The Role of a Quinone Methide in the Sequence Specific Alkylation of DNA

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Abstract: Oligonucleotide–naphthoquinone conjugates were prepared and examined for use as inducible, site-directed alkylating agents of DNA. Reaction was found to be sequence specific and under control of either biomimetic reduction or near-UV irradiation. Both conditions induced the formation of a transient and highly electrophilic intermediate consistent with a quinone methide. Enzymatic reduction of 5-((mesyloxy)methyl)- and 5-(bromomethyl)naphthoquinone derivatives produced cross-linking between a target and probe sequence, but the equivalent 5-(acetoxymethyl), 5-(hydroxymethyl) and 5-methyl analogues were predictably inactive. Conversely, irradiation of the 5-methylnaphthoquinone derivative produced cross-linking through a mechanism of photochemical enolization that was not available to the 6-methyl, 3-methyl, or unsubstituted analogues. Hydroxyl radical footprinting of the modified DNA demonstrated that guanine and cytosine were targets of alkylation.

Introduction

The emerging strategies for inhibiting gene expression by antisense and triplex oligonucleotides provide significant new opportunities in research and chemotherapy.[1] Translation or transcription of a chosen gene may be selectively attenuated by these techniques through the formation of a stable complex between a probe sequence and its single or double stranded complement.[2] Routine application in vivo still requires advances in the design of nucleic acid derivatives that demonstrate metabolic stability, cell permeability, and, most importantly, a very high but selective affinity for their intended target.

Noncovalent as well as covalent processes have been used to enhance binding between synthetic oligonucleotides and their biological complement. The thermal stability of duplex and triplex structures has been increased by linking intercalators to the termini of probe strands.[3] Likewise, target hybridization has been rendered irreversible by sequence specific alkylation using probe-electrophile conjugates. Nitrogen mustard,[4] aziridine,[5] and α-halocarbonyl[6] groups have been evaluated for this process, and all provided selective albeit slow modification (>10 h) of target sequences. The use of functional groups with greater intrinsic reactivity and more timely modification has also been attempted but found to lack sufficient stability.[7]

An alternative approach for covalently stabilizing antisense and triplex associations has relied on functional groups with an inducible rather than intrinsic reactivity. In these examples, highly reactive intermediates have been generated in situ for rapid modification of predetermined sequences of DNA. The most common method of activation, near-UV irradiation, has been used to trigger target-selective reaction of azidoproflavin,[8] azidoacetophenone,[9] psoralen,[10] and naphthoquinone.[11] Alkylation[12] and anion dependent[13] triggers have also been reported, but these procedures are not particularly suitable for routine

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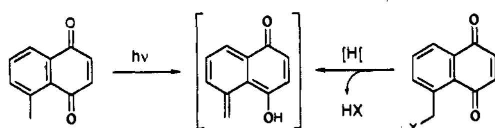
Scheme 1



application in vivo. In contrast, biological reduction[14] represents a well-established method of activation in vivo as illustrated by the therapeutic mechanism of widely prescribed drugs such as mitomycin[15] and the anthracyclines, adriamycin and daunomycin.[16] Our laboratory has recently utilized an equivalent process for controlling the sequence specific alkylation of DNA by an oligodeoxynucleotide–naphthoquinone conjugate.[17] Mechanistic studies of this reaction are now presented and compared to parallel investigations on a complementary photochemical process.

Preliminary analysis of the 5-methyl- and 5-((mesyloxy)-methyl)-1,4-naphthoquinone–oligodeoxynucleotide conjugates established their ability to modify complementary stands of DNA irreversibly.[11,17] Reaction were initiated by irradiation or reduction, respectively, and modification of the targets was complete within 30 min. These latent groups were originally chosen for their potential to form a highly electrophilic quinone methide (Scheme 1),[18,19] and the significance of this proposed intermediate has since been evaluated. An accurate description of the reaction pathway is a prerequisite for advancing the design and construction of the next generation of bioreductive reagents to be used in antisense and triplex procedures. Complete chemical analysis of the naphthoquinone–DNA alkylation product was not possible due to the general alkaline lability of naphthoquinone and its derivatives.[11] However, the chemical and structural specificity for reaction has been examined and found most consistent with quinone methide generation.

Results and Discussion

Synthesis of an Oligodeoxynucleotide–Naphthoquinone Conjugate. Our initial choice of a latent appendage was based on its (i) inability to impede cell permeation, (ii) low affinity for DNA, (iii) ease and flexibility of synthesis, (iv) standard coupling to a variety of site-directing components, and, most importantly, (v) biocompatible activation. Naphthoquinones satisfied all of these criteria, although their alkaline lability precluded direct coupling to a nascent oligodeoxynucleotide through phosphoramidite chemistry.[20] Instead, *N*-hydroxysuccinimide esters of the desired species were condensed onto an oligodeoxynucleotide (**O2**, Table 1) containing a hexamethyleneamino linking group at its 5′-terminus (Scheme 2).[11,17]

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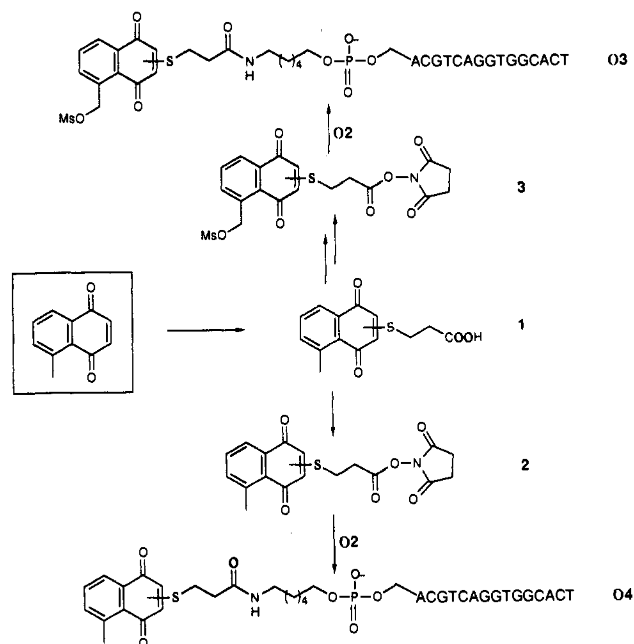
Table 1. Sequences of Oligodeoxynucleotides

no.	oligodeoxynucleotide sequences
O1	5′d(ACGTCAGGTGGCACT)
O2	5′H_2N-$(CH_2)_6$-d(ACGTCAGGTGGCACT)
C1	5′d(AGTGCCACCTGACGTCTAAG)
C2	5′d(AGTGCCACCTGACGTGAG)

Scheme 2



Preparation of the naphthoquinone derivatives began with a Michael addition of 3-mercaptopropionic acid followed by oxidation with ambient oxygen to yield **1**.[21] Formation of the activated ester in each case was accomplished with a stoichiometric amount of *N*-hydroxysuccinimide and a carbodiimide in DMF. Modification at the benzylic position was introduced by photolytic bromination using NBS, AIBN, and dibenzoyl peroxide in refluxing CCl_4. Surprisingly, a reaction did not proceed in the absence of any one of these three common initiators, light, AIBN, and peroxide. The desired bromomethyl product was converted to its mesylate analogue by treatment with silver mesylate and to its hydroxyl derivative by reflux in 40% dioxane.[18] The hydroxymethyl derivative in turn was transformed to its acetoxy derivative by reaction with acetic anhydride and 4-(dimethyl-amino)pyridine. Finally, the desired oligodeoxynucleotides (**O3**–**O10**) were purified by reverse-phase HPLC (Table 2).

