DNA Damage by *tert*-Butoxyl Radicals Generated in the Photolysis of a Water-Soluble, DNA-Binding Peroxyester Acting as a Radical Source

Waldemar Adam,[†] Günther N. Grimm,*[,†] Chantu R. Saha-Möller,[†] Francesco Dall'Acqua,[‡] Gorgia Miolo,[‡] and Daniela Vedaldi[‡]

Institute of Organic Chemistry, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany, and Institute of Pharmaceutical Sciences, University of Padova, Via Marzolo 5, I-35131 Padova, Italy

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The photolysis of the water-soluble perester **1** leads to *tert*-butoxyl radicals as confirmed by EPR studies with the spin trap 5,5-dimethylpyrroline *N*-oxide (DMPO). In the presence of DNA, oxidative cleavage of the latter was demonstrated by the formation of strand breaks in supercoiled pBR 322 DNA and by a substantial decrease of the melting temperature of *salmon testes* DNA. Guanidine, released from, for example, oxazolone and oxoimidazolidine on base treatment, was observed with *calf thymus* DNA and 2′-deoxyguanosine. These DNA modifications were effectively inhibited by the radical scavenger di-*tert*-butylcresol or the hydrogen atom donor glutathione. Photosensitization by the arene chromophore was excluded since the corresponding ester **2** caused no DNA damage, nor were the photoproducts of the perester **1** active. The efficacy of the perester **1** in oxidizing DNA derives from the fact that the *tert*-butoxyl radicals are photolytically generated in the immediate vicinity of the DNA, due to electrostatic binding of the cationic perester to the DNA, as confirmed by fluorescence measurements. These results demonstrate that the photolysis of perester **1** provides a suitable source of *tert*-butoxyl radicals in aqueous media, a necessary prerequisite for biochemical investigations.

Introduction

Oxygen-centered radicals are abundantly formed during the oxidative metabolism. Such reactive species conduct important biological functions, e.g., in the respiratory chain or in the lipid peroxidation, only to mention a few prominent ones. Nevertheless, radicals also exhibit cytotoxicity if the cellular defense mechanisms are impaired or if the flux of radicals is significantly increased. Indeed, in the past decades, much evidence on the importance of oxidative damage of biomolecules [oxidative stress (*1*, *2*)] in inflammation (*3*), aging (*4*−*6*), mutagenesis, carcinogenesis (*1*, *7*), and also HIV expression (*8*) has accumulated. In this context, the role of hydroxyl radicals, which may be formed by the metal-catalyzed decomposition of hydrogen peroxide [Fenton reaction (*9*)] or in the reaction of hydrogen peroxide with superoxide [Haber−Weiss reaction (*10*)], has been intensively studied. In contrast, only little attention has been paid to alkoxyl radicals, although their formation has been repeatedly demonstrated in cellular processes (*11*−*13*). An effective source is the autoxidation of polyunsaturated fatty acids which result in lipid hydroperoxides which on photolytic or metal-catalyzed decomposition lead to hydroxyl and alkoxyl radicals (*14*−*17*). The latter oxyl species are short-lived and preferentially cyclize to carbon-centered epoxyallylic radicals, which may react with molecular oxygen to form peroxyl radicals (*18*). Nevertheless, alkoxyl radicals were detected upon incubation of hydroperoxides with the heme enzyme cytochrome P450 (*11*−*13*, *19*, *20*) and proposed to be involved in the DNA-damaging activity (*21*, *22*) by lipid hydroperoxides (*23*, *24*). Recently, it has also been shown that UV irradiation of mouse and even human skin resulted in the formation of lipid-derived alkoxyl radicals and contributed to photoinduced aging and carcinogenesis (*25*). Alarmingly, alkoxyl radicals have been detected in cigarette smoke and, thus, may contribute to cell damage and diseases (*26*).

The generation of alkoxyl radicals in cellular systems but the lack of information on their cytotoxic activity compels us to investigate the oxidation of biomolecules, in particular DNA. Most recently, we have shown through chemical model studies that alkoxyl radicals, generated in the photolysis of *N*-alkoxypyridinethiones, induce DNA strand breaks (*27*). Furthermore, *N*-aroyloxy-2-pyridinethiones were reported as DNA-photocleaving reagents (*28*). In this study, we have employed the water-soluble perester **1** as the photochemical radical source **(***29***)** to evaluate its applicability in photobiological studies. Besides its strand-cleaving activity in pBR 322 and *salmon testes* DNA, we also have examined the guanine base oxidation in *calf thymus* DNA and 2′-deoxyguanosine (dG) and demonstrate that *tert*-butoxyl radicals are effective oxidants of such biomolecules.

* To whom correspondence should be addressed. Telephone: +49-931-888-5340, 5339. Fax: +49-93-888-4756. E-mail: adam@chemie.uni-wuerzburg.de.
[†] University of Würzburg.
[‡] University of Padova.

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Materials and Methods

Materials. Methanol and acetonitrile, both HPLC grade, were purchased from Acros Chimica (Brüggen, Germany). Citric acid was obtained from Riedel-de-Haen (Seelze, Germany); HF/pyridine (70% HF) and 4-methylbenzoyl chloride were acquired from Aldrich (Steinheim, Germany). 5,5-Dimethylpyrroline *N*-oxide (DMPO), 2,6-di-*tert*-butyl-4-methylphenol, glutathione, guanidine hydrochloride, sodium acetate solution (3 M in water), ammonium formate, and *tert*-butylperbenzoate were supplied by Fluka Chemie AG (Buchs, Switzerland). *Calf thymus* DNA and 2′-deoxyguanosine, ethidium bromide for biochemical use, boric acid, and dibenzoyl peroxide were purchased from Merck KGaA (Darmstadt, Germany). Supercoiled pBR 322 DNA (form I, MW of 2.9×10^6 Da, 4365 bp) was obtained from Pharmacia Biotech Europe GmbH (Freiburg, Germany). Agarose was acquired from Serva Feinbiochemica GmbH (Heidelberg, Germany). The sodium salt of 1,2-naphthoquinone-4-sulfonic acid, bromophenol blue gel-loading solution, tris(hydroxymethyl)aminomethane (Tris base), and *salmon testes* DNA were supplied by Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany).

