A PyreneDihydrodioxin with Pyridinium “Arms”: A Photochemically Active DNA Cleaving Agent with Unusual Duplex Stabilizing and Electron Trapping Properties

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Graphical abstract

ABSTRACT

Pyrenedi hydrodioxins (PDHD) comprise effective DNA intercalation agents that are masked *ortho*-quinones, which can be released by near ultraviolet or visible irradiation. We have studied the binding and photoreactions of chiral dipyridinium PDHDs with herring sperm DNA and an 11-mer duplex DNA containing all 10 basepair steps. Binding affinities to herring sperm DNA were determined for purified enantiomers ($K_b = 1.6 \pm 0.15 \times 10^5$ and $2.3 \pm 0.2 \times 10^5 \text{ M}^{-1}$). UV-melting experiments using the 11-mer DNA revealed significant stabilization of duplex DNA,

($\Delta T_m = 11.5^\circ$ and $\Delta T_m = 15.3^\circ$ C). Both enantiomers linearized (double-strand cleavage) supercoiled Φ X174 plasmid DNA with high efficiency. PDHDs have specificity to cleave and/or damage DNA duplexes at Gs and have preferable binding to GG DNA sites.

A full range of transient spectroscopy from the ultrafast femtosecond to the microsecond domains has been applied in the study of this system. These studies have revealed a novel mechanism for quinone release via the pyrene radical cation and the entrapment of the released electron by the coordinated action of the two pyridinium rings. These same studies have shown that the released pyrenequinone can photochemically initiate the further release of pyrenequinone. Thus, this reaction is autocatalytic and can be initiated with visible light.

KEYWORDS: PyreneDihydrodioxin; Schönberg-Mustafa Reaction; Autocatalytic Quinone Release; Dipyridinium-Electron Complex; DNA Binding Agents; Photochemical DNA Cleavage

INTRODUCTION.

Materials that interact with DNA duplexes via intercalation have found widespread application in the study of DNA [1]. The intercalation process involves the insertion of a planar molecule between DNA basepairs, which results in a decreased helical twist and lengthening of the DNA [2]. Usually, this distortion of the double helix structure by an intercalator provides greater thermal stability of the overall complex compared to the naked duplex due to π -stack interactions, hydrogen-bonding, van der Waals interactions, as well as hydrophobic interactions[3].

DNA intercalators have attracted particular attention due to their antitumor activity and ability to manipulate DNA. They provide significant structural modifications to the DNA double helix and may inhibit DNA replication, as well as, gene transcription [4]. These unique properties have many important applications, for example, as artificial nucleases and in gene-targeted chemotherapy [5]. The genotoxicity of non-covalent interactions have been demonstrated [5a,6]. Indeed, there are a number of multivalent DNA-binding drugs that contain an intercalating polycyclic ring and a carbohydrate or peptide moiety that binds to the minor groove of the duplex. The non-intercalating portion can form several hydrogen bonds or electrostatic interactions with DNA, thus contributing to the stability of the complex. The detection and characterization of multivalent modes of binding may result in the formulation of better rules for rational drug design. Improved drug binding affinity and the ability to discriminate larger DNA sequences may allow one to target unique genome sites [7].

Photochemically activated molecules represent a special group of DNA intercalation agents which are capable of cleaving double-stranded DNA. There are numerous examples of DNA-binding drugs that serve as cleaving agents already in use in molecular biology. These include metal complexes, organometallic complexes, and organic compounds such as enediynes, nitrosubstituted aromatics, halogen containing compounds, riboflavins, naphthalimide derivatives and certain anthraquinone derivatives [8]. Although there are several possible cleavage mechanisms and types of damage that they cause, all photochemical cleavers offer advantages over regular DNA cleaving agents. These advantages include the prior binding of the molecule to the DNA before activation and the ability to control the photoactivation in both a spatial and temporal manner. Also, light serves as a selective reagent; usually photocleaving agents are activated at wavelengths longer than 300 nm, the spectral region where nucleic acids and most proteins do not absorb light.

A particularly effective family of intercalation agents is that of pyrene[9], which has been widely used to incorporate a variety of units into DNA/RNA and as the foundation of photocleaving agents. Pyrenedihydrodioxins (PDHD) are photoreactive masked *ortho*-quinones. These materials are easily prepared in a classical organic photoreaction known as the Schönberg-Mustafa reaction (Scheme 1) [10].

Thus, visible light irradiation of *ortho*-quinones of polycyclic aromatic hydrocarbons in the presence of olefins leads to dihydrodioxin (DHD) formation. This reaction is reversed by irradiation with shorter wavelength (short wavelength visible or UV light) that reforms the original *ortho*-quinone and olefin [11]. In previous studies, this quinone releasing property of dihydrodioxins has been investigated as a possible tool for DNA cleavage [12]. These observations make masked *ortho*-quinone derivatives promising DNA photocleaving agents, since they might be derivatized with a variety of substituents that target them to specific DNA sites. The introduction of different substituents on the masking unit can dramatically affect the binding mode and cleavage efficiency of these photocleaving agents. However, little is known about PDHD-DNA binding properties and their sequence specificity, as well as the behavior of photoreleasedquinone in the presence of DNA.

A new compound, *trans**N,N*-dimethyl-10,11-di(4-pyridinium)-10,11-dihydro-9,12-dioxabenz[*e*]pyrenetetrafluoroborate salt (PDHD, **1**), has been synthesized from the dipyridine analog **2** (Scheme 2) in an effort to study these questions. This compound exists in two enantiomeric forms (SS, RR). These enantiomers have been found to have exceptional DNA binding and double-strand cleaving properties. They are differentiated based on their retention

times on a chiral HPLC column (see supplementary information section) as follows: PDHD I (first fraction eluted) and PDHD II (second fraction eluted). In this study, we describe and compare the unusual DNA binding and cleaving properties of these PDHDs.

2. MATERIALS AND METHODS.

All chemicals were obtained from commercial suppliers, were of analytical grade, and used without further purification unless otherwise noted. Detailed descriptions of the synthesis of enantiomeric PDHDs and separation of enantiomers on chiral HPLC column are available in supplemental data. PDHD stock solutions (100 μ M) were prepared in CB buffer (10 mM sodium cacodylate trihydrate, 100 mM NaCl, 0.5 mM EDTA, pH = 7.1) using the empirically determined extinction coefficient $\epsilon_{343} = 77459 \text{ M}^{-1} \text{ cm}^{-1}$.

2.1 Synthetic DNA Oligonucleotides:

DNA oligomers used in this work included the 11-mer or 10 base-pair-step (10 bps) (5'-ATCGACCAAGC-3'/3'-TAGCTGGTTCG-5'), the 12-mer (5'-GTTAGTATATGG-3'/3'-CAATCATATACC-5'), the 22-mer (5'-ATCGACCAAGCTAGCTGGTTCG-3'/3'-TAGCTGGTTCGATCGACCAAGC-5') and the 38-mer (5'-ATAAGTAAATAAACAATCAACAAAGCAACCAATAGACU-3'/3'-TATTCATTTATTTGTTAGTTGTTTCGTTGGTTATCTCA-5'). These oligomers were purchased from DNA Integrated Technology (www.idtdna.com) and purified on 20% denaturing PAGE in the presence of 7 M UREA, using standard crush and soak protocol to extract DNA from gel slices [13]. DNA strands were annealed at a concentration of 100 μ M of each strand in CB buffer by heating to 100° C and slow cooling to room temperature. The concentration of purified stock solutions was determined by absorbance at 260 nm using extinction coefficients calculated by the supplier: $\epsilon_{260} 211,100 \text{ M}^{-1} \text{ cm}^{-1}$ (11-mer DNA), $244,600 \text{ M}^{-1} \text{ cm}^{-1}$ (12-mer DNA), $419,600 \text{ M}^{-1} \text{ cm}^{-1}$ (22-mer DNA), and $762,200 \text{ M}^{-1} \text{ cm}^{-1}$ (38-mer DNA) at 25° C.

