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Photochemical Cleavage of DNA by Nitrobenzamides Linked to 9-Aminoacridine†

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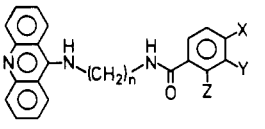
ABSTRACT: Nitrobenzamido ligands linked to the DNA intercalator 9-aminoacridine via poly(methylene) chains induce single-strand nicks in DNA upon irradiation with long-wavelength ultraviolet light ($\lambda \geq 300$ nm). Optimal photocleavage activity was found for the reagent 9-[[6-(4-nitrobenzamido)hexyl]amino]-acridine. Removal of the acridinyl ligand or changing the position of the nitro group from the 4- to the 2-position caused a 10-fold decrease in photocleavage efficiency, whereas a change to the 3-position caused a 30-fold reduction. The DNA cleavage was 5-fold enhanced by subsequent piperidine treatment and showed some sequence dependency with predominant cleavage at G and T residues. Furthermore, significant differences in cleavage preference were observed when the poly(methylene) linker length was changed.

Photochemical single-strand cleavage of DNA has been observed with several DNA binding reagents. The DNA photocleavage by methylene blue (Friedman & Brown, 1978; OhUigin et al., 1987), acridine orange (Freifelder et al., 1961; Bowler et al., 1984), or porphyrins (Praseuth et al., 1986) proceeds by an oxidative mechanism via singlet oxygen or via electron transfer, whereas the photocleavage by transition metal complexes of bleomycin or triphenanthrolines occurs via either singlet oxygen or a radical mechanism depending on the metal ion (Chang & Meares, 1984; Subramanian & Meares, 1985, 1986; Barton, 1986; Fleischer et al., 1986). Furthermore, a chemical DNA cleaving reagent has been developed by linking EDTA to the DNA intercalator methidium (Dervan, 1986), and this reagent has been used to footprint the binding of proteins to DNA in vitro (Van Dyke & Dervan, 1983) and in situ (Cartwright & Elgin, 1984). Such synthetic "nucleases", could be very useful tools in molecular biology, especially for studying protein–DNA interactions in vivo.

Table I



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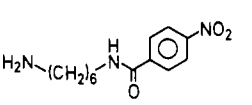

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reagent	X	Y	Z	*n*	photocleavage act.[a]
1a	NO_2	H	H	4	90
1b	NO_2	H	H	6	100
1c	NO_2	H	H	8	80
1d	H	NO_2	H	6	3
1e	H	H	NO_2	6	10
1f	NO_2	NO_2	H	6	10
1g	NO_2	H	NO_2	6	75
1h	H	H	H	6	<1
2					10

[a] The relative photocleavage activity was determined from the reagent concentration that under otherwise identical conditions, as specified in the legend to Figure 1, gave 50% relaxation of the pUC 19 DNA.

We are presently designing novel "photonucleases" based on previous work with polyfunctional DNA-intercalating 9-aminoacridine derivatives (Buchardt et al., 1984, 1987; Nielsen, 1982, 1985; Nielsen et al., 1983, 1984; Jeppesen et

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al., 1988) and are presenting some of the results in this paper.

The reagent design (Table I) is based on linking a DNA photocleaving ligand, in this case a nitrobenzamido group, to a 9-aminoacridinyl group via a poly(methylene) chain. The 9-aminoacridinyl group assures high affinity for DNA, and the nitrophenyl ligands which can abstract hydrogen and/or donate oxygen photochemically serve successfully as DNA cleavers (Buchardt et al., 1987).

Materials and Methods

Chemistry. 4-Nitrobenzoyl chloride was obtained from Aldrich and 9-phenoxyacridine was prepared as previously described (Dupré & Robinson, 1945). ^{1}H NMR spectra were recorded on a JEOL FX 90Q spectrometer, solvent DMSO, with TMS as internal standard, and mass spectra on a Masslab VG 12-250 quadrupole mass spectrometer fitted with a VG FAB source and probe. The elemental analyses were performed by Preben Hansen, The H. C. Ørsted Institute.

