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Photochemistry and Photobiology of Furocoumarin Hydroperoxides Derived from Imperatorin: Novel Intercalating Photo-Fenton Reagents for Oxidative DNA Modification by Hydroxyl Radicals*

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ABSTRACT

Photochemical and photobiological properties of the imperatorin-derived furocoumarin hydroperoxides 1a, 1a′, 2a and 2a′ have been investigated. Irradiation (350 nm) of the hydroperoxide 2a′ afforded the alcohol 2b (2%), a diastereomeric mixture of the hydroxy epoxide 2c (40%; diastereomeric ratio = 80:20) and the epoxide 2d (8%). The formation of these products was rationalized in terms of homolysis of the hydroperoxide bond initiated by intramolecular energy transfer from the photoexcited furocoumarin chromophore. The quantum yields for the photolytic decomposition of hydroperoxides were estimated to be in the range of 0.03–0.85 and decreased in the order 2a ≫ 2a′ ≫ 1a′ ≥ 1a. The involvement of hydroxyl radicals in these reactions was established by trapping experiments with benzene and spectroscopic evidence was obtained by EPR spin trapping with 5,5-dimethylpyrroline-*N*-oxide. Fluorescence titration, DNA melting and linear dichroism studies of furocoumarins indicated that these compounds undergo efficient complexation and also intercalation into the DNA. The binding parameters *K* (intrinsic binding constant) and 1/n (frequency of binding sites) of complexes between furocoumarin derivatives and DNA were determined to be in the range of 3900–23 900 M^{-1} and 0.017–0.045. The photoreaction of 1a′ and 1b′ with 2′-deoxyguanosine (dGuo) afforded exclusively 7,8-dihydro-8-oxo-2′-deoxy-guanosine (8-oxodGuo), presumably through singlet oxygen, which was formed in a type II photooxidation process. In contrast, the hydroperoxide 2a oxidized dGuo to oxazolone as major and 8-oxodGuo as minor products through hydroxyl radicals, which were generated from 2a under photolytic conditions. Interestingly, the photoreactions of furocoumarins with salmon testes DNA showed that the highly reactive (ϕ = 0.85) hydroperoxide 2a is also most efficient in inducing the mutagenic DNA oxidation product 8-oxodGuo. Hence, the novel furocoumarin hydroperoxide 2a constitutes the first intercalating photo-Fenton reagent and serves as convenient hydroxyl radical source for genotoxicity studies.

INTRODUCTION

The importance of oxidative DNA damage (1) in mutagenesis, carcinogenesis, aging and various related diseases prompted in the last decade intensive investigations of the chemical, biochemical and biological aspects of DNA oxidations caused by reactive oxygen species such as hydroxyl and alkoxyl radicals (2,3), singlet oxygen (4) and superoxide ion (3), which are involved in oxidative stress (5). Among the reactive oxygen species, hydroxyl radicals are implicated as the key oxidizing reagents of DNA and other biological molecules (2,5,6). Therefore, it is of great importance to elucidate their role in oxidative cell damage. The conventional chemical sources of hydroxyl radicals, for example H_2O_2/Fe^{2+} (Fenton reaction) (7) or radiolysis of aqueous solutions (8) are not properly suited for biochemical or biological investigations because they generate, besides hydroxyl radicals, other reactive oxygen species (8). In recent years, phthalimide hydroperoxides were developed as photochemical hydroxyl radical sources (photo-Fenton reagents) (9,10), which circumvent the use of transition metals and γ irradi-

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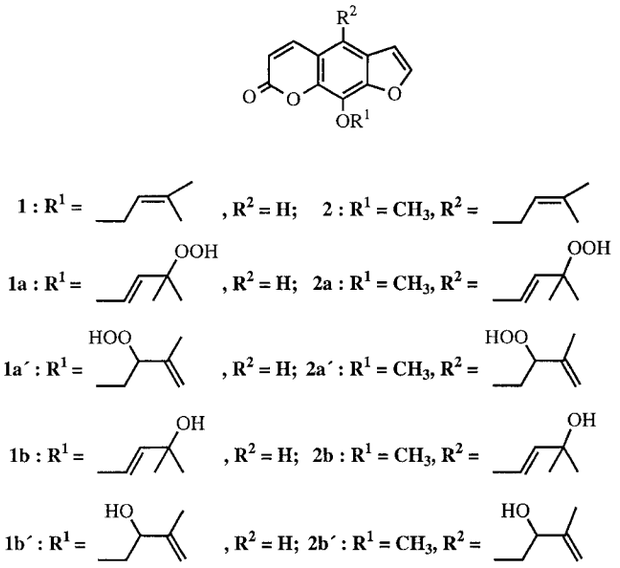

Figure 1. Chemical structures of the furocoumarins investigated.

ation in genotoxicity studies (11). Moreover, heterocyclic *N*-oxides (12) and *N*-hydroxypyridinethiones (13,14) were reported as nonperoxidic photochemical sources of hydroxyl radicals. However, an efficient, clean and facile source of hydroxyl radicals is still required for biochemical and biological studies.

In this context, our strategy has been to incorporate the photolabile hydroperoxide group into an intercalating chromophore such as furocoumarin (15), which absorbs in the UVA region ($\lambda \geq 350$ nm) to provide an effective hydroxyl radical source for the investigation of oxidative DNA damage. Recently we reported (16,17) that the furocoumarin hydroperoxides **1a** and **1a′** as well as **2a** and **2a′**, which are readily available by photooxygenation (18) of imperatorin (**1**) or the alloimperatorin derivative **2** (Fig. 1), cause single-strand breaks (SSB)§ and endonuclease-sensitive DNA modifications in supercoiled bacteriophage *PM2* DNA upon UVA irradiation ($\lambda = 360$ nm). To establish that furocoumarin hydroperoxides generate hydroxyl radicals under photolytic conditions and they intercalate into the DNA, we have studied in detail the photochemical and intercalating properties of the furocoumarin derivatives **1** and **2**. Furthermore, to elucidate the chemical nature of the DNA modifications and to provide mechanistic insight into the oxidation of nucleic acids by furocoumarin hydroperoxides upon irradiation (350 nm), also salmon testes DNA and 2′-deoxyguanosine (dGuo) were employed. Here we report that the hydroperoxides derived from imperatorin (**1**) and alloimperatorin derivative **2** are, indeed, effective photochemical sources of hydroxyl radicals and they undergo intercalation into the DNA. Hence, these novel furocoumarin hydroperoxides constitute the first intercalating, photo-Fenton (10) reagents and upon UVA irradiation they induce in salmon testes DNA significant amounts of the mutagenic (19–21) DNA oxidation product 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodGuo).

§*Abbreviations:* dGuo, 2′-deoxyguanosine; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; d.r., diastereomeric ratio; FPG, formamidopyrimidine-DNA glycosylase; 4-HO-8-oxodGuo, 4*R** and 4*S** diastereomers of 9-(2-deoxy-β-D-*erythro*-pentofuranosyl)-4-hydroxy-8-oxo-7,8-dihydroguanine; LD, linear dichroism; 8-MOP, 8-methoxypsoralen; mp, melting point; NQS, 1,2-naphthoquinone-4-sulfonic acid; oxazolone, 2,2-diamino-[(2-deoxy-β-D-*erythro*-pentofuranosyl)-4-amino]-5(2*H*)-oxazolone; 8-oxodGuo, 7,8-dihydro-8-oxo-2′-deoxyguanosine; RPR; Rayonet photoreactor; SSB, single-strand breaks.