Benzene methanaminium-4-[[(1,1-dimethylethyl)dioxyl]-carbonyl]-*N*,*N*,*N*-triethyl chloride (1) and benzene methanaminium-4-(methoxylcarbonyl)-*N*,*N*,*N*-triethyl chloride (2) were prepared according to the known six-step synthesis (*29*−*31*) by starting from 4-methylbenzoyl chloride. The ester **2** was an intermediate in this sequence.

EPR Studies. These were carried out in a flat quartz cell on a Bruker EPR 300 spectrometer (Bruker, ER 160FC). The following EPR spectrometer settings were applied: microwave power, 20 mW; modulation amplitude, 0.52 G; time constant, 1.28 ms; scan rate, 200 G/83.9 s; and receiver gain, 2×10^4.

Perester 1 in Benzene. To a solution of the water-soluble perester **1** (1.00 mg, 2.91 μmol) in 750 μL of benzene and 100 μL of acetonitrile was added 10 μL of the radical trap DMPO (89.5 μmol, final concentration of 105 mM) immediately before the irradiation experiments. Irradiation was carried out in the quartz cell at 350 nm for 20 min in a Rayonet photoreactor. Subsequently, the reaction mixture was submitted to EPR spectroscopy. As a control experiment, a solution of DMPO (105 mM) in a mixture of 750 μL of benzene and 100 μL of acetonitrile was irradiated under the same conditions and EPR-spectrally analyzed.

***tert*-Butyl Perbenzoate (3) in Benzene.** Two microliters (2.07 mg, 10.6 μmol) of perester **3**, 10 μL of the radical trap DMPO (89.5 μmol, final concentration of 89.5 mM), and 988 μL of benzene were irradiated at 300 nm for 10 min in a Rayonet photoreactor and EPR-spectrally analyzed.

Dibenzoyl Peroxide (4) in Benzene. A sample of 2.57 mg (10.6 μmol) of diacyl peroxide **4**, 10 μL of the radical trap DMPO (89.5 μmol, final concentration of 89.5 mM), and 990 μL of benzene were treated under the same conditions described above.

Perester 1 in Water. The water-soluble perester **1** (1 mM) and 89.5 mM DMPO (50 μL of a 20 mM solution in water, 50 μL of acetonitrile, 890 μL of water, and 10 μL of DMPO) were irradiated in the quartz cell at 300 nm for 10 min in a Rayonet photoreactor and subsequently EPR-spectrally analyzed.

Perester 3 in Water. The experiment was carried out as described above (perester **3** was added as a 20 mM solution in acetonitrile).

Determination of the Binding Constant. The fluorescence spectra were recorded on a Perkin-Elmer LS50B luminescence spectrometer. The excitation wavelength was 315 nm, and the emission was recorded in the range of 390−600 nm. Excitation and emission slits were set to 5 nm. An 8.72×10^{-5} M aqueous solution of perester **1** (free perester, solution F) was prepared from 210 μL of a stock solution of the perester (2.91×10^{-3} M) in water and 6.79 mL of sodium chloride/EDTA solution [2 mM NaCl and 1 mM EDTA (pH 7)]. A second solution (complexed perester, solution C) contained 90 μL of a stock solution of perester **1** (2.91×10^{-3} M) in water (final concentration of 8.72×10^{-5} M), 2.545 mL of a 5×10^{-3} M solution of *salmon testes* DNA, 2 mM sodium chloride, 1 mM EDTA (final concentration of 4.24×10^{-3} M), and 365 μL of a solution of 2 mM sodium chloride and 1 mM EDTA. The fluorescence of both solutions was recorded. Afterward, the solution of the complexed compound (solution C) was added successively in 20 to 200 μL portions (up to 4 mL) to the solution of the free compound (solution F), and after each addition, the fluorescence spectra were recorded. (The use of a solution of DNA and substrate instead of DNA alone prevents the fluorescence decrease due to dilution). From the fluorescence decrease upon addition of DNA, the values of r (number of molecules bound per nucleotide divided by the DNA concentration) and c (amount of free ligand) were calculated according to the procedure reported by Peacocke and Serrett (*32*), with the help of a computer program. From the r and c values, the binding constant K of the perester **1** and DNA was determined (two independent experiments) by an iterative procedure according to McGhee and von Hippel (*33*) (eq 2).

Modification of pBR 322 DNA. The reactions were carried out under atmospheric conditions in Eppendorf tubes with supercoiled pBR 322 DNA (10 mg/L) in 5 mM KH_2PO_4 buffer (pH 7.4). The samples with a 10 μL final volume were prepared from 3 μL of a stock solution of pBR 322 DNA [33.3 mg/L in 15.7 mM KH_2PO_4 buffer (pH 7.4)] and 0.5 μL of a 20 mM solution of perester **1** or ester **2** in water (final concentration of 1 mM). Perester **3** and diacyl peroxide **4** were added as a 20 mM solution in acetonitrile, and the final acetonitrile concentration was 20%. The samples were irradiated for 2 or 10 min from above in open Eppendorf tubes at 0 °C in a Rayonet photoreactor (300 nm), equipped with twelve 300 nm UV lamps (RPR 3000, 21 W, Southern New England Ultraviolet Co., Branford, CT).

In the control experiments, 2,6-di-*tert*-butyl-4-methylphenol (di-*tert*-butylcresol, final concentration of 10 mM) was added as a 20 mM solution in acetonitrile and glutathione (final concentration of 5 mM) as a 20 mM water solution.

Determination of Strand Breaks by Gel Electrophoresis. After irradiation of the above samples, 2.5 μL of bromophenol blue gel-loading solution was added to the samples. An 8-μL aliquot of the resulting mixture was transferred to a 1% agarose gel stained with 0.5 mg/L ethidium bromide. Subsequently, electrophoresis was carried out in Tris buffer [18 mM Tris base, 18 mM boric acid, and 10 mM EDTA (pH 8)] at 78 V for 3 h, in a Pharmacia horizontal apparatus (GNA 100), which was equipped with a gene power supply (GPS 200/400). The DNA spots were detected by exposure to a UV transilluminator (366 nm) and recorded by photography with a Herolab EASY 429K camera, which was connected to a personal computer, equipped with a Herolab EASY software program. The ratio of open circular DNA relative to the total amount of DNA was determined from the light intensities of the spots. The number of single-strand breaks per DNA molecule (4×10^3 base pairs) was calculated as described in the literature (*34*).

