., Laval, J., & Dizdaroglu, M. (1992) *Biochemistry 31*, 106−110.

Cadet, J., Berger, M., Decarroz, C., Wagner, J. R., van Lier, J. E., Giont, Y. M., & Vigny, P. (1986), *Biochimie 68*, 813−834.

Cadet, J., Anselmino, C., Douki, T., & Voituriez, L. (1992) *J. Photochem. Photobiol. B: Biol. 15*, 277−298.

Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H., & Goeddel, D. V. (1983) *Nature 302*, 33−37.

Cheng, K. C., Cahill, D. S., Kasai, H., Nishimura, S., & Loeb, L. A. (1992) *J. Biol. Chem. 267*, 166−172.

Chung, M.-H., Kiyosawa, H., Ohtsuka, E., Nishimura, S., & Kasai, H. (1992) *Biochem. Biophys. Res. Commun. 188*, 1−7.

Coohill, T. P., Peak, M. J., & Peak, J. G. (1987) *Photochem. Photobiol. 46*, 1043−1050.

Foote, C. S. (1979) in *Biochemical and Clinical Aspects of Oxygen* (Caughey, W. S., Ed.) pp 601−626, Academic Press, New York.

Foote, C. S. (1991) *Photochem. Photobiol. 54*, 659.

Hattori-Nakakuki, Y., Nishigori, C., Okamoto, K., Imamura, S., Hiai, H., & Toyokuni, S. (1994) *Biochem. Biophys. Res. Commun. 201*, 1132−1139.

International Agency for Research on Cancer (1992) *IARC monographs on the evaluation of carcinogenic risks to humans, Solar and UV radiation*, Vol., 55, International Agency for Research on Cancer, Lyon, France.

Ito, K., Yamamoto, K., & Kawanishi, S. (1992) *Biochemistry 31*, 11606−11613.

Ito, K., Inoue, S., Yamamoto, K., & Kawanishi, S. (1993) *J. Biol. Chem. 268*, 13221−13227.

Johnson, J. L., Hamm-Alvarez, S., Payne, G., Sancar, G. B., Rajagopalan, K. V., & Sancar, A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2046−2050.

Jones, C. A., Huberman, E., Cunningham, M. L., & Peak, M. J. (1987) *Radiat. Res. 110*, 244−254.

Kasai, H., Nishimura, S., Kurokawa, Y., & Hayashi, Y. (1987) *Carcinogenesis 8*, 1959−1961.

Kasai, H., Yamaizumi, Z., Berger, M., & Cadet, J. (1992) *J. Am. Chem. Soc. 114*, 9692−9694.

Kochevar, I. E., & Dunn, D. A. (1990) in *Bioorganic Photochemistry I* (Morrison, H., Ed.) pp 273−315, John Wiley & Sons, New York.

Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499−560.

Moan, J. (1980) *Acta Chem. Scand. B 34*, 519−521.

Nichol, C. A., Smith, G. K., & Duch, D. S. (1985) *Annu. Rev. Biochem. 54*, 729−764.

Peak, M. J., Peak, J. G., & Carnes, B. A. (1987) *Photochem. Photobiol. 45*, 381−387.

Pfaum, M., Boiteux, S., & Epe, B. (1994) *Carcinogenesis 15*, 297−300.

Pierceall, W. E., Goldberg, L. H., Tainsky, M. A., Mukhopadhyay, T., & Ananthaswamy, H. N. (1991) *Mol. Carcinogen. 4*, 196−202.

Reszka, K., & Sealy, R. C. (1984) *Photochem. Photobiol. 39*, 293−299.

Sancar, A., & Sancar, G. B. (1988) *Annu. Rev. Biochem. 57*, 29−67.

Schallreuter, K. U., Wood, J. M., Pittelkow, M. R., Gutlich, M., Lemke, K. R., Rodl, W., Swanson, N. N., Hitzemann, K., & Ziegler, I. (1994) *Science 263*, 1444−1446.

Setlow, R. B., Grist, E., Thompson, K., & Woodhead, A. D. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6666−6670.

Shibutani, S., Takeshita, M., & Grollman, A. P. (1991) *Nature 349*, 431−434.

Steenken, S. (1989) *Chem. Rev. 89*, 503−520.

Sugiyama, H., & Saito, I. (1996) *J. Am. Chem. Soc. 118*, 7063−7068.

Suzuki, F., Han, A., Lankas, G. R., Utsumi, H., & Elkind, M. M. (1981) *Cancer Res. 41*, 4916−4924.

Symons, M. C. (1987) *J. Chem. Soc., Faraday Trans. 1 83*, 1−11.

Tyrrell, R. M., & Keyse, S. M. (1990) *J. Photochem. Photobiol. B: Biol. 4*, 349−361.

van der Schroeff, J. G., Evers, L. M., Boot, A. J. M., & Bos, J. L. (1990) *J. Invest. Dermatol. 94*, 423−425.

Yamamoto, K., & Kawanishi, S. (1989) *J. Biol. Chem. 264*, 15435−15440.

Yamamoto, K., Inoue, S., Yamazaki, A., Yoshinaga, T., & Kawanishi, S. (1989) *Chem. Res. Toxicol. 2*, 234−239.

BI9620161