MATERIALS AND METHODS

General aspects. Infrared spectra were recorded on a Perkin-Elmer infrared spectrophotometer 1420 and the ^1H and ^{13}C NMR spectra were run on a Bruker WM 250 or a Bruker AC 200 NMR spectrometer. Elemental analyses were performed in the Microanalytical Laboratory of the Institute of Inorganic Chemistry, University of Würzburg. For TLC runs, Polygram SIL G/UV_{254} foils from Machery and Nagel (Düren, Germany) were used. The hydroperoxides were detected by 10% aqueous KI solution and for the other products a 5% ethanolic solution of molybdophosphoric acid was employed. Purifications by column chromatography were conducted on silica gel (63–200 μm) from Woelm (Erlangen, Germany) with an adsorbent/substrate ratio of 50:1. Commercial solvents and reagents were purified according to procedures found in the literature. Petroleum ether of boiling range 30–50°C was used.

Chemicals. The furocoumarin hydroperoxides **1a**, **1a′**, **2a** and **2a′** were prepared as described previously (18) by photooxygenation of imperatorin (**1**) and alloimperatorin derivative **2**. The corresponding alcohols were prepared from hydroperoxides by reduction with triphenylphosphine. 2′-Deoxyguanosine and 1,2-naphthoquinone-4-sulfonic acid and (NQS) were purchased from Sigma Chemical Co. (St. Louis, MO). 7,8-Dihydro-8-oxo-2′-deoxyguanosine was synthesized by hydrogenation of 8-(benzoyloxy)-2′-deoxyguanosine (22) and 2,2-diamino-[(2-deoxy-β-D-*erythro*-pentofuranosyl)-4-amino]-5(2*H*)-oxazolone (oxazolone) was prepared by γ irradiation or benzophenone-sensitized photooxidation of dGuo (23). The 4*R** and 4*S** diastereomers of 9-(2-deoxy-β-D-*erythro*-pentofuranosyl)-4-hydroxy-8-oxo-7,8-dihydroguanine (4-HO-8-oxodGuo) were obtained from methylene blue-mediated singlet oxygen reaction of dGuo (24).

Salmon testes DNA was purchased from Sigma and dissolved in buffer solution (pH 7.0), which contained 2 m*M* NaCl and 1 m*M* EDTA, unless mentioned otherwise. The concentrations of DNA solutions were determined by using an extinction coefficient of 6600 M^{-1} cm^{-1} at 260 nm and are expressed in terms of nucleotide equivalents per liter. The sample hypochromicity (>40%) was determined according to Marmur and Doty (25).

Phototransformation of 2a. A solution of furocoumarin hydroperoxide **2a** (105 mg; 0.330 mmol) in dry acetonitrile (70 mL) was purged with nitrogen gas for 30 min. The solution was irradiated with a Rayonet photoreactor (RPR) at 350 nm for 3 h in a Pyrex vessel at 22°C. The solvent was removed by rotary evaporation (0°C, 18 Torr) and the product mixture was separated by silica gel chromatography. By eluting with a 1:3 mixture of petroleum ether and ethyl acetate, 2 mg (2%) of the alcohol **2b**, 42 mg (40%) of a diastereomeric mixture of hydroxy epoxide **2c** (diastereomeric ratio [d.r.] 80:20), 8 gm (8%) of the epoxide **2d**, melting point (mp) 118–120°C [literature (26) mp 117–120°C] and 26 mg (25%) of undefined higher molecular weight material were obtained.

Alcohol **2b**: IR (KBr) ν_{max} 3500–3200 (br), 3100, 2940, 2900, 1700, 1680, 1600, 1570, 1455, 1360, 1295, 1150 and 1130 cm^{-1}; ^1H NMR ($CDCl_3$, 250 MHz) δ 1.51 (s, 6H), 1.55 (s, 1H, D_2O exchangeable), 4.20 (s, 3H), 6.21 (d, 1H, J = 16 Hz), 6.29 (d, 1H, J = 10 Hz), 6.86 (d, 1H, J = 2 Hz), 6.93 (d, 1H, J = 16 Hz), 7.63 (d, 1H, J = 2 Hz) and 8.00 (d, 1H, J = 10 Hz); ^{13}C NMR ($CDCl_3$, 63 MHz) δ 30.1 (q), 61.4 (q), 71.4 (s), 106.4 (d), 113.9 (s), 114.0 (d), 119.5 (d), 122.4 (s), 124.5 (s), 131.8 (s), 139.4 (d), 141.3 (s), 145.7 (d), 146.4 (d), 147.5 (s) and 160.3 (s). Analysis calculated for $C_{17}H_{16}O_5$: C, 67.99; H, 5.37. Found: C, 67.66; H, 5.29.

*R*S**-**2C**: IR (KBr) ν_{max} 3600–3200 (br), 3100, 2940, 2900, 1715, 1605, 1570, 1450, 1410, 1370, 1150 and 1120 cm^{-1}; ^1H NMR ($CDCl_3$, 250 MHz) δ 1.22 (s, 3H), 1.37 (s, 3H), 2.77 (d, 1H, J = 2 Hz, D_2O exchangeable), 3.17 (d, 1H, J = 7.5 Hz), 4.23 (s, 3H), 5.09

(dd, 1H, J_{AB} = 7.5 Hz, J_{AX} = 2 Hz), 6.35 (d, 1H, J = 10 Hz), 6.97 (d, 1H, J = 2 Hz), 7.65 (d, 1H, J = 2 Hz) and 8.31 (d, 1H, J = 10 Hz); ^{13}C NMR ($CDCl_3$, 63 MHz) δ 19.7 (q), 24.7 (q), 61.1 (s), 61.4 (q), 66.7 (d), 68.9 (d), 106.2 (d), 114.3 (d), 114.7 (s), 122.5 (s), 125.2 (s), 132.8 (s), 140.9 (d), 141.5 (s), 146.5 (s), 147.3 (d) and 159.8 (s).

*R*R**-**2C**: IR (KBr) ν_{max} 3600–3200 (br), 3100, 2940, 2900, 1710, 1600, 1570, 1455, 1370 and 1295 cm^{-1}; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.32 (s, 3H), 1.50 (s, 3H), 2.53 (d, 1H, J = 2 Hz, D_2O exchangeable), 3.04 (d, 1H, J = 8.2 Hz), 4.18 (s, 3H), 5.11 (dd, 1H, J_{AB} = 8.2 Hz, J_{AX} = 2 Hz), 6.25 (d, 1H, J = 10 Hz), 7.10 (d, 1H, J = 2 Hz), 7.60 (d 1H, J = 2 Hz), 8.15 (d, 1H, J = 10 Hz); ^{13}C NMR ($CDCl_3$, 63 MHz) δ 18.8 (q), 24.6 (q), 60.4 (s), 61.5 (q), 66.0 (d), 69.6 (d), 106.7 (d), 113.9 (s), 114.2 (d), 124.5 (s), 124.6 (s), 131.0 (s), 141.0 (d), 143.5 (s), 146.2 (d), 153.9 (s) 159.9 (s). Analysis (for the mixture) calculated for $C_{17}H_{16}O_6$: C, 64.55; H, 5.06. Found: C, 64.19; H, 5.00.

In an independent experiment, the photolysis of *trans*-**2a** (50 mg; 0.160 mmol) was carried out in acetonitrile (12 mL) in the RPR at 350 nm under argon atmosphere for 1 h at 0°C. The solvent was removed by rotary evaporation (0°C, 18 Torr) and the crude reaction mixture was analyzed by ^{1}H NMR spectroscopy by using 1,4-dichlorobenzene as an internal standard in $CDCl_3$. The *cis*-**2a** and a diastereomeric mixture of **2c** (d.r. = 83:17) were formed in a ratio of 74:26 and a total mass balance of 84% at 79% conversion of *trans*-**2a**.