N-[6-[(tert-Butoxycarbonyl)amino]hexyl]-4-nitrobenzamide. (*tert*-Butoxycarbonyl)hexanediamine hydrochloride (Hansen et al., 1982) (2.7 g, 11 mmol) was dissolved in water (50 mL) containing sodium hydrogen carbonate (3.1 g, 43 mmol), and ethyl acetate (100 mL) was added. Subsequently, 4-nitrobenzoyl chloride (2.5 g, 13 mmol) was added slowly and the reaction mixture was stirred for 18 h at room temperature. After this the organic phase was isolated, washed with 5% sodium hydrogen carbonate (100 mL) and water (2 × 50 mL), and dried over anhydrous magnesium sulfate, and the solvent was evaporated, in vacuo. Recrystallization from ethyl acetate (ca. 30 mL) yielded 3.1 g (80%); mp 132–133 °C. Anal. Calcd for $C_{18}H_{27}N_3O_5$: C, 59.11; H, 7.45; N, 11.50. Found: C, 58.92; H, 7.58; B, 11.46.

9-[[6-(4-Nitrobenzamido)hexyl]amino]acridine (**1b**) *Hydrochloride and Compound* **2** *Hydrochloride. N*-[6-[(*tert*-Butoxycarbonyl)amino]hexyl]-4-nitrobenzamide (1 g, 2.7 mmol) was dissolved in 1 M HCl in acetic acid (20 mL). After 1 h at room temperature the solvent was removed, in vacuo, to give compound **2** hydrochloride in almost quantitative yield. This was dissolved in phenol (10 g) and heated to 80 °C. Subsequently, 9-phenoxyacridine (0.74 g, 2.7 mmol) was added and the temperature was increased to 120 °C for 0.5 h, after which the cooled mixture was extracted with ether (3 × 50 mL). The remaining crystalline material (1.3 g) was isolated and recrystallized by dissolution in boiling methanol (20 mL), which after filtering and cooling was poured into ether (100 mL). Yield after three such purifications: 0.73 g (55%); mp 215–216 °C. Anal. Calcd for $C_{26}H_{26}N_4O_3$·HCl·0.5H_2O: C, 63.99; H, 5.78; N, 11.48; Cl, 7.27. Found: C, 63.59; H, 5.59; N, 11.50; Cl, 7.39. FAB-MS: MH^+ = 443 (M_r 442).

Compounds **1a** and **1c–h** were prepared analogously to **1b**. They all showed the expected ^{1}H NMR spectra. **1a**·HCl·0.5H_2O, mp 255–257 °C. Anal. Calcd for $C_{24}H_{22}N_4O_3$·HCl·0.5H_2O: C, 62.68; H, 5.26; N, 12.41; Cl, 7.71. Found: C, 63.04; H, 5.07; N, 12.18; Cl, 7.70. FAB-MS: MH^+ = 415 (M_r 416). **1c**·HCl·1.5H_2O, mp 148–151 °C. Anal. Calcd for $C_{28}H_{30}N_4O_3$·HCl·1.5H_2O: C, 62.97; H, 6.42; N, 10.49; Cl, 6.64. Found: C, 63.16; H, 5.96; N, 10.40; Cl, 6.80. FAB-MS: MH^+ = 471 (M_r 470). **1d**·HCl·1.5H_2O, mp 142–145 °C. Anal. Calcd for $C_{26}H_{26}N_4O_3$·HCl·1.5H_2O: C, 61.72; H, 5.98; N, 11.07; Cl, 7.01. Found: C, 61.67; H, 5.74; N, 10.97; Cl, 7.46. **1e**·HCl·3.5H_2O, mp 120–125 °C (decomp). Anal. Calcd for $C_{26}H_{26}N_4O_3$·HCl·3.5H_2O: C, 57.62; H, 6.32; N, 10.34; Cl, 6.54. Found: C, 57.52; H, 5.25; N, 10.23; Cl, 6.94. FAB-MS: MH^+ = 443 (M_r 442). **1f**·HCl·H_2O, mp 169–174 °C. Anal. Calcd for $C_{26}H_{25}N_5O_5$·HCl·H_2O: C, 57.62; H, 5.21; N, 12.92; Cl, 6.54. Found: C, 57.91; H, 5.15; N, 12.87; Cl, 6.65. FAB-MS: MH^+ = 488 (M_r 487). **1h**·HCl·1.5H_2O. Anal. Calcd for $C_{26}H_{27}N_3O$·HCl·1.5H_2O: C, 67.73; H, 6.79; N, 9.12. Found: C, 67.21; H, 6.45; N, 8.85. FAB-MS: MH^+ = 397 (M_r 398).

