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Sequence-Specific Photomodification of DNA by an Oligonucleotide-Phenanthrohydrodioxin Conjugate

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We introduce a new member of a family of photochemically active oligonucleotide conjugates. A Phenanthrohydrodioxin (PDHD)-based agent was synthesized and covalently linked to a 5'-end of the 9-mer oligonucleotide via a hexamethylene linker. The conjugate hybridized to a complementary 30-nucleotide-long target and efficiently cleaved it in a sequence specific manner. Up to 67% of target was specifically damaged (51% cross-links and 16% direct cleavage). While the photosensitizer alone nonspecifically damaged only Gs in a single-stranded target, its conjugate cross-linked to and damaged also A, T, and C sites in a target in agreement with duplex and triplex formation.

INTRODUCTION

Therapeutic potential of covalently modified oligonucleotides has driven the search for agents that can be coupled to DNA, are easy to control, and damage specific DNA target sites (1). One of the most frequently used classes of such agents has been photosensitizers since they offer the advantage of controlling the timing of the chemical inactivation event (2).

Several photoreactive groups have been covalently linked to oligonucleotides and their analogues (3). For example, psoralens (Pso)¹ that are able to form inter-strand cross-links or monoadducts in double-stranded DNA (4), often have been used as photosensitizers coupled to oligonucleotides. Pso-pN₁₃ was covalently linked to target DNA and irreversibly inhibited *bla* gene transcription in *Escherichia coli* in vitro (5). Psoralen-methylphosphonate conjugate cross-linked with rabbit globin mRNA 50–100% and specifically inhibited translation to the extent of 40–60% at oligomer concentrations of 5–20 μ M (6). Pso-pN₁₆ inhibition of *Dra*I digestion of HIV-I DNA was 95% at 7 μ M oligonucleotide concentration (7, 8). Pso-pN₁₀ induced 100-fold more frequent mutations in a specific region of the λ genome (9). After UV irradiation, Pso-pN₁₅ cross-linked and inhibited transcription of the α -subunit of the interleukin 2 receptor in vivo (10).

Another class of photosensitizers, porphyrins, linked to oligonucleotides, produced various types of photodamage on a complementary target DNA (11–13). This included oxidation of G and cross-linking of the porphyrin conjugate to the target sequence. Both reactions gave 57% yield of total modification (20% oxidized G, and 37%

cross-linking). Guanines located close to the porphyrin macrocycle were the most altered (11). Up to 7% of target DNA was cross-linked, and up to 23% was cleaved by the conjugate of sapphyrin, an extended porphyrin that damages DNA upon irradiation with 300 and >620 nm light (13). The conjugates of 3-azidoflavine (14) and *p*-azidophenacyl (15) were photo-cross-linked to the single- and double-stranded 27-mers and induced cleavage after piperidine treatment. A small fraction of ellipticine derivative photo-cross-linked with double-stranded DNA (16). Eosin has been linked to oligonucleotides and produced damage in double-stranded targets (17). Proflavin-linked oligonucleotide was able to induce 12% cleavage after irradiation followed by piperidine treatment (18). Uranyl UO₂²⁺ photooxidation induced cleavage at the ends of oligodeoxynucleotides. However, its conjugates failed to do so (19). Aryl azide/biotin/oligonucleotide derivative cross-linked to its target and was used to quantitate triple-helix formation (20).

Considering potential benefits of photosensitizer-DNA conjugates, it is important to search for and study new kinds of photoreactive groups that could give high yields of specific target DNA inactivation.

The purpose of this investigation was to prepare a PDHD-DNA conjugate and investigate its sequence-specific DNA damage potency. PDHDs were designed as photoactivatable DNA-damaging agents (21–26), and this study is a first attempt to couple them to a molecule that will give them sequence specificity.

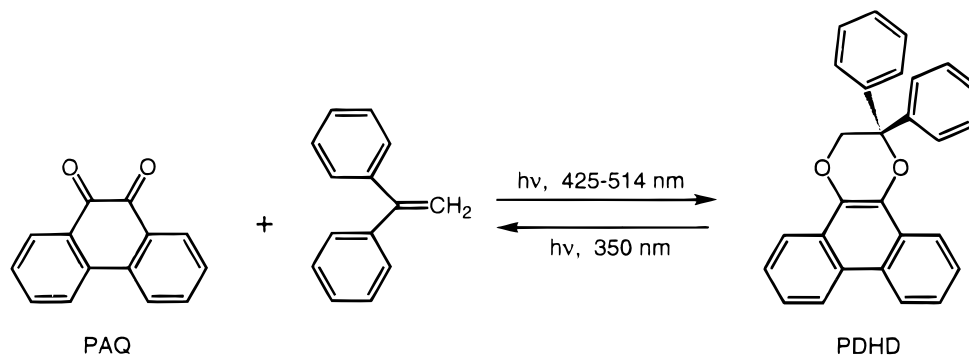
PDHDs are masked *o*-quinones: they can be made from and release phenanthrenequinone (PAQ) when irradiated with 425–514 and 350 nm light, respectively (Scheme 1) (21). This release of parent PAQ is believed to be responsible for DNA damage and cleavage. Several PDHD analogues carrying ionic groups to make them soluble in water have been prepared. PDHDs with cationic groups bind to DNA with typical binding constants $K_{\text{ass}} = 2.5 \times 10^4 \text{ M}^{-1}$. They show hyperchromism and a 3–7 nm bathochromic shift in the presence of DNA which is consistent with intercalation and/or groove binding. Cationic PDHDs efficiently relax supercoiled Φ X 174 FR I DNA upon irradiation with low doses of 365 nm light under both oxygen-rich and oxygen-free conditions. Cationic PDHDs inactivate up to 4 logs of

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¹ Abbreviations: PDHD, phenanthrohydrodioxin; Pso, psoralen; PAQ, phenanthrenequinone; DTT, dithiothreitol; TCA, trichloroacetic acid; THF, tetrahydrofuran; σ , standard deviation; SCP, sodium chloride in sodium phosphate buffer.

Scheme 1. Schönberg Reaction: Forward Reaction with Visible Light (425–514 nm), Reverse Reaction with UV Light (350 nm)

Vaccinia virus. Results of transient spectroscopy, quenching of DNA cleavage with dithiothreitol (DTT), and irradiation of PDHDs in the presence of cytochrome *c* and superoxide dismutase support a mechanism of DNA damage which involves the release of excited PAQ that is capable of hydrogen abstraction or electron transfer and the involvement of superoxide and other radicals (21, 27). Other mechanisms of DNA damage by cationic PDHDs, including involvement of singlet oxygen, are possible. The relative efficiencies of different DNA-cleaving mechanisms for different PDHDs is being investigated and will be a subject of a separate publication.

