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2′-Deoxyguanosine (DG) Oxidation and Strand-Break Formation in DNA by the Radicals Released in the Photolysis of *N-tert*-Butoxy-2-pyridone. Are *tert*-Butoxyl or Methyl Radicals Responsible for the Photooxidative Damage in Aqueous Media?

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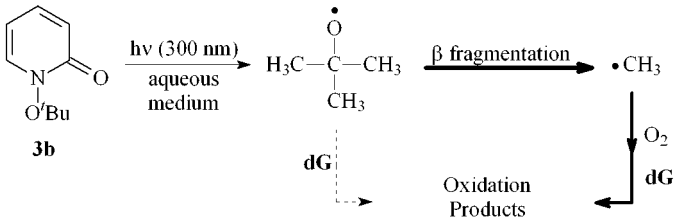
ABSTRACT



The photolysis of pyridone 3b (*photo-Fenton* reagent) in benzene releases *tert*-butoxyl radicals, which have been trapped by DMPO and EPR-spectrally identified. In aqueous solution, however, the fragmentation of the *tert*-butoxyl into methyl radicals prevails and the former radicals are of no direct consequence in the photooxidation of 2′-deoxyguanosine (dG) and pBR 322 DNA. The photooxidative damage of nucleic acids is caused by the oxyl radical species generated from the methyl radicals with oxygen.

Alkoxyl radicals, endogenously formed during the autoxidation of polyunsaturated fatty acids, have been suspected to cause cell damage and, consequently, speculated to be involved in aging and carcinogenesis.[1−3] With the aim of assessing the reactivity of alkoxyl radicals toward biomolecules such as DNA, photochemical alkoxyl-radical sources (so-called *photo-Fenton* reagents) have been developed.[4−8] In particular, the pyridinethione **1**[6,7] and the perester **2**[4,5] have been employed for this purpose. Indeed, we have previously reported that on photochemical activation of both the pyridinethione **1** and the perester **2**, *tert*-butoxyl radicals are

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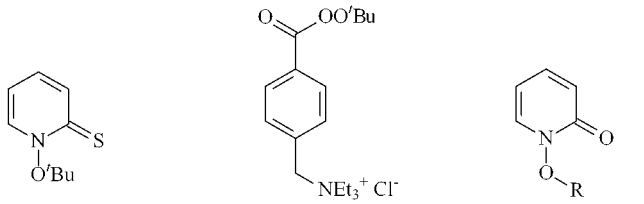
released.[4,6] In the case of the perester **2**, strand-break formation and guanine oxidation in DNA and **dG** have been attributed to the *tert*-butoxyl radicals.[4] In particular, on short-time (2−20 min) irradiation of the highly photoactive pyridinethione **1** the observed strand breaks in pBR 322 DNA have been assigned to the released alkoxyl radicals.[6] In contrast, the prolonged photolysis of the pyridinethione **1** affords photoproducts, which were shown to act as efficient photosensitizers for the guanine oxidation in **dG** and DNA.[7] This photosensitized electron-transfer process outweighs the oxyl-radical-mediated oxidation of nucleic acids and makes such a *photo-Fenton* reagent unsuitable for photobiological investigations.[7]



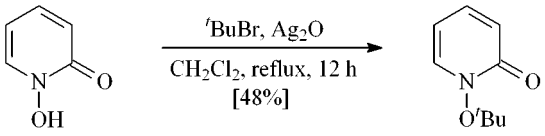
1 **2** **3a** R = H, **3b** R = ^{t}Bu

It has been established through transient spectroscopy that pyridone **3a** represents an efficient photochemical hydroxyl-radical source.[9] Indeed, we have recently demonstrated that the pyridone **3a** generates photochemically hydroxyl radicals without any photosensitization of DNA by the photoproducts.[10] Thus, in view of this advantageous photochemical behavior of the pyridone chromophore, we have examined the corresponding *tert*-butoxy derivative **3b** to assess whether the latter would produce *tert*-butoxyl radicals on photolysis and, thereby, be suitable for photobiological studies on the oxidative DNA damage by alkoxyl radicals. Our present results disclose that the photolysis of the *tert*-butoxy-substituted pyridone **3b** does release efficiently *tert*-butoxyl radicals (the quantum yield of its decomposition ϕ_{dec} was determined to be 0.17); however, in aqueous media, the latter cleave predominantly into methyl radicals, which either directly (no O_2) or indirectly (with O_2) oxidize 2′-deoxyguanosine (**dG**) and induce strand breaks in DNA.