Plasmid Relaxation Assay. pUC 19 DNA was transformed into *Escherichia coli* JM 101 and isolated by the alkaline extraction procedure (Birnboim & Doly, 1979). In a typical experiment 0.3 μg of DNA in 10 μL of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) was mixed with the desired amount of reagent dissolved in 1 μL of dimethyl sulfoxide (it was checked that the presence of 10% dimethyl sulfoxide did not influence the cleavage activity) and irradiated in Eppendorf tubes from above at room temperature by using Philips TL fluorescent light tubes at a distance of 15 cm [TL 40 W/03, λ ~420 nm (25-nm bandwidth), 20 J m^{-2} s^{-1}; TL 20 W/09N, λ ~ 365 nm (~30-nm bandwidth), 22 J m^{-2} s^{-1}; TL 20 W/12, λ ~ 300 nm (~30-nm bandwidth), 24 J m^{-2} s^{-1}]. The DNA analyzed by electrophoresis in 1% agarose in 0.5 × TBE buffer (45 mM Tris–borate, 0.5 mM EDTA, pH 8.3).

Quantitation of the photonicking was performed by densitometric scanning of photographs of the ethidium-stained gels. The staining of relaxed DNA was found to be 1.5 times that of supercoiled plasmid. The results were corrected for this difference in staining intensity.

Photocleavage of ^{32}P-End-Labeled DNA Fragments. The 90 and 232 bp *Eco*RI–*Pvu*II fragments of the plasmid pUC 19 were used to study DNA cleavage at the nucleotide level. The fragments were 3′-end-labeled at the *Eco*RI site (using [α-^{32}P]dATP and the Klenow DNA polymerase fragment) and purified according to standard techniques (Maniatis et al., 1982). In a typical cleavage reaction 0.1–0.2 pmol of end-labeled fragment was mixed with 0.5 μg of calf thymus DNA in 100 μL of TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA). After addition of reagent (dissolved in H_2O, 5 μL) the samples were irradiated from above with 300-nm light for 30 min by using the Philips TL 20W/12 lamp. The DNA was recovered by ethanol precipitation and treated with 1 M piperidine at 90 °C for 20 min followed by precipitation with 10 volumes of 1-butanol. The DNA pellet was washed with 70% EtOH and lyophilized. The samples were subsequently fractionated on 8% polyacrylamide/50% urea sequencing gels and the cleavage products visualized by autoradiography.

Results

The DNA photocleaving properties of the (nitrobenzamido)acridines were tested in a plasmid-relaxation assay as exemplified in Figure 1. The combined results, which are presented in Table I, show that the photocleaving activity is dependent on the presence of the nitro group (compare **1h** with the rest). It is heavily dependent on the position of this nitro group, 4-NO_2 > 2-NO_2 > 3-NO_2 (compare **1b**, **1d**, and **1e**), and it is slightly influenced by the length of the linker [$(CH_2)_n$] between the DNA intercalator, 9-aminoacridinyl, and the nitrobenzamido group (n = 6 > 4 > 8, **1a–c**). The presence of the 9-aminoacridinyl group is also important (**1b** versus **2**), and this is not due to an altered DNA conformation caused by intercalation since no enhancement of photocleavage by reagent **2** was detected in the presence of 9-aminoacridine even at DNA-saturating concentrations of the acridine (results not shown). The presence of a second nitro group does not enhance the photocleavage efficiency (**1g**) and, in the case of the 3,4-dinitro derivative (**1f**), even decreases it. 9-[[(4-Nitrobenzamido)hexyl]amino]acridine (**1b**) was found to be the most efficient, showing activity in the micromolar concentration range (Figure 1) corresponding to 1 reagent/30 bp, resulting in 1 cleavage/3000 bp (i.e., ~1% efficiency). The quantum

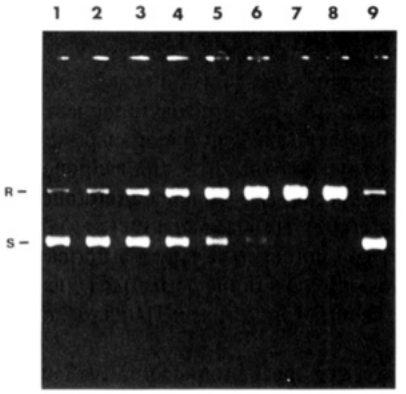


FIGURE 1: Relaxation of supercoiled pUC 19 DNA by photocleavage. pUC 19 DNA (0.3 μg) was incubated in 10 μL of TE with various amounts of reagent **1b** and irradiated for 30 min at 300 nm. The samples were analyzed by gel electrophoresis in 1% agarose (0.5 × TBE). The gel was stained with ethidium bromide and photographed to observe fluorescence (red filter, 320-nm excitation). The concentrations of **1b** were 0, 0.5, 1, 2, 5, 10, 20, 40, and 0 μM, respectively (lanes 1–9). R, relaxed circular plasmid; S, supercoiled circular plasmid.