2.2 Commercially Prepared Herring Sperm DNA (HS DNA).

HS DNA was obtained from Sigma-Aldrich Co. (www.sigmaaldrich.com) and dissolved in double deionized water (dd H₂O) containing 50 mM NaCl with further dialysis for 24 h at 4° C against a buffered solution (pH 7.1, sodium cacodylate). The removal of protein was monitored using the ratio of Abs (260 nm) / Abs (280 nm) [14]. A stock solution of HS DNA (1 mol/L in nucleotide phosphates) was prepared by directly dissolving DNA in dd H₂O, and quantified using $\epsilon_{260} = 6,600 \text{ M}^{-1} \text{ cm}^{-1}$, as described in the literature [7b].

2.3 Φ X174 Plasmid RF I 5386 Basepair DNA.

Φ X174 was purchased from New England BioLabs Inc. (> 90% supercoiled form, 1 mg/mL) (www.neb.com) cat # N3021L.

2.4 Photochemical Synthesis of DipyridineDihydrodioxin (2).

A solution of 0.0895 g (0.77 mmol) of pyrene-4,5-quinone in 150 mL of benzene and 0.2060 g (1.13 mmol) of *trans*-1,2-bis(4-pyridyl)ethylene was purged with Ar and placed in a Rayonet Photochemical Reactor. The reaction mixture was irradiated at 419 nm in a water bath with the temperature kept at freezing point of benzene +4° C. After 4 hrs of irradiation the color of the reaction mixture was yellow. The benzene was evaporated and 150 mL of methanol added. Immediate formation of a white powder-like crystals was observed. The solution was left for 24 hours in the dark. The crystals were collected by filtration (1st filtration) and washed with a small amount of CHCl₃. The collected crystals were partially redissolved in 7 mL of CHCl₃ with slight heating. The insoluble material was again collected by filtration (2nd filtration). These collected crystals again were partially dissolved in 3 mL of CHCl₃ and the insoluble material collected by filtration (3rd filtration). TLC analysis (ethyl acetate/ hexane 1/5) of this third set of crystals indicated that no starting materials were present, and afforded a single striking spot of **2**. These crystals were combined with the residue from evaporation of the CHCl₃ wash solution to afford 0.082 g of **2**. The CHCl₃ solutions from the 1st and 2nd filtrations were combined and TLC indicated the presence of pyrenequinone, *trans*-1,2-bis(4-pyridyl)ethylene and **2**. Column chromatography of this material (ethyl acetate /hexane 1/5 and then, after the first two bands were collected, CH₃OH/CHCl₃) afforded 0.059g of **2** for a combined yield 43% (0.141 g, 0.340 mmol) of a mixture of *R,R*- and *S,S*-isomers.

UV 0.01 mg/mL in CHCl₃ (calculated ϵ of **2** at 250 nm is 33316 M⁻¹cm⁻¹), **Fig. S1**.

¹H NMR (300 MHz, CDCl₃) (**Fig.S2**) δ 8.65 ppm (d, J =4.70 Hz, 4 H), 8.46 (d, J =7.8 Hz, 2 H), 8.19 (d, J =7.53 Hz, 2 H), 8.12 (s, 2 H), 8.05 (dd, J =7.69 Hz, J' =7.69 Hz, 2 H), 7.21 (d, J =5.54 Hz, 4 H), δ 5.23 (s, 2 H); ¹H NMR (500 MHz, DMSO-d₆) δ 8.58 (d, J =5.9 Hz, 4 H), 8.39 (d, J =7.8 Hz, 2 H), 8.29 (d, J =7.6 Hz, 2 H), 8.18 (s, 2 H), 8.09 (dd, J =7.7 Hz, J' =7.7 Hz, 2 H), 7.44 (d, J =5.9 Hz, 4 H), 5.94 (s, 2 H); ¹³C NMR (75 MHz, DMSO-d₆) δ 149.1, 143.9, 132.8, 129.7, 126.6, 125.7, 123.6, 123.5, 121.7, 119.3, 117.0, 76.5.

C₂₈H₁₉N₂O₂; HRMS (ESI Q-TOFMS) calculated for C₂₈H₁₉N₂O₂ 415.1447, found 415.14421;

It should be mentioned that this reaction works much better on the smaller scale, since the yield of this reaction was as high as 78% (0.025 g (0.077 mol) of pyrenequinone, 0.412 g (0.226 mmol) of *trans*-1,2-bis(4-pyridyl)ethylene, reaction time of 1 hour, but that procedure included only one filtration with

further recrystallization of the product **2** from CH₃OH. Also, the color of that reaction mixture was only slightly yellowish. Still the NMR quality was acceptable.

2.5 Separation of 10, 11 DiPyridylPDHD (2) Enantiomers.

2.5.1 Analytical Separation (Fig. S7).

Aliquots of 100 mg of 10, 11-DiPyridylPDHD di-(R)-(-)-10-Camphorsulfonic acid salt in methanol-water mixture (9:1) in 30 mL were injected and eluted with methanol-water (9:1) (isocratic mode, solvent velocity 1 mL/min, Detection: wavelength-260 nm and 280 nm, run total time 3000 sec, cycle time-10 s, Background correction-None). Two fractions were collected: 1st Fraction (Isomer 1)-*t_r* from 500-1000 s and 2nd Fraction (Isomer 2)-*t_r* from 2000 to 2500 s (**Fig. S7**). Both fractions were vacuum dried and CD spectra were measured (**Fig. S8**).

2.5.2 Preparative Separation.

Aliquots of 2.0 mg of the (R)-camphorsulfonic salt in 250 mL of solvent as above (isocratic mode, solvent velocity 3 mL/min and as above). Two fractions were collected: 1st Fraction (Isomer 1)-*t_r* from 6,000 to 10,000 s. and 2nd Fraction (Isomer 2)-*t_r* from 22,000 to 30,000 s.

2.6 Preparation of N,N-Dimethyl-*trans*-10,11-Di(4-pyridinium)-10,11-dihydro-9,12-dioxabenz[e]pyrene Tetrafluoroborate Salt, **1**.

To a stirred solution of 10, 11-DipyridylPDHD **2** (racemic mixture or of resolved enantiomers of **PDHDI** or **PDHD II**) (0.120 g, 0.29 mmol) in CH₂Cl₂ (300 mL freshly distilled for P₂O₅) at room temperature was added solid trimethyloxoniumtetrafluoroborate (0.257 g, 1.74 mmol). The reaction mixture was refluxed for 36 h at 40° C at which time a finely divided precipitate had formed. The solvent was evaporated and the solid residue recrystallized from acetonitrile-diethyl ether (9/1) to yield a fine white powder of **1** (0.135 g, 0.22 mmol, 75% yield). Additional purification on Bio-Rex[®]70 Cation Exchange Resin using acetonitrile/deionized water = 20/80 afforded analytically pure *N,N*-dimethyl *trans* 10,11-di(4-pyridinium)-10,11-dihydro-9,12-dioxabenz[e]pyrenetetrafluoroborate salt, **1**: ¹H NMR (500 MHz, acetonitrile-D₃) (**Fig. S9**): δ 8.19 (d, *J* = 7.0, 4H), 8.10 (dd, *J* = 7.5 Hz, *J'* = 1 Hz, 2H), 7.93 (dd, *J* = 7.5 Hz, *J'* = 1 Hz, 2H), 7.81 (s, 2H), 7.76 (dd, *J* = 7.5 Hz, *J'* = 7.5 Hz, 2H), 7.72 (d, *J* = 7.0 Hz, 4 H), 5.96 (s, 2H), 4.30 (s, 6H); ¹³C NMR (100.6 MHz, acetonitrile-D₃) (**Fig. S10**): δ 146.9, 134.0, 132.2, 128.7, 127.9, 127.2, 126.9, 126.3, 125.3, 122.3, 119.2, 76.7, 49.3; HRMS (ESI QTIFMS) (**Fig. S11**), calculated ; C₂₈H₂₀N₂O₂²⁺ *m/z* 208.0752, found *m/z* 208.0765; calculated for C₃₀H₂₄N₂O₂²⁺·BF₄¹⁻ *m/z* 531.18668, found *m/z* 531.18633; calculated for C₃₀H₂₄N₂O₂²⁺ *m/z* 222.09134, found *m/z* 222.09141.

2.7 Circular Dichroism (CD) Spectropolarimetry.

CD measurements of resolved PDHD enantiomers, **PDHD I** and **PDHD II**, were performed with an AVIV model 62A DS spectropolarimeter (Aviv Associates, Lakewood, NJ) equipped with a thermoelectrically controlled cell holder and a cuvette with a path length of 0.1 cm. The wavelengths studied ranged from 240 to 400 nm. Scans of DNA duplexes (~50 µM) were recorded at 1nm intervals. A scan of the buffer alone was recorded to provide a baseline.