Cis-**2a**: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.10 (s, 6H), 4.27 (s, 3H), 6.25 (d, 1H, J = 12 Hz), 6.32 (d, 1H, J = 10 Hz), 6.60 (d, 1H, J = 12 Hz), 6.76 (d, 1H, J = 2 Hz), 7.66 (d, 1H, J = 2 Hz) and 7.97 (d, 1H, J = 10 Hz).

Cis–trans photoisomerization of ***2b***. A solution of *trans*-**2b** (10.1 mg; 0.034 mmol) in $CDCl_3$ (1 mL) was irradiated in the RPR at 350 nm under argon atmosphere at 22°C. The reaction mixture was monitored by ^{1}H NMR spectroscopy. After 2.5 h irradiation, a photostationary *cis/trans* mixture (80:20 ± 5) was formed.

Cis-**2b**: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.22 (s, 6H), 4.26 (s, 3H), 6.15 (d, 1H, J = 12 Hz), 6.34 (d, 1H, J = 10 Hz), 6.44 (d, 1H, J = 12 Hz), 6.76 (d, 1H, J = 2 Hz), 7.66 (d, 1H, J = 2 Hz) and 7.96 (d, 1H, J = 10 Hz).

Quantum yields. The quantum yields for the decomposition of the hydroperoxides were determined by potassium ferrioxalate actinometry (27). The decomposition of the hydroperoxides was monitored by ^{1}H NMR spectroscopy and/or by iodometry. A solution of the hydroperoxide (2.64 m*M*) in acetonitrile was irradiated in the RPR (λ = 350 nm) at 22°C. The solvent was removed under reduced pressure (0°C, 18 Torr) and the decomposition of hydroperoxide was quantified by iodometry or by ^{1}H NMR spectroscopy by using hexamethyldisiloxane as an internal standard.

Hydroxyl radical trapping experiments. A solution of furocoumarin hydroperoxide **2a** (3.16 mg; 0.01 mmol) in benzene (70 mL) was irradiated in the RPR at 350 nm for 3 h at 22°C under a nitrogen gas atmosphere. The solvent was removed by rotary evaporation (0°C, 18 Torr), the residue was dissolved in dichloromethane (1 mL) and analyzed by gas chromatography (column OV 1701 [I.D. = 0.25 nm]; N_2 [0.75 kg/cm^2]; air [1.1 kgm/cm^2], H_2 [1.1 kgm/cm^2]; injector and detector temperature 200 and 220°C; oven temperature 400°C; naphthalene was used as an internal standard). Phenol was formed in 1.6% yield (t_R: 3.87 min).

In another experiment, **2a** (3.16 mg; 0.01 mmol) and adamantane (13.6 mg; 0.10 mmol) in acetonitrile (70 mL) were irradiated with an RPR (350 nm) for 3 h at 22°C. The solvent was removed by distillation and the residue was extracted with *n*-pentane (500 mL). After rotary evaporation (0°C, 18 Torr) of the solvent, the residue was analyzed for adamantane oxidation products by gas chromatography (injector and detector temperature 200 and 250°C; initial and final oven temperature 70 and 100°C with a temperature/time gradient of 1°C/min). By using dodecane as an internal standard, there were obtained 1-adamantanol (330 nmol, 3.3%, t_R: 23.06 min), adamantanone (165 nmol, 1.65%, t_R: 23.76 min) and 2-adamantanol (30 nmol, 0.3%, t_R: 29.13 min).

EPR studies. To a solution of furocoumarin hydroperoxide **2a** (10 μL of 6 m*M*) or **2a′** (10 μL of 12 m*M*) in acetonitrile was added 840 μL of water, 100 μL of sodium cacodylate buffer (20 m*M*) and 50 μL of 1 *M* 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). The reaction mixture was irradiated in the RPR at 350 nm for 15 min at 22°C and analyzed by EPR spectroscopy for the HO-DMPO adduct on a Bruker ESR 300 spectrometer.

Fluorescence measurements. Fluorescence measurements were carried out on a Perkin-Elmer LS-5 spectrofluorometer. The fluorescence titrations were performed in a buffer solution (pH 7.0) that contained 2 m*M* NaCl and 1 m*M* EDTA, at a constant furocoumarin concentration (3 μg/mL) by adding in batches a stock solution of DNA (4 m*M*), which contained 3 μg/mL furocoumarin.

The data of fluorescence quenching experiments were plotted according to the Stern–Volmer equation (Eq. 1), where I_o and I are the fluorescence intensities in the

$$I_o/I = 1 + K_q[DNA] \quad (1)$$

absence and presence of DNA. K_q is the Stern–Volmer quenching constant, which is a measure of the efficiency of fluorescence quenching by DNA. In the fluorescence enhancement experiments, the fluorescence intensities were quantified by plotting I/I_o as a function of the DNA concentration, which exhibit the high sensitivity of the fluorescence yields to the DNA concentration.

Computation of the binding parameters. The binding parameters of the complexes between the furocoumarin derivatives and DNA were determined from the fluorescence titration data. The method of computation involved an iterative procedure on the McGhee and von Hippel equation (Eq. 2) (28),

$$\frac{r}{c} = K(1 - nr)\left[\frac{1 - nr}{1 - (n - 1)r}\right]^{n-1} \quad (2)$$

where r is the extent of ligand molecules bound per unit amount of macromolecule at a free ligand concentration of c (*M*), *K* is the intrinsic binding constant to an isolated site, n is the number of nucleotides occluded by a furocoumarin molecule and 1/n is the frequency of binding sites. A program based on the least-squares method of the Taylor series expansion of the above equation was recycled until *K* and n changed by less than 1% to give final values, with a calculated binding isotherm at 5% saturation increments.

DNA melting studies. The thermal denaturation temperature (T_m) of DNA (0.45 m*M*) and in the presence of furocoumarin (20 μg/mL) with and without irradiation was determined on a Perkin-Elmer 571 spectrophotometer, which was equipped with a jacketed cuvette holder for this purpose. The absorbance of DNA at 260 nm was monitored with the rise of temperature in 2°C interval. The absorption data were then plotted as a function of temperature. Under these conditions, the average T_m value of DNA without additive is 58°C.

Linear flow dichroism (LD) measurements. A buffered (pH 7.0) DNA solution (3.8 m*M*), which contained 2 m*M* NaCl and 1 m*M* EDTA, was used for the LD measurements, either in the absence or presence of furocoumarin derivate (35 μg/mL). Orientation of the furocoumarin and DNA samples was achieved by employing a flow cell with an outer rotating cylinder. The LD values, which are the differential absorption of light polarized parallel ($A_\parallel$) and perpendicular ($A_\perp$) to the flow direction in the cell, were measured on a converted Jasco J500 circular dichroism spectrometer. The measuring device was designed by Wada and Kozawa (29). A constant shear gradient of 1000 s^{-1} was used for recording the LD spectra and the base line was taken at zero gradient.

Irradiation of dGuo with furocoumarins. To a solution of dGuo (1 m*M*) in water (2 mL) was added furocoumarin dissolved in ethanol (final concentration of ethanol was 1 vol%). The reaction mixture was irradiated with a black-light lamp (350 nm) in Pyrex tubes at 0°C for 0–60 min at a distance of 10 cm. The oxidation products of dGuo were analyzed as described below.

Irradiation of salmon testes DNA with furocoumarins. To a solution of DNA (50 μg/mL) in 5 m*M* phosphate buffer (pH 7.0) was added the furocoumarin dissolved in ethanol (final concentration of ethanol was 1 vol%). The reaction mixture was irradiated in an Eppendorf vessel with a black-light lamp (350 nm) at a distance of 10 cm at 0°C for 0–90 min. The modified DNA was digested with nuclease P1 and alkaline phosphatase and subsequently analyzed for 8-oxodGuo.