No information was available about sequence specificity of PDHDs. Due to the small size, little if any specificity was expected. Because of the relatively low DNA binding affinity [10 times lower than ethidium bromide, 1000 times lower than tripyridyl ruthenium (27)] PDHDs are good candidates for use as DNA conjugates since they should allow sequence-specific recognition by the targeting oligonucleotide, increasing its binding slightly, but not so much as to alter the site-specificity provided by the sequence-recognizing oligonucleotide group.

EXPERIMENTAL PROCEDURES

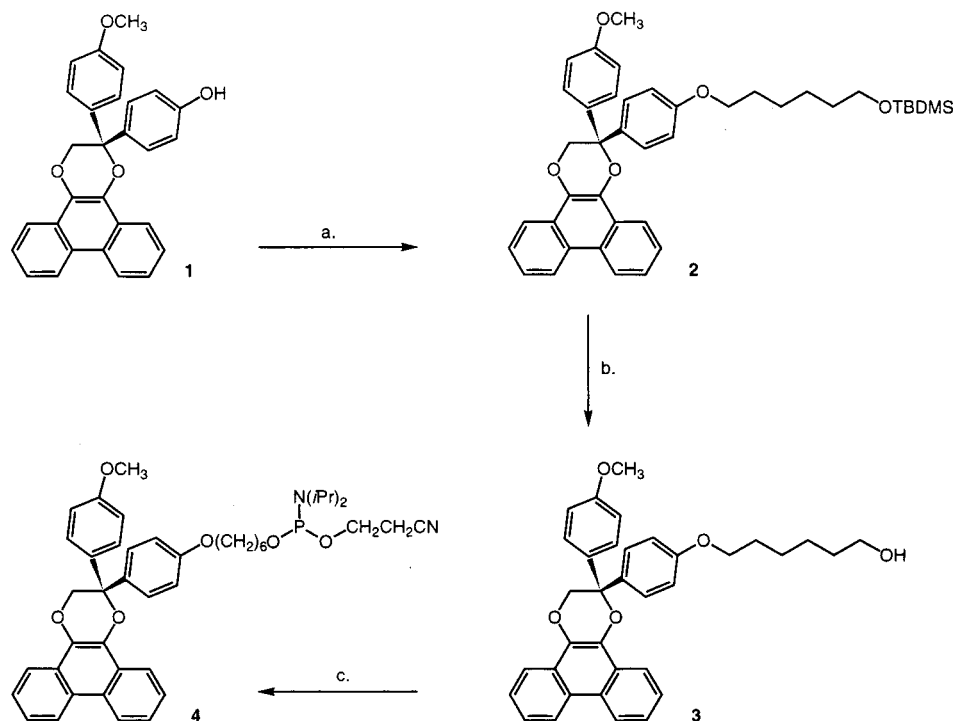
Preparation of Oligonucleotides. Regular oligonucleotides were synthesized on PerSeptive Biosystems Expedite Synthesizer using standard phosphoramidite chemistry at the DNA Core Facility at the University of Cincinnati. All oligos were purified by reverse-phase columns and PAGE and dissolved in water to make a 20 μ M solution. Target DNA was labeled at the 5'-end using [γ - 32 P]ATP and T4 polynucleotide kinase. Its purity and labeling efficiency were checked by 20% polyacrylamide gel electrophoresis.

Synthesis of 2-[4-(6-(*tert*-Butyldimethylsilyloxy)-hexyloxy)phenyl]-2-[4-methoxy-phenyl]-2,3-dihydrophenanthro-[9.10-b]-1,4-dioxin (2, Scheme 2). Dihydrodioxin **1** (Scheme 2) (0.0827 g, 0.19 mmol) was placed in a 5 mL one-necked flask and dissolved in 1.5 mL of HPLC-grade acetonitrile, followed by the addition of 0.06 g (0.23 mmol) 18-crown-6, 0.032 g (0.23 mmol) of potassium carbonate, and 0.078 g (0.23 mmol) of 6-(*tert*-butyldimethylsilyloxy)-1-iodohexane. The reaction was allowed to stir under nitrogen for 12 h and was then diluted with chloroform. The solids were removed by filtration and the solvent was evaporated. The crude reaction mixture was purified by rotary chromatography (7.5% ethyl acetate, 92.5% hexanes). The product **2** was isolated as a colorless oil. Product $C_{41}H_{48}O_5Si$; molar mass: 648.9 g/mol; yield 0.0993 g (0.153 mmol), 80.5%. 1H NMR ($CDCl_3$) δ (ppm): 8.56 (m, 2H), 8.43 (d, $J = 7.5$

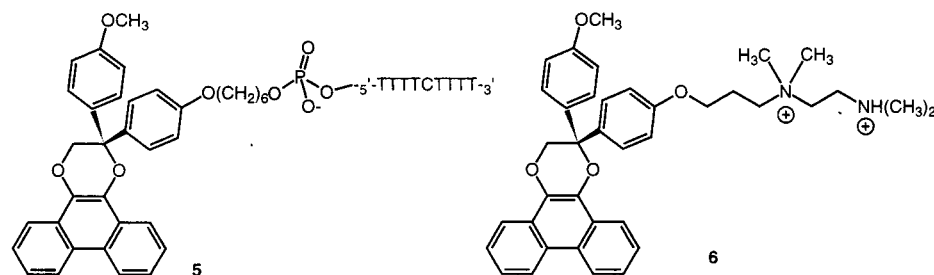
Hz, 1H), 8.12 (m, 1H), 7.67–7.42 (m, 8H), 6.83 (d, $J = 8.6$ Hz, 2H), 6.81 (d, $J = 8.6$ Hz, 2H), 4.75 (s, 2H), 3.88 (t, $J = 6.5$ Hz, 2H), 3.74 (s, 3H), 3.59 (t, $J = 6.2$ Hz, 2H), 1.72 (m, 2H), 1.54–1.35 (m, 6H), 0.88 (s, 9H), 0.03 (s, 6H). ^{13}C NMR ($CDCl_3$) δ (ppm): 158.8, 133.4, 133.1, 132.8, 132.5, 128.2, 128.1, 127.9, 126.9, 126.7, 126.6, 126.5, 126.2, 124.7, 122.4, 122.3, 120.8, 120.7, 114.3, 113.8, 79.3, 70.4, 67.8, 63.1, 55.2, 32.8, 29.2, 25.9, 25.8, 25.6, 18.4, –5.3. IR ($CHCl_3$): 2933, 2858, 1633, 1610, 1583, 1512, 1462, 1335, 1251, 1178, 1089, 1009, 978, 834 cm^{-1} . MS (m/z) 440, 208, 180, 152, 133 (base peak).