Enzyme Dependence of Bioreductive Activation. The concept of bioreductive alkylation was first suggested by studies on a series of simple benzoquinones and naphthoquinone derivatives[14,18] and later used to explain the biological activity of a wide variety of natural products.[14–16,18] Reduction in vivo can be supported by a number of enzymatic systems including NAD(P)H dehydrogenases and reductases[22] which in turn initiate the formation of a transient species that is subject to attack by DNA bases. Numerous biochemical[14–16] and biomimetic studies[23] have characterized the intermediate electrophile as a quinone methide or related semiquinone. The naphthoquinone component of **O3** should yield a similar intermediate and thus function as an inducible and sequence-selective alkylating agent.

(21) Michael addition produced an inseparable mixture of regioisomers coupled at the 2- and 3-position of the quinone [Cameron, D. W.; Feutrill, G. I.; Griffiths, P. G. *Aust. J. Chem.* **1981**, *34*, 1513–1532]. This mixture was used in all subsequent transformations since it would not effect the mechanism or specificity of reaction.

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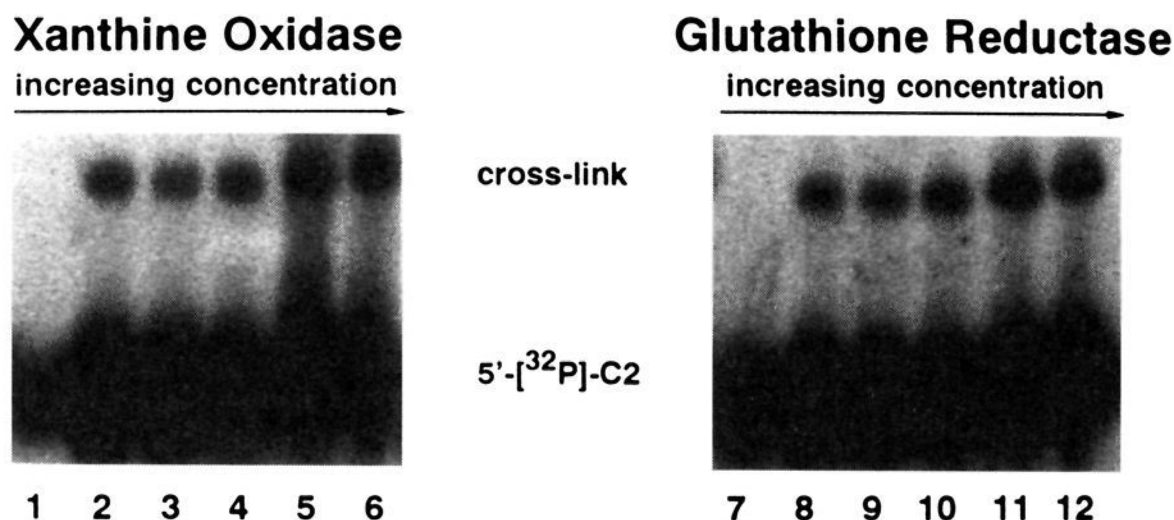
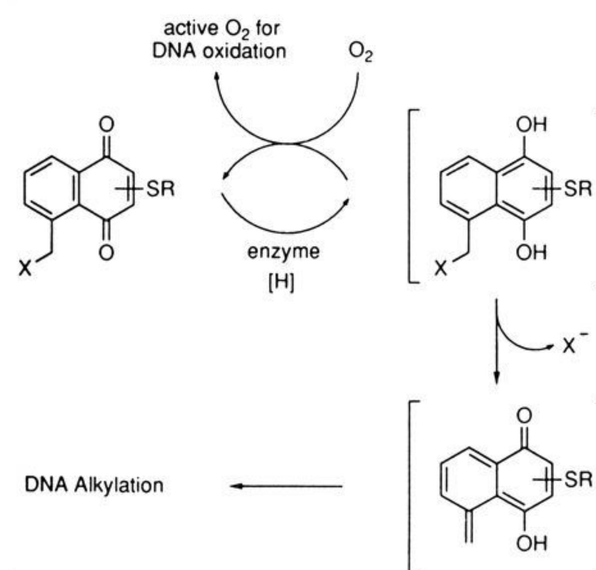


Figure 1. Enzyme dependence of target-probe cross-linking. Equimolar concentrations (2.2 μM) of target and reactant oligodeoxynucleotides, **5'-[^{32}P]-C2** and **O3**, respectively, were incubated at room temperature (buffered at pH 7 with 100 mM potassium phosphate) for 30 min. The annealed duplex was then treated with increasing concentrations of xanthine oxidase (lanes 1–6) in the presence of xanthine (100 μM) and EDTA (150 mM): lane 1, 0 units; lane 2, 1.75×10^{-5} units; lane 2, 1.75×10^{-4} units; lane 3, 1.75×10^{-3} units; lane 4, 1.75×10^{-2} units; lane 6, 1.75×10^{-1} units. Alternatively, the annealed duplex was treated with glutathione reductase in the presence of NADPH (100 μM) and EDTA (150 mM) (lanes 7–12): lane 7, 0 units; lane 8, 2.0×10^{-5} units, lane 9, 2.0×10^{-4} units; lane 10, 2.0×10^{-3} units; lane 11, 2.0×10^{-2} units; lane 12, 2.0×10^{-1} units. In all cases, reaction was allowed to proceed for 30 min and then diluted 10-fold, dialyzed, lyophilized, and resuspended in 80% formamide for electrophoresis.

Table 2. Structures of Naphthoquinone-Linked Oligodeoxynucleotides

no.	R_1	R_2	R_3
O3	H	H	CH_2OMs
O4	H	H	CH_3
O5	H	H	CH_2Br
O6	H	H	CH_2OH
O7	H	H	CH_2OAc
O8	H	CH_3	H
O9	CH_3	H	H
O10	H	H	H

Scheme 3



As expected, simple hybridization between the oligodeoxynucleotide derivative **O3** and its complement **C2** (Table 1) did not yield the high molecular species associated with target strand alkylation.[17] This species was only evident after the reaction mixture was treated with a reductant. Formal one- and two-electron reduction of **O3** in the presence of cytochrome *c* reductase/NADH and $NaBH_4$, respectively, provided a product that was identified as the alkylated, cross-linked duplex by comparison to standards on denaturing polyacrylamide gel electrophoresis.[17] The enzyme appeared to catalyze a direct reduction of the naphthoquinone moiety since target alkylation was unaffected by the presence of an electron carrier, methyl viologen (10 mM).