DNA Melting Studies. For the determination of the DNA melting point, a solution of *salmon testes* DNA (4.55×10^{-4} M) in a sodium chloride/EDTA solution [2 mM NaCl and 1 mM EDTA (pH 7)] was heated to 100 °C, and the UV absorption at 260 nm was determined every 2 °C in 1 mm cuvettes, sealed with Teflon stoppers, by employing a Perkin-Elmer 550S UV/vis spectrophotometer, connected with a temperature programmer (0.5 °C/min). The effect of perester **1** and ester **2** on the melting point of DNA was determined after addition of the compounds as 2.91×10^{-3} M stock solutions in water (final concentration of 8.72×10^{-5} M). The melting point of DNA was also determined after irradiation for 10 min with and without perester **1** or ester **2**, employing a Philips TL 12 lamp (290−340 nm, maximum at 310 nm). Finally, the change in the absorbance divided by the maximum absorbance ($\Delta A/A$) was plotted versus the temperature; the inflection point in the curve represents the DNA melting point.

Oxidation of *Calf Thymus* DNA. A typical reaction mixture (300 μL) contained 0.1 mg/mL (62.5 μM) calf thymus DNA (30 μL of an aqueous solution with a concentration of 1 mg/mL) and 625 μM perester **1** or ester **2** (9.38 μL of a 20 mM aqueous solution) in 5 mM phosphate buffer (pH 7, 7.5 μL of a 200 mM solution). In the inhibition experiments, 2,6-di-*tert*-butyl-4-methylphenol (final concentration of 2 mM) was added as solution in acetonitrile (30 μL of a 20 mM solution).

The solutions were irradiated in glass vials in a Rayonet photoreactor with 300 nm light (sixteen 300 nm UV lamps, RPR 3000, 21 W, Southern New England Ultraviolet Co.) at 10 °C under normal atmospheric conditions. After photolysis, an 80 μL aliquot was used for 8-oxoGua analysis and a 200 μL aliquot for analysis of guanidine-releasing products. Both solutions were extracted with ethyl acetate (2 × 160 or 2 × 400 μL), and the DNA was precipitated by addition of 8 or 20 μL of aqueous sodium acetate (3 M, pH 5, final concentration of 273 mM) and 3 volumes of cold ethanol (−50 °C). The samples were stored for 10 h at −50 °C. After centrifugation (3 min, 15 000 rpm), the liquid phase was removed by means of a pipet and the precipitated DNA was dried for 40 min at 20 °C/10^{-2} Torr. The samples for the quantification of guanidine-releasing products were dissolved in 100 μL of water and stored for 24 h at room temperature (ca. 20 °C). Derivatization and HPLC analysis were performed as described in the HPLC section. For the quantification of 8-oxoGua, the dried DNA was hydrolyzed by treatment with 12 μL of HF/pyridine (70% HF) for 30 min at 37 °C. The brown solution was neutralized by addition of ca. 15 mg of calcium carbonate and the mixture suspended in 200 μL of water and vigorously shaken for 20 min. After centrifugation and separation of the liquid phase, the residue was washed with 200 μL of water, and the combined aqueous solutions were after lyophilization (ca. 20 °C, 10^{-2} Torr) dissolved in 80 μL of water prior to the quantitative determination of 8-oxoGua by HPLC analysis with UV and electrochemical (EC) detection, as described in the HPLC section.

Oxidation of 2′-Deoxyguanosine (dG). A typical reaction mixture (200 μL) contained 0.5 mM dG and 5 mM perester **1** (50 μL of a 20 mM aqueous solution) in 5 mM phosphate buffer (pH 7, 5 μL of a 200-mM solution). In the inhibition experiments, 2,6-di-*tert*-butyl-4-methylphenol (final concentration of 5 mM) was added as a 20-mM solution in acetonitrile. Ester **2** (final concentration of 5 mM) was added as a 20-mM stock solution in water. The samples (200 μL) of perester **3** and diacyl peroxide **4** (each 1 mM; 10 μL of a 20 mM stock solution in acetonitrile) contained 0.5 mM dG (25 mM solution) and 20% acetonitrile. The solutions were irradiated in glass vials in a Rayonet photoreactor with 300-nm light (sixteen 300 nm UV lamps, RPR 3000, 21 W, Southern New England Ultraviolet Co.) at 10 °C under normal atmospheric conditions, and the deoxyguanosine conversion and 8-oxodG formation were determined directly on the oxidation mixtures as described in the HPLC section. For the determination of guanidine-releasing products, 100 μL was taken from the reaction mixtures, extracted with ethyl acetate (2 × 200 μL), and lyophilized (ca. 20 °C, 10^{-2} Torr). Afterward, the residue was redissolved in 100 μL of water. Derivatization and HPLC analysis were performed as described in the HPLC section. In the time-dependent decomposition studies of dG, the perester concentration was 8 mM; the other conditions were as described above.

Photodecomposition of Perester 1. The time dependence of the photodecomposition of perester **1** was monitored by NMR spectroscopy on a Bruker 200 MHz NMR spectrometer. A solution of the perester (8 mM) in D_2O was irradiated in a Rayonet photoreactor with 300 nm light, and the decomposition of the perester **1** was calculated against hexamethyldisiloxane (1 μL, 0.764 mg, 4.71 μmol) as an internal standard by means of the decrease of the *tert*-butyl peak at δ 1.36 ppm.

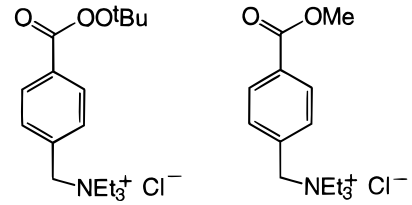
HPLC Analysis. The HPLC analytical system consisted of Bischoff HPLC pumps, model 2200 (Bischoff GmbH, Leonberg, Germany), equipped with a Rheodyne loop injector, model 7125 (Berkeley, CA). For the detection of the DNA bases, a Waters 994 photodiode array detector (Waters GmbH, Eschborn, Germany) was used, connected in series with an ESA Coulochem model 5100A electrochemical detector, supplied with a model 5011 highly sensitive analytical cell (ESA, Inc., Bedford, MA) for the detection of 8-oxoGua and 8-oxodG. The guanidine-releasing products were, after fluorescence marking, detected with a Shimadzu model RF-551 spectrofluorometric detector (Bischoff GmbH). All HPLC solvents were passed through a 0.45 μm Sartorious cellulose filter before being used.