Synthesis of 2-[4-(6-Hydroxyhexyloxy)phenyl]-2-[4-methoxy-phenyl]-2,3-dihydrophenanthro-[9.10-b]-1,4-dioxin (3, Scheme 2). In a 5 mL round-bottom flask, 0.0993 g (0.153 mmol) of **2** was dissolved in distilled THF. The solution was cooled in an ice bath, then 0.184 mL of a 1 M solution of tetrabutylammonium fluoride in hexanes (0.184 mmol) was added dropwise via syringe. The reaction mixture was allowed to stir for 4 h at 0 $^{\circ}C$. Thin-layer chromatography showed complete disappearance of the protected alcohol. The solution was diluted with 10 mL of chloroform and washed twice with water. The organic layer was collected and filtered through magnesium sulfate, and the solvent was evaporated. Purification was achieved by rotary chromatography (70% ethyl acetate, 30% hexanes). The product was isolated as a highly viscous oil. Product $C_{35}H_{34}O_5$; molar mass 534.7 g/mol; yield 0.0664 g (0.124 mmol), 81.2%. 1H NMR ($CDCl_3$) δ (ppm): 8.56 (m, 2H), 8.43 (d, $J = 8.3$ Hz, 1H), 8.11 (m, 1H), 7.67–7.41 (m, 8H), 6.83 (d, $J = 8.3$ Hz, 2H), 6.79 (d, $J = 8.3$ Hz, 2H), 4.74 (s, 2H), 3.86 (t, $J = 6.3$ Hz, 2H), 3.72 (s, 3H), 3.61 (t, $J = 6.4$ Hz, 2H), 1.77–1.49 (m, 8H), 1.27 (s, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 159.2, 158.7, 133.4, 133.1, 132.8, 132.5, 128.2, 127.8, 126.9, 126.7, 126.6, 126.4, 126.2, 124.8, 122.5, 122.4, 120.8, 120.7, 114.3, 113.7, 79.3, 70.4, 67.7, 62.8, 55.2, 32.7, 29.2, 25.9, 25.5. IR ($CHCl_3$): 3622, 3080, 2938, 2881, 1633, 1610, 1512, 1454, 1350, 1335, 1250, 1178, 1118, 1089, 1040, 949, 832 cm^{-1} .

Synthesis of 2-[4-(6-([*N,N*-Diisopropyl][cyanoethoxy]phosphoxy-hexyloxy)phenyl)-2-[4-methoxy-phenyl]-2,3-dihydrophenanthro-[9.10-b]-1,4-dioxin (4, Scheme 2). A solution of 0.0296 g (0.055 mmol) of **3** and 0.019 mL (0.11 mmol) *N,N*-diisopropylethylamine in 0.5 mL of distilled methylene chloride was placed in a 2 mL microscale flask. The flask was purged with argon, then 0.013 mL (0.058 mmol) of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite was added via syringe. The reaction was stirred at room temperature for 10 min, then diluted with chloroform, and the organic layer was washed twice with small portions of water. The organic solution was filtered through magnesium sulfate,

Scheme 2. Synthesis of PDHD–Phosphoramidite Precursor 4^a

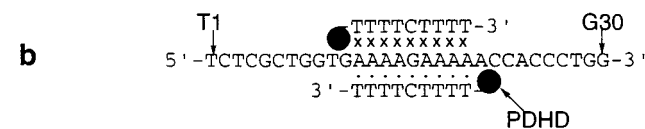
- a. I(CH₂)₆OTBDMS, K₂CO₃, 18-crown-6
 b. Bu₄N⁺F⁻
 c. ClP[N(iPr)₂]OCH₂CH₂CN, EtN(iPr)₂



^a **1**, phenanthrohydrodioxin (PDHD) derivative; **2**, 2-[4-(6-(*tert*-butyldimethylsilyloxy)hexyloxy)phenyl]-2-[4-methoxy-phenyl]-2,3-dihydrophenanthro-[9.10-b]-1,4-dioxin; **3**, 2-[4-(6-hydroxyhexyloxy)phenyl]-2-[4-methoxy-phenyl]-2,3-dihydrophenanthro-[9.10-b]-1,4-dioxin; **4**, 2-[4-(6-([*N,N*-diisopropyl][cyanoethyloxy]phosphoroxy-hexyloxy)phenyl)-2-[4-methoxy-phenyl]-2,3-dihydrophenanthro-[9.10-b]-1,4-dioxin; **5**, the PDHD conjugate; and **6**, water soluble photosensitizer PDHD.

Scheme 3^a

30A 5' -TCTCGCTGGTGAAGAAAAGAAAACCACCCTGG-3'
a 30T 3' -AGAGCGACCACCTTTTCTTTTGGTGGGACC-5'
9T 5' -TTTTCTTTT-3'



^a (a) Sequences of the target DNA (**30A**) and the complementary oligonucleotides **30T** and **9T**; (b) alignment of the target and the conjugates. The bottom strand binds to the target in antiparallel orientation via Watson–Crick base-pairing. The third strand binds in parallel orientation via Hoogsteen base-pairing (**5**). The bases in the target are numbered from the 5'-end.

and the desired product **4** was isolated by rotary chromatography (50% ethyl acetate, 48.5% hexanes, 1.5%

triethylamine). Product C₄₄H₅₁N₂O₆P; molar mass 734.9 g/mol; yield 0.0338 g (0.046 mmol), 83.6%. ¹H NMR (CDCl₃) δ (ppm): 8.56 (m, 2H), 8.43 (d, *J* = 8.2 Hz, 1H), 8.11 (m, 1H), 7.67–7.41 (m, 8H), 6.83 (d, *J* = 8.4 Hz, 2H), 6.80 (d, *J* = 8.7 Hz, 2H), 4.74 (s, 2H), 3.89–3.49 (m, 11H, –OCH₃, 2 –CH=, 3 –CH₂), 2.57 (t, *J* = 6.3 Hz, 2H), 1.75–1.25 (m, 8H), 1.15 (dd, *J* = 6.5 Hz, *J* = 4.6 Hz, 12H). ¹³C NMR (CDCl₃) δ (ppm): 159.1, 158.7, 133.3, 133.1, 132.8, 132.5, 128.1, 126.9, 126.7, 126.6, 126.5, 126.1, 124.7, 122.4, 122.3, 120.8, 120.7, 117.6, 114.2, 113.7, 79.3, 70.4, 67.7, 63.6, 63.4, 58.4, 58.1, 55.1, 43.1, 42.8, 31.1, 31.0, 29.7, 29.1, 25.7, 25.6, 24.6, 24.5, 24.4, 20.3, 20.2. ³¹P NMR (CDCl₃) δ (ppm): (external standard, H₃PO₄) 148.0. IR (CHCl₃): 3080, 2968, 2936, 2248, 1633, 1610, 1512, 1461, 1335, 1250, 1179, 1088, 1040, 978 cm⁻¹. MS (*m/z*) (*M* + *H*)⁺ observed, 735.3; calculated: 735.3. After its synthesis, the phosphoramidite **4** was tested on TLC plates for its stability in 30% ammonia and 20 mM I₂ in