A series of distinct enzymatic systems were next examined in order to identify the generality of inducing DNA modification. The first enzyme examined, cytochrome *c* reductase, is most often associated with one-electron reduction of its substrates, and alkylation induced under these conditions may be analogous to the one-electron activation observed for mitomycin.[24] Other enzymes such as glutathione reductase are thought to catalyze quinone reduction primarily by a single two-electron step.[25] This enzyme was equally efficient at promoting the modification of the target sequence in the presence of NADPH (Figure 1). Suitable reduction of the naphthoquinone moiety was also not limited to nicotinamide dependent enzymes. The combination of xanthine oxidase and xanthine[24,26] was also effective at inducing the covalent reaction of the naphthoquinone conjugate DNA (Figure 1). Enzymes lacking a tightly bound redox cofactor were incapable of inducing this process; lactic dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase both catalyze direct hydride transfer from NADH to bound substrates and neither were able to initiate alkylation by the naphthoquinone derivative.

Formation of a Transient Electrophile for DNA Alkylation. Preliminary data first identified the transient nature of the intermediate responsible for sequence specific modification of DNA.[17] Reduction of **O3** prior to addition of its complement **C2** did not yield detectable cross-linking. Furthermore, a noncomplementary oligodeoxynucleotide, 5'-[^{32}P]-d(CATGCGCTACCCGTG), was not alkylated by **O3** in the presence of a reductant. The reactive species formed by **O3** therefore did not persist long enough to interact with freely diffusing strands of DNA; instead, it was likely trapped by solvent and intramolecular reaction. Successful modification then required the simultaneous presence of a reducing system, target strand, and complementary probe with its attached latent appendage. The overall yield of alkylation is expected to reflect a competition between nucleobase and solvent (H_2O) addition to the intermediate quinone methide.

Oxygen could have potentially trapped and reoxidized the transient dihydroquinone but no evidence for this was observed (Scheme 3). The yield of target alkylation was not affected by the presence or absence of oxygen.[17] Previously, oligonucleotide

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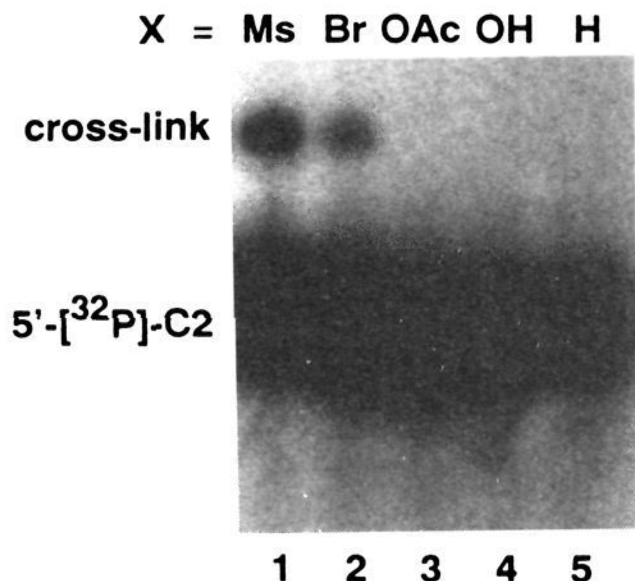
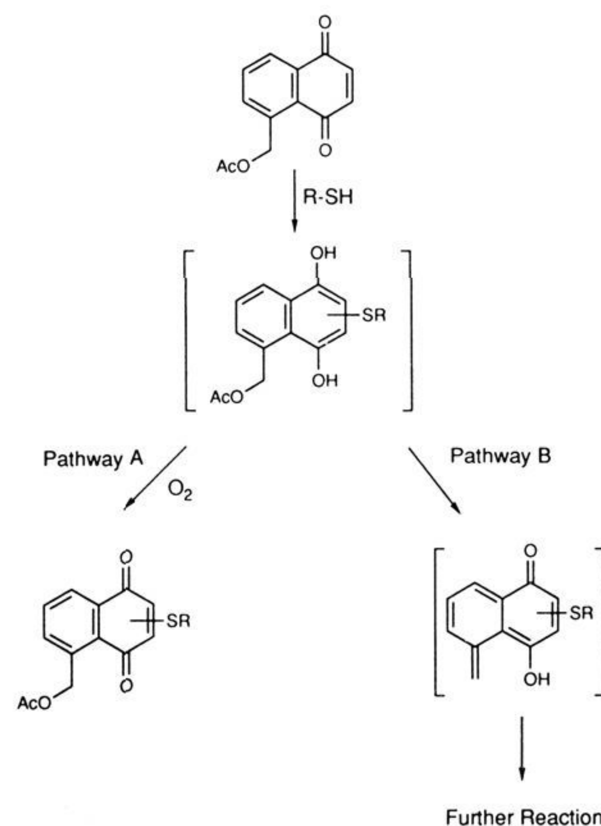


Figure 2. Effect of the leaving group on target-probe cross-linking. The target oligodeoxynucleotide **5'-[^{32}P]-C2** was alternatively incubated with equimolar concentrations (2.2 μM) of **O3** (lane 1), **O5** (lane 2), **O7** (lane 3), **O6** (lane 4), and **O4** (lane 5) in 100 mM potassium phosphate pH 7 for 30 min under ambient conditions. Reaction was then initiated by addition of cytochrome *c* reductase (1 mg/mL) and NADH (100 μM), incubated for an additional 30 min and analyzed by gel electrophoresis and autoradiography.

Scheme 4



conjugates of naphthoquinone[27] and anthraquinone[28] had been prepared for site-specific oxidation of nucleic acids in the presence of oxygen and reductants. The derivatives described here did not exhibit this alternative activity. No spontaneous and alkaline-induced fragmentation of DNA was detected suggesting that activated oxygen species did not significantly accumulate under the described conditions. Characteristic fragmentation was also not apparent when conversion of dihydroquinone to quinone methide was suppressed or blocked (see below and Figure 2).

Substituent Effects on Target Alkylation. Under reductive conditions, quinone methide formation necessitates elimination of a benzylic leaving group (Scheme 3). Consequently, generation of this electrophilic intermediate and subsequent alkylation of DNA should be highly dependent on the nature of the leaving group. To examine this property, the MsO group of **O3** was sequentially replaced with Br, AcO, HO, and H (**O4–O7**, Table 2). Enzymatic reduction of the resulting probe–target duplexes demonstrated that interstrand alkylation decreased in the order MsO > Br > AcO, HO, H (Figure 2). Both the MsO and Br substituents form stable anionic leaving groups, and both **O3** and **O5** effected sequence-specific modification. In contrast, HO and H substituents were not expected to undergo elimination, and no reaction was observed for **O4** and **O6**.

The acetoxy derivative **O7** was also unable to support target alkylation. The inactivity of this species might have been attributed to the inability of the AcO group to be expelled from the reduced quinone or alternatively to its spontaneous hydrolysis to form the unreactive hydroxyl derivative. This hydrolysis was considered unlikely, however, since the AcO group remained inert during the addition of 3-mercaptopropionic acid to 5-(acetoxymethyl)-1,4-naphthoquinone (Scheme 4). In this example, the 5-acetoxymethyl moiety was maintained in the product (pathway A) and not subject to hydrolysis, elimination (pathway B), or substitution.