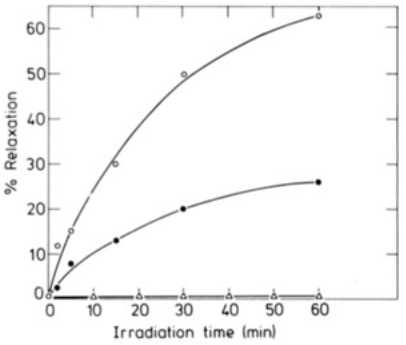


FIGURE 2: Irradiation and wavelength dependence of the photocleavage. Samples containing 0.3 μg of pUC 19 and 10 μM **1b** in 10 μL of TE were irradiated for the times indicated and analyzed by agarose gel electrophoresis as described in the legend to Figure 1. Fluorescent light tubes (see Materials and Methods) emitting at 300 nm (○), 365 nm (●), or 420 nm (△) were used.

efficiency for photonicking of pUC 19 DNA (2.7 kb) by **1b** is estimated as 10^{-5} nicks/absorbed photon from the extinction coefficient at 300 nm of **1b** (ϵ = 7600 M^{-1} cm^{-1}), the pathway of the light in the sample (1 mm), the measured light intensity of the lamp (4.4×10^{15} quanta s^{-1} cm^{-2}; irradiated area = 0.12 cm^2), and the initial rate of the photocleavage (Figure 2). Furthermore, the photocleavage was most efficient with light ($\lambda \sim 300$ nm; Figure 2) overlapping the strongest absorption band of the nitrobenzamido group [λ_{max} = 270 nm, ϵ_{270} = 11 800, ϵ_{300} = 4400 for *N*-methyl-4-nitrobenzamide, and λ_{max} = 267 nm, $\epsilon_{267} \sim 51\,800$, $\epsilon_{300} \sim 7600$ for **1b** (Figure 3)].

The presence of the $^1O_2$ quencher N_3^- (10 mM) (Haag & Mill, 1987) did not have any effect on the photocleavage by reagent **1b**. Neither had the presence of dithiothreitol (10 mM, data not shown) nor saturation of the reaction mixture with O_2 or N_2 (by vortexing of the mixture in the Eppitube filled with O_2 and N_2, respectively; repeated twice).

The influence of various buffer conditions on the DNA photonicking activity of **1b** was examined in order to be able to evaluate the potential of the nitrobenzamides in biological systems. As shown in Table II, Na^+ or Mg^{2+} concentrations in the physiological range have a slight inhibitory effect on the photonicking. This is probably due to a decreased affinity of the reagent to DNA under these conditions, a phenomenon characteristic for the binding of cationic intercalators to DNA (Wilson & Jones, 1981). Variations in pH, in the range

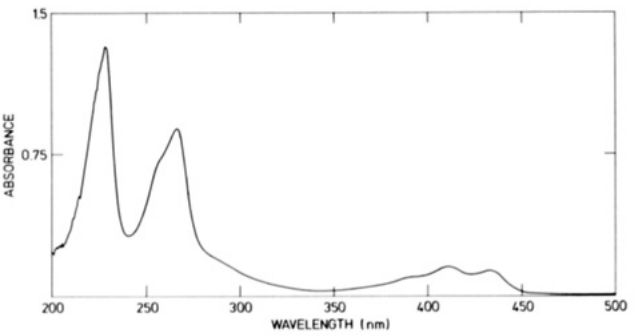


FIGURE 3: Absorption spectrum of reagent **1b** (17 μM) in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 10% dimethyl sulfoxide. There were no significant differences in the absorption spectra of reagents **1a–g** in the 300–450-nm range.

Table II: Photocleavage as a Function of Added Salts

conditions	rel photocleavage by reagent **1b**[a]	conditions	rel photocleavage by reagent **1b**[a]
TE (pH 7.4)	100	+1 mM $MgCl_2$	79
+50 mM NaCl	57	+10 mM $MgCl_2$	45
+100 mM NaCl	54		

[a] Calculated from the degree of plasmid relaxation by using a 10 μM solution of **1b** and 60-min irradiation at 300 nm.

4.5–8.5 (phosphate buffer) or in the buffer (Tris, phosphate, acetate), did not have any influence on the photonicking efficiency of **1b**.