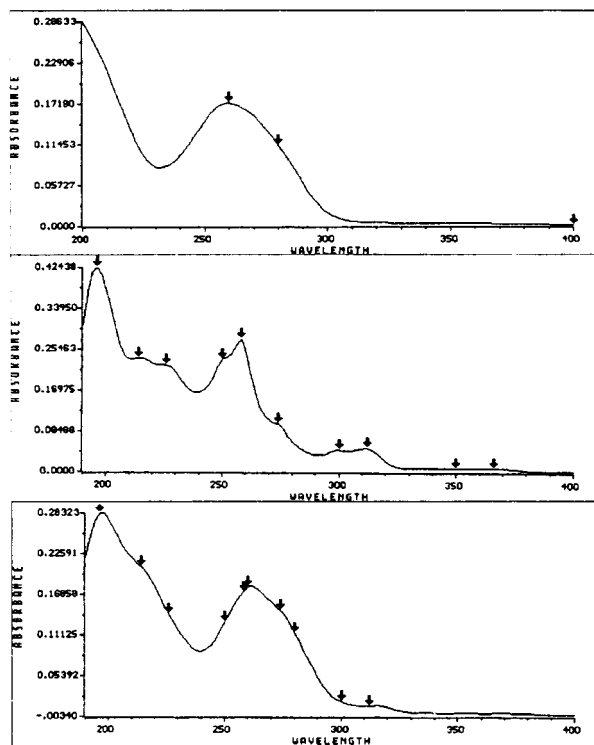


Figure 1. Absorbance spectra of 1 μM 9T DNA (top panel), 5 μM PDHD 6 (middle panel), and 1 μM conjugate 5 (bottom panel).

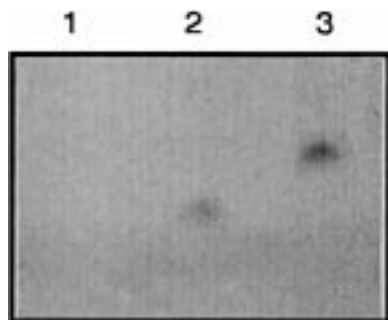


Figure 2. A photograph of the UV-shadowing experiment. Lane 1: 1 nmol of PDHD 6; lane 2: 1 nmol of PDHD 6 + 1 nmol of 9T DNA, gray spot; lane 3: 1 nmol of the conjugate 5, bright blue spot.

THF/pyridine/water, then carefully dehydrated, and dissolved in acetonitrile to make a 0.1 M solution.

Synthesis of an Oligonucleotide-PDHD Conjugate (5, Scheme 2). The 9-mer TTTTCTTTT was synthesized in the DNA Core Facility at the University of Cincinnati by standard synthesis on a 1 μM scale using a ABI 394 synthesizer with the following changes: an easily deprotected phosphoramidite of dC was used, and, after the 5'-end deprotection step of the oligonucleotide, TCA was washed from the column with acetonitrile for 2 min (4 times longer than usual) to minimize exposure of the photosensitizer to acid. The coupling of this resin-bound oligonucleotide to PDHD-phosphoramidite was performed in 0.5 M tetrazole in acetonitrile for 3 min (6 times longer than usual) to increase coupling efficiency. Instead of standard 0.1 M I_2 , 20 mM I_2 in THF/pyridine/water was used for the oxidation step. The detritylation step, since it involves the use of TCA, was excluded at the end of the program. After cleaving from the column in aqueous ammonia, the conjugate was deprotected for 1 h at room temperature, purified using a reversed-phase

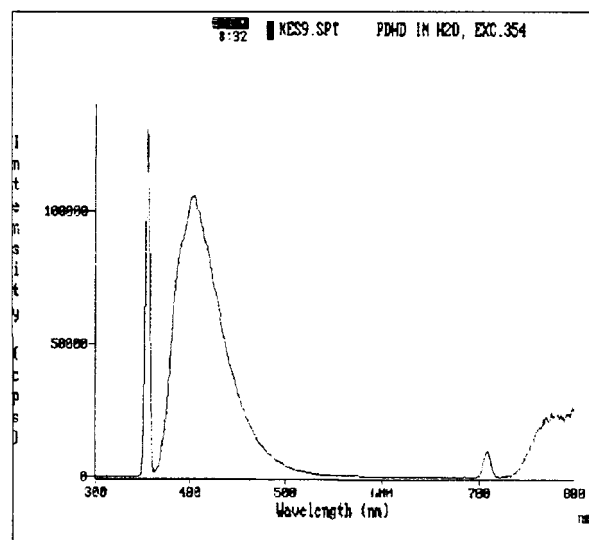


Figure 3. Fluorescence spectra of 2.5 μM PDHD, excitation at 354 nm.

cartridge, and checked using PAGE. The conjugate's UV-vis and fluorescence spectra were recorded with a Hewlett Packard 8452A Diode Array Spectrophotometer and SPEX 1680 0.22M Double Spectrometer, respectively. Mass spectrum of the conjugate was taken on PerSeptive Biosystems Voyager-DE MALDI-TOF as the sum of 64 nitrogen laser shots at 336 nm using 3-hydroxypicolinic acid as a matrix.

Unless stated otherwise, all chemicals for syntheses were purchased from Aldrich. Solvents were purified according to standard procedures. ^1H and ^{13}C and ^{31}P -NMR spectra were recorded with a Bruker AC 250. IR spectra were recorded with a Perkin-Elmer 1600 Series FTIR in a solution cell (path length, 0.2 mm). Low resolution mass spectra were taken with a Hewlett-Packard HP 5995, electron impact 70 eV or a Finnigan Mat LCQ Ion Trap, high-resolution mass spectra were obtained on a Kratos MS 801-DS 55. Unless stated otherwise, preparative chromatography separations were performed using centrifugal chromatography with a Chromatotron and plates were coated with E. Merck silica gel 60-PF254.

UV-Melting Experiments. The change of absorbance at 260 nm was measured with a Cary 219 Spectrophotometer interfaced to an IBM computer with an Intel 80386 processor that also controlled a Haake A80 circulating water bath and collected temperature data from a thermocouple using custom software. The sample chamber was purged with N_2 . DNA was dissolved in SCP (1 M NaCl, 10 mM sodium phosphate, pH 7.4) buffer, heated to 98 $^\circ\text{C}$, and cooled slowly, at 0.5 $^\circ\text{C}/\text{min}$ rate, to 4 $^\circ\text{C}$.