Alternative Formation of the Quinone Methide. An independent method for generating the proposed intermediate was used to support its role in site-specific alkylation of DNA. This was necessary since the leaving group dependence above would not easily differentiate between two mechanisms, direct benzylic substitution, and elimination/addition (via quinone methide formation), stimulated by reduction. 5-Methyl-1,4-naphthoquinone had previously been shown[19] to tautomerize under irradiation to a quinone methide intermediate equivalent to that implicated in the reaction of **O3** (Scheme 1). The chemical competence of this intermediate to alkylate DNA was subsequently demonstrated by the ability of the 5-methylnaphthoquinone conjugate **O4** to cross-link with its complement **C1** after photolysis (10 min, $\lambda > 345$ nm).[11] Irradiation of **O4 + C2** also formed the expected cross-linked product, and this was directly shown to correspond to the species formed by chemical or enzymatic reduction of **O3 + C2**.[17] Moreover, the photochemical process was similarly sequence specific, transient in nature, and independent of O_2.[11]

Careful interpretation of the photochemical results required further investigation since the excited-state quinone could still have alkylated target DNA through a diverse assay of pathways. The naphthoquinone appendage could have participated in [2 + 2] cycloaddition[29] and electron transfer/radical recombination[30] as well as photoenolization.[19] For simplicity, these pathways are illustrated with cytosine and its tautomer (Scheme 5), but other nucleobases would also likely react. Only the photoenolization mechanism is strictly dependent on a methyl substituent β to the carbonyl, and hence its contribution to DNA cross-linking can be distinguished from the action of its structural isomers and derivatives lacking a methyl substituent.

Oligodeoxynucleotide conjugates **O8–O10** (Table 2) were therefore synthesized and irradiated in the presence of the complementary sequence **C1**. A basal level of alkylation was common to all of these derivatives (Table 3) suggesting a universally low level of reaction for all quinones or their decomposition products.[11] Only the 5-methyl analogue **O4** exhibited reasonable efficiency for cross-linking. More than 70% of the product in this example required a methyl group to be in position for photoenolization and quinone methide formation.

In addition, selective alkylation of **C1** was the only reaction detected after irradiation. Thymine is particularly susceptible to oxidation by naphthoquinone photosensitization[31] but this was not evident under the conditions of these experiments. At least one of the characteristic products, thymine glycol, would have

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Scheme 5

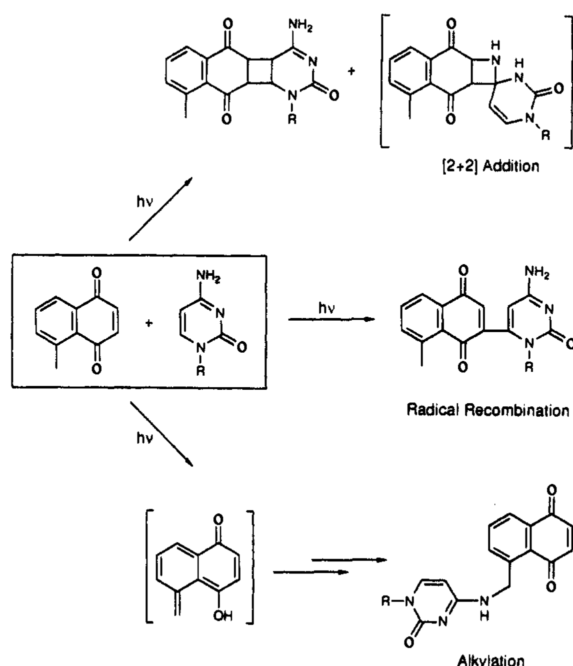

Table 3. Alkylation of Target Oligodeoxynucleotide **C1**

S—oligodeoxynucleotide

no.	R_1	R_2	R_3	%C1 Alkylated
O4	H	H	CH_3	27
O8	H	CH_3	H	6
O9	CH_3	H	H	8
O10	H	H	H	<6

led to strand fragmentation after alkaline treatment.[32] No spontaneous or alkaline-induced fragmentation was observed for **C1** in the presence of the naphthoquinones above. Thus, irradiation of **O4**, like reduction of **O3**, serves to modify DNA with a single dominant specificity for sequence, structure and reaction.

Site of Alkylation on Target Strands. The oligodeoxynucleotide model system used in this investigation was chosen to examine potential reaction of both double- and single-stranded regions of DNA. To identify the specific sites of base alkylation, the cross-linked product was purified and subjected to variety of diagnostic procedures.[11] The only species formed after exposing the cross-link to acid, heat or base was a derivative that migrated with the initial target strand **C1** on gel electrophoresis.[11] This result indicated that hydrolysis had occurred at the quinone or linker moiety rather than at the modified base or its attached phosphodeoxyribose moiety.

We subsequently employed a highly effective method of footprinting that is based on hydroxyl radical cleavage of DNA[33] and has been adapted by Hopkins for characterizing cross-links in duplex DNA.[34] Interstrand modification is determined in this manner by the fragmentation site at which scission products from the control and alkylated DNA coalesce. For reaction of **C1** and

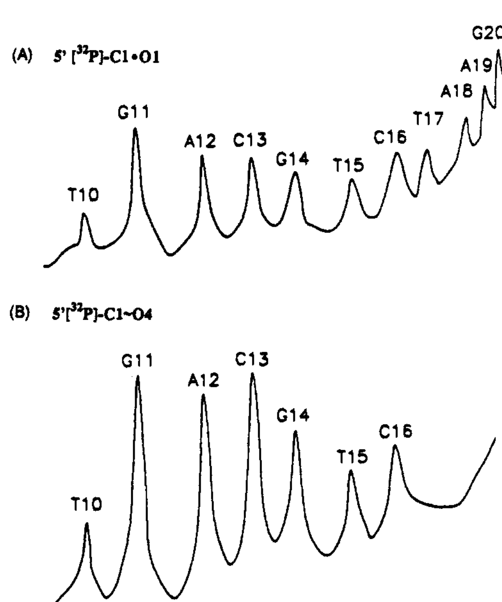

Figure 3. Densitometric scans representing hydroxyl radical-generated footprints of hybridized and cross-linked DNA. (A) Fragmentation of a control sample was compared to (B) the fragmentation of the alkylated product formed by irradiation and purified as described in the text.

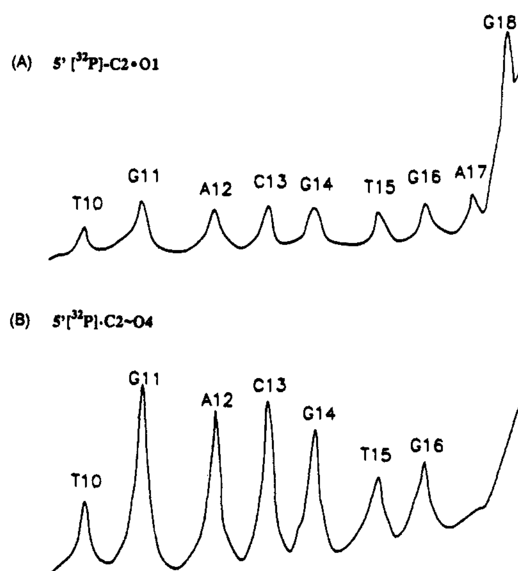

Figure 4. Densitometric scans representing hydroxyl radical–generated footprints of hybridized and cross–linked DNA. (A) Fragmentation of a control sample was compared to (B) the fragmentation of the alkylated product formed by irradiation and purified as described in the text.