The separation of the DNA bases, as well as that of 2′-deoxyguanosine (dG), was achieved on a 250 mm × 4.6 mm (i.d.) Eurospher 100-C18 7 μm column (Knauer GmbH, Berlin, Germany) by using a mixture of 50 mM sodium citrate buffer (pH 5) and methanol (90:10 for the DNA bases and 8-oxoGua; 80:20 for dG and 8-oxodG) at a flow rate of 1 mL/min. For electrochemical detection, the oxidation potential was set at 350 mV for 8-oxoGua and 450 mV for 8-oxodG.

The guanidine released from, for example, oxazolone and oxoimidazolidine on base treatment was detected by fluorescence labeling. To 100 μL of the reaction solution were added 38 μL of 1 N NaOH and 20 μL of an aqueous solution of 1,2-naphthoquinone-4-sulfonic acid (5 mg/mL). The mixture was kept at 65 °C for 10 min in the dark, which resulted in the release of guanidine, and its subsequent addition to 1,2-naphthoquinone-4-sulfonic acid yielded the fluorescent Schiff base product. The reaction mixtures were acidified by addition of 42 μL of 1 N HCl and separated on a 250 mm × 4.6 mm (i.d.) Eurospher 100-C18 5-μm column with a mixture of methanol and 25-mM ammonium formate (20:80, 1 mL/min) as the eluent and subsequently detected spectrofluorometrically (λ_{ex} = 355 nm, λ_{em} = 405 nm). Quantification was performed by using a solution of 5 mg/L guanidine hydrochloride (52.3 μM) as an external standard, which was treated with NaOH, 1,2-naphthoquinone-4-sulfonic acid, and HCl, as described above.

Results

The water-soluble peroxy ester [4-(*tert*-butyldioxycarbonyl)benzyl]triethylammonium chloride (**1**) was synthesized according to procedures described in the literature (*29*−*31*). Perester **1** was employed in this work as a photochemical source of alkoxyl radicals to investigate the oxidative DNA damage by such reactive species. In these studies, the photolytically persistent ester **2** was also investigated in parallel as a control.



Absorption Characteristics. Perester **1** and ester **2** show for the arene chromophore a UV absorption maximum at 277 nm in aqueous solution. The extinction coefficients were 1×10^3 L mol^{-1} cm^{-1} in both cases.

Release of Radicals upon Irradiation. The formation of alkoxyl radicals upon irradiation (300 nm) of perester **1** was substantiated by spin trapping experiments and subsequent EPR spectroscopy. For this purpose, the perester **1** (2.91 μmol) was irradiated in the

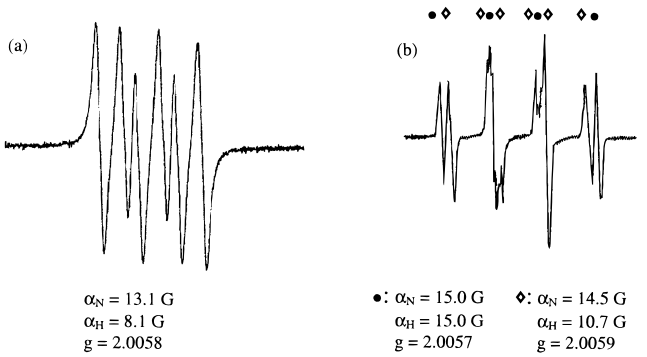
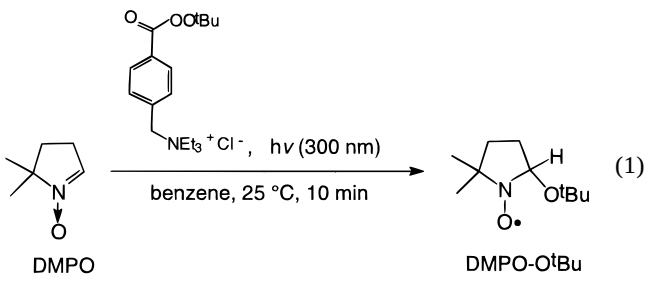


Figure 1. EPR spectra of the DMPO−O^{t}Bu adduct formed in the photolysis (300 nm) of peresters **1** und **3** in benzene (a) and water (b). In the latter case, the adduct of methylperoxyl radicals (◇) was also detected in addition to that of *tert*-butoxyl radicals (●).

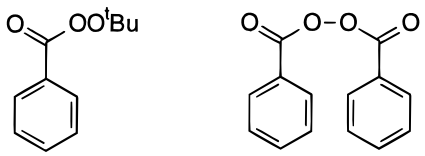
presence of DMPO (89.5 μmol) acting as a radical scavenger (eq 1).



(1)

The EPR spectrum of the radical adduct showed a doublet-of-triplets pattern with the characteristic hyperfine coupling constants (α_N = 13.1 G and α_H = 8.1 G) for the known (*35*) adduct of *tert*-butoxyl radicals and DMPO (Figure 1).

In contrast, ester **2** (51.4 μM) in H_2O (30 °C) gave upon irradiation (300 nm) for 3 h no radical adducts in the trapping experiments with DMPO. The acyloxyl radical, which is also released in the homolytic cleavage of the O−O bond, has not been detected under the applied conditions. For this purpose, *tert*-butyl peroxy benzoate (**3**) and dibenzoyl peroxide (**4**) were irradiated in the presence of DMPO.



3 **4**

In the case of perester **3**, a strong signal for the DMPO−O^{t}Bu adduct (Figure 1) was detected, whereas for the diacyl peroxide **4**, only a very weak signal was obtained. However, when the trapping experiments were performed in water for both peresters **1** and **3**, besides the *tert*-butoxyl adduct, the methylperoxyl−radical adduct of DMPO (α_N = 14.5 G und α_H = 10.7 G) was also observed (*36*). The latter adduct is formed by β-cleavage of the *tert*-butoxyl radical which generates methyl radicals, which are trapped by diffusion-controlled ($k > 2 \times 10^9$ M^{-1} s^{-1}) reaction with molecular oxygen, and the resulting peroxyl radical is subsequently spin-trapped by DMPO (*37*−*40*).