Analyses of guanine oxidation products. The 8-oxodGuo was analyzed by using a Dual model LC-4B/LC-17 A(T) amperometric detector (Bioanalytical System Inc., West Lafayette, IN) at an oxidation potential of 650 mV. The separation of 8-oxodGuo was

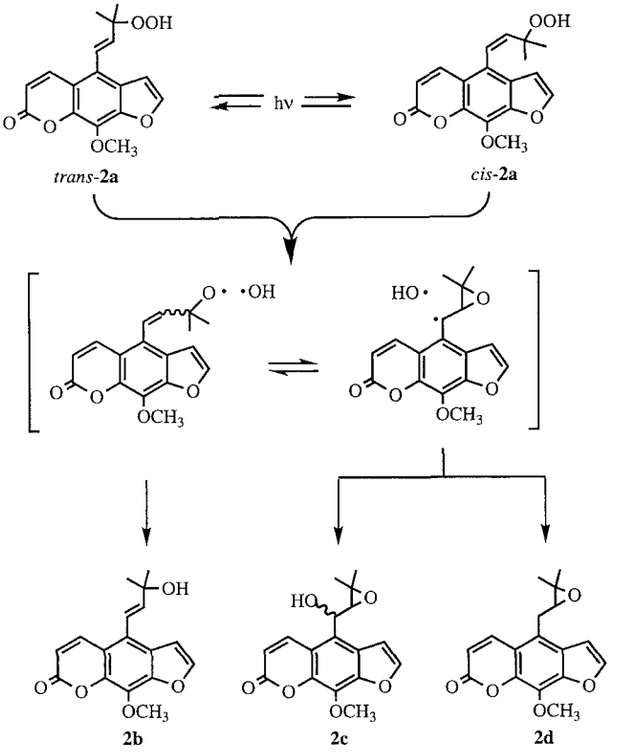

Figure 2. Chemical structures of the photoproducts of furocoumarin hydroperoxide **2a**.

achieved on a 250 × 4.6 mm (i.d.) Eurospher 100-C18 7 μm column by using a mixture of 0.05 *M* citrate buffer (pH 5.0) and methanol (80:20) (30,31). Quantification was achieved by means of external calibration with a standard solution of 8-oxodGuo.

The oxazolone, which releases guanidine upon alkaline treatment (23), was determined by employing a modified method described for the postcolumn detection of guanidine (32). For this purpose, 50 μL of NaOH (1 *M*) was added to 100 μL dGuo photolysate sample. The reaction mixture was kept in a water bath at 65°C for 10 min and 10 μL of freshly prepared NQS (8 mg/mL) was added. After 2 min at 65°C, the solution was neutralized with 55 μL of HCl (1 *M*) and the fluorescent product was separated on a C-18 reversed-phase column by using a 15:85 mixture of methanol and ammonium formate (0.025 *M*) as eluent. The excitation and emission wavelengths of the fluorescence detector (F 1000, Merck, Darmstadt, Germany) were set at 355 and 405 nm.

For the quantitative analysis of the two diastereomers of 4-HO-8-oxodGuo (type II photooxidation products), a 250 × 4.6 mm (i.d.) amino group-substituted silica gel 5 μm Lichrocart column (Merck, Darmstadt, Germany) was used by eluting with 20% of 0.05 *M* ammonium formate solution and 80% acetonitrile (33). The two diastereoisomers were monitored by means of an L4000 UV detector (Merck, Darmstadt, Germany) at 230 nm and the quantification was achieved by means of external calibration with a standard solution of 4-HO-8-oxodGuo.

RESULTS

Photochemistry

Of the furocoumarin hydroperoxides **1a**, **1a′**, **2a** and **2a′**, the derivative **2a** was the most active DNA-damaging agent as inferred from in the endonuclease assays (16). Therefore, as a representative example, the photochemistry of the hydroperoxide **2a** was studied in detail to corroborate the formation of hydroxyl radicals in the photolysis of this hydroperoxide and to provide mechanistic insight for their generation. Thus, irradiation of **2a** at ambient temperature for 3 h afforded the alcohol **2b** (2%), a diastereomeric mixture of hydroxy epoxide **2c** (40%; d.r. = 80:20) and the epoxide **2d** (8%), along with 25% of undefined higher molecular weight material (Fig. 2). Interestingly, when **2a** was irradiated at 0°C for 1 h, *cis*-**2a** and the hydroxy epoxide **2c** (d.r. = 83:17) were formed in a ratio of 74:26 with a mass balance of 84%. Irradiation of the corresponding alcohol **2b**, on the other hand, resulted after 2.5 h in a photostationary *cis/trans* mixture of 80:20 ± 5. The structures of the products were characterized by spectral data, elemental analyses and in some cases by independent synthesis. The stereochemistry of the major diastereomer of the hydroxy epoxide **2c** was assigned tentatively as *R*S** and of the minor one as *R*R** by comparison of their NMR data with that of the literature-known (3*R**4*S**)- and (3*R**4*R**)-configured epoxy alcohols (34).

Table 1. Quantum yields of the photodecomposition of furocoumarin hydroperoxides*

Hydroperoxide†	ε‡	ϕ§
1a	1580	0.03 ± 0.02
1a′	1350	0.07 ± 0.03 (0.06 ± 0.02)
2a	2530	0.85 ± 0.04 (0.96 ± 0.10)
2a′	2250	0.34 ± 0.05

*Based on potassium ferrioxalate actinometry (27).
†Furocoumarin hydroperoxides (2.64 m*M*).
‡Molar absorption coefficient (M^{-1} cm^{-1}) at λ_{max} 360 nm.
§Calculated for the disappearance of hydroperoxide in acetonitrile monitored by ^{1}H NMR spectroscopy, the values in parentheses were determined by iodometry.

Because great differences in the DNA damaging efficiency of the furocoumarin hydroperoxides (in the order **2a** > **2a′** ≫ **1a** ≥ **1a′**) were observed (16), we have determined the quantum yields (ϕ) for the photolytic decomposition of these hydroperoxides by ferrioxalate actinometry (27) (Table 1). The decomposition of the hydroperoxides was monitored by ^{1}H NMR spectroscopy by using hexamethyldisiloxane as an internal standard and/or by iodometry. As the data in Table 1 reveal, the quantum yields of the hydroperoxides coincide with their DNA damaging efficiency; thus, the most effective hydroperoxide **2a** possesses the highest ϕ (0.85 ± 0.04), while ϕ = 0.07 ± 0.03 was observed for the least reactive **1a′**.

Hydroxyl radical trapping studies

To provide direct evidence for the formation of hydroxyl radicals in the photolysis of furocoumarin hydroperoxides, trapping experiments with benzene, adamantane and DMPO were performed. Photolysis (λ = 350 nm) of the hydroperoxide **2a** (3.16 mg, 10 μmol) in benzene as solvent for 3 h under a nitrogen gas atmosphere afforded phenol in 1.6% yield (based on hydroperoxide consumption). However, we could not detect any oxidation products of adamantane, in contrast to the literature report (11,12), when **2a** was irradiated in the presence of adamantane under nitrogen gas at-

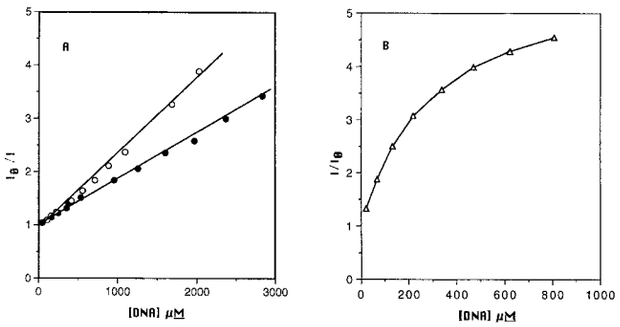

Figure 3. Fluorescence quenching (A) of furocoumarins **1a′** (open circles; 9.9 μ*M*; λ_{ex} = 302 nm and λ_{em} = 482 nm) and **1b′** (filled circles; 10.5 μ*M*; λ_{ex} = 304 and λ_{em} = 502 nm) and fluorescence enhancement (B) of furocoumarin **2a** (open triangles; 9.5 μ*M*; λ_{ex} = 336 and λ_{em} = 511 nm) as a function of DNA concentration.