O4, the first base extended from the duplex, C16, was modified by the naphthoquinone appendage (Figure 3). Similarly, G16 was modified in reaction of **C2** and **O4** (Figure 4). Modification of guanine and cytosine is consistent with reaction of a quinone methide. Such intermediates modify only the *exo*-amino groups of DNA[23b,24,35] and should therefore form products inert to strand

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scission effected by alkaline conditions. In contrast, alkylation by direct displacement occurs at the sites of greatest nucleophilicity.[36] The most notable result of these conditions is modification of guanine N7 leading to alkaline lability of the target strand.[37]

A positional selectivity for reaction at the first unpaired base as illustrated by the naphthoquinone moiety has also been observed for oligonucleotide-directed psoralen derivatives.[38] The origins of this characteristic have not yet been determined since neither the psoralen[10b] nor the naphthoquinone appendages appear to interact strongly with their respective duplexes prior to covalent reaction. For example, the thermal melting temperatures of **O1** + **C1** and **O9** + **C1** were experimentally indistinguishable (T_m = 44 ± 1 °C). Oligodeoxynucleotide conjugates containing an appendage such as acridine in contrast form duplex and triplex structures with enhanced thermal stability that in part reflects stacking and intercalation of the acridine.[3]

Conclusion

Synthetic oligodeoxynucleotides and their derivatives provide a general method for delivering reactive reagents to a chosen nucleotide sequence. Species exhibiting a latent rather than intrinsic activity may be particularly useful in chemotherapy since nonspecific modification would be minimized. The naphthoquinone derivatives examined in this investigation illustrate such an activity by requiring bioreductive activation prior to DNA alkylation. This process is common to a variety of antitumor drugs that generate intermediate quinone methides, and an equivalent species is likely formed by suitably prepared naphthoquinones. This mechanism is supported by the characteristic leaving group dependence of the alkylation induced by enzymatic reduction. Complementary evidence is provided by the unique photochemical reactivity of the 5-methylnaphthoquinone derivative. The reported mechanistic and structural requirements of target modification currently serve as the basis for constructing more potent compounds for site-directed derivatization of nucleic acids.

Experimental Section

Materials. 2-Methyl-1,4-naphthoquinone (menadione), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (CDI), 1,3-dicyclohexylcarbodiimide (DCC), di-*tert*-butyl dicarbonate (BOC-anhydride), 2-(((*tert*-butoxycarbonyl)oxy)imino)-2-phenylacetonitrile (BOC-ON), and *tert*-butyldimethylsilyl chloride were purchased from Aldrich. 2,2′-Azobis(2-methylpropionitrile) (AIBN) and silver methanesulfonate were purchased from Kodak. 4-(Dimethylamino)pyridine (DMAP) was a gift from NEPERA, Inc. Adenosine 5′-triphosphate (ATP), 2-(*N*-morpholino)ethanesulfonic acid (MES), 3-(*N*-morpholino)propanesulfonic acid (MOPS), β-nicotinamide adenine dinucleotide (NADPH), xanthine, cytochrome *c* reductase, xanthine oxidase, glutathione reductase, lactic dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase were purchased from Sigma. T-4 Kinase was purchased from Bethesda Research Laboratories. γ-[^{32}P]-ATP was purchased from Amersham (3000–5000 Ci/mmol). All other chemicals and reagents were obtained in the highest commercial grade available and used without further purification unless otherwise stated.

β-((5-Methyl-1,4-naphthoquinonyl)thio)propionic Acid (1).[21] 3-Mercaptopropionic acid (0.69 mmol, 65 μL) was dissolved in 8 mL of water, cooled in an ice bath, and added over 20–30 min to an ethanolic solution (15 mL) of 5-methyl-1,4-naphthoquinone[39] (0.69 mmol, 120 mg) cooled in an ice bath.[40] The reaction mixture was allowed to gradually warm to room temperature over 1 h before the ethanol was evaporated. The crude product was purified via silica gel flash chromatography (2:1 hexanes/ethyl acetate with 1% v/v acetic acid) to yield 134 mg of product (70% yield): ^{1}H NMR (acetone-d_6), δ 2.70 (s, 3H), 2.81 (t, *J* = 7.2 Hz, 2H), 3.20 (t, *J* = 7.2 Hz, 2H), 6.72 (s, 1H), 7.62 (d, *J* = 7.5 Hz, 1H), 7.71 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.96 (d, *J* = 7.5 Hz, 1H); LRMS *m/z* (rel intensity) 276 (M$^+$, 31) 203 (100), 115 (68), 90 (41), 89 (76).

***N*-Hydroxysuccinimide Ester of β-((5-Methyl-1,4-naphthoquinonyl)thio)propionic Acid (2).** *N*-Hydroxysuccinimide (72 μmol, 8.3 mg) and β-((5-methyl-1,4-naphthoquinonyl)thio)propionic acid (**1**, 72 μmol, 20 mg) were dissolved in 80 μL of DMF and cooled in an ice bath. CDI (72 μmol, 13.8 mg) was added, mixed once, and then placed at 4 °C overnight. During this time the product precipitated out. Water (2 mL) was added to complete the precipitation. The crude product was isolated by centrifugation and washed with 100 mM potassium phosphate (pH 7.5) to remove unreacted starting material. The precipitate was then further washed with water, dried under high vacuum, and purified via silica gel flash chromatography (2:1 hexanes/ethyl acetate) to yield 20 mg of the desired product (81%): ^{1}H NMR (CD_3CN) δ 2.74 (s, 4H), 2.86 (s, 3H), 3.07 (t, *J* = 7.2 Hz, 2H), 3.19 (t, *J* = 7.2 Hz, 2H), 6.61 (s, 1H), 7.51 (d, *J* = 7.5 Hz, 1H), 7.71 (dd, *J* = 7.5, 7.5 Hz, 1H), 8.03 (d, *J* = 7.5 Hz, 1H); LRMS *m/z* (rel intensity) 373 (M$^+$, 28), 203 (29), 115 (36), 89 (29), 55 (100).

