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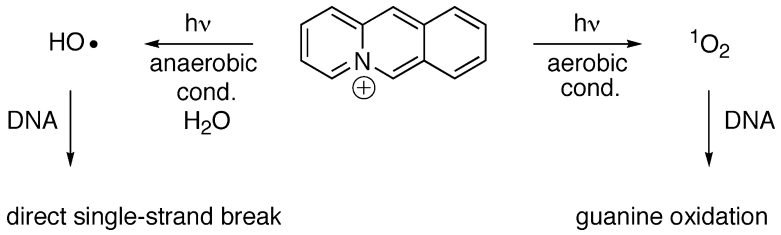

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Studies on the Mechanism of the Photo-Induced DNA Damage in the Presence of Acridizinium Salts—Involvement of Singlet Oxygen and an Unusual Source for Hydroxyl Radicals

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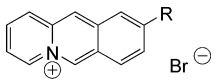
Abstract: Mechanistic investigations of the photoinduced DNA damage by acridizinium salts (*4a*-azoniaanthracene derivatives) are presented. Irradiation of 9-bromoacridizinium in the presence of defined double- and single-stranded DNA oligomers under aerobic conditions leads to both frank strand breaks and alkali-labile sites as determined by polyacrylamide gel electrophoresis (PAGE). The extent of the DNA damage increases significantly in D_2O and occurs selectively at guanosine residues. These observations reveal the formation of singlet oxygen ($^1O_2$) as reactive species, which oxidizes the DNA bases, above all the guanine bases. Further evidence for $^1O_2$ formation was obtained from laser-flash spectroscopic investigations, which show intersystem crossing (S_1 to T_1) of the excited states of the parent acridizinium and of the 9-bromo- and 9-amino-substituted derivatives. The resulting triplet state is efficiently quenched by oxygen ($k_q > 10^9$ $s^{-1}M^{-1}$) to yield $^1O_2$. Under anaerobic conditions, no significant alkali-labile lesions are observed, but frank strand breaks are induced; however, to lesser extent than under aerobic conditions. The DNA damage is suppressed in the presence of a radical scavenger, namely *t*-BuOH, and hydroxyl radicals are shown to be the reactive intermediates by trapping experiments with terephthalic acid. Moreover, the intercalated acridizinium molecules are not involved in the DNA damage reactions. The intercalated acridizinium salt leads to a primary PET reaction with the DNA bases; however, a fast BET transfer is proposed that regains the dye and the DNA, so that the excited intercalated dye does not contribute significantly to the overall DNA damage.

Introduction

The damage of DNA, mostly oxidative decay, by exogeneous molecules is an important event in cellular systems.[1] Although enzymatic repair of damaged sites is possible, the destruction of the DNA may also lead to cell death, mutagenesis, carcinogenesis, or aging in the absence of an efficient repair mechanism.[2] Thus, these irreversible reactions may have an important influence on the physiological function of the DNA, namely the gene expression. Among the classes of compounds that react with DNA, many cationic organic dyes are known to induce frank strand breaks or base modifications in DNA upon irradiation.[3] If this process is highly selective, such photoinduced DNA damage offers a promising tool to destroy DNA on purpose, for example in unwanted cell tissue such as cancer cells or in contaminated donor-blood samples. In fact, this methodology is already well established in photodynamic chemotherapy.[4]

We have observed recently that the acridizinium derivatives **1a**−**c** and derivatives thereof bind to DNA mainly by intercalation and that the irradiation of plasmid DNA (pBR322) in the presence of **1a**−**c** leads to DNA damage as shown by agarose-gel electrophoresis.[5] Interestingly, no sigificant differences have been observed under anaerobic and aerobic conditions.



1a: R = H
1b: R = Br
1c: R = NH_2

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Also, it has been shown that the aerobic DNA damage in the presence of **1a**−**c** is mainly due to frank strand breaks and takes place preferentially at the guanosine residues of the DNA. Preliminary mechanistic considerations were based on the assumption that an acridizinium-based intermediate may abstract hydrogen atoms from the sugar residue of the DNA backbone, which leads to the observed strand break; but so far, no experimental evidence was found for this assumption. Notably, it has been observed by Blazek et al. that even singlet oxygen can induce frank strand breaks in plasmid DNA,[6] so that our preliminary experiments do not exclude the involvement of this reactive intermediate under aerobic conditions. Moreover, the influence of other oxygen-containing intermediates have not been investigated in detail, so far. Thus, the distinct mechanism of the photoinduced DNA damage in the presence of acridizinium derivatives still needed to be clarified, because the knowledge of this mechanism is required to improve the efficiency and the selectivity of the DNA-damaging process by this class of molecules. To elucidate the parameters that govern the course of the photoinduced DNA damage by acridizinium salts, photochemical and photophysical studies were performed with the derivatives **1a**−**c** and will be presented herein.

Experimental Section

A. DNA-Damage Experiments. 1. General. Acridizinium salts **1a**−**c** were prepared according to literature procedures.[7] Buffer solutions were prepared with Nanopure water from a Barnsted NANOpure water system and salts from Fluka (BioChemika, Ultra for molecular biology) and Merck. [γ-^{32}P]ATP was ordered from Pharmacia and T4 polynucleotide kinase from New England Biolabs. Oligonucleotides were purchased from Microsynth (PAGE grade), plasmid pBR322 (MW 2.9 10 Da, 4365 bp) from MBI Fermentas, and peptides from Bachem. Oligonucleotides were further purified by HPLC (Series 1050 from Hewlett-Packard) on a reverse phase column (LiChrospher 100 RP-18e, 5 μm, 250 × 4 mm from Merck). Solvents were TEAA buffer (0.1 M TEAA) and acetonitrile (HPLC grade, Gebr. Machler AG) with a gradient of 6% to 20% acetonitrile within 40 min. Oligonucleotide concentrations were determined by the absorbance at 260 nm on a Perkin-Elmer Bio-Lambda II UV−vis spectrophotometer. Buffer for gel electrophoresis: 1X TBE derived from 10× TBE (pH 8.3; 1 M tris(hydroxymethyl)aminomethane, 1 M boric acid, 20 mM EDTA).

2. Mass Spectrometry. Identification of oligonucleotides was carried out by MALDI-TOF MS (Vestec Benchtop II) with 2,4-dihydroxyacetophenone as matrix using a laser wavelength of 337 nm and an acceleration voltage of 25 kV.

3. Melting Temperatures. Melting temperatures for the double strand experiments were measured on the Perkin-Elmer Bio-Lambda II UV−vis spectrophotometer equipped wih a PTP-6 Peltier unit. Both strands (500 pmol each) were dissolved in buffer (1 mL, pH 7.4, 25 mM K_2HPO_4/KH_2PO_4, 50 mM NaCl). Melting temperatures are the average of 2 cycles: **2:3** (21 bp) 66.6 °C; **4:5** (33 bp) 78.2 °C.

4. Radioactive Labeling. Oligonucleotides (16−24 μL, 1.6−2.4 nmol) were radioactively labeled at 5′-ends with [γ-^{32}P]ATP (5 μL, 15 pmol, 50 μCi) by T4 polynucleotide kinase (2 μL, 20 units) in a solution of kinase buffer (4 μL, 70 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol) and water (added to a final volume of 40 μL). Incubation for 45 min at 37 °C followed by purification with Mini Quick Spin Columns G-25 from Roche. For double strand experiments the radioactively labeled strand together with the complementary strand were heated to 85 °C for 5 min. After slow cooling to room-temperature annealing was achieved.