Determination of the Binding Constant. To examine whether the perester **1** binds with DNA, a prerequisite for the formation of radicals in the immediate proximity of DNA, the binding parameters had to be determined. For this purpose, the interaction of aromatic molecules with DNA may be studied by their fluorescence quenching upon addition of DNA (*41*, *42*). Perester **1** exhibits fluorescence emission on excitation at 315 nm with a maximum at 408 nm. The influence of DNA on the fluorescence intensity of perester **1** was monitored by addition of various amounts of a mixture of *salmon testes* DNA and perester **1** (4.24×10^{-3} M DNA and 8.72×10^{-5} M **1**) to a solution of pure perester **1** (8.72×10^{-5} M), and this process was found to decrease the fluorescence intensity (Figure 2a) significantly. From these fluorescence titration experiments, the values of r (amount of complexed ligand/DNA concentration) and c (amount of free ligand) were calculated according to the procedure reported by Peacocke and Serrett (*32*). These experimental data were scrutinized in terms of a Scatchard plot (Figure 2b). From the r and c values, the binding constant K for binding of perester **1** with DNA was determined to be 48 500 ± 1500 M^{-1} (two independent experiments) by an iterative procedure according to McGhee and von Hippel (*33*) (eq 2),

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

where n is the number of covered DNA−lattice residues.

Formation of Strand Breaks in Supercoiled pBR 322 DNA. The DNA-cleaving activity of perester **1** was assessed with supercoiled pBR 322 DNA, which yields the open circular form due to strand breaks. The supercoiled and open circular forms can be separated by gel electrophoresis, and the number of single-strand breaks (ssb) is calculated according to the literature (*34*). Indeed, perester **1** (1 mM) induced 1.23 ssb per DNA molecule (4×10^3 bp) in supercoiled pBR 322 DNA (Figure 3, lane 1) upon UV irradiation (λ = 300 nm) compared to 0.19 ssb in the irradiation of DNA alone under the same conditions (Figure 3, lane 6).

To gain further mechanistic insight into the involvement of radicals, the photolysis was carried out in the presence of the radical scavengers di-*tert*-butylcresol (Figure 3, lane 2) and the hydrogen donor glutathione (Figure 3, lane 3). Both additives significantly decreased the amount of strand breaks.

The activity of the chromophore itself was assessed by using ester **2** (1 mM), which possesses the same chromophore as perester **1** but is photochemically persistent. Upon irradiation in the presence of supercoiled DNA, only a small increase in strand breaks (0.23 ssb per 4×10^3 bp) compared to that of the blank (0.19) was observed (Figure 3, lane 4). The photolysate of perester **1** did not cause any significant number of strand breaks (0.21 ssb per 4×10^3 bp) in pBR 322 DNA upon irradiation (Figure 3, lane 5), as determined by the use of preirradiated solutions (300 nm, 12 h) of radical source **1**.

To assess the reactivity of *tert*-butoxyl versus benzoyloxyl radicals, we also utilized *tert*-butyl peroxy benzoate (**3**) and benzoyl peroxide (**4**) as photochemical radical sources in the DNA cleavage studies (Figure 4). While the *tert*-butoxyl radical source **3** was quite effective in the formation of DNA strand breaks (Figure 4, lanes 1 and 2), the benzoyloxyl radical source **4** displayed only a

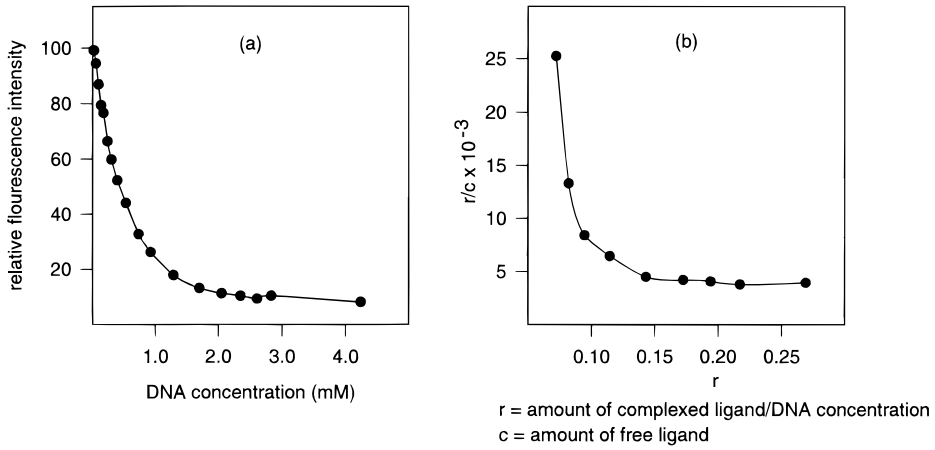


Figure 2. Fluorescence decrease (a) upon addition of *salmon testes* DNA and a Scatchard plot (b) of perester **1**. The values of *r* and *c* were determined by fluorescence quenching experiments.

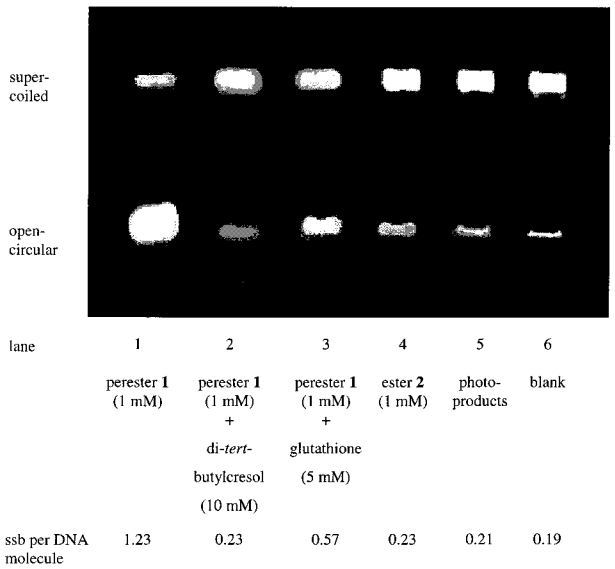


Figure 3. Gel electrophoretic analysis of single-strand breaks [ssb per DNA molecule (4×10^3 bp)] generated in pBR 322 DNA [10 mg/L in 5 mM KH_2PO_4 buffer (pH 7.4): irradiation (300 nm, 0 °C, 2 min) of perester **1** (1 mM, lane 1) and **1** in the presence of di-*tert*-butylcresol (10 mM, lane 2) and glutathione (5 mM, lane 3) as radical scavengers, irradiation of ester **2** (1 mM, lane 4) and **2** in the presence of the photoproducts of perester **1** (preirradiation of 1 mM perester **1** for 12 h at 300 nm, lane 5), and as a blank the DNA alone was irradiated (lane 6), where 2 min of irradiation (300 nm) did not lead to any significant amounts of open-circular DNA. The amount of open circular DNA was determined from the fluorescence intensity of the spots (error ±15%).