β-((5-((Mesyloxy)methyl)-1,4-naphthoquinonyl)thio)propionic Acid. Acetic anhydride (ca. 2 mL) was added dropwise to dissolve **1** (50 mg, 0.181 mmol) in refluxing CCl_4 (6 mL). NBS (35 mg, 0.199 mmol) was then added, and the reaction mixture was photolyzed for 10 min. Benzoyl peroxide (BPO) (7 mg) and AIBN (7 mg) were next added, and the mixture was once again photolyzed for 10 min. This process of adding BPO and AIBN (7 mg each) and photolyzing for 10 min was repeated twice until the ^{1}H NMR of the reaction mixture indicated the formation of the 5-bromomethyl moiety (δ = 5.01 ppm). The solvent was evaporated, and the crude mixture was redissolved in CH_3CN (3 mL). Silver mesylate (150 mg, 0.718 mmol) was then added, and the reaction mixture was stirred at room temperature for 5 h. Solvent was once again removed, and the residue was purified via silica gel flash chromatography (2:1 hexanes/ethyl acetate with 1% HOAc) to yield 25 mg of product (37%): ^{1}H NMR (Acetone d_6) δ 2.71 (t, *J* = 7.2 Hz, 2H), 3.09 (t, *J* = 7.2 Hz, 2H), 3.14 (s, 3H), 5.75 (s, 2H), 6.65 (s, 1H), 7.79 (dd, *J* = 7.8, 7.2 Hz, 1H), 7.94 (d, *J* = 7.8 Hz, 1H), 8.14 (d, *J* = 7.2 Hz, 1H).

***N*-Hydroxysuccinimide Ester of β-((5-(Mesyloxy)methyl)-1,4-naphthoquinolinyl)thio)propionic Acid (3).** *N*-Hydroxysuccinimide (72 μmol, 8.3 mg) and the mesyloxy derivative above (72 μmol, 25 mg) were dissolved in 80 μL of DMF and cooled in an ice bath. CDI (72 μmol, 14 mg) was added, mixed once, and then incubated at 4 °C for 3 h. The product was purified as described for the synthesis of **2** to yield 11 mg (34%): ^{1}H NMR ($CDCl_3$) δ 2.86 (s, 4H), 3.08 (t, *J* = 7.2 Hz, 2H), 3.17 (s, 3H), 3.21 (t, *J* = 7.2 Hz, 2H), 5.77 (s, 2H), 6.67 (s, 1H), 7.83 (dd, *J* = 7.8, 7.8 Hz, 1H), 7.98 (d, *J* = 7.8 Hz, 1H), 8.18 (d, *J* = 7.8 Hz, 1H); LRMS M$^+$ 467.

***N*-Hydroxysuccinimide Ester of β-((5-(Bromomethyl)-1,4-naphthoquinonyl)thio)propionic Acid (4).** A procedure equivalent to that described for the synthesis of the mesyloxy derivative was used to brominate **2** (50 mg) to form the 5-bromomethyl derivative (48% crude yield estimated by ^{1}H NMR) and an equal amount of a ring brominated derivative. Separation of these two compounds via silica gel flash chromatography was quite difficult, but a small amount (3 mg) of the desired material was purified: ^{1}H NMR ($CDCl_3$) δ 2.86 (s, 4H), 3.07 (t, *J* = 7.2 Hz, 2H), 3.18 (t, *J* = 7.2 Hz, 2H), 5.05 (s, 2H), 6.65 (s, 1H), 7.50 (d, *J* = 7.5 Hz, 1H), 7.60 (dd, *J* = 7.5, 7.5 Hz, 1H), 8.01 (d, *J* = 7.5 Hz, 1H).

5-(Hydroxymethyl)-1,4-naphthoquinone.[18b] NBS (537 mg, 3.03 mmol) and BPO (68 mg) were added to a solution of 5-methyl-1,4-naphthoquinone[39] (513 mg, 3 mmol) in CCl_4 (30 mL). The reaction mixture was heated to reflux and photolyzed for 10 min with a sun lamp. After an additional hour of reflux, AIBN (20 mg) was added to the mixture, and it was once again photolyzed for 10 min. This process of alternatively adding dibenzoylperoxide and AIBN and irradiating (10 min) was repeated twice more until all of the NBS had been consumed. The reaction mixture was then filtered, evaporated, and redissolved in 10 mL of dioxane. Water (15 mL) was added, and the reaction mixture was refluxed for 30 min. The product was finally extracted in $CHCl_3$ and purified by silica gel flash chromatography (3:1 hexanes/ethyl acetate) to yield 272 mg (48%): ^{1}H NMR ($CDCl_3$) δ 4.95 (s, 2H), 6.95 (s, 2H), 7.73 (dd, *J* = 7.5, 6.9 Hz, 1H), 7.85 (d, *J* = 7.5 Hz, 1H), 8.09 (d, *J* = 6.9 Hz, 1H).

β-((5-(Hydroxymethyl)-1,4-naphthoquinonyl)thio)propionic Acid (5).[21] 3-Mercaptopropionic acid (0.83 mmol, 7 μL) was dissolved in 3 mL of water, cooled in an ice bath, and added over 20–30 min to a solution of 5-(hydroxymethyl)-1,4-naphthoquinone (0.104 mmol, 20 mg) in ethanol (10 mL) also cooled in an ice bath. The reaction mixture was allowed to warm gradually to room temperature over 30 min before the ethanol

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was evaporated. The residue was purified via silica gel flash chromatography (1:1 hexanes/ethyl acetate with 1% v/v acetic acid) and yielded 22 mg of product (48%): ^{1}H NMR (acetone-d_6) δ 3.20 (t, J = 7.8 Hz, 2H), 3.36 (t, J = 7.8 Hz, 2H), 5.08 (s, 2H), 6.79 (s, 1H), 7.86 (dd, J = 7.8, 8.1 Hz 1H), 8.00 (d, J = 7.8 Hz, 1H), 8.22 (d, J = 8.1 Hz, 1H).

N-Hydroxysuccinimide Ester of β-((5-(Hydroxymethyl)-1,4-naphthoquinonyl)thio)propionic Acid (6). A procedure equivalent to that described for the synthesis of **2** was used to convert **5** (28 μmol, 8 mg) to the desired product (5 mg, 46%): ^{1}H NMR (CD_3CN) δ 2.80 (s, 4H), 3.20 (t, J = 7.8 Hz, 2H), 3.36 (t, J = 7.8 Hz, 2H), 5.08 (s, 2H), 6.79 (s, 1H), 7.86 (dd, J = 7.8, 8.1 Hz 1H), 8.00 (d, J = 7.8 Hz, 1H), 8.22 (d, J = 8.1 Hz, 1H).

5-(Acetoxymethyl)-1,4-naphthoquinone. Acetic anhydride (18 μL, 0.193 mmol) and DMAP (3 mg, 0.02 mmol) were added to a solution of 5-(hydroxymethyl)-1,4-naphthoquinone (33 mg, 0.175 mmol) in CH_3CN (5 mL). The reaction mixture was stirred for 15 min before the solvent was evaporated, and the crude product was extracted with H_2O/$CHCl_3$. The organic layer was washed with potassium phosphate (100 mM, pH 7.5) to remove excess acid and then evaporated. The residue was purified through a pad of silica to yield 23 mg (47%) of the product: ^{1}H NMR ($CDCl_3$) δ 2.19 (s, 3H), 5.65 (s, 2H), 6.92 (s, 2H), 7.76 (dd, J = 7.8, 7.5 Hz, 1H), 7.82 (d, J = 7.8 Hz, 1H), 8.09 (d, J = 7.5 Hz, 1H).