5. Maxam−Gilbert-Sequencing. The radioactively labeled oligonucleotide (10 pmol) and dimethyl sulfate (2 μL) were let to stand in buffer (50 μL, pH 7, 20 mM Na_2HPO_4/NaH_2PO_4) for 15 min at room temperature. After adding a piperidine solution (200 μL, 1M) the mixture was heated to 90 °C for 15 min and dried afterward (Savant, Speed Vac Plus).

6. Photolysis. DNA strands (200−300 pmol) and acridizinium salt **1b** (10−100 nmol) were irradiated for 10 min in buffer (200 μL, pH 7.4, 25 mM K_2HPO_4/KH_2PO_4, 50 mM NaCl) by an ORIEL 68810 photolysis stand with an Osram high-pressure mercury arc lamp (500 W) and a 360 nm cutoff filter (Schott). Irradiations were carried out in poly(methyl methylacylate) cuvettes from Semadeni at 25 °C. For aerobic conditions samples were irradiated without further treatment. For anaerobic experiments argon was bubbled through the solution for 10 min prior to irradiation. Due to its photolability the acridizinium salt was added as late as possible.

7. Piperidine and Ammonia Treatment. Three samples (30 μL each) were taken from the solution after irradiation. Piperidine solution (300 μL, 1 M) was added to the first samples and concentrated ammonia (300 μL, 35%) to the second sample. Samples were heated at 90 °C for 30 min (piperidine) or 60 min (ammonia) under aerobic conditions, respectively. The third sample was analyzed without further treatment (frank strand cleavage). All samples were freeze-dried (Savant, Speed Vac Plus).

8. PAGE. Irradiation products were dissolved in water (30 μL) and loading buffer (30 μL: 3 μL 10× TBE and 27 μL formamide, dyed with bromophenol blue and xylene cyanol FF) and separated by polyacrylamide gel electrophoresis. Electrophoresis was performed on a Life Technologies Model 2 equipped with a Pharmacia potentiostatic power unit. Denaturing gels (15%) were cast from urea (50 g, 0.8 mol), Nanopure water (10 mL), 10× TBE buffer (10 mL) and Accu Gel 40 (30 mL, National Diagnostics). After degassing the sample, the polymerization was initiated by addition of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (100 μL) and ammonium persulfate solutions (100 μL, 10%). Before loading the gel, radioactivity levels were measured with a scintillation counter (Liquid Scintillation Analyzer Tri-Carb 2000CA from Packard) to make sure that material with the same intensity was loaded to each lane. After gel electrophoresis at 1500 V for 2−3 h the gel was transferred to filter paper (Whatman 3 MM Chr), dried (3 h, 60 °C, on a SpeedGel SG 210D from Savant) and exposed to a phosphor screen (Molecular Dynamics) for 15−20h.

9. Scanning and Evaluation. The screen was transferred to the phosphorimager (Storm 820 from Molecular Dynamics) and scanned. Quantification was performed using the software ImageQuant.

10. Peptide Cleavage. Pro-Pro-Pro (0.5 μmol) and acridizinium salt **1b** (2.4 μmol) were irradiated for 1 and 2 h in 200 μL buffer (pH 7.4, 25 mM K_2HPO_4/KH_2PO_4, 50 mM NaCl) as described above. Samples of the irradiation solution (30 μL) were analyzed by HPLC (Waters Alliance 2690 Separation module, 996 photodiode array detector) on a reversed-phase column (LiChrospher 100 RP-18e, 5 μm, 250 × 4 mm from Merck). The solvents were Nanopure water and acetonitrile (HPLC grade) with a gradient of 0% to 10% of acetonitrile for 15 min followed by a gradient from 10% to 100% in 30 min. Separation was carried out at room temperature with UV-detection at 210 nm.

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B. Trapping of Hydroxyl Radicals. A stock solution of acridizinium tetrafluoroborate **1a-BF$_4$** (2.67 mg, 10.0 μmol) and disodium terephthalate (2.10 mg, 10.0 μmol) in water (10 mL) was diluted (1:10) with water. Under argon-gas atmosphere, 10 mL of this solution (10^{-4} M) were irradiated for 2 h at room temp. (Rayonet photoreactor, λ_{ex} = 350 nm). A sample of 750 μL was taken from the reaction mixture in time intervals of 20 min, and treated with 100 μL of aqueous NaOH (2 M). This sample was analyzed by fluorescence spectroscopy (λ_{ex} = 323 nm, slit$_{ex}$ corresponding to a band-pass of 5.0 nm; slit$_{em}$ corresponding to a band-pass of 5.0 nm). The formation of hydroxyterephthalic acid was monitored by the emission band of the corresponding anion at 438 nm. The assignment of this fluorescence signal to hydroxyterephthalic acid was supported by comparison with an authentic sample.[8] Separate samples of **1a** or terephthalic acid were also irradiated under identical conditions and subsequently treated with base. Spectrofluorimetric analysis showed no emission band that is superimposable with the one for hydroxyterephthalic acid. In control experiments, acridizinium **1b** and terephthalic acid were irradiated separately to show that the fluorescence band at λ_{max} = 438 nm does not appear under these conditions.

C. DNA-Cleavage Experiments in the Presence of Terephthalic Acid. Under anaerobic conditions, solutions (10 μL) of pB*R*322 plasmid DNA (10 mg/L), 9-bromoacridizinium bromide (**1b**, 5×10^{-5} M), and terephthalic acid (as disodium salt; a: 10^{-4} M, b: 10^{-3} M, c: 10^{-2} M, and d: 10^{-1} M) in buffer solution (5.0 mM KH_2PO_4, 50 mM NaCl, pH = 7.4) were irradiated with a Rayonet photoreactor at λ = 350 nm for 15 min at room temperature. For comparison, plasmid DNA was irradiated under the same conditions for 15 min in the absence of the acridizinium and terephthalic acid. This latter DNA sample did not show significant damage.

DNA-strand breaks were determined directly from the photolyzate by agarose-gel electrophoresis with ethidium bromide as indicator. The spots were detected by exposure to an UV transilluminator and recorded with a Herolab EASY 429 K camera. The amounts of damaged open-circular (relaxed) and remaining supercoiled DNA were determined from the light intensities of the spots. A coefficient of 1.66 was used to correct the lower efficiency for the ethidium bromide binding to supercoiled plasmid than to open-circular form.[9]

For a qualitative comparison, the photoinduced DNA damage by methylene blue was checked under identical conditions. Under anaerobic conditions, the extent of DNA damage is slighly smaller than with **1a**, whereas under aerobic conditions, the extent of DNA damage by methylene blue is between the one of **1a** and **1b**.

D. Photophysical Experiments. 1. General. Distilled water was deionized by employing Millipore MilliQ equipment. Calf thymus DNA (Merck) was used as purchased and dissolved in deionized water (ca. 1 mg/mL). The DNA concentrations (in nucleic base) was determined by UV spectroscopy (ϵ_{260} = 6600 $M^{-1}cm^{-1}$). UV spectra were measured on a Hitachi U3200 and the emission spectra were recorded on a Perkin-Elmer LS50. All experiments with DNA were performed in phosphate buffer (10 mM, pH = 7.0, from K_2HPO_4 and KH_2PO_4). Laser-flash experiments with acridizinium salts without DNA addition were performed in water, if not stated otherwise.