mosphere. On the other hand, the photolysis under atmospheric conditions afforded 1-adamantanol (3.3%), 2-adamantanol (0.3%) and adamantanone (1.7%), which are photooxidation products rather than hydroxyl radical trapping products. Furthermore, EPR spectral evidence for the generation of hydroxyl radicals was obtained by spin trapping with DMPO. Thus, on irradiation (350 nm) of **2a** in the presence of DMPO in a 94:1 (vol/vol) mixture of water and acetonitrile, the characteristic (35) 1:2:2:1 quartet ($a_N = a_H$ = 14.90 ± 0.03 G and g = 2.0053) was observed for the resulting nitroxyl radical. A similar spectrum was also obtained for the hydroperoxide **2a′**.

Formation of molecular complexes with DNA

In order to establish whether the furocoumarin hydroperoxides examined herein undergo binding with DNA, a prerequisite for photoactivity directly in the DNA matrix (15,36), we have determined the binding parameters of the furocoumarin derivatives **1a′**, **1b′** and **2a**. The formation of a molecular complex between salmon testes DNA and furocoumarin hydroperoxide **1a′** and its alcohol **1b′** was confirmed by a strong quenching of their fluorescence as reported earlier for other intercalating furocoumarins (15,36,37). Thus, the fluorescence intensity of **1a′** and **1b′** (3 μg/mL) decreased rapidly with the increase in concentration of salmon testes DNA. The DNA titration data are plotted according to the Stern–Volmer equation (Eq. 1) as shown in Fig. 3A. The Stern–Volmer fluorescence quenching constants were calculated as 1300 M^{-1} for **1a′** and 836 M^{-1} for **1b′**. In contrast to **1a′** and **1b′**, a strong enhancement of the fluorescence was observed for the hydroperoxide **2a**. Thus, the addition of DNA to a solution of **2a** (3 μg/mL) showed a strong enhancement of fluorescence intensity, which was quantified by plotting I/I_0 as a function of the DNA concentration (Fig. 3B).

The binding parameters of the complexes between furocoumarins and DNA were determined by fluorescence titration experiments. From the titration data, the values of r and c (for definition *cf.* Eq. 2) were determined as described by Peacocke and Skerrett (38). The binding isotherms were calculated according to the method of McGhee and von Hippel (Eq. 2) (28) by using the data points of the Scatchard plot (39). From these isotherms, binding constants as well as

Table 2. Photophysical properties and binding parameters of the furocoumarins with salmon testes DNA*

Furocoumarin	Abs† (λ_{max}, nm)	Fl‡ (λ_{max}, nm)	T_m§ (°C)	K‖ (M^{-1})	1/n¶ 10^{-2}
1a′	300	487	60	6130	1.7
1b′	302	499	71	3900	2.2
2a	318	525	52	23900	4.5
8-MOP#	300	440	—	740	12.8

*Calculated according to McGhee and von Hippel (28).
†Absorption maxima in H_2O.
‡Fluorescence in H_2O (λ_{ex} was 301, 309 and 334 nm for **1a′**, **1b′**, and **2a**).
§Temperature at which the double helix denatures into single-stranded DNA after irradiation of DNA (0.45 m*M*) in the presence of furocoumarin (20 μg/mL) with a black-light lamp (350 nm) for 1 h; T_m = 58°C for DNA with and without irradiation and also with furocoumarins in the dark.
‖Intrinsic binding constant.
¶Frequency of binding sites.
#Taken from the literature (36).

number of binding sites were estimated and are shown in Table 2. The furocoumarin derivatives examined herein form molecular complexes with DNA more efficiently than 8-methoxypsoralen (8-MOP). The binding constant K was estimated to be 23900 M^{-1} for the highly reactive hydroperoxide **2a** and 6130 M^{-1} for the least reactive **1a′**. The binding affinity of the furocoumarins toward DNA was in the order **2a** ≫ **1a′** > **1b′** and is much higher than that of 8-MOP (K = 740 M^{-1}). However, the frequency of binding sites is in the range of 0.017–0.045, which is *ca.* three-times lower than for 8-MOP.

DNA melting studies

Intercalation of the furocoumarins into the DNA (in the dark) (40) and interstrand crosslink formation during irradiation are known to increase the DNA melting temperature (T_m) (15), *i.e.* the temperature at which the double helix denatures into single-stranded DNA. Thus, DNA melting studies under irradiation provide evidence for intercalation through crosslinking because the latter requires that the furocoumarin penetrates into the interior of the DNA double helix. Because the extinction coefficient of DNA at 260 nm in the double-helical form is much lower than in the single-stranded form, melting of the helix leads to an increase in the absorption at this wavelength. Thus, the helix-to-coil transition temperature can be determined by monitoring the absorbance of the DNA bases at 260 nm as a function of temperature (40).

As shown in Table 2, the T_m of DNA was increased from 58°C (without additive) to 60°C in the presence of the furocoumarin hydroperoxide **1a′** under irradiation conditions at a 1:1 molar ratio of DNA and **1a′**. In the presence of the corresponding alcohol **1b′** under similar conditions, the T_m of DNA was significantly increased to 71°C. The stabilization of the DNA by the furocoumarins **1a′** and **1b′** under irradiation may be due to the formation of crosslinks, which inhibit the deformation of the double-stranded DNA. In contrast, irradiation of DNA in the presence of highly reactive

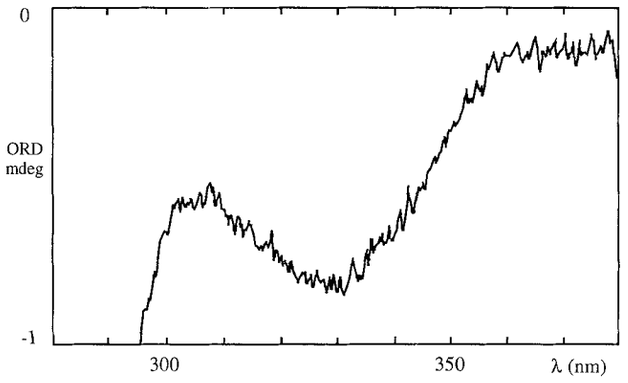

Figure 4. Linear flow dichroism of the **2a**–DNA complex formed in the dark at [DNA] = 3.8 m*M* and [**2a**] = 0.11 m*M* in an optical cell of 1 mm pathlength, measured on a Jasco J500 circular dichroism spectrophotometer converted for LD. A constant gradient of 500 s^{-1} was used for recording the LD spectrum and the baseline was taken at zero gradient.

hydroperoxide **2a** decreased the T_m of DNA to 56°C. The destabilization of DNA by **2a** under irradiation conditions is possibly caused by hydroxyl radicals, which were generated from **2a** photolytically. The DNA melting temperature (T_m = 58°C) did not change in the presence of furocoumarins in the dark.

LD measurements

Further evidence for the intercalation of the furocoumarin derivatives was obtained by LD (36,37,41) measurements on the DNA solution (3.8 m*M*) in the presence of furocoumarin (3 μg/mL). With this technique, the long and stiff molecule of DNA orients during the flow and a characteristic negative dichroism of the macromolecule is observed (29,41). When a planar molecule intercalates into the DNA, it assumes an ordered position similar to that of purine and pyrimidine bases. Thus, a negative dichroism for the chromophore of the intercalator (different from that of DNA) is displayed when its transition moment is polarized in parallel with the purine and pyrimidine bases (29).