β-((5-(Acetoxymethyl)-1,4-naphthoquinonyl)thio)propionic Acid (7).[21] 3-Mercaptopropionic acid (0.1 mmol, 9 μL) was dissolved in 3 mL of water, cooled in an ice bath, and added over 20–30 min to a solution of 5-(acetoxymethyl)-1,4-naphthoquinone (0.1 mmol, 23 mg) in ethanol (10 mL) also cooled in an ice bath. The reaction mixture was allowed to warm to room temperature over 45 min during which time a yellow precipitate separated out. The solid product was isolated (13 mg, 39% yield), and the reaction solvent evaporated to yield additional product (15 mg, crude). The residue was purified via silica gel flash column chromatography (1:1 hexane/ethyl acetate with 1% v/v acetic acetic) to yield an additional amount of the desired product (8 mg, 24%, total yield = 63%): ^{1}H NMR (acetone-d_6) δ 2.16 (s, 3H), 2.75 (t, J = 6.9 Hz, 2H), 3.24 (t, J = 6.9 Hz, 2H), 5.59 (s, 2H), 6.77 (s, 1H), 7.82–7.87 (m, 2H), 8.07 (d, J = 7.5 Hz, 1H).

N-Hydroxysuccinimide Ester of β-((5-(Acetoxymethyl)-1,4-naphthoquinonyl)thio)propionic Acid (8). A procedure equivalent to that described for the synthesis of **2** was used to convert **7** (36 μmol, 12 mg) to the desired product (8 mg, 52%). ^{1}H NMR (CD_3CN) δ 2.20 (s, 3H), 2.86 (s, 4H), 3.07 (t, J = 7.2 Hz, 2H), 3.20 (t, J = 7.2 Hz, 2H), 5.64 (s, 2H), 6.64 (s, 1H), 7.51–7.79 (m, 2H), 8.11 (d, J = 7.5 Hz, 1H).

β-((3-Methyl-1,4-naphthoquinonyl)thio)propionic acid (9). 3-Mercaptopropionic acid (0.275 mL, 3.18 mmol) was dissolved in 6 mL of water, cooled in an ice bath, and added over 20–30 min to a solution of 2-methyl-1,4-naphthoquinone (2.9 mmol, 0.5 gm) in ethanol (15 mL) at 4 °C. The reaction mixture was allowed to warm gradually to room temperature over 6 h. After an additional 14 h, the ethanol was evaporated, and the solid residue was dissolved in CH_2Cl_2 (7 mL). The product was then extracted into 1 M $NaHCO_3$ (10–12 mL), precipitated with addition of 6 M HCl, and isolated by filtration. The solid was washed free of excess HCl with cold water and finally dried under vacuum to yield 658 mg (82%): mp 160–161 °C (lit. mp 160 °C);[40] ^{1}H NMR (acetone-d_6) δ 2.2 (s, 3H), 2.6 (t, 2H), 3.3 (t, 2H), 7.7 (m, 2H), 7.9 (m, 2H); LRMS m/e 276 (M^+).

N-Hydroxysuccinimide Ester of β-((3-Methyl-1,4-naphthoquinonyl)thio)propionic Acid (10). DCC (37 mg, 180 μmol) was added to a solution of N-hydroxysuccinimide (21 mg, 180 μmol) and **9** (50 mg, 180 μmol) in 1 mL of DMF at 4 °C. This reaction mixture was then allowed to warm to room temperature and remain at that temperature for 40 h. The dicyclohexyl urea was precipitated by addition of CH_2Cl_2 (2 mL). The liquid phase was washed with 1 M $NaHCO_3$ to remove unreacted starting material, and the solvent was evaporated. The remaining residue was purified by silica gel flash column chromatography (hexanes/ethyl acetate, 2:1) to yield 20 mg (30%) of the desired product: ^{1}H NMR (acetonitrile-d_3), δ 2.26 (s, 3H), 2.68 (s, 4H), 2.97 (t, 2H), 3.37 (t, 2H), 7.7 (m, 2H), 8.0 (m, 2H); LRMS m/e 373 (M^+).

β-((6-Methyl-1,4-naphthoquinonyl)thio)propionic Acid (11).[21] A procedure equivalent to that described for the synthesis of **9** was used to convert 6-methyl-1,4-naphthoquinone (120 mg, 0.69 mmol) to 139 mg of the desired product in 75% yield: ^{1}H NMR (CD_3CN) δ 2.49 (s, 3H), 2.74 (t, J = 6.9 Hz, 2H), 3.12 (t, J = 6.9 Hz, 2H), 6.65 (s, 1H), 7.56–7.95 (m, 3H); LRMS m/z (rel. int.) 276 (M^+ 6), 149 (57), 69.1 (100).

N-Hydroxysuccinimide Ester of β-((6-Methyl-1,4-naphthoquinolinyl)thio)propionic Acid (12). A procedure equivalent to that described for the synthesis of **10** was used to convert **11** (72 μmol, 20 mg) to 21 mg of the desired product in 80% yield: ^{1}H NMR (CD_3CN) δ 2.49 (s, 3H), 2.86 (s, 4H), 3.07 (t, J = 6.6 Hz, 2H), 3.20 (t, J = 6.6 Hz, 2H), 6.60 (s, 1H), 7.49–8.01 (m, 3H).

β-((1,4-Naphthoquinonyl)thio)propionic acid (13). A procedure equivalent to that described for the synthesis of **9** was used to convert 1,4-naphthoquinone (110 mg, 0.69 mmol) to 127 mg of the desired product in 70% yield: ^{1}H NMR (acetone-d_6) δ 2.81 (t, J = 6.9 Hz, 2H), 3.23 (t, J = 6.9 Hz, 2H), 6.76 (s, 1H), 7.82–8.07 (m, 4H).

N-Hydroxysuccinimide Ester of β-((1,4-Naphthoquinonyl)thio)propionic Acid (14). A procedure equivalent to that described for the synthesis of **10** was used to convert **13** (74 μmol, 20 mg) to 22 mg of the desired product in 84% yield: ^{1}H NMR (acetone-d_6) δ 2.88 (s, 4H), 3.22 (t, J = 7.2 Hz, 2H), 3.39 (t, J = 7.2 Hz, 2H), 6.84 (s, 1H), 7.82–8.15 (m, 4H).

Oligodeoxynucleotide Preparation. All oligodeoxynucleotides were synthesized via standard solid-phase cyanoethyl phosphoramidite chemistry. The hexamethyleneamino linker was attached in the last step of the synthesis of **O2** using a monomethoxytrityl protected precursor (Clontech) that was designed for the routine protocols of automated coupling. The protecting group was removed by treating the crude product with 80% acetic acid for 30 min under ambient conditions. When necessary, oligodeoxynucleotide samples were purified by C-18 reverse-phase chromatography. The crude aminolinker material was used directly in the following couplings.

Conjugation of the 5-((Mesyloxy)methyl)naphthoquinone Derivative to the Aminolinker-DNA (O3). The N-hydroxysuccinimide ester **3** (1.0 mg) was dissolved in 25 μL of DMF and added to a crude preparation of **O2** (A_{260} = 6 absorbance units of aminolinker material, 44 pmol) dissolved in 25 μL MOPS (pH 7.5, pH 7.5) and 25 μL DMF and kept undisturbed at room temperature for about 3 h. Analysis and purification utilized reverse-phase HPLC (C-18 Spherex column, 250 × 4.6 mm, Phenomenex) eluted with a linear gradient of 5% CH_3CN in 47.5 mM triethylammonium acetate (pH 6) to 25% CH_3CN in 37.5 mM triethylammonium acetate over 30 min (1 mL/min). The product **O3** was isolated in 25% yield estimated by the recovery of A_{260} units. Linkage was established by an A_{260}/A_{330} ratio of 12.