2. Laser Flash Photolysis/ Transient Absorption Spectroscopy. The laser flash photolysis setup has been previously described.[10] An excimer (Lumonics EX-510, 308 nm), or a Nd:YAG laser (Spectra Physics, GCR-12, 266 or 355 nm) were used as excitation sources at fixed wavelengths. A Coherent Infinity OPO laser was used as a tunable laser source for excitations above 420 nm. A pulsed Xe-arc lamp was used for the monitoring beam and at least 6 transient decays were averaged for each experiment. Spectra were obtained by collecting decay traces at different wavelengths and averaging the ΔA values between defined time windows for each kinetic decay trace. The samples were contained in 7 × 7 mm Suprasil cells and, unless otherwise stated, the samples were de-oxygenated by bubbling nitrogen for 20 min. Samples in the presence of oxygen were measured either in air ([O_2] = 0.3 mM) or by saturating the solution with oxygen ([O_2] = 1.4 mM).[11] The concentration of acridizinium salts was such that the absorption at the excitation wavelengths was between 0.2 and 0.4 for a path length of 7 mm. Since the addition of DNA to acridizinium salts results in a significant hypochromic effect, the corresponding acridizinium-DNA mixtures were also adjusted such that acridizinium absorption was between 0.2 and 0.4 in the presence of DNA. Oxygen was removed from quencher stock solutions by bubbling nitrogen for 20 min, and the appropriate quencher volume was added to the sample solution using a gastight syringe.

3. Fluorescence Lifetimes. Fluorescence decay times were measured on a single-photon-counting apparatus (Edinburgh Instruments, model 199S) at 25 °C. The maximum number of counts collected for the channel of highest intensity were between 4000 and 6000. Fluorescence was measured under oxygen-free conditions with an excitation wavelength of 358 nm. The emission was detected at the wavelengths corresponding to the maximum of the fluorescence spectra. The precision of the mean lifetimes was ca. 0.1 ns. Measurements were performed for DNA-dye ratios of 0, 5, and 10. The acridizinium concentration remained constant (10^{-5} M). The data were processed by iterative techniques and they were further treated by literature methods.[12]

Results

A. DNA-Damage Experiments. Recently, two of us have shown that photolysis of an aqueous solution of acridizinium salts **1a**−**c** at 350 nm leads to DNA cleavage of plasmids pBR322 and pUC18, respectively.[5a] The strand scission occurred not only in the presence but also in the absence of oxygen, which was a very surprising result. To understand this reaction, experiments were carried out with shorter oligomers whose sequences were varied. We chose derivative **1b** for our further studies on the photoinduced DNA damage, because in our first experiments this compound exhibited the largest cleavage yield.

We started with two fragments of plasmid pUC18, the 21mer **2**:**3** and the 33mer **4**:**5** (Figure 1). Irradiation (360 nm cutoff filter) in the presence of acridizinium salt **1b** (concentration ratio of **1b**:DNA base pair = 1.6 and 16, respectively), and quantification of the decrease of the radiolabeled strands **2** and **4**, respectively, gave the results shown in Figure 2.

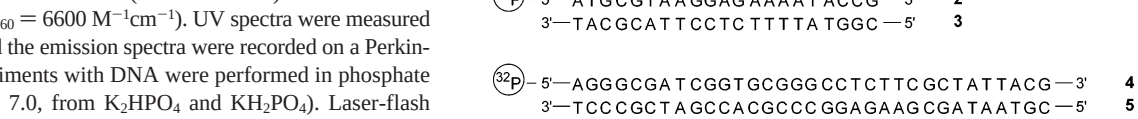

Figure 1. Double strands **2:3** and **4:5**.

The data demonstrate that frank DNA strand scission occurs not only in large plasmids, as shown before,[5a] but also in short oligomers such as the 21mer **2**:**3** or 33mer **4**:**5**. Under the reaction conditions described in Figure 2, the cleavage efficiencies under aerobic conditions are similar or slightly higher than under anaerobic conditions. But subsequent treatment with ammonia dramatically enhanced the cleavage yield differences between aerobic and anaerobic irradiations. Whereas the DNA

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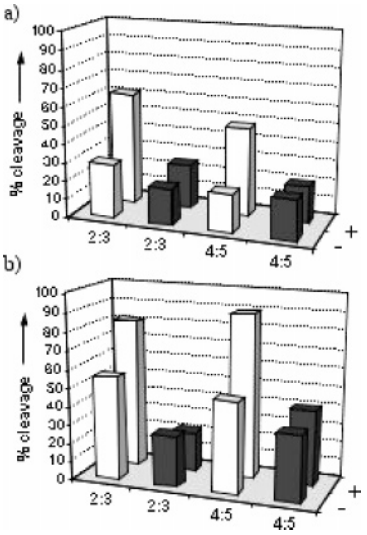

Figure 2. Photolysis of double strands **2:3** and **4:5** for 10 min at 360 nm under aerobic (white columns) and anaerobic (black columns) conditions. Strand cleavage is shown before (−) and after (+) ammonia treatment. Ratio of acridizinium salt **1b** to bases was 1.6 (a) or 16 (b).

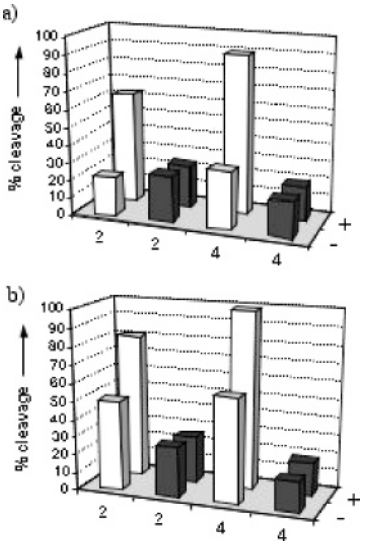

Figure 3. Photolysis of single strands **2** and **4** for 10 min at 360 nm under aerobic (white columns) and anaerobic (black columns) conditions. Strand cleavage is shown before (−) and after (+) ammonia treatment. Ratio of acridizinium salt **1b** to bases was 1.6 (a) or 16 (b).

scission yields increased by 35−50% during ammonia treatment after aerobic irradiation, further cleavage could not be observed by ammonia treatment after anaerobic irradiation. Interestingly, similar results as with DNA double strands were observed with DNA single strands **2** and **4**, respectively (Figure 3).

Thus, intercalation of the acridizinium salt **1b** into double stranded DNA is not a prerequisite for DNA photocleavage. We assume that photoexcited acridizinium salt **1b** generates reactive intermediates that induce the DNA strand scission and, under aerobic conditions, the formation of ammonia-labile sites. It is known that photoexcited acridizinium salts convert triplet oxygen ($^3O_2$) into singlet oxygen ($^1O_2$),[13] and this reactive intermediate may induce DNA strand cleavage[14] and oxidize guanine (G)[15] leading to base labile sites.[1c,16] In accord with

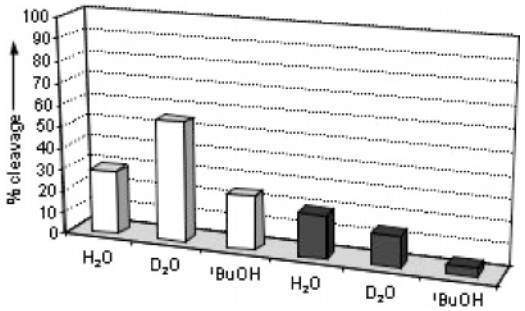

Figure 4. Photolysis of single strand **2** for 10 min at 360 nm under aerobic (white columns) and anaerobic (black columns) conditions. Irradiation was carried out in H_2O, D_2O and H_2O containing 0.2 mol % of *t*-BuOH. Ratio of acridizinium salt **1b** to bases was 1.6.