A negative LD was observed between 310 and 370 nm for the derivative **2a** (Fig. 4), which indicates that **2a** intercalates into the DNA and it assumes a position parallel to the DNA bases (29). Similarly, a negative LD between 310 and 360 nm was obtained for the compounds **1a′** and **1b′** in the presence of DNA. These results are in accordance with those of the intercalating furocoumarins reported earlier 29,41).

Oxidation of dGuo and DNA by furocoumarin hydroperoxides

Recently we reported (16,17) that the furocoumarin hydroperoxides **1a**, **1a′**, **2a** and **2a′** efficiently caused SSB and formamidopyrimidine-DNA glycosylase (FPG)-sensitive oxidative modifications in supercoiled bacteriophage *PM2* DNA upon irradiation. To establish the chemical nature of the oxidative damage and to provide mechanistic insight into the DNA oxidation, we investigated in detail the formation of DNA oxidation products such as 8-oxodGuo (24,30,31,42),

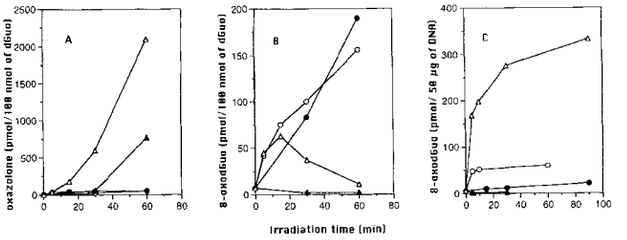

Figure 5. Formation of oxazolone (A) and 8-oxodGuo (B) in dGuo and 8-oxodGuo in salmon testes DNA (C) photoinduced by the furocoumarins as a function of time. Irradiation of dGuo (1 m*M*) in H_2O (2 mL) and DNA 50 μg/mL in 5 m*M* phosphate buffer (1 mL) with the furocoumarins by a black-light lamp (350 nm) at 0°C. Furocoumarins **1a′** (30 μ*M*, open circles), **1b′** (30 μ*M*, filled circles); **2a** (10 μ*M*, open triangles) and **2b** (10 μ*M*, filled triangles). Error *ca* ±10% of the stated values.

oxazolone (23,33,43) and the 4*R** and 4*S** diastereomers of 4-HO-8-oxodGuo (24,33) by the hydroperoxides **1a′** and **2a** and, for comparison, their alcohols **1b′** and **2b** in the mononucleoside dGuo and in salmon testes DNA.

The data for the time-dependent formation of oxazolone and 8-oxodGuo in dGuo by photolytic decomposition of furocoumarins **1a′**, **1b′**, **2a** and **2b** are presented in Fig. 5A,B. Oxazolone (2.1 nmol in 100 nmol of dGuo after 60 min irradiation) is the main oxidation product induced by the furocoumarin **2a** in dGuo. The corresponding alcohol **2b** also yielded oxazolone (0.77 nmol) but after longer irradiation (Fig. 5A), whereas **1a′** and **1b′** did not produce any oxazolone (detection limit *ca.* 0.05%). In contrast, **1a′** and **1b′** are very efficient in forming 8-oxodGuo (Fig. 5B) and its formation increased with irradiation time. The hydroperoxide **2a** also produced 8-oxodGuo but to a less extent and the formation of the latter decreased at longer irradiation time. The corresponding alcohol **2b,** however, did not generate any 8-oxodGuo.

In order to examine the concentration effect of the furocoumarins on the formation of oxazolone and 8-oxodGuo, irradiation of dGuo at various concentrations of furocoumarin hydroperoxides **1a′** and **2a′** was carried out for 10 min. Figure 6A shows that the oxazolone is again the main dGuo oxidation product caused by **2a**. The formation of oxazolone increases with increasing concentration of the hydroperoxide **2a** and reaches a plateau at 10 μ*M* concentration of **2a**. In contrast, the yield of the minor product 8-oxodGuo decreased at higher concentration (>10 μ*M*) of **2a**. Irradiation of furocoumarin **1a′** in the presence of dGuo yielded 8-oxodGuo as main oxidation product and its formation increased with increasing concentration of **1a′**. However, no oxazolone (a hydroxyl radical-mediated and type I photooxidation product of dGuo) (43) was produced by **1a′** even at 30 μ*M* concentration and after 60 min irradiation.

To establish what type of reactive oxygen species are involved in the oxidation of dGuo by furocoumarins upon irradiation, specific additives such as sodium azide (singlet oxygen quencher) (44) and sodium formate (hydroxyl radical scavenger) (45) were employed. Additionally, the effect of D_2O in place of H_2O was investigated to evaluate the role of singlet oxygen in the formation of oxidation products of dGuo, in particular 8-oxodGuo, which can be formed in a

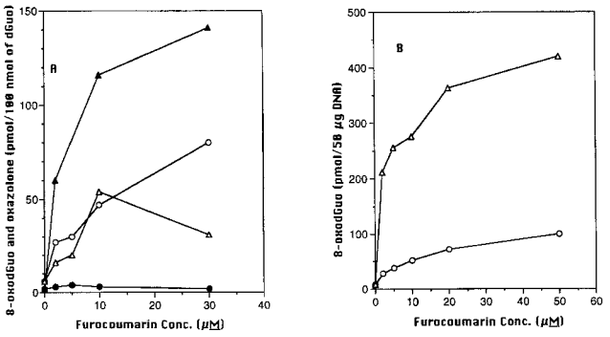

Figure 6. Formation of 8-oxodGuo and oxazolone in dGuo (A) and 8-oxodGuo in salmon testes DNA (B) photoinduced by furocoumarins as a function of concentration. Irradiation of furocoumarins with dGuo (1 m*M*) for 10 min in H_2O (2 mL) and DNA (50 μg/mL) in 5 m*M* phosphate buffer (1 mL) for 30 min by a black-light lamp (350 nm) at 0°C. Code for 6A: 8-oxodGuo in dGuo generated by **1a′** (open circles) and **2a** (open triangles); oxazolone in dGuo by **1a′** (closed circles) and **2a** (closed triangles); code for 6B: 8-oxodGuo in DNA by **1a′** (open circles) and **2a** (open triangles). Error *ca* ±10% of the stated values.

type II photooxidation process (24,43). The results are shown in Table 3.

The formation of 8-oxodGuo by the hydroperoxide **1a′** and its alcohol **1b′** was about 8-fold enhanced in D_2O *vs* H_2O (Table 3, entries 1 and 4), which corresponds with the 10-fold longer lifetime of singlet oxygen in D_2O (46). However, a small D_2O effect on 8-oxodGuo yield was observed in the case of the hydroperoxide **2a** (Table 3, entry 5). Furthermore, 8-oxodGuo formation by **1a′** was inhibited by *ca.* 60% (entry 2) in the presence of sodium azide (1 m*M*), whereas sodium formate did not affect the formation of 8-oxodGuo nor of oxazolone (entry 3).

Once it was established that furocoumarin hydroperoxides readily oxidize 2′-deoxyguanosine (dGuo) to oxazolone and 8-oxodGuo, we investigated the photooxidation of DNA by the hydroperoxides **1a′** and **2a**. These novel intercalating photo-Fenton reagents are effective in producing 8-oxodGuo in salmon testes DNA upon irradiation. The time (Fig. 5C) and concentration profiles (Fig. 6B) clearly reveal that the hydroperoxide **2a** is more efficient in inducing the mutagenic (19–21) DNA oxidation product 8-oxodGuo than the derivative **1a′**. A maximum yield of 8-oxodGuo (1.8% based on hydroperoxide consumption) was achieved at 20 μ*M* concentration of **2a** after 30 min irradiation. In contrast to the hydroperoxides **1a′** and **2a**, their alcohols **1b′** and **2b** did not yield any significant amounts of 8-oxodGuo under similar photolysis conditions. Without irradiation, the hydroperoxides **1a′** and **2a** did not oxidize the DNA.