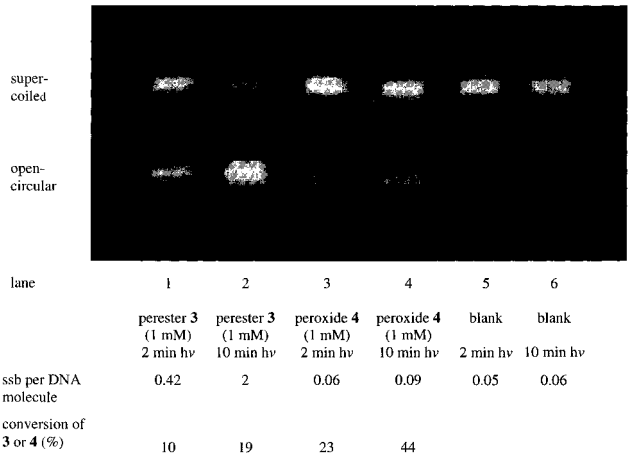


Figure 4. Gel electrophoretic analysis of single-strand breaks [ssb per DNA molecule (4×10^3 bp)] generated in pBR 322 DNA [10 mg/L in 5 mM KH_2PO_4 buffer (pH 7.4)]: irradiation (300 nm, 0 °C, 2 min) of 1 mM perester **3** for 2 min (lane 1) or 10 min (lane 2), irradiation of 1 mM peroxide **4** for 2 min (lane 3) or 10 min (lane 4), and as a blank the DNA alone was irradiated as well for 2 (lane 5) or 10 min (lane 6). The blank value of open circular DNA without irradiation was ca. 0.04; the amount of open circular DNA was determined from the fluorescence intensity of the spots (error ±10%).

moderate activity (Figure 4, lanes 3 and 4). The conversion of the radical sources **3** and **4** under the applied conditions was monitored by HPLC which revealed that the diacyl peroxide **4** decomposed twice as fast as the perester **3** (Figure 4).

DNA Melting Studies. The physical or chemical interaction between a substance and DNA may stabilize or destabilize the DNA structure, which results in an increase or decrease of the DNA melting point (the temperature at which the double-stranded DNA denatures into single strands). The melting of DNA may be detected by the increase of the UV absorption, since the absorbance of the single-stranded DNA is higher than that of double-stranded (*43*). Perester **1** (8.72×10^{-5} M) decreased the melting point of *salmon testes* DNA (4.55×10^{-4} M) from 62 to 60 °C without irradiation. The destabilization was much more significant on irradiation (300 nm) for 10 min, for which the melting point decreased from 59.3 °C for the irradiated DNA alone to 55.7 °C for DNA in the presence of perester **1** (Figure 5a). In contrast, ester **2**, which has the same chromophore but does not generate radicals on irradiation, led to a decrease of only 1 °C (Figure 5b) upon irradiation (300 nm) for 10 min under the same conditions.

Guanine Oxidation in *Calf Thymus* (CT) DNA. Since the photolysis of the perester **1** induced DNA strand breaks, it was of interest to establish its efficacy in oxidizing the DNA bases. For this purpose, perester **1** [625 μM in 5 mM phosphate buffer (pH 7)] was irradiated (300nm, 30 min) in the presence of *CT* DNA (62.5 μM guanine), and 1.46 ± 0.15% guanidine-releasing products (*44*), presumably the guanosine oxidation products oxazolone and oxoimidazolidine, were formed.

The radical scavenger di-*tert*-butylcresol (final concentration of 2 mM, 32 equiv) was employed as a mechanistic probe, which significantly decreased (82%) the formation of guanidine-releasing products (0.26 ± 0.1 vs 1.46 ±

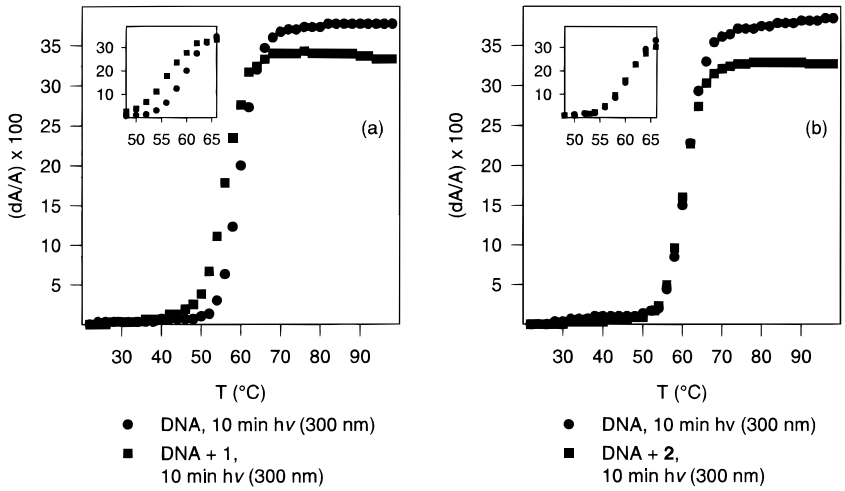


Figure 5. DNA melting after irradiation (300 nm) for 10 min with and without perester **1** (a) and ester **2** (b).

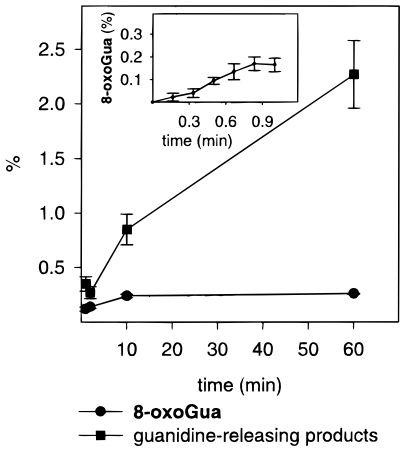


Figure 6. Time dependence of the formation of guanidine-releasing products and 8-oxoGua from *CT* DNA [62.5 μM guanine and 5 mM phosphate buffer (pH 7)] upon irradiation (300 nm) in the presence of perester **1** (625 μM). The blank values when DNA was irradiated alone (already subtracted) were ca. 0.1% for guanidine-releasing products and 0.2% for 8-oxoGua.