Maxam and Gilbert Chemical Sequencing. The target DNA was sequenced using standard procedures (28) with the following changes: both, GA and TC reactions with formic acid and hydrazine, respectively, were incubated for 2 h at room temperature, and the treatment with 10% piperidine was performed at 90 $^\circ\text{C}$ for 60 min for GA and for 50 min for TC reaction.

Photochemical Cleavage Reaction. Radioactively labeled target DNA at 10 nM concentration was mixed with PDHD or PDHD-conjugate at 0–10 μM in 20 μL of SCP buffer. Carrier calf thymus DNA (2 μg) was included where indicated. Samples were heated at 55 $^\circ\text{C}$ for 1 min, cooled to 4 $^\circ\text{C}$ at the rate of 1 $^\circ\text{C}/\text{min}$ in a thermal cycler, and incubated for 1 h at 4 $^\circ\text{C}$. All of the

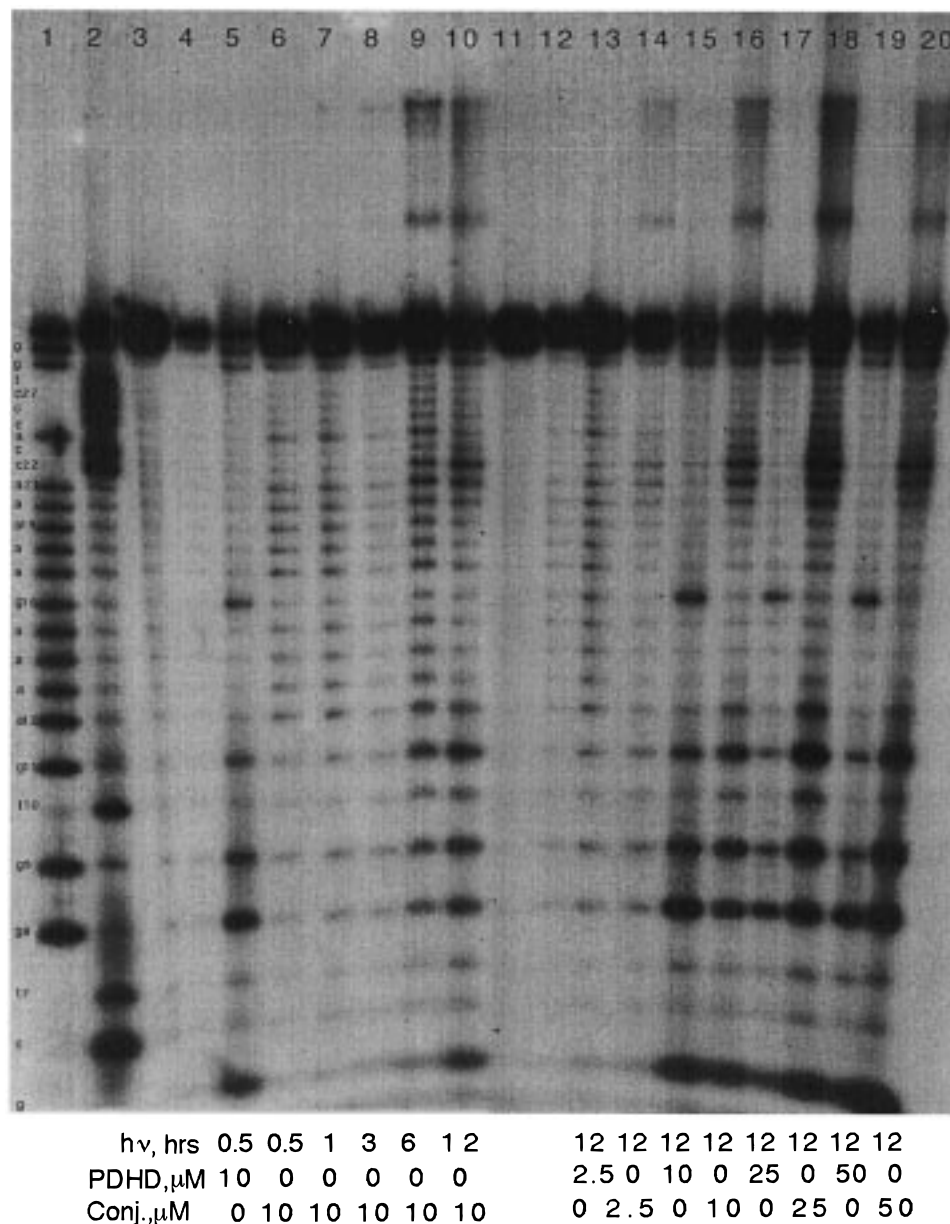


Figure 4. Irradiation of the target **30A** DNA with and without PDHD **6**/conjugate **5** for different times and concentrations. Lanes: (1) GA Maxam–Gilbert reaction of the target, (2) TC Maxam–Gilbert reaction, (3 and 12) target irradiated for 12 h and treated with piperidine (as the rest of the lanes, unless otherwise indicated), (4 and 11) target not irradiated and not treated, (5–10 and 13–20) target irradiated ($h\nu$) for indicated times with indicated concentrations of PDHD or PDHD-conjugate.

above were performed with dimmed lights. Samples were placed on Parafilm on a glass table surrounded by SCP buffer and enclosed in a Pyrex glass dish. Samples were irradiated for 1–12 h at 4 °C in a RAYONET Preperative Type RS Photochemical Reactor equipped with a cooling fan and heated-cathode 350 nm emission lamps from Southern New England Ultraviolet Co. Irradiance at the sample was 2 mW/cm². The reaction was stopped by turning off the lamps. Radicals were quenched by addition of 2 μ L of 0.1 M DTT. DNA was treated in 10% piperidine for 1 h, triple-lyophilized, redissolved in 10 μ L of 80% formamide loading buffer, and run on 20% polyacrylamide, 8 M urea gel. After electrophoresis, the gel was covered with saran wrap and exposed to Kodak X-ray film. Films were scanned using a Bio-Rad GS-670 imaging densitometer. The data were processed on an IBM computer with an Intel 80386 processor using Molecular Analyst software.