2.8 UV-Vis Absorption Spectroscopy.

UV-Vis spectra and melting curves were measured using a 100 CARY-BIOUV-Visible spectrophotometer (Varian Inc.) equipped with a thermoelectrically controlled cell holder and a cuvette with a 1.00 cm path length. UV-Vis titrations were conducted at 20° C in CB buffer by manually injecting 1-10 µL aliquots of HS DNA or oligonucleotide DNA solution into 1 mL of PDHD sample at 10 µM concentration in the same buffer. The resulting DNA concentration ranged from 0 to 225 µM. After each injection, the absorption spectrum was recorded between 200 and 500 nm.

A detailed description of the transient spectroscopy facilities used in this work is provided in the Supplementary Information Section.

2.9 Optical Melting Experiments.

Absorbance vs. temperature profiles were measured at 260 nm on CARY-BIO UV-Visible spectrophotometer (Varian Inc.) equipped with a thermoelectrically controlled cell holder, and a 1.00 cm path-length cuvette heated at a rate of 0.5° C/min. DNA melting temperatures (T_m), determined by UV melting in the presence and absence of varying amounts of PDHD enantiomers were used to calculate the binding constant of PDHD to DNA oligonucleotide duplex at the DNA melting temperature (K_b at T_m), using the equation derived by Crothers [15]

$$1/T_m^\circ - 1/T_m = (R/n\Delta H_{wc}) * \ln(1 + K_{T_m}\alpha) \quad (1)$$

where T_m° is the UV melting temperature of DNA and T_m is the melting temperature of the DNA/PDHD complex. ΔH_{wc} is the theoretically calculated enthalpy of DNA at 10 µM concentration in presence of 110 mM NaCl. R is the gas constant, K_{T_m} is the PDHD binding constant at T_m and α is the free drug activity estimated as one half of the total PDHD concentration. The size of the drug-binding site, n, is determined from UV titration experiments.

2.10 Φ X 174 Plasmid DNA Photocleavage Assay.

Supercoiled plasmid DNA stock solution (1 $\mu\text{g}/\mu\text{L}$) was diluted in CB buffer to final concentration of 0.03 $\mu\text{g}/\mu\text{L}$. The samples were prepared by adding enantiomerically pure PDHD to plasmid DNA in the dark to achieve a final concentration of 1 mol PDHD per 50 bp DNA. Samples were rapidly mixed in Eppendorf tubes and incubated at ambient temperature for 30 min. PDHD/DNA complexes were photolyzed in Eppendorf tubes placed on ice at a distance of 20 cm from a He/Cd CW Laser system (56 series Melles Griot Co.) producing 442 nm light with 78 mW of power. For air-free samples, 100 μL vials containing the sample were out-gassed on ice for 10 min with ultrapure argon via a sterile 16-gauge needle before irradiation. Irradiation was carried out from 0 to 420 seconds in 30 s intervals. Φ X174 supercoiled plasmid DNA was used as a control and irradiated in the absence of PDHD for 420 s. Irradiated samples were subsequently mixed with 0.4 volume of loading dye (30% glycerol, 1X TAE and 0.1% bromophenol blue) vortexed and loaded on 1% agarose gels to resolve the conversion of supercoiled plasmid (RFI) to circular (RFII) or linear form (RFIII) [16]. The gel was run at 50V for 3 hours in 1X TAE buffer (40mM TrisHCl, 20mM acetic acid, and 1mM EDTA, pH =8) and then was stained for 30 min in GelRed from Biotium Inc. (6 μL of 10,000X solution in water dissolved in ~ 300-350 mL of 1X TAE buffer). The relative quantities of the supercoiled (RFI), nicked (RFII), and linear (RFIII) DNA were calculated by integrating the area of each band using the Image J software (<http://rsbweb.nih.gov/ij/>).

Single strand breaks (n_1) were quantitated by calculating the fraction of supercoiled replicative form of ϕ X 174 remaining after irradiation according to the method of Alabugin [17].

$$RFI = \exp [-(n_1 + n_2)] \quad (2)$$

Where the average number of double strand breaks (n_2) per molecule was obtained using the following equation

$$n_2 = RFIII / (RFI + RFII) \quad (3)$$

where RFI, RFII and RFIII are the supercoiled, circular/nicked and linear forms of plasmid DNA, respectively.

2.11 Sequence Selectivity of a DNA Oligonucleotide.

Deoxyoligonucleotides, 22-mer and 38-mer, were ^{32}P -5'-end-labeled with T4 polynucleotide kinase (New England Biolabs Inc.) and $[\gamma\text{-}^{32}\text{P}]$ ATP (PerkinElmer Co.) according to a described procedure [18]. The end-labeled DNA strands were hybridized to the complementary strand (2.5 μM strand concentration) in 10 mM sodium cacodylate buffer, pH 7.0 in the presence of 100 mM NaCl.

The 22-mer DNA duplex was incubated with PDHD enantiomers (50 μM) at 37° C for 1 hour. Aliquots of 40 μL were subjected for DNA photocleavage using 442 nm He-Cd laserlight as described above for 10 min. After piperidine treatment (94° C, 20 min), the samples were resuspended in formamide loading dye (50% formamide, 10 mM EDTA 0.01% bromophenolblue), loaded onto a 20% denaturing polyacrylamide sequencing gel, and electrophoresed at 1800 V for approximately 3 h. DNA fragments were visualized using the Storm phosphorimager (Amersham, Storm 860). The 38-mer DNA duplex samples, after incubation with **PDHD 1(-)** for 5 min at 37° C, were irradiated at 442 nm and directly subjected to 15% polyacrylamide gel electrophoresis. The piperidine treatment step was not applied.

2.12 DNase I Footprinting Assay.

DNA 38-mer was used in this assay. The ^{32}P -5'-end-labeled 38-mer DNA 2 μL was combined with 4 μL of **PDHD 1(-)** in 10 mM CB buffer. Individual reactions were performed in 1.5 mL Eppendorf tubes with increasing concentrations of **PDHD 1(-)**. The final concentration of the **PDHD** enantiomer covered the range of 0.01 – 100 μM . The mixtures were incubated in the dark for 2 hours to obtain equilibrium at room temperature. The cleavage reaction was initiated by adding 2 μL of DNase I (Promega Co.) (0.05 U/mL) and incubating for 8 min at room temperature. The reaction was stopped by transferring the tubes to dry ice followed by lyophilization with a SpeedVac for 40 min. Then, the dry products were dissolved in 5 μL formamide loading dye solution and loaded onto 15% denaturing polyacrylamide sequencing gel, and electrophoresed at 1700 V for approximately 2 hours. DNA fragments visualization was performed using phosphor imaging screen and the Storm phosphorimager (Amersham, Storm 860).

3. RESULTS AND DISCUSSION

3.1 Theoretical PDHD/DNA Binding Studies.

PDHD1 might exist in either the diequatorial or diaxial conformation (**Fig. 1A**). Apparently, the diaxial conformation is easily accessed due to the significant steric and electrostatic repulsion between the two proximate positively charged pyridinium rings in the diequatorial conformation. This diaxial conformation would seem to be ideally suited for binding in the major groove of duplex DNA. In this

conformation the pyrene ring could intercalate into the base-pair stack while the positively charged pyridinium “arms” are held in closely proximity to the negatively charged phosphate backbone strands of the DNA, **Fig. 1B**.

While it is not certain which enantiomer, SS or RR, would fit most effectively into the major groove of the DNA, extensive calculations similar to previously described molecular dynamics/molecular mechanics calculations [19] indicate the SS enantiomer usually forms more stable complexes than the RR enantiomer at various sites in duplex B-DNA.