The *tert*-butoxy-substituted pyridone **3b** was synthesized from the hydroxy derivative **3a** by addition of *tert*-butyl bromide in the presence of Ag_2O as catalyst (Scheme 1).

Upon photolysis of a benzene solution of pyridone **3b** in the presence of **DMPO**, the generated *tert*-butoxyl radicals were trapped in form of the **DMPO** adduct, as confirmed by EPR spectroscopy.[6,11,12] This substantiates that NO bond cleavage took place to release *tert*-butoxyl radicals (Figure 1).

Scheme 1



3a **3b**

Since photobiological studies with nucleic acids are usually carried out in aqueous solutions, water was used instead of

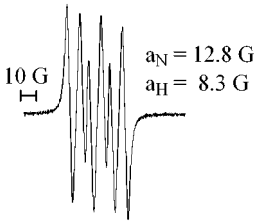


Figure 1. EPR spectrum of **DMPO** spin adducts observed upon irradiation (300 nm, 10 °C, 5 min) of pyridone **3b** in benzene.

benzene as solvent for the EPR studies. On photolysis of the pyridone **3b** in water under the exclusion of molecular oxygen, besides the expected *tert*-butoxyl radicals, also methyl radicals were trapped by **DMPO** (Figure 2). The

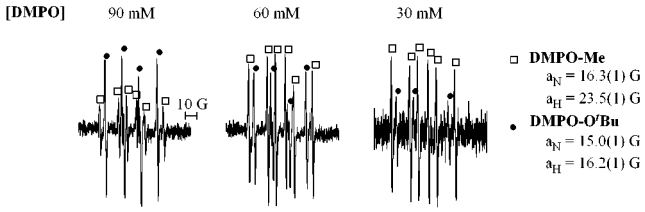


Figure 2. EPR spectra observed in the photolysis (300 nm, 20 °C, 10 min) of pyridone **3b** (3.00 mM) as a function of **DMPO** concentration in aqueous solution under the exclusion of molecular oxygen.

corresponding coupling constants for both radical adducts are identical to those reported in the literature.[13]

It is known that in aqueous solutions *tert*-butoxyl radicals rapidly ($k_\beta = 1.5 \times 10^6$ s^{-1}) fragment into methyl radicals and acetone (Scheme 2).[13,14] We observe that when a high **DMPO** concentration of 90 mM is employed, mainly *tert*-butoxyl radicals are trapped, whereas the methyl-radical adducts are formed in minor amounts; however, this ratio is reversed when the **DMPO** concentration is decreased from 90 mM to 30 mM (Figure 2). Thus, at 30 mM [**DMPO**] only traces of the *tert*- butoxyl radicals are trapped, whereas the signals of the methyl-radical adduct of **DMPO** (**DMPO**-

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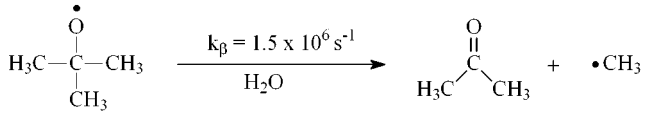
Scheme 2



Me) dominates in the EPR spectrum. Consequently, upon photolysis of the pyridone **3b** in water, a mixture of *tert*-butoxyl and methyl radicals is generated, with their proportion depending on the [**DMPO**]. At the lower **DMPO** concentration, the extent of β cleavage is higher and less **DMPO** adduct is formed with the *tert*-butoxyl radicals.