The base and sequence specificity of the DNA photocleavage by the nitrobenzamides was analyzed by using a ^{32}P-end-labeled DNA fragment. The results presented in Figure 4 show that photocleavage is most pronounced (lane 4) at some T and G residues, but it is also apparent that the photoreaction is not base specific since the DNA is not cleaved at all T and G residues, and cleavage is also occurring at some of the C residues. It is furthermore observed that the DNA cleavage is 5-fold enhanced upon subsequent treatment with piperidine at 90 °C without any apparent change in sequence/base specificity (lane 4 versus lane 5). The superiority of the 4-nitro derivatives over the 2- or 3-nitro derivatives in DNA photocleavage (Table I) is confirmed by the experiments using a specific DNA fragment (Figure 4b).

Changing the linker length between the acridine and the nitrobenzamido group significantly influences the sequence pattern of the photocleavage (Figures 4b and 5). Reagent **1c** (n = 8) exhibits pronounced T preference whereas reagent **1a** (n = 4) shows the least base preference (Figure 5).

Alkaline-labile sites are induced in the DNA by 300-nm irradiation alone at some C residues in polypyrimidine tracts (Figure 4, lanes 2). We tentatively ascribe this cleavage to the formation of 6,4 pyrimidine–pyrimidone adducts (Johns et al., 1964) or their precursors (Franklin et al., 1982).

Discussion

Because of the importance of the 9-aminoacridinyl ligand for the DNA photocleavage efficiency by the nitrobenzamides, we believe that the DNA photocleavage by reagents **1** is performed by the intercalation complex.

The detailed cleavage mechanism is not yet known. It is highly unlikely that $^1O_2$ participates due to lack of influence of the oxygen concentration as well as the lack of quenching by the azide ion. It is conceivable that radical chemistry is involved since it is wellknown that radicals are formed upon irradiation of aromatic nitro compounds (Döpp, 1975; Levy & Cohen, 1979). Such a mechanism is also compatible with