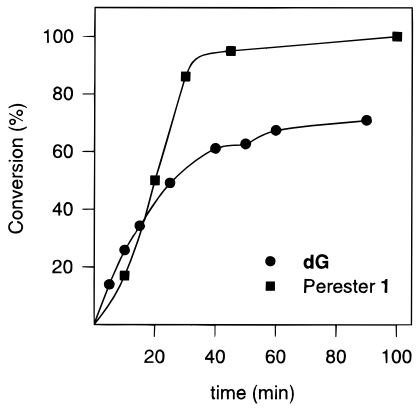


Figure 7. Time dependence of the photodecomposition (300 nm) of perester **1** (8 mM in D_2O, monitored by NMR analysis) and conversion of dG (four times) upon irradiation (300 nm) of perester **1** (0.5 mM dG and 8 mM perester **1** in 5 mM phosphate buffer at pH 7, 300 nm, and 10 °C).

0.15% without a scavanger). To assess the photosensitizing activity of the arene chromophore, ester **2** [625 μM in 5 mM phosphate buffer (pH 7)] was irradiated (300 nm, 30 min) in the presence of *CT* DNA (62.5 μM guanine). Ester **2** is scarcely active in DNA photooxidation, and merely 0.14 ± 0.1% guanidine-releasing products were formed.

In contrast to the reaction of hydroxyl radicals or type I and type II photooxidations (*45, 46*), the photolysis (300 nm) of perester **1** (625 μM) in the presence of *CT* DNA (62.5 μM guanine) yielded only very small amounts of 8-oxo-7,8-dihydroguanine, which did not increase on prolonged photolysis (Figure 6).

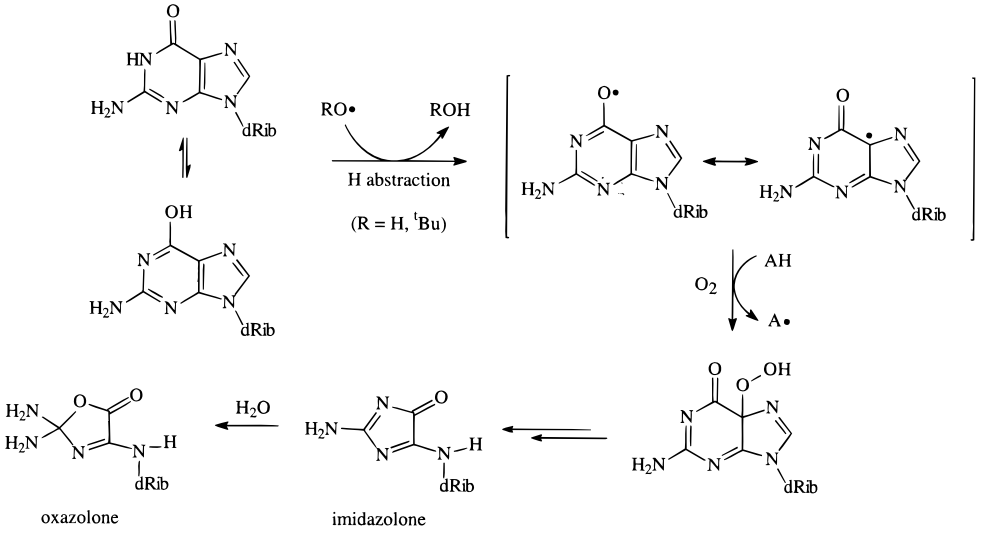
2′-Deoxyguanosine Photooxidation. Structural information on the reactive species responsible for the DNA damage in the irradiation of perester **1** was to be acquired through model studies with 2′-deoxyguanosine (dG). The photolysis (λ = 300 nm, 2 h) of perester **1** (5 mM) in the presence of 2′-deoxyguanosine (dG, 0.5 mM) resulted in a significant consumption (17.2 ± 2.5%) of dG. Alkaline treatment released 3.6 ± 0.5% guanidine, which was quantified by HPLC after fluorescence labeling with 1,2-naphthoquinone-4-sulfonic acid (*44*). Higher concentrations of perester **1** (10 mM) did not lead to more conversion of dG (17.7 ± 1%), and the amount of guanidine-releasing products remained the same (3.7 ± 0.3%). In all cases, 8-oxodG was not observed by HPLC analysis (electrochemical detection at 450 mV).

The involvement of radicals in this photoreaction was again tested with radical scavengers. Thus, the addition of di-*tert*-butylcresol (5 mM) led to a significant decrease (60%) in dG consumption, as did glutathione (5 mM), for which a 33% decrease of dG conversion was found.

To evaluate the effect of photosensitization by the arene chromophore [type I or type II reactions (*45*)], ester **2** (10 mM) was irradiated in the presence of dG. Ester **2** was much less active since only 3.4 ± 1.6% of the dG conversion was obtained compared to 17.2 ± 2.5% for perester **1**. Furthermore, the conversion of dG (monitored by HPLC analysis) was compared with the time dependence of the UV-induced decomposition of perester **1** (monitored by NMR spectroscopy). The data (Figure 7) reveal that dG conversion terminated when all perester **1** was consumed.

Also, in the dG oxidation studies, the *tert*-butyl peroxy benzoate (**3**) and benzoyl peroxide (**4**) were employed to evaluate the relative efficiency of *tert*-butoxyl versus benzoyloxyl radicals. Again, the *tert*-butoxyl radical source **3** was much more effective (8 vs 0.5%) than the benzoyloxyl **4** in converting dG and in the formation of guanidine-releasing products (1.3 vs 0.1%).