^{32}P– 5′—TTTTTTTTTTTTTTTTTTTT—3′	**6**
^{32}P– 5′—TTTTTTTTTTGTTTTTTTTT—3′	**7**
^{32}P– 5′—TTTTGTTTTTGTTTTTGTTTT—3′	**8**
^{32}P– 5′—TGTTGTTGTTGTTGTTGTTGT—3′	**9**
^{32}P– 5′—GTGGTGGTGGTGGTGGTGGTG—3′	**10**

Figure 5. Single strands **6**−**10** with varying G-content.

this suggestion is our finding that under aerobic conditions the cleavage yield of **2** doubles in D_2O, which increases the lifetime of $^1O_2$.[14,15d] Under anaerobic conditions nearly no change was observed. However, addition of *t*-BuOH, which traps HO• radicals,[17] suppressed the strand scission under anaerobic conditions but had no effect on the aerobic photocleavage (Figure 4).

All these experiments clearly demonstrate that the photoexcited acridizinium salt **1b** yields different reactive species, presumably mainly $^1O_2$ in the presence, and HO• radicals in the absence of oxygen. For $^1O_2$ it is known that it attacks guanines with high selectivity.[18] We therefore have increased the guanine content in 21mers **6**−**10** and photocleaved the strands with **1b** in the presence and in the absence of oxygen.

The results are summed up in Figure 6. The efficiency of aerobic cleavage increased before and after ammonia treatment with the numbers of guanines in the strands. Almost no cleavage was observed under these conditions for strands containing no guanines but adenines (data not shown). Thus, the DNA strand cleavage with acridizinium salt **1b** in the presence of oxygen is highly selective toward guanine.

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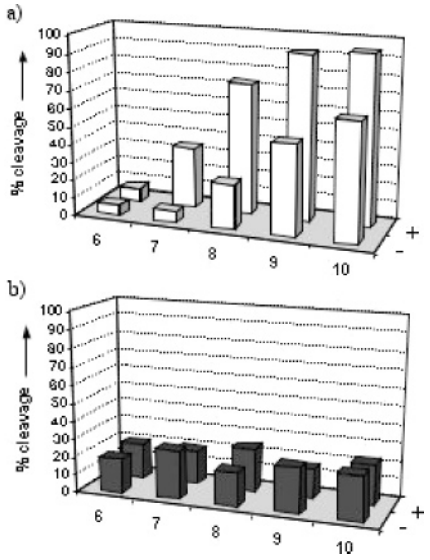

Figure 6. Photolysis of single strands **6**−**10** for 10 min at 360 nm under aerobic (a) and anaerobic (b) conditions. Strand cleavage is shown before (−) and after (+) ammonia treatment. Ratio of acridizinium salt **1b** to bases was 1.6.

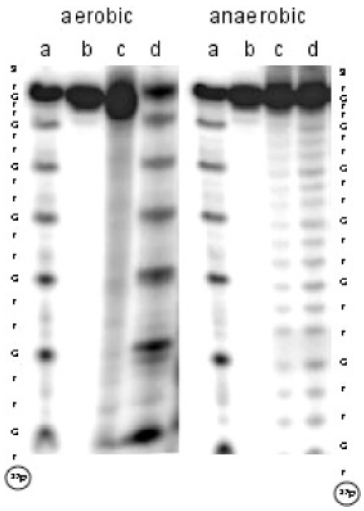

Figure 7. Photolysis of single strand **9** for 10 min at 360 nm under aerobic and anaerobic conditions. Ratio of acridizinium salt **1b** to bases was 1.6. The lanes show Maxam−Gilbert Sequencing (a), strands before irradiation (b), after irradiation (c) and subsequent ammonia treatment (d).

In contrast, photolysis of **1b** in the absence of oxygen cleaves DNA strands completely unselectively. We have shown this not only with strands **6**−**10** (Figure 6) but also with other adenine and cytosine containing 21mers (data not shown). This striking difference in the cleavage selectivity is demonstrated also by the gel electrophoresis chromatograms of Figure 7. Under aerobic irradiation of oligomers **9** in the presence of acridizinium salt **1b** and subsequent ammonia treatment, cleavage occurs only at the guanine sites, whereas under anaerobic conditions, cleavage occurs unselectively at all positions.

B. Trapping of Hydroxyl Radicals. The DNA-cleavage experiments indicate that even under anaerobic conditions, a reactive radical intermediate other than singlet oxygen is formed, which may induce frank strand breaks in DNA. The DNA-damage experiments give evidence that the hydroxyl radical may be the reactive species under these conditions. In fact, trapping experiments showed that during irradiation of acridizinium

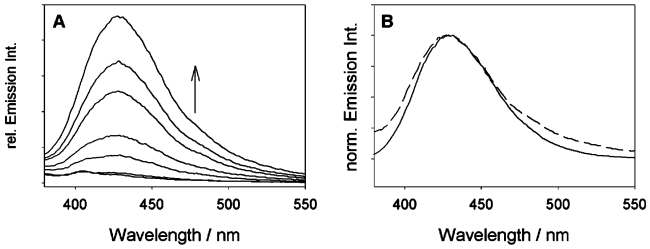

Figure 8. **A**: Formation of hydroxyterephthalic acid upon irradiation of **1a** as tetrafluoroborate salt and terephthalic acid in water (λ = 350 nm, arrow indicates the increase of the emission band after irradiation times of 0, 15, 40, 60, 85, 105, and 120 min, λ_{ex} = 322 nm). **B**: Comparison of the emission band from **A** (solid line) with the one of an authentic sample of hydroxyterephthalic acid (dashed line).

Table 1. Dependence of DNA Damage on the Presence of Terephthalic Acid

c (terephthalic acid)[a]/M	10^{-4}	10^{-3}	10^{-2}	10^{-1}
DNA damage[b,c]/%	45	34	27	11

[a] As disodium dicarboxylate; [b] Determined after irradiation for 15 min (λ = 350 nm) of a mixture of **1b** ($c = 10^{-5}$ M^{-1}**)**, terephthalic acid and plasmid pBR322 (10 mg/l) in buffer solution under anaerobic conditions, DNA damage given as percentage of open-circular plasmid; [c] error = ±10%.

derivatives in aqueous solution hydroxyl radicals are intercepted with terephthalic acid, whose trapping product, i.e., the hydroxyterephthalic acid, could be detected by fluorescence spectroscopy, i.e., characteristic emission signal of its corresponding anion (Figure 8A).[19] The assignment of this fluorescence band was made by comparison with the one of an authentic sample (Figure 8B). The experiments also reveal a steady increase of the emission intensity with increasing reaction times. Note, that tetrafluoroborate was used as inert counterion to avoid byproducts resulting from reactions with bromide. Further evidence for the involvement of hydroxyl radicals in the anaerobic DNA damage was obtained from DNA-cleavage experiments with plasmid pBR322 in the presence of terephthalic acid, because the DNA damage is significantly suppressed with an increasing concentration of the acid (Table 1).

C. Photophysical Studies. 1. Characterization in Homogeneous Solution. Laser-flash photolysis experiments were performed with the acridizinium derivatives **1a**−**c** in buffered solutions under anaerobic conditions and in the presence of oxygen. The objective of these experiments was to identify the transients in the absence and presence of DNA and to determine from kinetic studies the reactivity of transient species toward DNA.