An example of this type of comparison is shown in **Fig. 2**. In this example, the diaxial conformation of the SS enantiomer binds as shown in **Fig.2**. While the RR enantiomer adopts a diequatorial conformation which leads to a much less stable complex. This is the usual outcome of these comparisons. The SS enantiomer seems to cause far less distortion of the DNA duplex and seems to form much more stable complexes than does the RR enantiomer. However, it must be noted that many different types of complexes have been found in these calculations depending upon the base-pair sequence and the proximity of the pyrene intercalation to the end of the duplex strand. Complexes have even been calculated in which both pyridinium rings are bonded to the same DNA strand. Extensive further theoretical studies are going to be required in order to determine the optimum base-pair sequence for PDHD binding. What can be said at this point is that a single PDHD binds to a three or four base-pair length of duplex DNA. Thus, while a duplex DNA strand can bind to more than one PDHD, these theoretical considerations indicate that the DNA duplex will become saturated when the strand binds to approximately one PDHD/3-4 base pairs of DNA. This limit is consistent with experimental binding studies, *vide infra*. Finally, this PDHD/DNA binding model is unusual in that it includes both coordinated intercalative and electrostatic binding modes, which should greatly stabilize the DNA duplexes to which the PDHD binds. This model will help the reader interpret the results described in the remainder of this paper.

3.2 Circular Dichroism (CD) Spectropolarimetry.

As indicated above, the enantiomers of the **PDHD1** can be resolved by preparative HPLC on a chiral column and the purified enantiomers displayed the CD spectra shown in **Fig. 3** (see Supplementary Information).

3.3 UV-Vis Spectroscopy of PDHD/DNA Binding

We have measured the concentration-dependent binding of each enantiomer and the racemate. Binding studies have been conducted by monitoring the UV-Vis absorbance spectrum of the enantiomers upon titration of HS DNA or 10 bps DNA (**Fig.4**). The addition of DNA induces a hypochromic effect (> 40% observed in all cases) and produces a bathochromic shift (>10 nm observed in all cases) in the spectrum, which indicates that both enantiomers intercalate into the DNA helix [20]. The magnitudes of hypochromism are usually correlated with the strength of the intercalative binding interaction. In order to compare the binding strengths of PDHD enantiomers, the intrinsic binding constant (K_b) was calculated according to the previously reported procedures [21]. When the ratio of bound PDHD to DNA base pairs is relatively low, the intrinsic binding constant K_b may be determined from a double-reciprocal plot of the changes in the apparent extinction coefficient of PDHD and DNA concentration as follows:

$$1 / \Delta \varepsilon_{ap} = 1 / \Delta \varepsilon K_b [DNA] + 1 / \Delta \varepsilon \quad (4)$$

Where $\Delta \varepsilon_{ap} = |\varepsilon_a - \varepsilon_f|$, $\Delta \varepsilon = |\varepsilon_b - \varepsilon_f|$. The apparent extinction coefficient, ε_a , is obtained by calculating $A_{obs}/[PDHD]$. ε_f and ε_b are extinction coefficients of PDHD free and PDHD fully bound form respectively. Multiplying by [DNA] puts the equation in the half-reciprocal plot form.

$$[DNA] / \Delta \varepsilon_{ap} = (1 / \Delta \varepsilon) * [DNA] + 1 / \Delta \varepsilon K_b \quad (5)$$

A plot of $[DNA] / \Delta \varepsilon_{ap}$ versus [DNA] will have a slope of $1 / \Delta \varepsilon$ and a y-intercept equal to $1 / (\Delta \varepsilon K_b)$. K_b is then given by the ratio of the slope to intercept. Since a double-reciprocal plot gives excessive weight to data points obtained at low [DNA], the half-reciprocal plot should generally be more accurate. The change in the absorbance of PDHD enantiomers with increasing DNA concentration was used to construct the half-reciprocal plot as shown in **Fig. 4 A** and **B** insets. The resulting intrinsic binding constants for PDHDs are listed in Table 1. Binding data suggest that PDHD enantiomers bind to either short oligomeric DNA or herring sperm DNA with high affinity. The magnitude of binding is comparable with those observed for known intercalators [22]. Remarkable hypochromic and bathochromic effects of PDHD in the presence of increasing amounts of DNA have been observed in the absorption spectra. **PDHDII** has a slightly larger binding constant than **PDHDI** and the racemic mixture.

3.4 Optical Melting Experiments.

To evaluate the effect of the bound PDHD enantiomers on the thermal stability of the DNA duplexes, UV melting experiments were conducted in the presence and absence of each PDHD

enantiomer. Ligands that bind more tightly to double-stranded DNA than to the single-stranded form induce an increase in the melting temperature of the host duplex. The ligand-induced increase in duplex melting temperature (ΔT_m) along with the ligand:DNA binding stoichiometry and the calorimetric transition enthalpy of the host duplex has been used extensively to calculate the ligand binding constant (K_b) [23].

Fig. 5 shows ligand-induced changes in the thermal stabilities of the 10bps DNA upon addition of PDHDs. In the absence of enantiomers, the T_m of 10 bps DNA was measured at $50.22 \pm 0.3^\circ\text{C}$. When the ratio 'r' of [PDHD]/[DNA] is sufficient to saturate the DNA duplex, the T_m was increased to $61.7 \pm 0.3^\circ\text{C}$ and $\Delta T_m = 11.5^\circ\text{C}$, $65.53 \pm 0.7^\circ\text{C}$ and $\Delta T_m = 15.3^\circ\text{C}$, and $63.49 \pm 0.4^\circ\text{C}$ and $\Delta T_m = 13.3^\circ\text{C}$ for **PDHDI**, **PDHDII** and **PDHDrac**, respectively.

These UV-melting titration data were used in conjunction with a binding site size (n) defined by UV-Vis absorbance titration, to estimate apparent ligand-duplex binding constants at T_m (K_{Tm}) using Equation 1. These data are summarized in Table 2, and suggest that **PDHDII** has a slightly stronger affinity for 10 bps DNA than **PDHDI** with values of $4.7 \times 10^5 \text{ M}^{-1}$ for **PDHDII**/DNA and $3.2 \times 10^5 \text{ M}^{-1}$ for **PDHDI**/DNA interaction. These observations are consistent with the binding constants obtained from UV-vis titration experiments, and confirm that **PDHDII** binds to DNA more strongly than does **PDHDI**. Binding site size or the number of DNA base pairs occupied by a single PDHD enantiomer is about the same for **PDHDII** (3.3 bp) and **PDHDI** (3.1 bp). Thus, one DNA molecule containing 10 base pair steps (10 bps DNA) can accommodate roughly 3 PDHDs simultaneously. However, for **PDHDrac** the binding site size is 4.2 which is greater than those for resolved enantiomers. This suggests that 10 bps DNA interact with only about 2.5 **PDHDrac** molecules.

In some cases, addition of PDHD to a duplex leads to more significant duplex stabilization as shown in **Fig. 6**. In this case, the 12-mer DNA 5'-GTTAGTATATGG-3'/3'-CAATCATATACC-5' has a lower GC/AT ratio than does the 11-mer DNA, 5'-ATCGACCAAGC-3'/3'-TAGCTGGTTCG-5', 12-mer GC/AT = 4/8 (2.33 hydrogen bonds/base pair) versus 11-mer GC/AT = 6/5 (2.54 hydrogen bonds/base pair). Thus, the 12-mer has the lower melting point, 39.4°C , than the 11-mer, 50.2°C , as might be expected. However, both duplexes are stabilized to about the same degree by the additions of PDHDs, **PDHDI** + 11-mer, $T_m = 63.5^\circ\text{C}$ and $\Delta T_m = 11.5^\circ\text{C}$, + 12-mer, 61.2°C and $\Delta T_m = 22.7^\circ\text{C}$, and **PDHDII** + 11-mer, $T_m = 65.5^\circ\text{C}$ and $\Delta T_m = 15.3^\circ\text{C}$, + 12-mer, 68.1°C and $\Delta T_m = 28.7^\circ\text{C}$. Thus, both enantiomers stabilize the duplex, but **PDHDII** is substantially more effective in enhancing the duplex stability. Inspection of data obtained by UV titration and UV-melting analysis reveal that the apparent

binding affinities of the PDHDs to 10 bps DNA and HS DNA follow the hierarchy: **PDHDII>PDHDI>PDHDrac**.

3.5 Φ X 174 Plasmid DNA Photocleavage Assay.

In this section, the extreme proficiency of PDHD in photochemically damaging DNA is shown. This damage can be triggered with either long-wavelength UV light or short-wavelength visible light. In the latter case, the initiating absorption is either very weak absorption of the PDHD or absorption of traces of pyrenquinone released by ambient UV light. The details of these alternative photoprocesses will be developed in the later sections of this paper.