Conjugation of the 5-(Bromomethyl)naphthoquinone Derivative to the Aminolinker-DNA (O5). A procedure equivalent to that described for synthesis of **O3** was used to produce **O5** in 31% yield (A_{260}/A_{330} = 15).

Conjugation of the 5-(Acetoxymethyl)naphthoquinone Derivative to the Aminolinker-DNA (O7). A procedure equivalent to that described for synthesis of **O3** was used to produce **O7** in 30% yield (A_{260}/A_{330} = 18).

Conjugation of the 5-(Hydroxymethyl)naphthoquinone Derivative to the Aminolinker-DNA (O6). A procedure equivalent to that described for synthesis of **O3** was used to produce **O6** in 30% yield (A_{260}/A_{330} = 19).

Conjugation of the 5-Methylnaphthoquinone Derivative to the Aminolinker-DNA (O4). A procedure equivalent to that described for synthesis of **O3** was used to produce **O4** in 34% yield (A_{260}/A_{330} = 23).

Conjugation of the 6-Methylnaphthoquinone Derivative to the Aminolinker-DNA (O8). A procedure equivalent to that described for the coupling of **O3** was used to produce **O8** in 30% yield (A_{260}/A_{330} = 19).

Conjugation of the 2-Methylnaphthoquinone Derivative to the Aminolinker-DNA (O9). A procedure equivalent to that described for synthesis of **O3** was used to produce **O9** in 36% yield (A_{260}/A_{330} = 23).

Conjugation of the Naphthoquinone Derivative to the Aminolinker-DNA (O10). A procedure equivalent to that described for synthesis of **O3** was used to produce **O10** in 30% yield (A_{260}/A_{330} = 28).

Preparation of 5'-[^{32}P]-Labeled Oligonucleotides.[41] Individual oligodeoxynucleotides target strands (**C1** and **C2**) (3.5 pmol) were incubated with γ-^{32}P-ATP (50 μC_i, 3000 C_i/mmol), kinase buffer (0.05 M Tris·HCl pH 7.6, 0.01 M $MgCl_2$, 5 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mM spermidine), and T-4 polynucleotide kinase (10 units) for 1 h at 37 °C. Reaction mixtures were then diluted to 2 mL with water and centrifuged in an amicon concentrator (10 000 MW cut-off) for 40 min (4 °C) to remove the excess salt and unincorporated γ-^{32}P-ATP. This process was repeated a second time to provide 4.7 μC_i of phosphorylated oligodeoxynucleotides in about 45% yield (based on oligodeoxynucleotide).

Methods. ^{1}H NMR spectra were determined using a General Electric QE 300 spectrometer (operating at 300 MHz for ^{1}H). UV/vis studies were conducted on a Perkin-Elmer λ-5 spectrometer. Photochemical

(41) Maniatis, T.; Fritsch, E. F.; Sambrook, J. *Molecular Cloning, A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, 1989.

brominations were performed with a 275-W Sears sunlamp (Sears, Roebuck and Co., no. 34-7105) or a 700-W Sperti sunlamp (Cooper Hewitt Electric Co., no. P-181-E). Flash chromatography was performed using 230–400 mesh chromatographic silica gel from Fisher Scientific.

Extinction Coefficients for Oligodeoxynucleotides. The oligodeoxynucleotides strand concentrations were calculated from absorbance at 260 nm and ϵ_{260} values estimated from the sum of nucleotide absorptivity as affected by adjacent bases.[42] The extinction coefficient for **9** at 260 and 330 nm was determined by dissolving a known quantity (1.2 mg) of the quinone in methanol, diluting 100-fold into 10 mM potassium phosphate buffer (10 mM), and determining the absorbance at the required wavelength ($\epsilon_{260} = 15$, $\epsilon_{330} = 3.09$ mM^{-1} cm^{-1}).

Target-Probe Hybridization and Alkylation. Redox active probes (2.2 μM) were annealed in the presence of target DNA (2.2 μM, containing 40 nCi of 5′-[^{32}P]-labeled sequence) and 100 mM potassium phosphate (pH 7) at 4 °C (60 min) or ambient temperature (30 min). Alkylation was initiated by addition of a reducing system. After 30 min, the reaction mixture was diluted 10-fold and dialyzed for at least 3 h against water. Equivalent concentrations of DNA were used in the photochemical studies. In this case, the oligodeoxynucleotides were added to 10 mM potassium phosphate pH 7 and annealed by heating to 70 °C and allowing them to cool to ambient temperature over 3–4 h. Photolysis (5 min) was carried out in Pyrex glassware at the focal point of a 150-W Xenon arc lamp (PRA Associates) using a 345-nm long pass band filter. Target modification was analyzed by denaturing polyacrylamide gel electrophoresis (20%, 7 M urea) and autoradiography. Autoradiograms were scanned and quantified using a LKB Ultrascan XL Laser Densitometer.

(42) Fasman, G., Ed.; *Handbook of Biochemistry and Molecular Biology-Nucleic Acids*, 3rd ed.; CRC Press: Boca Raton, FL, p 175.

Purification of target-Probe Cross-Link. The cross-linked DNA was separated preparatively by denaturing polyacrylamide gel electrophoresis (20%, 7 M urea), located by autoradiography, and extracted by standard crush and soak methods.[41] The crude residue was redissolved in a minimum amount of water (ca. 30 μL) and then the DNA was precipitated at −20 °C (3–8 h) after addition of NaOAc (30 μL, 7.5 M) and ethanol (180 μL). The purity of the isolated product was checked using the electrophoretic conditions described above.

Hydroxyl Radical Footprinting of the Alkylation Sites.[34] Duplex DNA and the cross-linked products (60 nCi) were treated with 1 mM $(NH_4)_2Fe(SO_4)_2$, 2 mM EDTA (pH 8), 1 mM sodium ascorbate, and 0.06% hydrogen peroxide for 3 min. The reaction was quenched by addition of 1 M thiourea (10 μL) and then lyophilized. The resulting fragmentation patterns of DNA were analyzed via denaturing polyacrylamide gel electrophoresis (20% and 25%, 7 M urea), autoradiography, and densitometric scanning. Data were initially plotted by the densitometer (GelScan XL, Ver 1.2) and then digitized for smoothing by Sigmaplot.

Thermal Melting Temperature (T_m) of a Target-Probe Duplex. Equal concentrations (3.7 μM) of **C1** and either **O1** or **O9** were added to 10 mM potassium phosphate (pH 7), heated to 70 °C, and then cooled slowly to 4 °C over 4 h. The samples were then equilibrated in a temperature controlled UV spectrophotometer and their absorbance at 260 nm was recorded over a range of temperatures (14–70 °C). The T_m values were estimated at $(^1/_2)(\Delta A_{260})$.

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