The photolysis of the pyridone **3b** in the presence of supercoiled pBR 322 DNA in phosphate buffer induced strand-break formation (35% open-circular DNA), as verified by gel-electophoretic analysis (Figure 3, lane 1). The

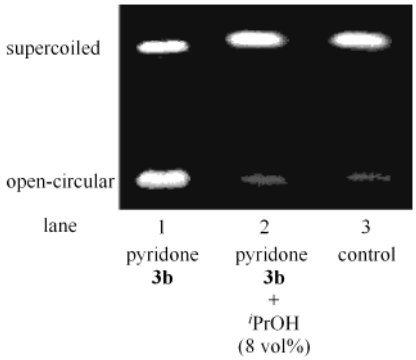


Figure 3. Gel-electrophoretic analysis of strand breaks generated in the photolysis (312 nm, 0 °C, 30 min) of supercoiled pBR 322 DNA (10 mg/L in 0.5 mM KH_2PO_4 buffer, pH 7.4) in the presence of pyridone **3b** (1.50 mM).

presence of isopropyl alcohol (8 vol %) as radical scavenger significantly (3%) suppressed the yield of open-circular DNA (Figure 3, lane 2). This inhibitory effect establishes that radicals participate in the photolytic strand-break formation by pyridone **3b**.

To assess whether guanine oxidation is caused by the photolysis of pyridone **3b**, 2′-deoxyguanosine (**dG**) was irradiated in the presence of the photochemical radical source **3b**. Complete photodecomposition of the latter (Figure 4) revealed that **dG** was converted to the extent of about 25% to yield mainly (15%) the guanidine-releasing products (GRP)[15] such as oxazolone, whereas **8-oxodG** was detected

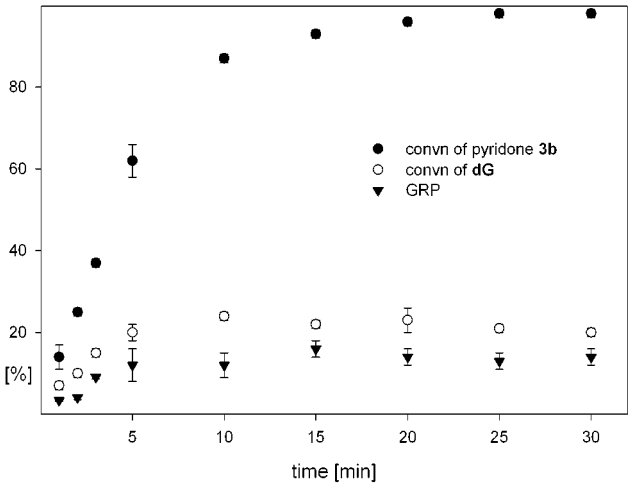


Figure 4. Time dependence of the **dG** (0.10 mM) conversion upon its irradiation (300 nm) in the presence of pyridone **3b** (0.50 mM) in phosphate buffer (5 mM, pH 7.0) at 10 °C; the error bars represent at least two independent runs.

in negligible (<0.1%) amounts (not shown). This is analogous to the oxidative behavior observed for the perester **2**, a photochemical *tert*-butoxyl-radical source which was shown to oxidize **dG** also predominantly to guanidine-releasing products (GRP).[4] Additionally, the time profile (Figure 4) of the **dG** oxidation reveals that after complete decomposition of pyridone **3b** (10−15 min) and prolonged irradiation, no further oxidation of **dG** takes place. Therefore, it is concluded that the radicals released in the photolysis of pyridone **3b** are responsible for the observed damage; sensitization by photoproducts may be ruled out as possible oxidation process. This favorable result encourages the use of the pyridone system as suitable *photo-Fenton* reagent for biological studies.