The ability of PDHD to cleave DNA upon irradiation was investigated using a supercoiled plasmid relaxation assay. The supercoiled form (RFI) of Φ X174 plasmid DNA was used in this study. The DNA cleavage efficiencies of each PDHD enantiomer, as well as that of the racemate, were determined using He-Cd CW laser light at 442 nm (78 mW). Conversion of supercoiled Φ X174 DNA into relaxed circular and linear forms, RFI, RF II and RF III, respectively, by this visible light irradiation was analyzed by 1% agarose gel electrophoresis. The relative amounts of the three DNA forms were determined using densitometric analysis of the gel electrophoresis bands after different irradiation times.

Irradiation of plasmid Φ X 174 in the presence of PDHD enantiomers generates linear DNA before the supercoiled DNA is entirely converted to the relaxed-circular forms (lanes 1–5; 10-14; 18-21) (**Fig.7**). Under conditions where all of the three DNA forms are present at the same time, one can gain a more thorough insight into the nature of the cleavage using the statistical test of Povirk [25]. This test assumes a Poisson distribution of strand breaks and calculates the average number of single-strand breaks ($ss-(n_1)$) and double-strand breaks ($ds-(n_2)$) per DNA molecule (Table 3). In all cases of plasmid DNA photochemical cleavage, the n_1/n_2 ratio decreases with increasing irradiation time and all three forms of DNA (supercoiled (RF I), circular/single-strand nicked (RF II), and linear/double-strand nicked within 5 base pairs of each other (RF III)) are present at the same time suggesting that ss and ds events are kinetically independent and that conversion to the linear form (RF III) occurs at a much slower rate than the initial scission to the relaxed form (RF II). However, at 30 – 180 s. of irradiation, the range of n_1/n_2 values is significantly smaller than the expected ratio for strictly random single-strand cleavage events. Φ X174 (5386 base pairs) should require 653 random single-strand cuts for a single linearization event to occur (double-strand nicking within 5 base pairs of each other) [26]. These data indicate that double-strand cleavage is particularly favorable with PDHD enantiomers and racemate. Thus, once the PDHD is activated, the reactive intermediate seems to remain strongly associated with the DNA, and can inflict multiple lesions in the DNA backbone in the region of its formation. By comparing the

n_1/n_2 values between PDHD enantiomers, one can see, as expected, that these values decrease with increasing irradiation times. **PDHDI** and **PDHDII** have very similar n_1/n_2 values, and strangely, the **PDHDrac** seems to be most effective in linearizing the Φ X 174.

3.5.1 Effect of Oxygen on DSCleavage Efficiency.

To investigate the role of molecular oxygen, the formation of Reactive Oxygen Species (ROS) and their impact on the efficiency of PDHD ds DNA cleavage, we have performed the same photochemical Φ X 174 cleavage experiments as described above, but under an inert argon atmosphere. The resulting agarose gel electrophoresis analyses are shown in **Fig.8** and Table 4 (as well as Fig. S16). Under aerobic conditions, the **PDHDrac** produced the most localized damage (lowest value of n_1/n_2), but **PDHDII** was most effective in producing localized damage under anaerobic conditions. Thus, the cleavage pattern was reversed under anaerobic conditions, where extremely localized damage was observed for all PDHDs, but most significantly for **PDHDII**, $n_1/n_2 = 5.23$.

Under anaerobic conditions, **PDHDII** showed better DNA linearization efficiency than **PDHDI** or **PDHDrac** (**Fig.9**): the n_1/n_2 range is 5.2 – 29.4 with less than 10% of supercoiled DNA remaining after 180 s of irradiation (**Fig.8**). Comparable n_1/n_2 values have been observed for iron bleomycin and lysine-enediynes conjugates [8f,17]. In addition, the presence of molecular oxygen significantly enhances the rate of DNA cleavage with n_1 (O_2 /no O_2): **PDHDI** = 5.62/3.46, **PDHDII** = 6.70/2.94, and **PDHDrac** = 6.48/2.82, but this ROS cleavage is more random with n_1/n_2 (O_2 /no O_2): **PDHDI** = 29.50/7.71, **PDHDII** = 32.73/5.23, and **PDHDrac** = 15.12/7.04.

One possible explanation for this behavior is that the PDHD generates highly mobile Reactive Oxygen Species (ROS) such as hydroxyl radicals [27,28] which may randomly cleave DNA sites remote from the PDHD intercalation site. If molecular oxygen is not present in the reaction mixture, the production of ROS will be limited or eliminated, and cleavage of the DNA will be localized to the immediate vicinity of the PDHD intercalation site.

3.5.2 Sequence Specific Cleavage of DNA by PDHD Enantiomers.

Sequence selectivity of DNA base damage induced by PDHDs was investigated using a DNA 22-mer duplex. The DNA was 32 P-5'-end-labeled and incubated with PDHD enantiomers in 10 mM sodium cacodylate buffer (pH 7.0) at 37 °C for 1 hour in the dark. Aliquots were exposed to 442 nm He/Cd laser light as described in the aforementioned plasmid photocleavage assay. The damaged nucleotides sites

were detected as cleavage bands generated by hot piperidine treatment and analyzed on 20 % denaturing PAGE, **Fig. 10 A**. This procedure afforded strong cleavage bands at the all DNA base pairs with little or no specificity as is shown in **Fig. 10 A**, lanes 2,4 and6. The intensities of cleavage bands are different for **PDHDII**,**PDHDI** and **PDHDrac**; irradiated **PDHDII**:DNA complex exhibited more significant DNA base damage than did irradiated **PDHDI**:DNA or **PDHDrac**:DNA complexes. Since piperidine breaks the DNA at a sugar with a modified base or a sugar without a base [29], these results indicate more or less random damage with exposure to the intense 442 nm laser light in the presence of PDHDs. The faint bands in the “unirradiated” lanes of A, lanes 1, 3 and 5, may be caused by ambient light and seem to be localized to guanosine and adenosine residues in **Fig. 10B**.

3.5.3 Binding Specificity.

The PDHD binding specificity was examined using DNA cleavage-protection mapping, better known as the footprinting assay. This technique has gained widespread popularity in pharmacology as a simple and reliable method for identifying binding sites for small molecules that interact with nucleic acids [30]. The results of a typical DNase I footprinting experiment using the ^{32}P -5'-end-labeled 38-mer synthetic DNA is shown in **Fig. 11**. These cleavage reactions were performed in the absence and presence of increasing concentrations of the **PDHD1** enantiomers. The products of a Maxam–Gilbert sequencing reaction on the same radiolabeled DNA molecules were also separated by electrophoresis in parallel to allow for identification of the sequences protected from nuclease cleavage. A region that is protected from digestion by the nuclease is clearly visible on this gel. Dose-dependent enhancements of cleavage rates relative to those in drug-free control lanes occur at sequences flanking the PDHD binding sites. The inhibition of cleavage is particularly strong at the 5'-G₁₀G₉ site. As expected, this high-affinity site contains GG step to which the PDHD apparently binds tightly. The footprint extends up to 3 base-pairs, suggesting the presence of overlapping binding sites which is in agreement with UV-titration data (n_{app} for **PDHD I** = 3.1).

3.6 Ultra-fast Transient Spectroscopy of PDHD Photochemistry.

3.6.1 Pyrene Radical Cation as an Intermediate in Pyrene Quinone Release.

In an effort to determine the mechanism(s) by which PDHD photochemically damages DNA, we have studied the ultrafast spectroscopy of PDHD photochemistry. Previous work with the 1,1-di-4-methoxyphenylDHD in the nanosecond regime, indicated that upon excitation the pyrene moiety readily lost an electron to form a pyrene radical cation with a very conspicuous intense absorption at 453 nm and

that this species survived into the microsecond time domain [11]. In the present work, we have extended these observations into the picosecond and femtosecond time domains. In this ultrafast work, irradiation of **PDHD1** in aqueous buffer at $pH = 7.4$ initiates very rapid electron transfer within the 200 fs-instrument response function determined by solvent contribution. This electron transfer affords the pyrene radical cation ($\lambda_{\max} = 450$ nm) and a species with $\lambda_{\max} = 530$ nm, which is assigned to the pyridinium radical in **3** in Scheme 3, *vide infra*. (**Fig. 12**) The radical cation peak at 450 nm persists into the μs time domain, but the 530-nm species is replaced by a very broad peak at ca. 600 nm within 10 ps. This very broad 600 nm-band seems best interpreted as due to aqueous solvated electrons [31] possibly spawned by the 530-nm pyridinium radical species. The best multi-exponential fit of the ΔA kinetic traces, Table 5, uses three components and a permanent offset, A_0 , to describe a long-lived signal. The fitting analysis suggests that a 1.28 ps component is dominant at 450 nm. Whereas, the 530 nm trace has two major components with time constants of 0.94 ps and 4.4 ps.