RESULTS AND DISCUSSION

The M13 genome was chosen as a target for the studies. The sequence AAAAGAAAA (between bases 5983 and 5992 of M13, traditional numbering) was selected for several reasons. It is a unique site in the genome. It is a potential site not only for double-strand but also for triple-strand formation. It is a part of the *lac i* gene which enables blue/white plaque screening for later studies. A 30-mer 5'-TCTCGCTGGTAAAAAGAAAACCACCCTGG-3' (**30A**, Scheme 3a) containing the target sequence AAAAGAAAA was synthesized and labeled with ³²P at the 5'-end. The conjugate that binds to such a target should have a polypyrimidine sequence 5'-TTTTTCTTTT-3' to be capable of both double- and triple-strand formation (Scheme 3b). The hexamethylene linker was used to give the photosensitizer PDHD some degree of freedom to interact with the target. Two oligonucleotides complementary to the target were syn-

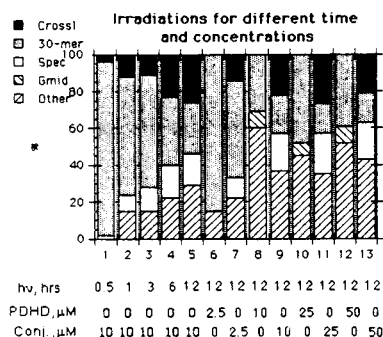


Figure 5. Quantitation of product formation for Figure 4. The amount of cross-links (Crossl), undamaged 30-mer (30-mer), specific cleavage in the region between bases A19 and C27 (Spec), damage to G16 (Gmid), and damage in the region between bases G8 and G11 (Other) was measured by scanning densitometry. Irradiation times and reagent concentrations are as indicated.

thesized, a 30-mer **30T** and a 9-mer **9T** (Scheme 3a) for the appropriate control experiments.

The PDHD derivative **1** (Scheme 2) was synthesized in a five step procedure (27). This synthesis will be reported elsewhere. Subsequent functionalization of **1** was achieved using an etherification method described by Fedorynski et al. (29). The reaction was carried out in the presence of potassium carbonate and 18-crown-6, allowing mild deprotonation of the phenolic compound **1**. The alkylating agent was 6-(*tert*-butyldimethylsiloxy)-1-iodohexane. It was prepared according to a published procedure by Heslin et al. (30). Introduction of the hexyl side chain yielded the PDHD derivative **2** (Scheme 2) in approximately 80% yield. Subsequent cleavage of the silyl ether by treatment with tetrabutylammonium fluoride afforded the alcohol **3** (Scheme 2) in good yield. This compound was then reacted with a 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (31) in the presence of *N,N*-diisopropylethylamine, yielding the desired PDHD-phosphoramidite **4** (Scheme 2) in 84% yield. Its structure was verified by complete spectral characterization. Due to the lability of the dihydrodioxin ring system, the M^+ ion of **4** could not be observed using EI or FAB. However, ESI provided the molecular ion peak, $(M + H)^+$.

The PDHD oligonucleotide conjugate **5** (Scheme 2) was prepared by standard synthesis of TTTTGTTTT at 1 μM scale and coupling this oligonucleotide to PDHD-phosphoramidite using special precautions and the manual adjustment of standard synthesis procedures to avoid any damage of the conjugate by light, acid, or harsh treatment during deprotection (see Experimental Procedures). The yield after purification through a reversed-phase column was 191 μg.

The PDHD dication **6** (Scheme 2) was prepared for comparison of the effects of the free photosensitizer versus the effects of the conjugate. This synthesis will be reported elsewhere.

The synthesis of the conjugate **5** was confirmed using UV-vis, fluorescence, PAGE, and MALDI-TOF. The 200–400 nm spectra of **9T** DNA alone, PDHD **6**, and the conjugate **5** were compared. The spectra of the conjugate has the peaks of both DNA and PDHD (Figure 1), indicating the presence of both substances in the product. Covalent linkage of PDHD to the oligonucleotide was confirmed by electrophoresis of PDHD **6**, PDHD **6** with **9T** DNA and the conjugate **5** on 8% polyacrylamide 8M urea gel (Figure 2). DNA and PDHD were visualized by UV-shadowing. As expected, PDHD in the first lane migrated out of the gel because it has a positive charge.

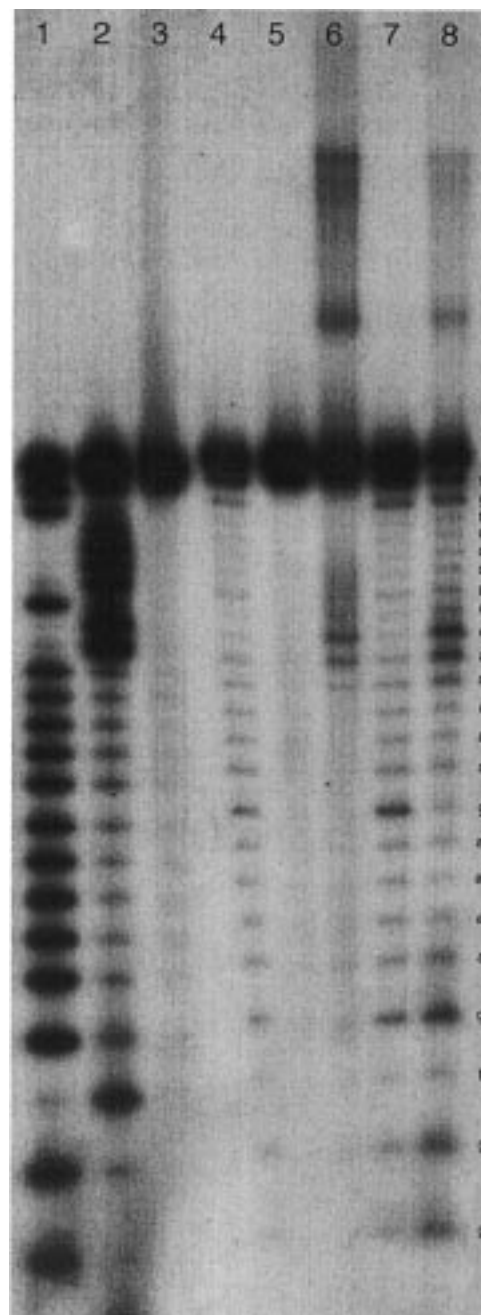


Figure 6. Irradiation of the target **30A** DNA with and without PDHD **6**/conjugate **5** with and without piperidine treatment. Lanes: (1) GA Maxam–Gilbert reaction of the target, (2) TC Maxam–Gilbert reaction, (3) target not treated, (4) target irradiated for 12 h (as the rest of the samples) and treated with piperidine, (5 and 6) without piperidine treatment, (7 and 8) with piperidine treatment, (5 and 7) with 10 μM PDHD, and (6 and 8) with 10 μM conjugate.