All acridizinium salts showed the formation of transients upon excitation (Figure 9, Table 2). A common feature was the bleaching in the spectral region where the precursor ground-states absorption occurred. The transient spectra for **1a** and **1b** were similar and did not change at different delays, suggesting that one transient was formed. The lifetimes of the transient were longer than 10 μs and were shortened when the concentration of the acridizinium precursor increased (see below). The transients were efficiently quenched by oxygen, and the quenching rate constants (k_q) were estimated from quenching plots ($k_{obs} = k_o + k_q$[quencher]) where the observed decay rate constants were determined for samples in air and when the solution were bubbled with either nitrogen or oxygen. The transient is efficiently quenched by oxygen with a quenching rate constant

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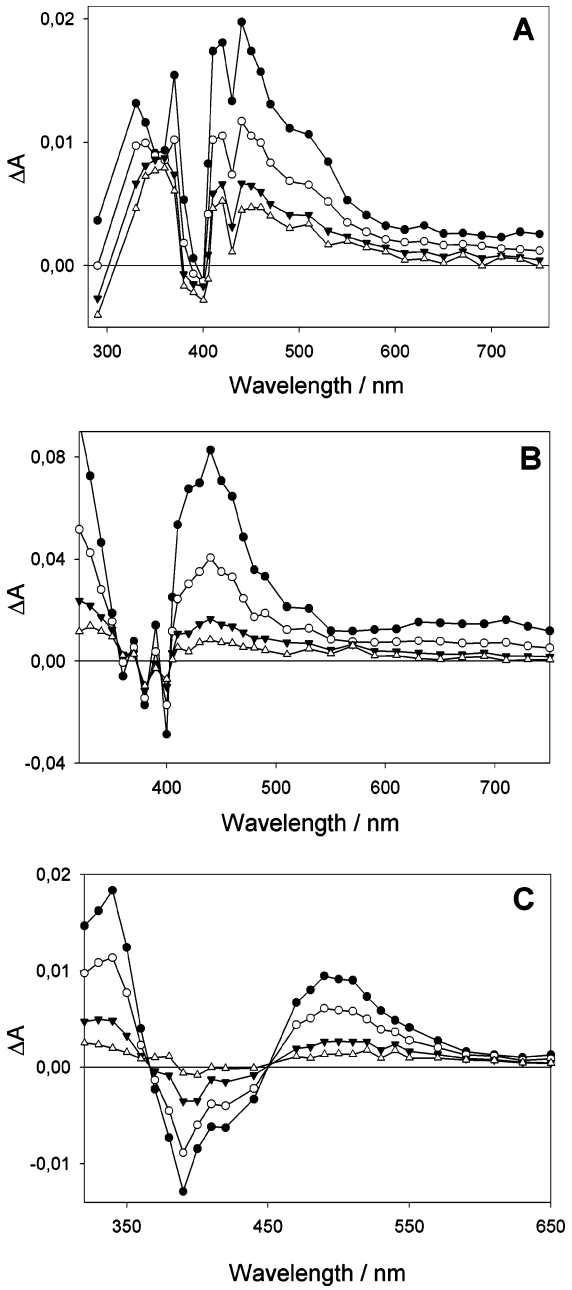

Figure 9. Transient absorption spectrum of acridizinium salts **1a**, **1b**, and **1c** in water [**A**: c(**1a**) = 10^{-4} M; λ_{ex} 308 nm; delays after the laser pulse of ●: 3 μs, ○: 14 μs, ▲: 36 μs, Δ: 73 μs; **B**: c(**1b**) = 10^{-4} M; λ_{ex} 308 nm; delays after the laser pulse of ●: 3 μs, ○: 13 μs, ▲: 32 μs, Δ: 70 μs; **C**: c(**1c**) = 5×10^{-5} M; λ_{ex} 455 nm; delays after the laser pulse of ●: 5 μs, ○: 25 μs, ▲: 74 μs, Δ: 146 μs].

(Table 2) that is somewhat smaller than for the diffusion controlled limit in water (6.5×10^9 M^{-1} s^{-1}).[11] The transients observed for **1a** and **1b** were assigned to their triplet excited states based on their reactivity toward oxygen.

In the case of the amino-substituted acridizinium **1c**, the transient spectra were dependent on the excitation wavelengths. Excitation at 308 and 355 nm led to the formation of two transients with different lifetimes, whereas only one transient was observed when **1c** was excited at 455 (Figure 9C). This transient showed a monoexponential decay and was efficiently quenched by oxygen (Table 2). In analogy to the results with **1a** and **1b** the transient was assigned to the triplet excited state

Table 2. Photophysical Data of the Excited Acridizinium Triplet and Electrochemical Data of Acridizinium Salts **1a**−**c**

	1a	**1b**	**1c**
τ [a,b]/μs	> 500	100	200
k_q $(O_2)^b$/ $s^{-1}M^{-1} \times 10^9$	1.4	3.6	1.4
$k_{sq}{}^b$/ $s^{-1}M^{-1} \times 10^8$	15	5.3	2.7
$k_q{}^{b,c}$ (ferrocene) /$s^{-1}M^{-1} \times 10^9$	18	14	9.9
$k_q{}^{b,c}$ (1,3-CHD)/$s^{-1}M^{-1} \times 10^6$	*d*	5	*d*
E_{0-0}/V	3.1[e]	3.1[e]	2.6[f]
E_{red}/V	−0.7[g]	−0.5[g]	−0.7[h]
E_{red}*/V [i]	2.4	2.6	1.9

[a] Determined by extrapolation to c(**1**) = 0 M; [b] λ_{ex} = 308 nm (**1a** and **1b**) and 455 nm (**1c**); [c] In CH_3CN; data for [1,3-CHD] ≤ 0.3 M; [d] No quenching by 1,3-CHD; [e] Ref 21; [f] Ref 7c; [g] Ref 28; counterion: ClO_4^-, in 0.1 M tetraethylammonium perchlorate in DMF; [h] Counterion BF_4^-, in DMF; [i] $E_{red}^* = E_{0-0} + E_{red}$.

of **1c**. The second transient observed when **1c** was excited at shorter wavelengths was not quenched by oxygen, and further assignment was not pursued. All experiments in the presence of DNA were performed by exciting **1c** at wavelengths longer than 400 nm to avoid the formation of transients different from the triplet excited state.

The triplet excited-state lifetimes of **1a**−**c** were shortened when the concentration of ground-state acridizinium was increased. This result indicates that the triplet states undergo self-quenching when an excited-state interacts with the ground state of the same molecule. The quenching plots were determined for acridizinium concentrations between 5×10^{-5} M to 4×10^{-4} M. The quenching plots were linear and the values for the self-quenching rate constants ranged from 3×10^8 $s^{-1}M^{-1}$ (**1c**) to 2×10^9 $s^{-1}M^{-1}$ (**1a**) (Table 2). The fact that the triplet states of the acridizinium salts undergo efficient self-quenching has to be considered when comparing the triplet lifetimes for experiments performed at different concentrations of these compounds. Table 2 shows the lifetimes for the triplet states extrapolated to "zero" concentration of acridizinium salts.