Furthermore, the apparent quantum yield for pyrenequinone release in aqueous solution upon continuous irradiation with visible light at 445 nm was determined to be quite low at only 0.017%. Since rigorously purified PDHD does not absorb at 445 nm, any absorption at this wavelength is due to the presence of small amounts of pyrenequinone released by ambient UV light. Therefore this low quantum yield is not a measure of quinone release from the direct irradiation of the PDHD, but of the early stages of an autocatalytic version of quinone release involving quinone excitation, *vide infra*. To a first approximation these data are consistent with the pyrene radical cation formation via the two routes shown in Scheme 3: a) Via direct excitation and d) via electron transfer to excited pyrenequinone) and decay via three routes: a) Pyrene radical cation/electron recombination in the initially formed charge separated species **3** to reform the starting **PDHD1**, b) fragmentation to form the pyrenequinone and dipyridinium stilbene **5**, and c) release of the pyridinium radical electron into the aqueous medium. The pyrene radical cation **4** might then recombine with the solvated electrons in a much slower process in the nanosecond time domain. It might be noted that the hydrated electron absorption might have come directly from the photolysis of **1** rather than via **3**, because transient absorption signals of hydrated electrons observed in femtosecond photolysis experiments in neat water are significantly smaller than for aqueous solutions of **1** under the same excitation conditions.

The same pyrene radical cation band ($\lambda_{\max} = 455$ nm) and weak band ($\lambda_{\max} = 520$ nm) are formed when **PDHD1** is irradiated in the presence of herring sperm DNA, **Fig. 13**. However, in this intercalative DNA environment the pyrene radical cation absorption band is attenuated significantly and shifted to the red just as was the **PDHD1** absorption band upon intercalation into DNA, **Fig. 4**. Also, in

this intercalative environment, the pyrene radical cation band decays more rapidly and the broad $\lambda_{\text{max}} = 600$ nm band attributed to hydrated electrons does not develop, **Fig. 13B**.

Even though the above interpretation is consistent with our results, we also considered a somewhat modified mechanism. In this mechanism, the initially excited Franck-Condon PDHD state reached upon absorption of a 350-nm photon would be a locally excited (LE) state, possibly of a π, π^* nature on the basis of a large extinction value in the lowest-energy, 340-nm absorption band ($\sim 15,000 \text{ M}^{-1} \text{ cm}^{-1}$). This LE state rapidly relaxes into the charge-transfer excited state (**3**, Scheme 3) observed at 200 fs, in addition to populating a close in energy charge-transfer-to-solvent (CTTS) excited state, which is responsible for ultrafast ejection of an electron into the aqueous solvent, leaving the radical cation **4** as the product. This electron transfer originates from the electronic state closer to the photolysis photon energy in comparison with **3**, and therefore, is less energetically demanding. Electrons are known to be ejected into water cavities on a sub-200 fs time scale [32], and start to absorb in the near-infrared region. On the picosecond time scale the electrons become thermalized and solvated [31,32b,c], and their absorption gradually shifts into the visible range to form the 720 nm band [33]. At ~ 2 ps, we begin to observe the short-wavelength (550-620 nm) absorption wing of this species which continues to undergo recombination with **4** on a 100-ps time scale. As evident from the above description, this mechanism is different in the nature of the nascent, initially excited state as well as the nature of the precursor state for electron ejection, but it leaves unchanged our main conclusion about the picosecond occurrence of species **3** and **4**. The importance of this PDHD as a cleaving agent justifies further transient absorption experiments utilizing time resolution better than 100 fs and probing in the near infrared range (700-1500 nm) to gain a full understanding of the photochemical reaction mechanism of this molecule.

3.6.2 Autocatalytic Release of Pyrene Quinone from PDHD 1.

There are several points worthy of note in the analysis of these data. The most conspicuous of which is the fact that many of these experiments were conducted with visible light that is not absorbed by **PDHD1** (**Fig. 14**). However, as indicated in **Fig. 10B**, there is sufficient long wavelength UV light in ambient light to release small amounts of pyrenequinone in the samples and this quinone readily absorbs visible light (**Fig. 14**). Thus, if the excited pyrenequinone can abstract an electron from **PDHD1** to form the radical cation **4**, then an alternative route for release of pyrenequinone with visible light might be possible (pathway d, Scheme 3). This possibility has been tested by microsecond transient spectroscopy in which pyrenequinone was excited with visible light in the presence of **PDHD1** (**Fig. 15**). Excitation of pyrenequinone alone at 445 nm affords intense absorption bands at 380, 460 and 760 nm (**Fig. 15A**) all of which decay completely in about 6 microseconds. The same bands are observed when

pyrenequinone is excited in the presence of **PDHD1** (**Fig. 15B**). However, in this case, an intense band at 450 nm remains following decay of the quinone bands. This 450 nm band is due to the pyrene radical cation **4** and persists into the microsecond time domain.

This very interesting result indicates that pyrenequinone released from **PDHD1** is autocatalytic; the release of pyrenequinone is catalyzed by the previously released quinone. This type of autocatalysis is especially intriguing when the reaction components are associated by intercalation within the same DNA duplex, Scheme 4. Thus, the released pyrenequinone is not appreciably soluble in water and will remain trapped in the DNA slot where it was generated. If other **PDHD1** molecules are intercalated in nearby duplex slots, as was the case in the aforementioned cleavage experiments, the released pyrenequinone is in an ideal position to abstract electrons from these proximate **PDHD1**s via electron transfer through the base pair stack as shown in Scheme 4 [34]. This autocatalytic process would catalyze the formation of additional pyrenequinone with much higher quantum yields than were observed in the absence of DNA and in the presence of very low pyrenequinone concentrations.

3.7 A Dipyridinium Electron Trap.

Another interesting observation in this ultrafast transient work is the observation of the weak band at 530 nm in the transient spectrum of **PDHD1** in aqueous medium in the absence of DNA (**Fig. 12A**). The simple explanation for this band is that it is associated with the pyridine radical in the charge-separated species **3**, Scheme 3. However, *N*-butyl pyridinium radical is reported to have a $\lambda_{\text{max}} = \text{ca. } 320 \text{ nm}$ [35] instead of the 530 nm band observed for **3**. Clearly, this 530 nm band is associated with the electron ejection from the pyrene moiety, since its formation coincides with that of the pyrene radical cation. However, it is not due to the formation of a simple pyridinium radical. Therefore, we have examined the ultrafast transient absorption spectroscopy of the less complex dimethyl 1,2-di-4-pyridinium ethane (**6**) when excited in the presence of the efficient electron donor DABCO, Scheme 4. This dipyridinium salt affords a prominent transient peak at 530 nm that decays in ca. 15 ps, **Fig. 16**.

These characteristics are completely consistent with those observed with **PDHD1**. The drastically different absorption properties of the mono- and dipyridinium salts are extremely interesting. Apparently the dipyridinium salts in **PDHD1** and **6** are acting as effective electron sinks via the cooperative bonding of both pyridinium moieties with the ejected electron. We have probed this type of electron entrapment by calculations of the absorption properties of the pyridinium conformations in

which the electron is coordinated by both pyridinium rings, **7a**, as opposed to incorporated into a single pyridinium ring, **7b**. The results of these calculations are summarized in Table 6 for the conformations **7a**, **7b**, and **7c** shown in **Fig. 17**. These calculations support the hypothesis that the two pyridinium rings act cooperatively to accommodate the ejected electron. At this time, the hypersurfaces of the dipyridinium-electron complex have not been thoroughly explored using high level calculations, but the observation of a distinct minimum in what would normally be considered a higher energy staggered conformation **7a** is very interesting. Finally, it should be noted that this electron-stabilizing dipyridinium geometry is not the most favorable geometry for **PDHD1** bound to DNA that is shown in **Figs. 1** and **2**, which require the diaxial conformation of the two pyridinium rings. Therefore, the weak band at 530 nm in the transient spectra of **PDHD1** in the presence of DNA (**Fig. 13**) must have another source or be due to **PDHD1** that is not intercalated into the DNA. The failure to generate solvated electrons in the presence of DNA also would seem to indicate that DNA provides a more stable environment for free electrons than does water. Such an environment might well be the π -system of base-pair stack. Clearly further experimentation will be necessary to firmly establish the nature of these coordinated electron traps.