Although it is electrostatically bound to DNA, it also migrated out of the second lane that contained both PDHD and **9T** DNA. The gray band in this lane is **9T** DNA. Only the lane containing the conjugate exhibited the strong dark blue (about 400–440 nm) fluorescence that is observed at 420 nm with PDHD alone (Figure 3). This proves that PDHD is covalently linked to **9T** DNA in the conjugate. Notably, the conjugate migrates more slowly on the gel because of the higher molecular weight and because of the lower charge to molecular weight ratio (PDHD is an approximate equivalent of one and a half nucleotides with no charge) (Figure 2). The covalent linkage was clearly confirmed by the MALDI-TOF spec-

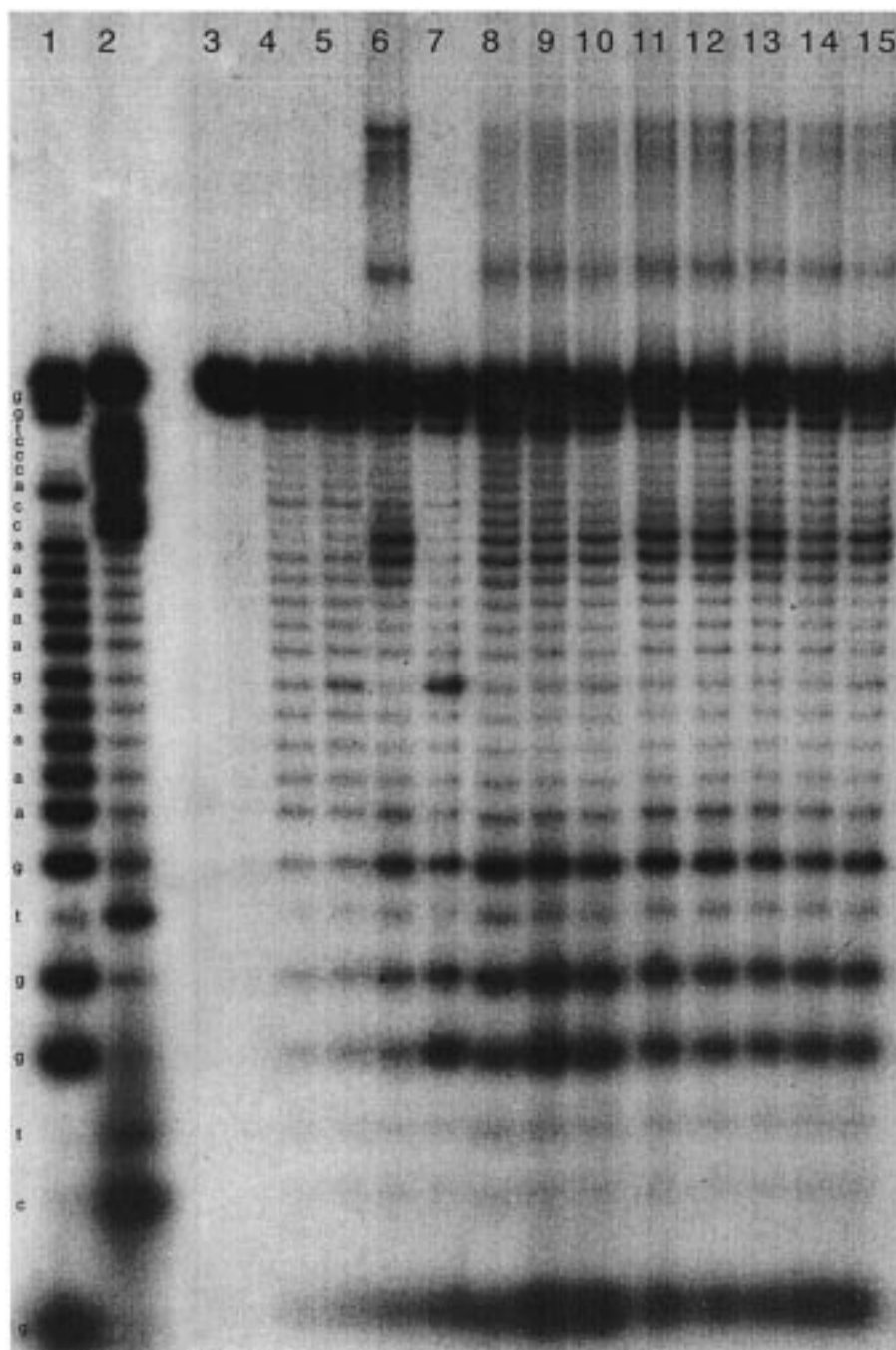


Figure 7. Irradiation of the target **30A** DNA with and without PDHD **6**/conjugate **5** in the presence of carrier DNA, at different concentrations of NaCl and different pH. Lanes: (1) GA Maxam–Gilbert reaction of the target, (2) TC Maxam–Gilbert reaction, (3) target not treated, (4–6) with 2 μg of calf thymus carrier DNA, (4) target irradiated for 12 h and treated with piperidine (as the rest of the lanes, unless otherwise indicated), (5 and 7) with 10 μM PDHD, (6 and 8–13) with 10 μM conjugate, (8) 0 M NaCl, (9) 0.1 M NaCl, (10) 0.25 M NaCl, (11) 0.5 M NaCl, (12) 0.75 M NaCl, (13) 1.0 M NaCl, pH 7.4, (14) pH 6.0, (15) pH 8.8.

trum of the conjugate [(m/z) (M – H) $^-$ observed, 3255.1; calculated, 3256.3].

To assess how PDHD in a conjugate affects DNA–DNA binding, UV-melting experiments were performed. **30A** was premixed with **30T**, **9T**, and a conjugate **5** at 1:1 molar ratio in SCP buffer, heated to 98 $^{\circ}\text{C}$, and cooled slowly to 4 $^{\circ}\text{C}$ to anneal. Multiple UV-melting curves were collected, and melting temperatures of duplexes were determined at the maximum of the calculated dA/dT curve [data not shown, (32)]. The melting temperature for the duplex of **30A** and **30T** was 80.8 $^{\circ}\text{C}$ ($\sigma = 0.1$ $^{\circ}\text{C}$). The duplex of **30A** and **9T** melted at 20.1 $^{\circ}\text{C}$ ($\sigma = 0.5$ $^{\circ}\text{C}$). The duplex of **30A** and a conjugate **5** had a melting temperature of 21 $^{\circ}\text{C}$ ($\sigma = 0.5$ $^{\circ}\text{C}$). This is the

expected result indicating very little, if any, stabilization of the duplex by the conjugated PDHD.