The magnitude of the triplet energies of the acridizinium derivatives **1a**−**c** were estimated from quenching experiments by using 1,3-cyclohexadiene (1,3-CHD, E_T = 219 kJ/mol) and ferrocene (E_T = 159 kJ/mol) as quenchers. Due to the low solubility of the quenchers in water, the experiments were performed in acetonitrile as solvent. In the case of **1c** its tetrafluoroborate salt was used to increase the solubility of this compound in acetonitrile. The triplet lifetimes of **1a** and **1c** were not significantly shortened in the presence of 1,3-CHD. The triplet state of **1b** was quenched by the diene and the quenching rate constant was estimated to be 5×10^6 s^{-1} M^{-1}. The triplet excited states of **1a**, **1b**, and **1c** were quenched with high efficiency by ferrocene (Table 2). These results suggest that the triplet energy is highest for **1b** than for **1a** and **1c**, because triplet **1b** is quenched inefficiently by 1,3-CHD, whereas the other two compounds are not quenched by this diene. All triplets are quenched by ferrocene with rate constants close to the diffusion controlled limit. Therefore, the triplet energies for **1a**−**c** are higher than 159 kJ/mol. The triplet energy for **1b** is lower than the energy for triplet 1,3-CHD because the quenching rate constant is at least 3 orders of magnitude lower than expected for a diffusional process. This estimate for **1a** is in good agreement with the published triplet energy value of 172 kJ/mol for this compound.[13]

2. Transient-Absorption Studies in the Presence of DNA. The transient absorption spectra of mixtures of acridizinium salts

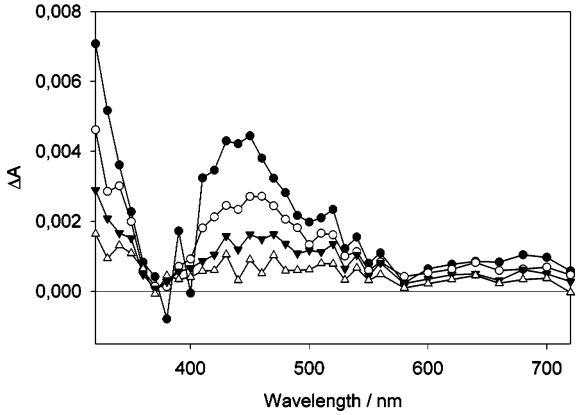

Figure 10. Transient absorption spectrum of 9-bromoacridizinium (**1b**) (10^{-4} M) in the presence of 10 molar equivalents (in bases) of DNA oxygen-free water (λ_{ex} = 308 nm; delays after the laser pulse of ●: 3 μs, ○: 11 μs, ▲: 28 μs, Δ: 62 μs)

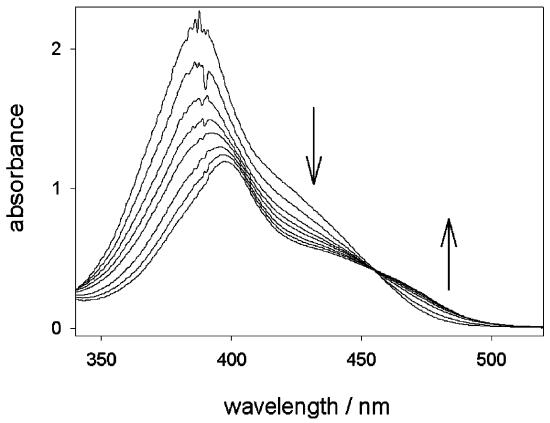

Figure 11. Spectrophotometric titration of ct DNA to **1c** (10^{-5} M); arrows indicate the increase or decrease of the absorption band with increase in DNA-acridizinium ratio up to 10:1.

with calf thymus DNA (ct DNA) at different ratios were investigated. The same transient spectra (λ_{ex} = 308 nm, anaerobic conditions) were observed for **1a** and **1b** in the presence of DNA (Figure 10 for **1b**) as in buffered solutions. The intensity of the absorption was smaller in the presence of DNA compared to samples in water with the same ground-state absorption at the excitation wavelength of the laser. The magnitude of this decrease was enhanced when the DNA concentration was increased. The lifetimes for the triplet states at the different DNA concentration were similar. These results show that no new transients were formed when **1a** and **1b** were excited in the presence of DNA, but the quantum yield for formation of the triplet excited state was decreased. The transient in the presence of DNA was assigned to the formation of triplet states in the aqueous phase, because the signal was quenched by oxygen with the same efficiency as the quenching in homogeneous solvent. The fact that no significant shortening was observed for the triplet lifetimes indicates that the excited state is not being quenched by DNA. To substantiate this lack of reactivity of the triplet state with DNA, quenching studies for triplet **1b** by guanosine-5′-monophosphate were performed. No decrease of the triplet lifetimes was observed for a concentration of 20 mM of the nucleotide. This result indicates that if the triplet state of **1c** is quenched by guanosine-5′-monophosphate the bimolecular quenching rate constant is less than 5×10^5 s^{-1} M^{-1}.

The absorption of **1c** is red shifted when this compound is bound to DNA and an isosbestic point is observed at 455 nm (Figure 11).[7c] Therefore, at wavelengths shorter than 455 nm the molar absorptivity of free **1c** is larger than DNA bound **1c**, whereas the reverse occurs at wavelengths longer than 455 nm. Solutions of **1c** in the presence of DNA were excited at 445, 455, and 465 nm to establish if different transients were formed when either the excitation of free or bound **1c** was enhanced. At the latter wavelength, most of the excited **1c** corresponds to that bound to DNA. At all excitation wavelengths a much smaller transient signal was observed for **1c** in the presence of DNA than in the absence of the nucleic acid. No new transients were detected. The small signals in the presence of DNA precluded any quantitative measurements at varying DNA concentrations.

3. Time-Resolved Fluorescence Spectroscopy in the Presence of DNA. Lifetime measurements for the singlet excited states of **1a** and **1c** in the absence and presence of DNA were determined. The objective of this experiment was to establish if the previously observed decrease of the fluorescence of compounds **1a**−**c** in the presence of DNA[5a] was due to dynamic or static quenching. The former quenching mechanism is related to the bimolecular reaction of the fluorophore and quencher, and leads to equal values for the ratio of intensities (I_o/I) and lifetimes (τ_o/τ) in the absence (I_o, τ_o) and presence of quencher (I, τ). Static quenching is expected to occur when the fluorophore and quencher are associated prior to excitation and only a decrease of the intensity ratio is observed.[20]

The fluorescence decays for **1a** and **1c** in aqueous solutions followed monoexponential kinetics. The lifetime of **1b** is too short to be detected within the limit of the spectrometer. The fluorescence lifetimes of **1a** and **1c** were determined to be 6 ns and 14 ns. The value of **1a** is similar to the one published earlier.[21] Addition of DNA led to fluorescence decays that did not follow a monoexponential behavior. The decays were fitted to the sum of two exponentials. One of the lifetimes recovered were similar to those observed for the acridizinium salts in buffered solution and the corresponding fluorescent species was assigned to the unbound compound in the aqueous phase. The second component had lifetimes of 1 ns and 3 ns for **1a** and **1c** respectively. This species was assigned to the DNA bound acridizinium salt.

To compare the ratio of fluorescence intensities with the ratio of lifetimes intensities in the absence and presence of DNA it is necessary to calculate the average lifetimes (eq 1)[22] from the nonmonoexponential decays in the presence of DNA.

$$\langle\tau\rangle = \frac{\sum_i^n a_i\tau_i}{\sum_i^n a_i} \qquad (1)$$

In the eq 1, n corresponds to the number of exponentials, a_i is the preexponential factor and τ_i is the lifetime for each species.