To elucidate the reaction intermediates involved in the DNA oxidation, the photoreaction of furocoumarins **1a′** and **2a** was carried out with DNA in the presence of additives such as D_2O and *tert*-butanol (hydroxyl radical scavenger) (16). The results are shown in Table 4. In the presence of 2% *tert*-butanol, the 8-oxodGuo formation by **1a′** was inhibited by *ca* 35% (Table 4, compare entries 1 and 3), whereas in the case of the significantly more reactive hydroperoxide **2a**, 30% (compare entries 4 and 6) inhibition was observed. Furthermore, control experiments revealed that the extent of 8-oxodGuo generation in these reactions was not influenced by D_2O (not shown).

Table 3. Effect of additives on the formation of 8-oxodGuo and oxazolone in dGuo photoinduced by furocoumarins*

Entry	Furocoumarin†	Additive‡	Oxidation products§,‖ 8-OxodGuo	Oxazolone
1	**1a′**	D_2O	7.9 ± 0.5	1.6 ± 0.1
2	**1a′**	NaN_3	0.4 ± 0.1	0.4 ± 0.2
3	**1a′**	HCO_2Na	1.0 ± 0.1	1.2 ± 0.2
4	**1b′**	D_2O	8.2 ± 0.6	n.d.¶
5	**2a**	D_2O	1.4 ± 0.1	1.8 ± 0.2

*Irradiation of dGuo (1 m*M*) with the particular furocoumarin in H_2O or D_2O (2 mL) by a black-light lamp (350 nm) at 0°C.
†**1a′** (30 μ*M*), **1b′** (30 μ*M*), **2a** (10 μ*M*).
‡NaN_3 (1 m*M*), HCO_2Na (5 m*M*).
§Relative to the corresponding measurement without additive, which is taken as unity (1.0).
‖Data are averaged over at least two independent runs (±SD).
¶Not determined.

DISCUSSION

Hydroxyl radicals constitute the most reactive oxygen species that are involved in oxidative stress (5). To elucidate their role in oxidative cell damage, effective hydroxyl radical sources are required. In recent years, photochemical hydroxyl radical sources (photo-Fenton reagents) (10,17) were developed, which circumvent the use of transition metals and γ irradiation in genotoxicity studies (11,16,17). Our present photochemical and photobiological studies with furocoumarin hydroperoxides reveal that these hydroperoxides, the first intercalating photo-Fenton reagents, oxidize nucleic acids effectively upon irradiation.

The photolysis of hydroperoxide **2a** afforded the allylic alcohol **2b** and the epoxides **2c** and **2d**. The formation of these photoproducts can be rationalized in terms of the homolysis of the hydroperoxide moiety initiated by intramolecular energy transfer from the photoexcited furocoumarin chromophore as shown in Fig. 2. The prerequisite exothermicity for efficient energy transfer is fulfilled in that the triplet energy E_T of the furocoumarin chromophore (ex-

Table 4. Effect of *tert*-butanol concentration on 8-oxodGuo formation in salmon testes DNA induced by furocoumarin hydroperoxides upon irradiation*

Furocoumarin†	*tert*-butanol (%)	8-OxodGuo‡ (pmol/50 μg DNA)
1a′	0.0	72
1a′	0.5	62
1a′	2.0	48
2a	0.0	364
2a	0.5	300
2a	2.0	254

*DNA (50 μg/mL) irradiated in 5 m*M* phosphate buffer (1 mL) with the particular furocoumarin and *tert*-butanol for 30 min by a black-light lamp (350 nm) at 0°C.
†**1a′** (30 μ*M*), **2a** (10 μ*M*).
‡Blank was 8 pmol/50 μg DNA, error *ca* ±10% of the stated values.

ample for 8-MOP, E_T = 63 kcal/mol) (47) is higher than that of the hydroperoxide moiety (E_T *ca.* 53 kcal/mol) (48,49). The initial furocoumarin alkoxy radical intermediate formed after the homolysis of the hydroperoxide bond either abstracts a hydrogen atom to give the product **2b** or cyclizes to the epoxy benzyl radical (50). The latter may react with the hydroxyl radical generated by the homolytic cleavage of the hydroperoxide bond to give the diastereometric mixture of hydroxy epoxide **2c** or abstracts one hydrogen atom to form the epoxide **2d** (Fig. 2).

The quantum yields for the decomposition of furocoumarin hydroperoxides (Table 1) correspond with their efficiency in inducing FPG-sensitive modifications in *PM2* DNA (16). Thus, the most effective hydroperoxide **2a** possesses the highest quantum yield (0.85 ± 0.04) for the decomposition of hydroperoxide functionality. The observed differences in the reactivity of the furocoumarins, *i.e.* **2a** > **2a′** ≫ **1a** ≥ **1a′**, may be due to their different efficiencies in the intramolecular energy transfer. As an alternative, electron transfer from the triplet-excited furocoumarin chromophore to the hydroperoxide moiety (48,49) also may generate the furocoumarin alkoxy and the hydroxyl radicals through peroxide bond homolysis and electron back transfer. Furthermore, the molar absorptivity (ε) of these furocoumarins also contributes to their reactivity differences, as the more reactive 8-MOP derivatives **2a** and **2a′** have double the molar absorption than that of the less reactive imperatorin derivatives **1a** and **1a′** (Table 1).

Direct evidence for the formation of hydroxyl radicals in the photolysis of the hydroperoxide **2a** was obtained by trapping experiments with benzene. The low yield of phenol (1.6%, relative to hydroperoxide) in the photoreaction of **2a** in benzene under a nitrogen gas atmosphere may be rationalized in terms of further oxidation of phenol by hydroxyl radicals to catechol and 1,4-hydroquinone (51) and preferential dimerization of the initially formed hydroxycyclohexadienyl radicals in the absence of oxygen (52). Although it was reported (9,10) that adamantane is oxidized by hydroxyl radicals, no oxidation products of adamantane could be detected when **2a** was photolyzed with adamantane in the absence of oxygen. However, the photolysis under atmospheric conditions afforded adamantane oxidation products, which were presumably formed by the reaction of molecular oxygen with the adamantyl radicals (51) generated by hydrogen abstraction by hydroxyl radicals from the adamantane. The definitive spectroscopic proof for the intermediacy of hydroxyl radicals in the irradiation of **2a** or **2a′** was obtained from the EPR spin trapping experiments with DMPO. The only detectable EPR signal was that assignable to the spin adduct between the hydroxyl radical and DMPO (35).

The DNA titration experiments showed that the fluorescence intensity of furocoumarin hydroperoxide **1a′** and its alcohol **1b′** was quenched (Fig. 3A) with increasing DNA concentration, which is in accord with previously reported furocoumarins (36,37). Surprisingly, a strong enhancement of the fluorescence of **2a** (Fig. 3B) was observed in the presence of DNA. The diverging effects on the fluorescence intensity of the structurally similar furocoumarins is intriguing and further investigations will be required to clarify this point. Possibly, similar to ethidium bromide (53) and gilvocarcin (54), the fluorescence emission of furocoumarin **2a**

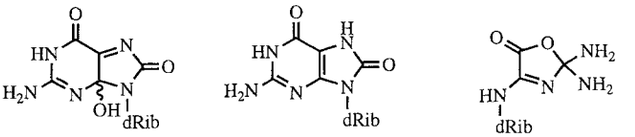

Figure 7. Chemical structures of various oxidation products of dGuo.

is enhanced because intercalation into DNA reduces self-quenching effects of the chromophore. Moreover, the *cis–trans* isomerization of **2a** might be efficiently restricted in the DNA matrix, which may also play a role.