Irradiation of the ss 30-mer target **30A** with and without a PDHD conjugate **5** and dication PDHD **6** was performed in SCP buffer for 1–12 h at 350 nm at about 2 mW/cm^2 at 4 $^{\circ}\text{C}$. The concentration of the radioactively labeled target was constant at 10 nM. The concentration of the conjugate and photosensitizer was varied from 0 to 10 μM . The reactants were premixed, heated to 55 $^{\circ}\text{C}$, and cooled to 4 $^{\circ}\text{C}$ at a rate of 1 $^{\circ}\text{C}/\text{min}$ and incubated at 4 $^{\circ}\text{C}$ for 1 h in the dark before irradiation. After irradiation, the radical reaction was quenched by 10 mM DTT. Then the sample was treated with 10% piperidine at 90 $^{\circ}\text{C}$ to cleave the single stranded target at damaged

bases. Results were analyzed by electrophoresis on denaturing polyacrylamide gels. To determine which bases were damaged, Maxam–Gilbert chemical sequencing was performed. Two controls were also included: the target that was not irradiated and the target that was irradiated and treated with piperidine, to determine the background cleavage that is not due to PDHD and PDHD conjugate.

The following can be concluded from Figure 4. Free PDHD damages specifically only Gs in ss DNA (lane 5 vs lane 3). In contrast, the conjugate specifically cleaves A21 and C22 (lane 10 vs lane 5). This can be explained only by ds formation (Scheme 3b). Notably, the conjugate also specifically damages T7, T10, and A12 (lane 18 vs lane 17), sites that are consistent with triplex formation (Scheme 3b). The conjugate protects from nonspecific cleavage G16, which is in the middle of the binding region (lane 16 vs lane 15). The conjugate nonspecifically damages other Gs (G8, G9, and G11 in lane 16 vs lane 15). The conjugate also forms cross-links with the target forming two new bands of higher MW (**30A** with a conjugate via ds formation and **30A** with two conjugates via triplex formation, lane 9).

Damage to the target was quantitated by scanning densitometry. The total amount of cross-links, undamaged 30-mer, specific cleavage in the region between bases A19 and C27, and damage of G16 and damage in the region between bases G8 and G11 was estimated (Figure 5). As time of irradiation was increased from 1/2 to 12 h, cross-links increased from 4 to 26%, specific damage increased from 2 to 17% (Figures 4 and 5). The irradiation time for all further studies was set at 12 h. The concentration of PDHD and the conjugate were varied in the range 2.5–50 μM . There was little specific damage and cross-links by the conjugate formed at 2.5 μM concentrations. These products increased at 10 μM concentrations. Further increases in concentration only increased nonspecific damage (Figures 4 and 5). Thus, the concentration of damaging agents was set at 10 μM for all further experiments.

When ss target was irradiated with PDHD or PDHD conjugate, but not treated with piperidine, little cleavage at Gs was observed with free PDHD (lane 5 vs lane 7, Figure 6), and there were more cross-links than cleavage with the conjugate (lane 6 vs lane 8, Figure 6). This indicates that PDHD is damaging Gs, but not cleaving them, and that the conjugate primarily forms cross-links that are later partially cleaved by piperidine.

When ss target was irradiated with a large amount of carrier DNA (2000 carrier, 1 target), PDHD cleaved the target severalfold less (lane 5 vs lane 7, Figure 7), indicating complete nonspecificity, while the conjugate cleaved with almost the same efficiency as it cleaved in the absence of carrier DNA (lane 6 vs lane 13, Figure 7). Specific cleavage increased when the concentration of NaCl was increased (0, 0.1, 0.25, 0.5, 0.75, and 1.0 M in lanes 8–13, Figure 7, and lanes 10–15, Figure 8). This is consistent with increased double-strand stability at higher salt concentrations. Slight variation of pH (7.4, 6.0, and 8.8 in lanes 13–15, Figure 7) did not affect cleavage significantly. Triplex cleavage was not detected under these conditions. The maximum site-specific modification achieved was 67% (51% of cross-links and 16% of direct cleavage) in a sample that was not treated with piperidine (lane 3, Figure 8).

Four separate irradiations of the target with the conjugate at standard conditions (10 nM target, 10 μM conjugate, 12 h of irradiation, piperidine treated) gave an average of 40% site-specific modifications: 23% ($\sigma =$

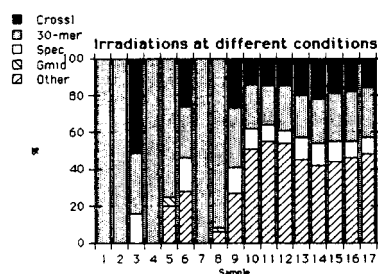


Figure 8. Quantitation of product formation for Figures 6 and 7. Samples: (1–3) DNA alone, with PDHD and with the conjugate, no piperidine treatment, (4–6) DNA alone, with PDHD and with the conjugate, piperidine treated, (7–9) DNA alone, with PDHD and with the conjugate, with 2 μg of calf thymus carrier DNA present, 10–15 NaCl concentrations of 0, 0.1, 0.25, 0.5, and 1 M, respectively, (15) pH 7.4, (16) pH 6, (17) pH 8.8.

3%) cross-links and 17% ($\sigma = 3\%$) direct cleavage (lanes 5 and 9, Figure 5, and lanes 6 and 15, Figure 8).

While the damage at double-strand formation sites was always consistent, the damage at triple-strand sites was observed in a few cases, in conditions where it was strongly favored. Some evaporation of the samples, even though compensated by the addition of water, may have affected product formation.

As compared to other photosensitizers that were conjugated to DNA (2, 3), PDHD seems to have desirable qualities. The wavelength of excitation is 350 nm, above the absorbance of DNA and proteins although still much shorter than the 700 nm that is more useful for phototherapy. PDHD conjugate gives high yields (up to 67%) of specific damage, which places it second after psoralens (6) in efficacy of damage. PDHD conjugate is capable of photo-cross-linking to or damaging all four bases in a target DNA, while most other sensitizers damage only a limited set of bases (4, 13). Besides the site-specific damage, the current generation of PDHD conjugates induce nonspecific damage of Gs around the targeted sequence. This can hopefully be eliminated by attaching the sequence-recognizing unit to the quinone rather than the olefin-derived moiety, thus preventing damage to nearby sequences through migration of released PAQ.

In conclusion, the photochemical cleavage studies prove that the PDHD–oligonucleotide conjugate binds to the complementary target. It efficiently and site-specifically cleaves the designated target, thus, the incorporation of the oligonucleotide sequence recognizing unit onto the PDHD converts a nonspecific photochemical reagent into a sequence specific one.

In future studies, the ss PDHD conjugate will be tested as a selective photochemical M13 virus inactivating agent by measuring inactivation of the *lac i* gene. A variety of improvements of PDHDs are currently under investigation.

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