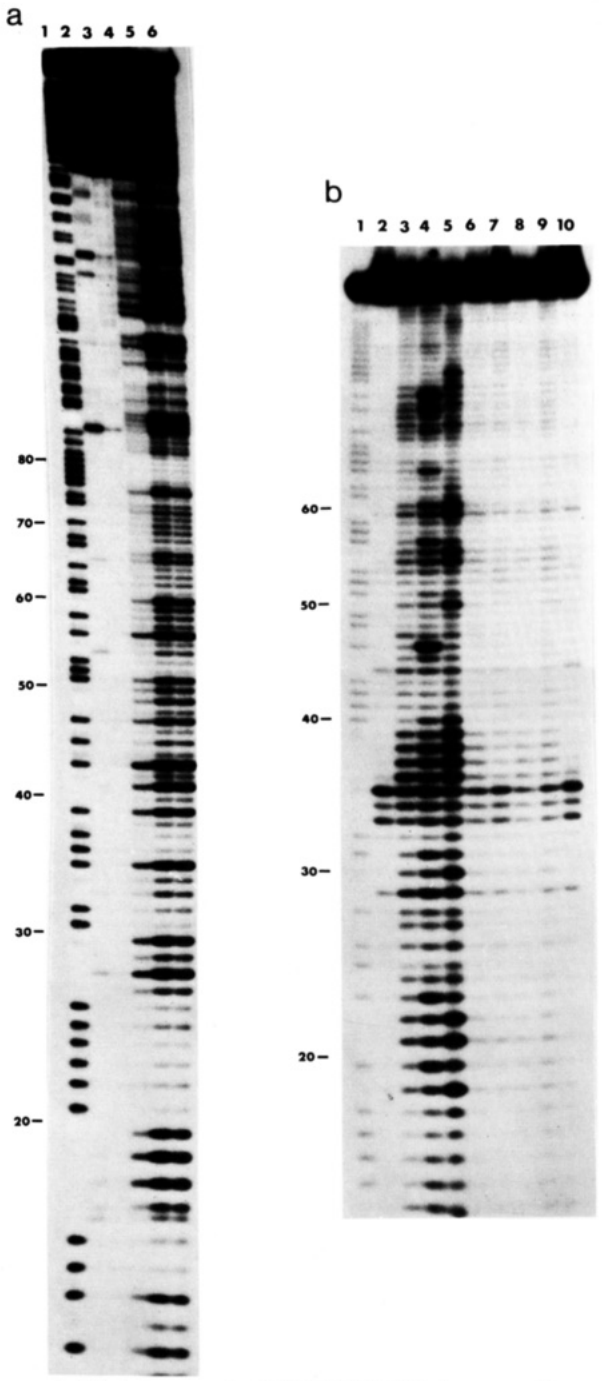


FIGURE 4: Photocleavage of a 3′-^{32}P-labeled DNA fragment. Sequence specificity of the photocleavage. (a) The 232 bp *Eco*RI(3′-^{32}P)–*Pvu*II fragment of pUC 19 was used with irradiation at 300 nm for 30 min. Lane 1: A + G sequence reaction. Lane 2: Irradiation background without reagent (piperidine treated). Lane 3: Control (40 μM **1b**), no irradiation (piperidine treated). Lane 4: Photocleavage with reagent **1b** (40 μM) without subsequent piperidine treatment. Lanes 5 and 6: Photocleavage with reagent **1b** (40 or 20 μM) and piperidine treatment. (b) The 90 bp *Eco*RI(3′-^{32}P)–*Pvu*II fragment of pUC 19 was used. Lane 1: A + G sequence reaction. All other samples were irradiated (300 nm, 30 min) and treated with piperidine. A 20 μM concentration of reagent was used in each case. Lanes 3–10: Reagents **1a**, **1b**, **1c**, **1d**, **1e**, **1f**, **1g**, and **2**, respectively. Lane 2: No reagent.