Scheme 1. Proposed Mechanism for the Formation of Guanidine-Releasing Products by Alkoxyl Radicals



Mechanistic Discussion

The significant DNA-cleaving activity of perester **1** is demonstrated by the formation of strand breaks (1.23 ssb per 4×10^3 bp) in supercoiled pBR 322 DNA (Figure 3) and the appreciable decrease of the DNA melting point (approximately 4 °C, Figure 5a) upon irradiation in the presence of perester **1**. Such DNA damage has been attributed to the involvement of radicals. For example, the decrease of the DNA melting point upon radical-induced formation of strand breaks has been demonstrated earlier for the strand-cleaving drug peplomycin (*47*), a derivative of the chemotherapeutic agent bleomycin. With *CT* DNA and 2′-deoxyguanosine (dG), we observed predominantly guanidine-releasing products. 8-OxoGua was detected in only low amounts, and it did not accumulate during the photolysis (Figure 6) (*48*). This is in contrast to hydroxyl radicals (*52*, *53*), which form 8-OxoGua in DNA, and to type I sensitizers [e.g., benzophenone (*54*)], which induce the formation of 8-OxoGua in DNA. In this context, it is significant to recall that with hydroxyl radicals the formation of 8-OxoGua in dG is only a minor process (*46*).

That radicals are, indeed, the active species in these DNA oxidations was confirmed by inhibition (cf. Results) with the radical scavenger di-*tert*-butylcresol (*55*, *56*) or the hydrogen donor glutathione (*57*, *58*). Furthermore, photosensitization reactions by the arene chromophore do not play a significant role, as was shown by the lack of DNA damage when the samples were irradiated in the presence of ester **2**. The latter possesses the same chromophore as perester **1**, but does not release radicals upon irradiation. Also, the photoproducts of perester **1** do not cause DNA strand breaks, which was established by the fact that a preirradiated solution was inactive (Figure 3) on further irradiation. In fact, a time profile of the photolysis revealed that the dG oxidation paralleled perester consumption (Figure 7).

All these experimental facts taken together establish that oxyl species, namely *tert*-butoxyl radicals, are responsible for the DNA damage. Indeed, in spin trapping experiments with DMPO, the *tert*-butoxyl−radical adduct was detected by EPR spectroscopy, as expected from peroxide bond homolysis in the cleavage of peroxyester **1**. However, benzoyloxyl radicals are necessarily formed as well, and the question as to what extent the latter participate in the observed DNA damage arises. In general, the reactivity of *tert*-butoxyl radicals for hydrogen abstraction is 3 orders of magnitude higher than for benzoyloxyl radicals (*59*). For example, the rate constants for α-hydrogen abstraction from tetrahydrofuran are 8.3×10^6 at 21 °C for *tert*-butoxyl radicals but only 2.5×10^3 at 25 °C for benzoyloxyl ones (*59*). As a matter of fact, the lower reactivity of benzoyloxyl versus *tert*-butoxyl radicals in the formation of DNA strand breaks was previously shown for *N*-benzoyloxy- versus *N*-alkoxypyridinethiones as photochemical sources for these radicals (*27*, *28*). Moreover, the control experiments with benzoyl peroxide in this study (Figure 4) convincingly demonstrate that benzoyloxyl radicals cause insignificant oxidative DNA damage. In analogy, we attribute the observed DNA oxidation and strand break formation by perester **1** mainly to *tert*-butoxyl rather than to benzoyloxyl radicals. Furthermore, although methylperoxyl radicals have been detected by trapping experiments with DMPO and subsequent EPR spectroscopy, they are not expected to contribute significantly in the DNA damage because they are much less reactive than alkoxyl radicals (*59*, *60*).

The slightly higher (ca. 2-fold) oxidative activity of the perester **1** compared to **3** in causing DNA strand breaks may be explained by the fact that the radicals are released in the immediate vicinity of DNA during the photolysis, since a binding constant of $48\,500 \pm 1500$ M^{-1} was determined by fluorescence titration experiments. For comparison, the binding constants of pyrimidine derivatives with a short cationic side chain are in the range of 3500−295000 M^{-1} (depending on the polarity of the molecules) (*61*). Such cationic substrates bind principally (*61*) through electrostatic interaction with the negatively charged groove, made up by the sugar-phosphate backbone of the DNA. Binding through intercalation into the DNA is unlikely for such a small arene-type perester **1** since effective classical intercalators require at least three condensed aromatic rings (*61*, *62*).

As for the mode of oxidative action of *tert*-butoxyl radicals, the formation of guanidine-releasing products from the guanine oxidation may be rationalized in terms of a mechanism established for the type-I oxidation of

guanine since the guanine in DNA or dG is known to yield the guanidine-releasing products (*44*) oxazolone and oxoimidazolidine upon hydrogen abstraction (*63*, *64*). Thus, the formation of guanidine-releasing products from the reaction of guanine with *tert*-butoxyl radicals may be rationalized mechanistically as shown in Scheme 1.

Alkoxyl radicals are less electrophilic than hydroxyl radicals (*65*, *66*) and sterically more encumbered such that hydrogen abstraction rather than addition to olefinic double bonds would be expected. In view of the strength of the ^{t}BuO−H bond [ca. 105 kcal/mol (*59*)], hydrogen abstraction by *tert*-butoxyl radicals from O−H or N−H bonds of the guanine base to generate the resonance-stabilized guanine radical is energetically feasible (*59*) (Scheme 1). Nevertheless, since the addition of *tert*-butoxyl radicals to double bonds [e.g., at C-4 or C-8 of guanine] cannot be excluded, an alternative pathway of generating guanine radicals would be an addition-elimination process as previously described for hydroxyl radicals (*2*).

Conclusions

This work qualifies the cationic perester **1** as a convenient and effective photochemical source of *tert*-butoxyl radicals for the study of oxidation reactions by alkoxyl radicals, as demonstrated presently for the oxidation of DNA. Compared to the Fenton-type reactions, the photochemical decomposition of peroxides prevents the involvement of metal ions and, thus, provides a clean method of radical formation. Further advantages of this radical source are (i) the ease of preparation, (ii) no photosensitization of DNA by the arene chromophore, (iii) the efficient electrostatic DNA binding through its cationic nature, (iv) the facile photocleavage of the peroxide bond, and (v) the relatively low reactivity of the benzoyloxyl partner radical. Since alkoxyl radicals are likely to be involved in oxidative stress in vivo (*21*−*24*), but little is known to date on their mode of action compared to that of the related hydroxyl radicals, some important biological applications are anticipated for the perester **1** as a photochemical radical source.

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