The stabilization of DNA in the presence of furocoumarin alcohol **1b′** under irradiation, as evidenced by the increase of its melting temperature (T_m) by 13°C (Table 2), is substantially more effective than the 2°C rise by the corresponding hydroperoxide **1a′**. This pronounced stabilization of DNA by furocoumarin **1b′** under irradiation may be attributed to the efficient formation of interstrand crosslinks. Because intercalation of furocoumarins into the DNA is a prerequisite for the photolytic generation of crosslinks, we infer that the furocoumarin derivatives **1a′** und **1b′** intercalate into the DNA. In contrast, the DNA was destabilized (by 6°C) under similar conditions in the presence of highly reactive hydroperoxide **2a**. This destabilization of the DNA structure in the presence of hydroperoxide **2a** under irradiation conditions derives presumably from the efficient generation of hydroxyl radicals in the DNA matrix, which lead to DNA strand breaks.

Although it was reported that the substitution at the C5 position of the psoralen hinders the intercalation (55), the results of LD measurements and DNA melting studies clearly indicate that the substituted furocoumarin hydroperoxides **1a** and **1a′** (at C8 position) and **2a** and **2a′** (at C5 and C8 position) undergo intercalation into the DNA, a prerequisite for photoactivity directly in the DNA matrix (15,36). The negative LD curves observed for the furocoumarins **1a′**, **1b** and **2a** in the presence of DNA is in accord with other intercalating furocoumarins (29,41).

The photooxidation of dGuo by hydroperoxide **1a′** afforded 8-oxodGuo as the main product, but no oxazolone was formed; in contrast, **2a** photoxidized dGuo mainly to the oxazolone. These results imply that different mechanisms are involved in the oxidation of dGuo by hydroperoxides **1a′** and **2a**, which can function as photosensitizers for nucleic acid oxidation and also as hydroxyl radical sources.

The photosensitized oxidation of dGuo may proceed either directly through electron transfer and hydrogen atom abstraction (type I photooxidation) (43) or indirectly (energy transfer to triplet oxygen) through singlet oxygen (type II photooxidation) (33). The type I photooxidation of dGuo forms mainly oxazolone (33,43), while the type II photooxidation (singlet oxygen) generates the two diastereomers 4*R** and 4*S** of 4-HO-8-oxodGuo (24) as major and 8-oxodGuo (33) as a minor product (structures as shown in Fig. 7). However, 8-oxodGuo can be produced as a minor product also by type I photooxidation of the DNA (56). It is well established that hydroxyl radicals generated by γ radiation also form oxazolone (23) and 8-oxodGuo (43). Because the hy-

droperoxide **1a′** and its alcohol **1b′** (the latter one cannot generate hydroxyl radicals) oxidize dGuo mainly to 8-oxodGuo and no oxazolone was detected, we assume that singlet oxygen (type II photosensitized oxidation) is involved in this photooxidation. Indeed, about eight-fold more 8-oxodGuo was formed by **1a′** and **1b′** in D_2O *vs* in H_2O (Table 3, entries 1 and 4), which is in accord with the singlet oxygen-mediated generation of 8-oxodGuo. Furthermore, 70% inhibition of 8-oxodGuo by sodium azide (Table 3, entry 2) also supports the involvement of singlet oxygen in the photosensitized oxidation of dGuo by **1a′** and **1b′**. However, all attempts to detect the diastereomeric mixture of 4-HO-8-oxodGuo (characteristic singlet oxygen product of dGuo) (24,33) in the reaction of **1a′** and **1b′** failed. Either these products are not formed or they do not persist under the reaction conditions. Support for the latter case was obtained when dGuo was irradiated with **1a′** and 3-carbethoxypsoralen or methylene blue. The yield of 4-HO-8-oxodGuo in the methylene blue photooxidation (irradiation at 350 nm, 60 min) decreased from 0.84 to 0.28% in the presence of **1a′** and in the case of 3-carbethoxypsoralen even from 3.74 to 0.52% (the experimental details are not presented here). In contrast to **1a′** and **1b′**, the hydroperoxide **2a** generated the oxazolone as major and 8-oxodGuo as minor oxidation product of dGuo (Figs. 5A,B and 6A), which indicates that hydroxyl radicals and/or type I process are involved in the photooxidation of dGuo by **2a**. Because the corresponding alcohol **2b** (cannot generate hydroxyl radicals) is ineffective in forming these products, and no significant deuterium isotope effect was observed on the yield of 8-oxodGuo in dGuo by **2a** (Table 3, entry 5), we conclude that in the case of **2a** hydroxyl radicals are the oxidizing agents of dGuo.

In view of its high mutagenicity, 8-oxodGuo is considered as one of the most biologically significant DNA oxidation products (16,17). Therefore, we have investigated the formation of 8-oxodGuo by the photo-Fenton reagent **2a** and for comparison its alcohol **2b**. The photoreactions of furocoumarins with salmon testes DNA showed that the highly reactive hydroperoxide **2a** is also most efficient in producing 8-oxodGuo in DNA (*cf.* Fig. 5B,C). Because the extent of 8-oxodGuo formation by **2a** was not influenced by D_2O and the corresponding alcohol **2b** formed only insignificant amounts of 8-oxodGuo in DNA under similar photolysis conditions (*cf.* Fig. 5C), the hydroxyl radicals produced by photosensitized homolytic hydroperoxide bond cleavage in **2a** are the most likely DNA oxidizing agents. For the hydroperoxide **2a**, a maximum yield of 8-oxodGuo (1.8% based on hydroperoxide consumption) was achieved at 20 μM concentration after 30 min irradiation (Fig. 6B). Prolonged irradiation (up to 90 min) did not significantly change the yield of 8-oxodGuo. The reason for the low yield (1.8%) of 8-oxodGuo relative to consumed hydroperoxide may be due to other DNA modifications such as strand breaks, base free sites and oxidation products of DNA bases other than 8-oxodGuo (8,57).

The hydroxyl radical scavenging experiments with *tert*-butanol (Table 4) indicate that the hydroxyl radicals are formed, at least partly, in the DNA matrix by photolysis of the DNA-intercalated furocoumarin hydroperoxides. This is substantiated by the quite inefficient inhibition of 8-oxodGuo formation (*ca.* 30–35%) in the photolysis of furocoumarin hydroperoxides **1a** and **2a** (Table 4) *vs* about 70% inhibition of this DNA damage by γ radiation (free hydroxyl radicals) (16), which confirms the intercalation of these hydroperoxides into the DNA.

In summary, these results demonstrate that the readily available intercalating and photosensitizing furocoumarin hydroperoxides serve as convenient sources for the generation of hydroxyl radicals. Irradiation of these novel photo-Fenton reagents with near UVA light leads efficiently to the mutagenic DNA oxidation product 8-oxodGuo. The advantage of this new type of photo-Fenton reagent resides in the fact that hydroxyl radicals can be generated directly in the DNA matrix by photosensitization and the concentration-dependent formation of DNA oxidation products such as 8-oxodGuo can be quantitatively assessed. Hence, the furocoumarin hydroperoxides, in particular the 8-MOP derivative **2a**, can be applied as an effective hydroxyl radical source for the investigation of biologically important reactions such as DNA oxidation and cleavage (58), protein degradation (59), crosslinking of biopolymers (60) and lipid peroxidation (61). With the help of this novel concept, *i.e.* employ intercalating, hydroxyl radical-releasing photosensitizers, the way has been opened to design more effective photoactive hydroxyl radical sources for biological studies.

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