It still needs to be determined whether the observed **dG** oxidation and DNA cleavage may be attributed to *tert*-butoxyl radicals, methyl radicals, or peroxyl radicals derived therefrom under aerobic conditions. To confirm the intervention of methyl radicals during the irradiation of the pyridone **3b** in the presence of **dG**, efforts were expended to detect 8-methyl-2′-deoxyguanosine (**8-MedG**), a known methyl-radical adduct of **dG**.[16−18] Indeed, in the photolysis of a mixture of **dG** and pyridone **3b**, under the exclusion of molecular oxygen, appreciable amounts of **8-MedG** (2.5%) were detected and identified by comparison of the MS, the UV spectrum, and the retention time (on HPLC column) with that of the authentic material synthesized according to the literature procedure.[19] Therefore, the fragmentation of the photolytically generated *tert*-butoxyl radicals into methyl radicals occurs in aqueous phosphate buffer, and when **dG** is present, but no O_2, the **8-MedG** adducts are produced. From the known rate constants for the reaction of *tert*-butoxyl radicals with the guanine base[14] and for the β fragmentation of the *tert*-butoxyl radical into methyl radicals,[13] it may be estimated (Scheme 3) that not more than 0.3% of the generated *tert*-butoxyl radicals react with **dG** (0.10 mM) and,

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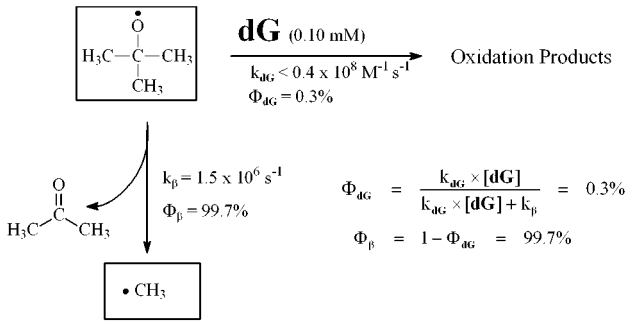
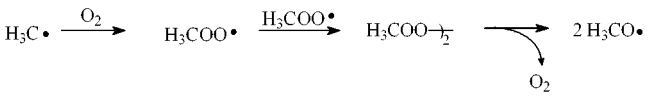
Scheme 3[a]



[a] ϕ_{dG} = efficiency of the **dG** oxidation. ϕ_β = efficiency for the β cleavage of the *tert*-butoxyl radical in water.

consequently, more than 99% undergo β cleavage to methyl radicals. Evidently, the fragmentation pathway (ϕ_β) of the *tert*-butoxyl radical into methyl radicals overwhelmingly dominates over the direct reaction with **dG** (ϕ_{dG}). Thus, the *tert*-butoxyl radicals, initially released in the photolysis of the pyridone **3b**, cannot be responsible for the presently observed and previously reported[4] **dG** photooxidation. It must be the methyl radicals that are formed by the *tert*-butoxyl-radical fragmentation, either directly by reaction with the guanine in **dG** and DNA or indirectly through the radicals derived from the methyl radical. Since methyl radicals are scavenged by molecular oxygen at diffusion-controlled rates to form methylperoxyl radicals, the latter or radicals derived from them (e.g., the methoxyl radical)[20] are the most likely damaging species (Scheme 4).

Scheme 4



On the basis of the present results it is concluded that *tert*-butoxyl radicals, despite their higher reactivity compared with the peroxyl radicals,[21,22] do not have a chance to react with **dG** and DNA in aqueous media, since the *tert*-butoxyl species cleaves too efficiently into methyl radicals. Any observed damage is ascribed to the latter, either directly by addition to the guanine base or indirectly through oxidation by the methylperoxyl radical obtained on O_2 trapping of the methyl radical. Also the methoxyl radicals generated from the latter by disproportionation may be involved.[20] Since under cellular conditions the concentration of oxygen is significantly lower than under aerobic aqueous conditions, the addition of methyl radicals to cellular DNA has been observed.[18] We advocate that future photobiological work on the oxidative propensity of *tert*-butoxyl radicals, and for that matter quite generally for alkoxyl radicals generated photochemically, thermally or enzymatically, should take into account the facile β cleavage in aqueous media.

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Supporting Information Available: Experimental Section with the details of the synthesis and the photolysis studies of the pyridone **3b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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