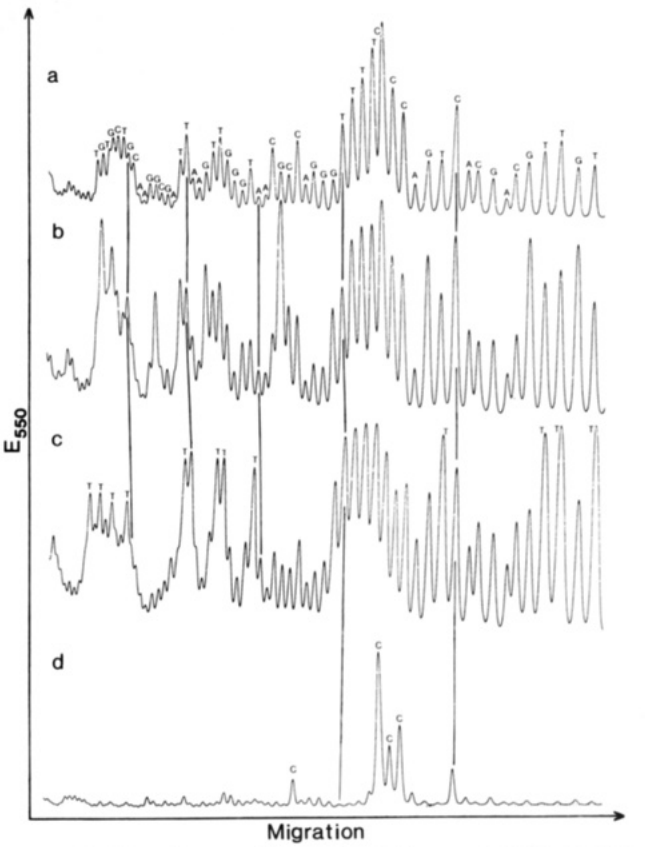


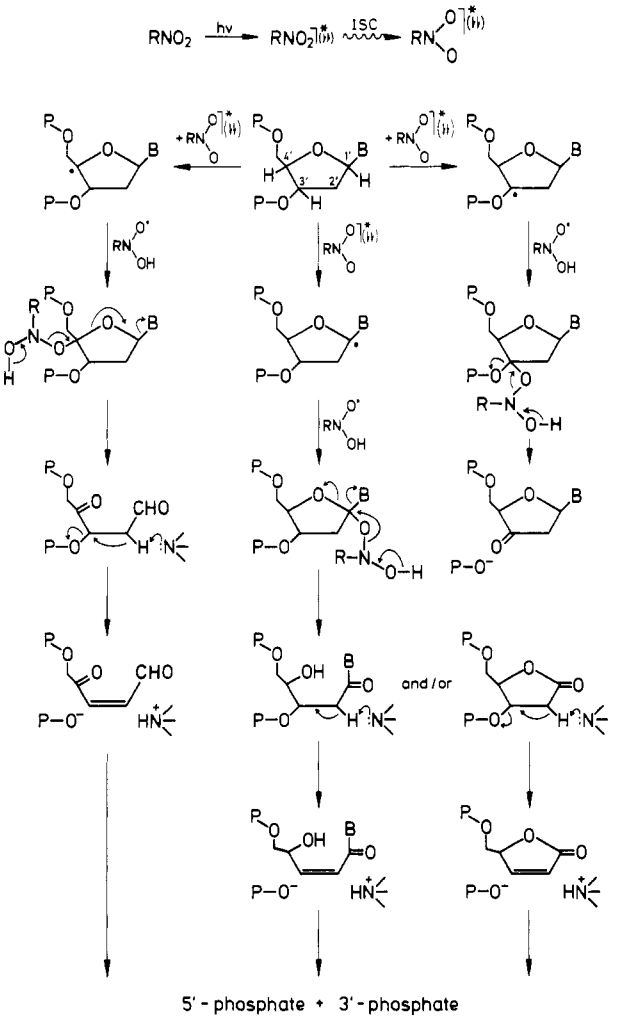
FIGURE 5: Photocleavage sites on the 90 base pair 3′-^{32}P-labeled *Eco*RI–*Pvu*II fragment of pUC 19. The densitometric scans of lanes 3, 4, 5, and 2 of the autoradiogram presented in Figure 4b are shown. (a) Reagent **1a**; (b) reagent **1b**; (c) reagent **1c**; (d) control, no reagent.

the observed differences between the 2-, 3-, and 4-nitrobenzamides. These differences correlate well with the relative photochemical hydrogen abstraction efficiencies of the 3- and 4-nitrophenyl moities (Hashimoto & Kano, 1972), whereas we suggest that the low efficiency found with the 2-nitrophenyl group is due to steric constraints. The mechanism for cleavage of DNA presumably involves a β-elimination in the deoxyribose moiety, thereby accounting for the catalytic effect of piperidine. Such a cleavage mechanism is also consistent with the formation of 5′-phosphate-containing DNA cleavage products indicated by the identical gel electrophoretic migration of the DNA fragments produced by the G + A sequence reaction and those produced by reagents **1a–c** (Figure 4).

We have recently found that a [[(diazocyclopentadienyl)-carbonyl]oxy]-9-aminoacridine derivative (Nielsen et al., 1983) analogous to **1b** photocleaves DNA practically without any sequence preference (Nielsen et al., 1988). It is therefore highly improbable that the sequence preference observed with reagent **1b** should be caused by any sequence preference of the acridine intercalation. On the other hand, if the cleavage were caused by diffusable radicals, much less base specificity would be expected since DNA cleavage by EDTA–Fe(II) complexes, which is proposed to proceed via hydroxyl radicals (D'Andrea & Haseltine, 1978; Hertzberger & Dervan, 1984), is essentially base and sequence independent (Dervan, 1986; Tullius, 1987).

We propose a mechanism involving hydrogen abstraction from the deoxyribose of the DNA backbone by the excited triplet state of the nitro group (Buchardt et al., 1987), thereby producing deoxyribose radicals, followed by oxygen transfer from the nitro group (Scheme I). It is known that photooxidation by aromatic nitro compounds can proceed via such a mechanism (Döpp, 1975). Reaction at the 3′-position (or the presumed less reactive 5′-position) would be expected to result in spontaneous cleavage of the DNA backbone whereas the products arising from hydrogen abstraction from the 1′- or 4′-position would be susceptible to alkaline hydrolysis via

Scheme I



β-elimination. Such a mechanism accounts for both spontaneous photonicking and the additional alkaline-labile photoproducts. The sequence dependence could (at least partly) be caused by a conformational differences along the DNA helix, especially with respect to the deoxyribose, maybe to some degree induced by intercalation. The conformation of the DNA in terms of, e.g., base tilt and twist angles is sequence dependent (Dickerson, 1983) and could render some of the deoxyribose units especially susceptible to attack.

The influence of the length of the poly(methylene) linker on the sequence preference could also reflect an effect of deoxyribose conformation. Model building indicates that a linker length of four (or eight, respectively) methylenes allows photoreaction with deoxyriboses at the intercalation site (or one base pair away, respectively) in the 3′–5′ direction and one (or four, respectively) base pair(s) away in the 5′–3′ direction, assuming an intercalation geometry with the 9-amino group of the acridine protruding into the minor groove (Hansen et al., 1983). If these considerations are correct, the information obtained by linker length dependent sequence-preferential DNA cleavage could be useful in the study of the conformational changes in the DNA B-helix close to intercalation sites.