4. CONCLUSIONS.

In this work, the binding properties of **PDHD1** to herring sperm DNA as well as to short synthetic DNA oligomers have been studied by spectroscopic methods. Both enantiomers of **PDHD1** bind to double stranded DNA with a high affinity. Remarkable hypochromic and bathochromic effects of enantiomers in the presence of increasing amounts of DNA have been observed in the absorption spectra. The enantiomer **PDHDII** has a slightly larger binding constant ($K_b = 2.3 \pm 0.8 \times 10^5 \text{ M}^{-1}$) than its optical isomer **PDHDI** ($K_b = 1.6 \pm 0.15 \times 10^5 \text{ M}^{-1}$). UV titration experiments reveal that these PDHDs intercalate into the DNA double helix. The ability of PDHDs to stabilize duplex DNA was confirmed by UV-melting studies. **PDHDII** enhances the melting temperature of 10bp duplex DNA by $\Delta T_m = 15^\circ\text{C}$ and, more surprisingly, it provides ΔT_m of almost 29°C for a duplex DNA 12-mer. This type of DNA binding is most effective since it implements both the hydrophobic intercalative bonding characteristic of many aromatic hydrocarbons and coordinated ionic bonding to both phosphate backbones. Furthermore, it reduces the ionic repulsion of the negatively charged phosphate backbones, inhibiting the separation of the two duplex chains.

Of particular significance, this **PDHD1** duplex stabilizer is photochemically active. Upon photochemical initiation with short wavelength visible light (442 nm), these PDHDs

linearizesupercoiled plasmid DNA with high efficiency. In addition, these PDHDstend to cleave DNA strands at random sites with intense laser light and at G and GG sites with weak ambient light. These processes occur via an autocatalytic mechanism in which traces of pyrenequinonephotochemically oxidize the PDHDs causing the further release of pyrenequinone. We are currently investigating the mechanism by which the released pyenequinonedamages DNA. It is important to note that there are both oxygen-dependent DNA cleavage mechanisms and oxygen-independent mechanisms at work here. In addition, this work has led to the observation of a most unusual electron transfer mechanism in which an ejected electron is trapped by the cooperative action of two pyridinium rings. We are currently investigating related systems in an effort to determine the scope of and to optimize this process.

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Scheme 1. Schönberg-Mustafa reaction: The forward reaction occurs with visible light (425-514 nm), while the reverse reaction occurs with UV light (350 nm).

Scheme 2. Synthesis of dipyridiniumpyrenedi hydrodioxin (PDHD) **1**.

Scheme 3. Pathways for pyrene radical cation **3** annihilation and alternative routes for pyrenequinone generation.

Scheme 4. Autocatalysis of pyrenequinone release from **PDHD 1** intercalated in DNA.

Scheme 5. Electron transfer from DABCO to dipyridinium salt **5** and cooperative binding of the electron by two pyridinium rings.

Figure 1.(A)Diequatorial and diaxialconformations of **PDHD1**. (B) Binding of **PDHD1** enantiomers (SS and RR) in the major groove of duplex DNA.

Figure 2.Binding of **PDHD1** enantiomers to a DNA decamer in an aqueous environment.

Figure 3. CD spectra of enantiomerically pure **PDHD1** enantiomers: isomer #1”-“ = **PDHDI** and isomer #2 “+” = **PDHDI**.

Figure 4. The absorption spectra of pure **PDHD1** enantiomers 10 μM and **PDHD1**–herring sperm (HS) DNA complexes in cacodylate buffer (10mM sodium cacodylate, 100mMNaCl, 0.5 mM EDTA, $p\text{H}$ 7.1) at 20°C. Panels **A** and **B** related to the enantiomers **PDHDII**, and **PDHDI** UV-Vis curves respectively with the upper maxima at 343 nm and with increasing concentration of HS DNA (25, 50, 75, 100, 125, 150, 175, 200 and 225 μM of DNA basepairs). Inserts are the half-reciprocal plots of $[\text{DNA}]/\Delta\epsilon_{\text{ap}}$ as a function of DNA concentration as determined from the absorption spectral data. The solid line represents the best linear fit of the data with a regression coefficient of > 0.995 .

Figure 5. Thermal denaturation of 10 μM 10bps DNA alone (leftmost curve in each plot) and in the presence of increasing concentrations of **PDHD1** isomers 1 μM , 5 μM , 10 μM , 15 μM and 20 μM . Saturation (r) occurs when the ratio of **PDHDI** to DNA reaches 1.5; **PDHDII** to DNA reaches 2; **PDHDracemate** to DNA reaches 1. First derivative melting curves for the transitions of 11-mer DNA in the absence and presence of saturating amounts of PDHD enantiomers are presented in the fourth graph. The r values for these curves are 1.5, 1.5 and 1.5 for **PDHDI**, **PDHDII** and **PDHDrac**, respectively.

Figure 6. First derivative plots of the melting point profiles of duplex dodecamer (12 bps): 5'-GTTAGTATATGG-3'/3'-CAATCATATACC-5', $T_m = 39.4^\circ\text{C}$, dodecamer with **PDHDI**, $T_m = 61.2^\circ\text{C}$ and $\Delta T_m = 21.8^\circ\text{C}$ and dodecamer with **PDHDII**, $T_m = 68.1^\circ\text{C}$ and $\Delta T_m = 28.7^\circ\text{C}$. Saturation was achieved at a ratio of PDHD: DNA of 2:1 for both enantiomers.

Figure 7. Photochemical cleavage of supercoiled $\phi\text{X } 174$ plasmid DNA ($0.033 \mu\text{g}/\mu\text{l}$) with **PDHD** enantiomers and racemic mixture ($1 \mu\text{M}$ of each) in cacodylate buffer (10 mM sodium cacodylate, 100 mM NaCl, 0.5 mM EDTA, $\text{pH } 7.1$). Each sample was irradiated with 442 nm He/Cd CW laser light (78 mW) at 18°C in the presence of molecular oxygen. Samples were then analyzed on a 1% agarose gel, electrophoresis plate. Lanes 1–8, are DNA-**PDHDI** complex with increasing irradiation times of $0 - 420 \text{ s}$; lanes 9–16 are DNA-**PDHDII** complex with increasing irradiation times of $0 - 420 \text{ s}$; lanes 17–24 are DNA-**PDHDrac** with increasing irradiation times of $0 - 420 \text{ s}$; lane 25 is DNA irradiated for 420 s , and lane 26 is DNA that was not irradiated.

Figure 8. Photochemical cleavage of supercoiled ϕ X 174 plasmid DNA (0.033 $\mu\text{g}/\mu\text{l}$) with **PDHDI** enantiomers and racemic mixture (1 μM of each) in cacodylate buffer (10 mM sodium cacodylate, 100 mM NaCl, 0.5 mM EDTA, pH 7.1) in absence of oxygen. Each sample was irradiated with 442 nm He/Cd CW laser light while maintaining its temperature at 18° C. Then the samples were analyzed on a 1% agarose gel, electrophoresis plate. Lanes 1–8, are DNA-**PDHDI** complex with increasing irradiation times from 0 – 420 s; lanes 9–16 are DNA-**PDHDII** complex with increasing irradiation times from 0–420 s; lanes 17–24 are DNA-**PDHDrac** with increasing irradiation times from 0–420 s; lane 25 is DNA alone irradiated for 420 s, and lane 26 is DNA alone with no irradiation.

Figure 9. Comparison of ds-breaks (n_2 value) caused by PDHD enantiomers and racemic mixture (**1**) as a function of irradiation time in absence of oxygen.