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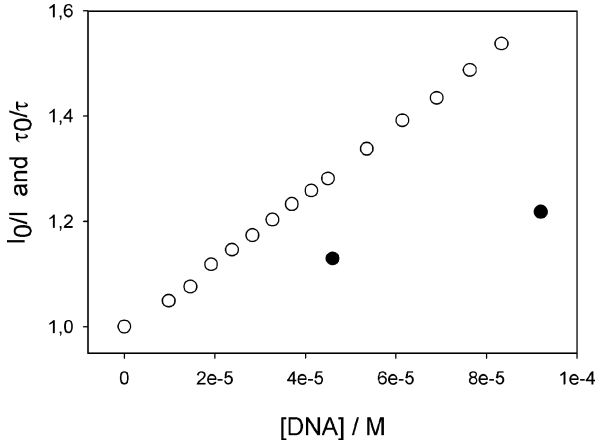


Figure 12. Relative emission intensity (○: I_0/I) and relative average fluorescence lifetime (●: $<\tau_0>/\langle t\rangle$) of aminoacridizinium **1c** upon addition of ct DNA.

The sum of the preexponential factors is defined as being unity. Notably, the steady-state fluorescence quenching (I_0/I) of **1c** by DNA is more efficient than the dynamic one ($<\tau_0>/\langle t\rangle$) as illustrated by the corresponding Stern−Volmer plots (Figure 12), presumably due to static quenching within the DNA-acridizinium complex.

Discussion

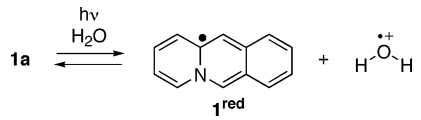
The experiments demonstrate that the efficiency and selectivity of the photoinduced DNA damage in the presence of acridizinium derivatives depends significantly on the oxygen concentration. Most notably, the reactive intermediate, which leads to DNA damage, is not the same when changing from aerobic to anaerobic conditions. It is known that $^1O_2$ selectively oxidizes guanine and selectively cleaves DNA strands at the guanine sites,[18] whereas HO• radicals lead to an unselective DNA strand scission.[23] We therefore conclude from our results that irradiation of acridizinium salts **1a**−**c** in the presence of oxygen yields $^1O_2$ as the reactive oxygen species, whereas in the absence of oxygen HO• radicals are generated. In the following, several aspects of these reaction pathways will be discussed in detail.

A. DNA Damage under Aerobic Conditions. The experiments provide evidence that the aerobic photoinduced DNA damage in the presence of acridizinium salts is mainly caused by singlet oxygen ($^1O_2$). Especially, the increased DNA damage in D_2O, in which the lifetime of $^1O_2$ is increased by a factor of ca. 14, confirms this assumption. Moreover, the preferential DNA damage on guanine residues and increasing strand breaks on base treatment are characteristic features of $^1O_2$-induced DNA damage. Thus, it is likely that the excited acridizinium acts, like other cationic dyes,[3b] as a singlet-oxygen photosensitizer. Since such a photosensitization is most efficient from the excited triplet state, a significant rate of intersystem crossing (ISC) is required in the excited state of the dye. In fact, the laser-flash experiments show that irradiation of the acridizinium derivatives **1a**−**c** generates significant amounts of triplet-excited species ^{3}**1***, which are efficiently quenched by molecular oxygen. The latter being transformed to $^1O_2$. It may be noted that previous attempts to determine acridizinium quenching by oxygen failed due to relative small signal intensities.[13]

The ISC from ^{1}**1*** to ^{3}**1*** is comparable to the one in anthracene, i.e., S_1 ($^1B_{2u}$) to T_3($^3B_{3u}$), and it has been shown to be dependent on the substitution pattern.[21] Most notably, the intercalated acridizinium does not generate $^1O_2$, because a fast ET transfer with the DNA bases prevents triplet formation, as shown by the fluorescence-quenching experiments. Thus, the intercalation of the dye is not required for DNA damage under aerobic conditions. Nevertheless, the intercalation is a dynamic process, so that there is always a sufficient amount of temporarily unbound dye, which may generate $^1O_2$. The bromo-substituted derivative **1b** exhibits more intense transient absorption bands, most likely because of an enhanced ISC by an internal heavy-atom effect of the bromo functionality. All investigated acridizinium salts show a concentration-dependent self-quenching, which is almost diffusion controlled. Thus, a variation of the observed transient lifetimes is due to different concentrations in the respective experiments. It may be assumed that the self-quenching is the result of an electron transfer between an excited dye and a ground-state molecule as proposed for methylene blue[24] or cresyl violet.[25] The resulting intermediates may not be detectable due to a fast electron back transfer. Also, the self-quenching may be a result of a photoinduced dimerization or aggregate formation as reported for anthracene derivatives [26] or squarine dyes.[27]

B. Formation of DNA Strand Breaks under Anaerobic Conditions. The most interesting result is the discovery that the irradiation of acridizinium salts in aqueous medium generates hydroxyl radicals, which may further damage DNA by hydrogen abstraction. The formation of these reactive intermediates most likely results from a photoinduced electron transfer between the excited acridizinium and water, which leads to the corresponding radical cation of water and the reduced acridizinium **1**^{red}. The formation of **1**^{red} has been proposed earlier during cathode reduction of **1a**.[28] Subsequent deprotonation of the water radical cation leads to the hydroxyl radical (Scheme 1). This proposal

Scheme 1



is consistent with the observation that the acridizinium salt with the largest excited-state reduction potential (i.e., **1b**, Table 2) results in a higher yield of DNA damage than the one with the lowest reduction potential (**1c**). Nevertheless, the different efficiencies of DNA damage may also be explained by the binding modes of **1a**−**c**. We have shown that **1c** binds to DNA exclusively by intercalation at high DNA-to-dye ratios, whereas **1a** and **1b** also bind to the DNA backbone.[5a] The intercalated dye does not contribute to the photoinduced hydroxyl-radical formation due to an efficient ET reaction with DNA bases, and groove-bound and unbound molecules are usually not involved in this ET reactions. Therefore, it may be possible that **1a** and **1b** generate more hydroxyl radicals than **1c** under identical

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conditions because more nonintercalated acridizinium molecules are available for the corresponding reaction with water.

Recently, hydroxyl radicals were reported to be formed in a similar PET process of an excited Ruthenium(III) complex,[29] ninhydrin,[30] or benzophenone[31] with water; but the proposal that free hydroxyl radicals are formed upon irradiation of quinones in water was shown to be erroneous.[32] In the case of acridizinium salts **1a**−**c**, however, we were able to intercept the hydroxyl radicals with terephthalic acid and observed characteristic inhibitory patterns with respect to the DNA damage in the presence of *t*-BuOH. Thus, the formation of so-called "crypto-hydroxyl radicals", which have been proposed as intermediates that do not exhibit typical characteristics of "free radicals" but which react like a hydroxyl radical, can be excluded.[33]

Considering our results with acridizinium derivatives, it cannot be excluded that other cationic dyes also give rise to the formation of hydroxyl radicals upon irradiation. Nevertheless, to the best of our knowledge, only hints for analogous reactions are found in the literature.[34] Presumably, this reaction pathway has been overlooked, since DNA-cleavage experiments are often performed under aerobic conditions and the reactive species under investigation is $^1O_2$, which is formed with significantly higher quantum yields. This point should also be considered in the case of acridizinium salts, because under aerobic conditions, hydroxyl radicals can also be formed along with the singlet oxygen and the former intermediate should therefore be detectable under these conditions. Nevertheless, the efficiency of photoinduced $^1O_2$ formation and subsequent DNA oxidation is significantly higher than the one of the hydroxyl-radical formation, as shown by the higher extent of DNA damage under aerobic conditions as compared to anaerobic conditions. Thus, hydroxyl radicals do not contribute significantly to the overall DNA damage under aerobic conditions.