Conclusion

We have shown that 9-[[(nitrobenzamido)polymethylene]amino]acridines induce single-strand nicks in double-stranded DNA in a light-dependent reaction. Thus the nitrobenzamido ligand can be used in the design of a new DNA photocleaving reagents. Such reagents include compounds like **1b**. This reagent may be regarded as a photochemical analogue of the well-described methidium–EDTA reagent (Hertzberger & Dervan, 1982; Dervan, 1986) which has been used as a DNA footprinting reagent [e.g., Van-Dyke and Dervan (1983) and Cartwright and Elgin (1984)]. Furthermore, it should be feasible to construct DNA site directed "photonucleases" by coupling nitrobenzamido ligands to oligonucleotides in analogy with the reagents based on EDTA (Boutorin et al., 1984; Moser & Dervan, 1987), aryl azides (Le Doan et al., 1987a; Praseuth et al., 1987), or porphyrins (Le Doan et al., 1987b).

Registry No. **1a**·HCl, 112757-45-6; **1b**·HCl, 112767-03-0; **1c**·HCl, 112757-46-7; **1d**·HCl, 115307-02-3; **1e**·HCl, 115307-03-4; **1f**·HCl, 112757-47-8; **1g**·HCl, 112757-48-9; **1h**·HCl, 115318-63-3; **2**·HCl, 112757-49-0.

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Arginyl-tRNA Synthetase from *Escherichia coli*, Purification by Affinity Chromatography, Properties, and Steady-State Kinetics†

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ABSTRACT: A Blue Sephadex G-150 affinity column adsorbs the arginyl-tRNA synthetase of *Escherichia coli* K12 and purifies it with high efficiency. The relatively low enzyme content was conveniently purified by DEAE-cellulose chromatography, affinity chromatography, and fast protein liquid chromatography to a preparation with high activity capable of catalyzing the esterification of about 23 000 nmol of arginine to the cognate tRNA per milligram of enzyme within 1 min, at 37 °C, pH 7.4. The turnover number is about 27 s^{-1}. The purification was about 1200-fold, and the overall yield was more than 30%. The enzyme has a single polypeptide chain of about M_r 70 000 and binds arginine and tRNA with 1:1 stoichiometry. For the aminoacylation reaction, the K_m values at pH 7.4, 37 °C, for various substrates were determined: 12 μM, 0.9 mM, and 2.5 μM for arginine, ATP, and tRNA, respectively. The K_m value for cognate tRNA is higher than those of most of the aminoacyl-tRNA synthetase systems so far reported. The ATP–PP_i exchange reaction proceeds only in the presence of arginine-specific tRNA. The K_m values of the exchange at pH 7.2, 37 °C, are 0.11 mM, 2.9 mM, and 0.5 mM for arginine, ATP, and PP_i, respectively, with a turnover number of 40 s^{-1}. The pH dependence shows that the reaction is favored toward slightly acidic conditions where the aminoacylation is relatively depressed.

Since the 1960s, the mechanism of a group of three small aminoacyl-tRNA synthetases, arginyl-, glutamyl-, and glutaminyl-tRNA synthetases, has been widely studied and discussed. These synthetases require the cognate tRNA for the ATP–PP_i[1] exchange reaction (Zubay, 1962; Ravel et al., 1964, 1965; Mitra & Mehler, 1966, 1967; Parfait & Grosjean, 1972; Gangloff et al., 1976; Char & Gopuiathan, 1986) and are very different from the majority of aminoacyl-tRNA synthetases. Until now, the true mechanism has not yet been clearly elucidated. Generally, two different mechanisms have been proposed: (a) the aminoacyl adenylates being formed as in the other synthetases, e.g., the arginyl-tRNA synthetase from *Neurospora crassa* (Nazario & Evans, 1974) and baker's yeast (Fersht et al., 1978); (b) a concerted mechanism without formation of the aminoacyl adenylates, e.g., the arginyl-tRNA synthetase from *Bacillus stearothermophilus* (Parfait & Grosjean, 1972) and from brewer's yeast (Thiebe, 1983). We consider it very important to study the detailed properties, including the interactions between the enzyme and substrates,

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[1] Abbreviations: ArgRS, arginyl-tRNA synthetase; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LeuRS, leucyl-tRNA synthetase; PMSF, phenylmethanesulfonyl fluoride; PP_i, inorganic pyrophosphate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; $tRNA^{Arg}$, arginine-specific tRNA.

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