Figure 10. Autoradiography of 20% denaturing PAGE of 5'-end³²P-ATP labeled 22-mer DNA fragments. **A.** Cleavage of the **PDHDI**:DNA complexes upon irradiation at 442 nm 80 mW (He/Cd laser system). Lanes 1,3 and 5 are controls in which no intentional irradiation was performed (complexes were kept in the dark); Lanes 2, 4, and 6 include **PDHDI**:DNA complexes that were irradiated for 10 min; Lane 7 is DNA alone irradiated for 10 min (control); lane G's is Maxam–Gilbert sequencing reaction specific for G's. **B.** Incubation of complexes at 37° C for 1 hour, samples were not kept in the dark. Lane G's and A's is Maxam–Gilbert sequencing reaction specific for A's and G's.

Figure 11. DNase I footprinting assay of **PDHDI** binding to radiolabeled 38-mer DNA. Lanes: G's and A's is Maxam–Gilbert sequencing reaction for G and A; Lane 0 to 100 μM are DNase I cleavage fragments of the DNA in presence of increasing concentration of **PDHDI** from 0 to 100 μM.

Figure 12. Transient absorption spectra of **PDHD1** in cacodylic buffer ($pH = 7.4$) at the excitation wavelength of 350 nm and at different time delays between excitation and probe pulses (A: 0.3 – 10 ps. B: 10 ps – 1 ns).

Figure 13. Transient absorption spectra of **PDHD1** and **PDHD 1/DNA** in cacodylic buffer ($pH = 7.4$) at the excitation wavelength of 350 nm and at different time delays: A) 0.5 picoseconds and B) 10 picoseconds.

Figure 14. Absorption spectra of **PDHD1** (black), pyrenequinone (red) and a mixture of **1** and pyrenequinone (blue) in 1:1 acetonitrile:water.

Figure 15. Transient spectra produced by 445 nm excitation of pyrenequinone alone in acetonitrile:water, 1:1 (μ s) (A) and of pyrenequinone in the presence of **PDHD1** in acetonitrile:water, 1:1 (μ s) (B). The microsecond time intervals following excitation are given in the legend.

Figure 16. Transient absorption spectra of 1,2-dipyridinium salt **6** in 40 mM DABCO and 40 mM Mcacodylic buffer excited at 330 nm.

Figure 17. Dipyridinium radical cation **7** geometries optimized at the M0522X/cc-pVDZ level of theory in water.

Table 1. Calculated apparent binding constants from UV-vis titration data. Errors of K_b are from three independent UV-titration experiments.

DNA	Enantiomers PDHD 1	apparent K_b
		(M^{-1})
	PDHD rac	$7.1 \pm 0.15 \times 10^4$
5' - ATCGACCAAGC - 3' 3' - TAGCTGGTTCG - 5' herring sperm	PDHD I	$1.8 \pm 0.08 \times 10^5$
	PDHD II	$3.2 \pm 0.12 \times 10^5$
	PDHD rac	No data
	PDHD I	$1.58 \pm 0.15 \times 10^5$
	PDHD II	$2.34 \pm 0.2 \times 10^5$

Table 2. PDHD1-induced DNA duplex thermal stabilization data (ΔT_m) for resolved enantiomers and binding constant at the DNA melting temperature (K_b at T_m).

DNA 11bp	Enantiomers PDHD 1	ΔT_m^a	K_b at T_m^b	α_f	n^c
		(K)	(M^{-1})	(μM)	(bp)
	PDHD rac	11.48 ± 0.3	$4.40 \pm 0.5 \times 10^5$	10	4.2
5' - ATCGACCAAGC - 3' 3' - TAGCTGGTTCG - 5'	PDHD I	13.27 ± 0.4	$3.19 \pm 1.1 \times 10^5$	10	3.1
	PDHD II	15.31 ± 0.3	$4.71 \pm 0.9 \times 10^5$	10	3.3

^a ΔT_m values were derived from UV melting profiles at 10 μM 11 mer (10 bps) DNA in the absence (T_m°) and presence of saturated amount of PDHD (T_m). Each T_m value is an average derived from three experiments, with the indicated errors corresponding to the average deviation from the mean.

^b Binding constants at T_m were determined using Equation 1, calculated duplex-to-single strand transition enthalpies (ΔH_{w-c}) at 110 mM Na^+ and the total strand concentration of 10 μM for the host duplex was - 7.5 kcal/mol per base pair determined by nearest – neighboring parameters [3b,24].

^c Apparent binding site size n was calculated from UV titration experiment (supplementary data).

Table 3. Statistical efficiency of single-strand n_1 and double-strand n_2 breaks caused by PDHD enantiomers and racemic mixture (**1**) as a function of irradiation time in the presence of oxygen.

Irradiation time (sec)	PDHD I			PDHD II			PDHDrac		
	ss-break (n_1)	ds- break (n_2)	n_1 / n_2	ss-break (n_1)	ds-break (n_2)	n_1 / n_2	ss-break (n_1)	ds- break (n_2)	n_1 / n_2
0	0.27	0.00	-	0.27	0.00	-	0.16	0.00	-
30	2.13	0.04	51.12	0.77	0.01	95.02	0.74	0.01	81.90
60	2.33	0.06	40.80	1.87	0.03	72.99	1.25	0.02	68.06
90	3.48	0.10	36.52	2.33	0.04	56.00	2.90	0.10	28.92
120	3.63	0.10	35.37	3.76	0.10	38.06	4.44	0.17	26.15
180	4.45	0.14	32.31	4.09	0.11	36.80	4.61	0.22	21.00
300	4.68	0.15	31.31	6.72	0.19	35.27	5.06	0.23	21.59
420	5.62	0.19	29.50	6.70	0.20	32.73	6.48	0.43	15.12

Table 4. Statistical efficiency of single-strand n_1 and double-strand n_2 breaks caused by PDHD enantiomers and racemic mixture (**1**) as a function of irradiation time in the absence of oxygen.

Irradiation time (sec)	no oxygen PDHD I			no oxygen PDHD II			no oxygen PDHD rac		
	ss-break (n_1)	ds-break (n_2)	n_1 / n_2	ss- break (n_1)	ds-break (n_2)	n_1 / n_2	ss- break (n_1)	ds- break (n_2)	n_1 / n_2
0	0.29	0.00	-	0.28	0.00	-	0.25	0.00	-
30	0.60	0.01	45.50	0.45	0.02	29.44	0.52	0.01	39.71
60	0.99	0.03	34.47	0.88	0.03	27.64	0.94	0.03	30.29
90	1.34	0.05	26.65	1.16	0.05	22.00	1.29	0.05	24.07
120	1.51	0.10	15.89	1.70	0.11	15.33	1.68	0.08	20.40
180	1.97	0.23	8.41	2.12	0.23	9.03	1.92	0.14	14.08
300	2.66	0.33	7.99	2.40	0.41	5.89	2.29	0.24	9.49
420	3.46	0.45	7.71	2.94	0.56	5.23	2.82	0.40	7.04

Table 5. Decay parameters for the 450 nm and 530 nm transients using the triple exponential mode:

$$\Delta\text{abs} = A_0 + A_1 \cdot \exp(-t/\tau_1) + A_2 \cdot \exp(-t/\tau_2) + A_3 \cdot \exp(-t/\tau_3).$$

λ_{max} (nm)	A_0 (%)	A_1 (%)	τ_1 (ps)	A_2 (%)	τ_2 (ps)	A_3 (%)	τ_3 (ps)
450	5.6±0.07	92±1	1.28±0.02	1.4±0.1	6.3±0.6	0.5±0.1	115±11
530	8.5±0.2	56±3	0.94±0.07	28±3	4.4±0.5	8.5±0.5	93±11

Table 6. Singlet-to-singlet vertical excitation transitions and absorption properties of **7a**, **7b** and **7c** in water.

species	λ , (nm)- TD-M052X/cc-pVDZ (f , oscillator strength)	λ , (nm) experiment
7a	1076 (0.0072) 866 (0.0033) 591 (0.0160) 305 (0.0001) 291 (0.0155) 286 (0.0002)	530
7b	905 (0.0016) 767 (0.0007) 501 (0.0004) 304 (0.0000) 290 (0.2000) 283 (0.0000)	-
7c	3889 (0.1205) 997 (0.0073) 883 (0.0063) 292 (0.0062) 289 (0.0013) 283 (0.0158)	-