Most notably, this method represents a useful addition to the most common methods for hydroxyl radical formation; i.e., the Fenton reaction or radiolysis of water.[35] Only few examples are known for UV-light-induced hydroxyl-radical generation. In one approach, the precursor molecules carry a hydroperoxy functionality,[36] which releases hydroxyl radicals upon irradiation. Nevertheless, the thermal and chemical lability of the peroxide may constitute a significant drawback of this method. Also, hydroxypyridone and derivatives thereof have been used as hydroxyl radical sources,[37] but these compounds do not exhibit a high affinity for DNA binding. Moreover, their absorption overlaps with the one of the DNA bases so that irradiation of the pyrones may also induce direct DNA damage, e.g., photodimerization of thymine bases. Thus, irradiation of acridizinium salt in aqueous solution offers a new and complementary method for the generation of HO• radicals under very mild conditions, especially at excitation wavelengths (>360 nm) at which the biomaterial does not absorb significantly. Considering the absorption bands of acridizinium derivatives the irradiation may even be performed with visible light. Thus, the hydroxyl-radical formation may be addressed selectively by the choice of the irradiation wavelength. Apparently, the DNA association of the acridizinium is not required for hydroxyl-radical generation. In contrast, intercalated acridizinium does not react with water because of a highly competing ET reaction with the DNA bases. Nevertheless, the high affinity of the dye toward DNA (10^4−10^5 M^{-1})[5a] provides a large concentration of dye in close vicinity of the DNA backbone that may generate the hydroxyl radicals directly at the reaction center of the DNA damage. In addition, we showed in preliminary experiments that a binding process is not necessarily required for efficient damage reactions with hydroxyl radicals. Thus, in preliminary experiments we have shown that the tripeptide Pro-Pro-Pro can be cleaved under these mild conditions. We will continue these experiments in order to study the selectivity of peptide cleavage.

C. Involvement of the Intercalated Dye in the DNA-Damage Reaction. Our experiments reveal that the major pathways in the acridizinium-induced DNA damage are the formation of hydroxyl radicals or singlet oxygen depending on the oxygen concentration of the sample. In both processes, the reactive intermediate is formed in an external reaction between the excited acridizinium ion and molecular oxygen or water, i.e., the association of the dye with DNA appears not to be necessarily required. Nevertheless, since acridizinium salts and derivatives thereof are good intercalators, it needs to be evaluated whether the singlet or triplet excited intercalated molecules also contribute to the overall DNA damage.

From our experiments, we exclude that the triplet excited state of the intercalated acridizinium ions plays a major role in degradation reaction, because in the presence of DNA, new transients or a quenching of the triplet, which would have indicated a reaction between the excited dye and DNA, were not observed. In addition, the triplet state is formed with a reduced yield and it is quenched by oxygen with the same efficiency as in the absence of DNA, suggesting that the triplet state is not protected by binding to DNA from the reaction with oxygen.

These results indicate that only the transient of the uncomplexed acridizinium is observed in the presence and in the absence of DNA, whereas the intercalated acridizinium does not contribute to the transient spectrum. Although it cannot be excluded that the intercalated triplet-excited dye reacts faster than the detection limit of the apparatus, i.e., within the laser pulse, we conclude from the additional fluorescence-spectros-

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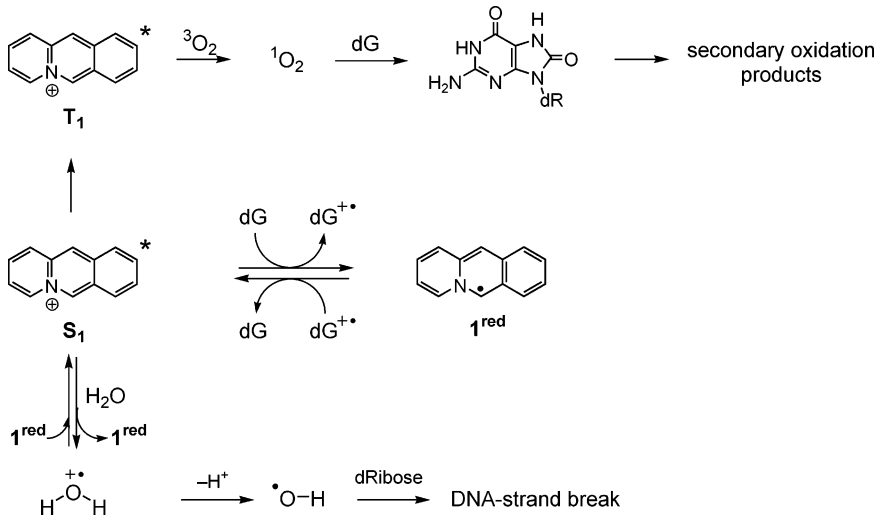
Scheme 2



copy experiments that the DNA-bound acridizinium does not form the excited triplet state at all upon irradiation.

The reluctance of acridizinium salts to form excited triplet state within DNA may be explained with the data from the steady-state and time-resolved fluorescence spectroscopy. Along with the minor quenching pathway that leads to the short fluorescence lifetime, another major quenching process takes place at such a rate that the corresponding fluorescence lifetime is too short to be detected within the time-resolution of the instrument. Since recent results show that ET reaction rates of intercalated dyes with DNA are in the femto-second time scale,[38] it may be concluded that the fast quenching results from an ET of the intercalated excited acridizinium with the DNA base pairs. Considering the reduction potential of acridizinium salts **1a**−**c** (Table 2) and the one of the nucleic bases, each singlet excited acridizinium derivative **1a**−**c** is able to oxidize the nucleic bases in an exergonic reaction. Therefore, a PET reaction is most likely responsible for the fluorescence quenching by DNA and, thus, for the lack of acridizinium triplet formation within DNA.

According to the discussion above, the radical $\mathbf{1}^{red}$ is formed as reactive intermediate within DNA upon irradiation, and it may be assumed that this compound also reacts with DNA. Nevertheless, since $\mathbf{1}^{red}$ corresponds to a π radical, a hydrogen abstraction reaction is very unlikely. Considering the relatively high reaction rate of electron back transfer (BET), which is in the femtosecond range and has recently been observed to be significant in complexes between thionine and DNA,[39] we also propose that the main reaction pathway following the initial PET reaction is the electron back transfer. A hole transfer through the DNA strand and subsequent damage of the guanine moieties, i.e., formation of 8-oxo-7,8-dihydroguanine, can be excluded because in this case, alkali labile lesions and a pronounced selectivity of the DNA damage at guanine sites should have been observed under anaerobic conditions. Thus, the excitation of the intercalated dye leads to no net reaction, i.e., no DNA damage, since the starting components are regained.

In summary, we investigated three initial photoprocesses upon irradiation of acridizinium salts in the presence of DNA and evaluated their relevance for the subsequent DNA damage (Scheme 2). Under aerobic conditions, triplet sensitization leads to $^1O_2$, which leads to an oxidative base modification of DNA, whereas under anaerobic conditions, an ET reaction with water generates hydroxyl radicals, which abstract hydrogen atoms from the DNA backbone and induces a direct strand cleavage. The intercalated acridizinium salt leads to a primary PET reaction with the DNA bases; however, a fast BET transfer regains the dye and the DNA, so that the excited intercalated dye does not contribute significantly to the overall DNA damage.

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