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Sequence-Specific Delivery of a Quinone Methide Intermediate to the Major Groove of DNA

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Abstract—Silyl-protected phenol derivatives serve as convenient precursors for generating highly electrophilic quinone methide intermediates under biological conditions. Reaction is initiated by addition of fluoride and has previously exhibited proficiency in DNA alkylation and cross-linking. This approach has now been extended to the modification of duplex DNA through triplex recognition and fluoride-dependent quinone methide induction. Both oligonucleotides of a model duplex were alkylated in a sequence specific manner by an oligonucleotide conjugate that is consistent with triplex association. Optimum reaction required the presence of the two complementary target sequences and a pH of below 6.5. In addition, one guanine in each strand adjacent to the triplex region was the predominant site of alkylation. The yield of modification varied from approximately 20% for the purine-rich strand to only 4% for the pyrimidine-rich strand. This surprising difference indicates that the linker between the recognition and reactive elements may limit productive interaction between the quinone methide and the reactive nucleophiles of DNA. Restricted orientation of this intermediate may also be responsible for the lack of target cross-linking at detectable levels.

Introduction

The Dervan laboratory was one of the first to demonstrate the ease by which sequence and conformation specific reagents can be developed for modification of nucleic acids.[1,2] Simply conjugating recognition and reactive elements together is often sufficient to achieve this feat, and its success has supplanted the need to integrate these elements by elaborate design and synthesis. From this new perspective, gene-specific modification has became a realistic goal. While applications in medicine await further development, applications in basic research have been immediate. Powerful tools for exploring nucleotide accessibility, proximity and conformation within complex nucleic acids and nucleic acid–protein structures have been achieved through this general approach. Investigations have explored a range of reactive elements including alkylating and oxidizing agents coupled to a series of ligands that alternatively associate with single-stranded DNA, the major or minor groove of DNA or nonclassical structures of polynucleotides.[2–5] Considerable effort in this area has, and continues to be, focused on the problems of target recognition. Far fewer advances have been reported with regard to new reactive elements, and most rely on a rather traditional array of structures and activities. Discovery and evaluation of additional reactive elements should enhance and expand the future success of modifying chosen nucleotide sequences in a selective and irreversible manner.

Two strategies have been employed to ensure that target association precedes covalent modification, a necessity for sequence-directed applications. Reagents contain either a moiety with low intrinsic or spontaneous reactivity to prevent competition from nonspecific background processes or an inert precursor with an ability to be transformed by an external signal into a highly reactive intermediate. The inducible nature of the latter technique not only offers the possibility of greatest control but also allows for a number of time-resolved experiments. Typical methods for initiating these types of reactions include irradiation with ultraviolet light[6–8] and addition of either an oxidant[9–11] or reductant.[12–14] One of the most promising strategies relies on chemical activation induced merely by association of a probe and its target.[15–21] Unfortunately, success in this area has been sporadic, and thus attention remains directed to conventional methods of activation.

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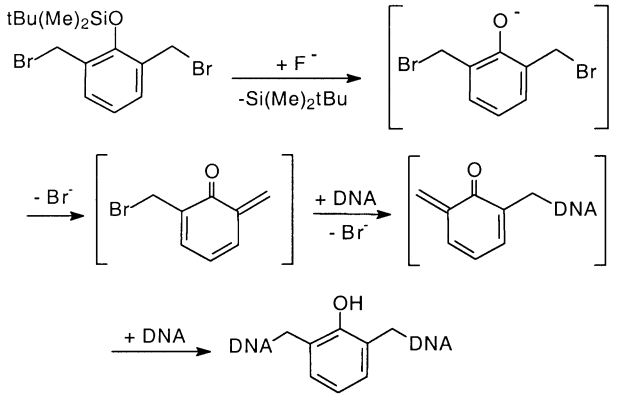
Our laboratory has been exploring the chemistry and application of quinone methides and now report their use in triplex-mediated alkylation of DNA. Interest in these highly electrophilic and reactive intermediates derives from their utility in synthesis[22–25] and their formation in nature as a result of xenobiotic metabolism.[26–31] A number of recent model studies have also begun to identify the fundamental properties of these species and should help to predict their biological activity in the future.[32–39] The first example of site-directed formation of a quinone methide was based on naphthoquinone–oligonucleotide conjugates that alternatively responded to photochemical and reductive signals.[40–42] Our attention soon shifted to a more convenient system based on a silyl-protected phenol that responded to the presence of fluoride. An oligonucleotide conjugate of this provided a simple method for alkylating targeted sequences of single-stranded DNA,[43] and nonconjugated derivatives have since been used to identify the innate reactivity of each nucleotide.[44,45] Addition of a second halomethyl group *ortho* to the phenol successfully supported cross-linking of DNA via tandem quinone methide generation in the presence of fluoride (Scheme 1).[46] Most recently, a bis(acetoxymethyl) derivative has been developed for routine coupling through its activated ester to any ligand containing a primary amine (Pande et al., in preparation). An oligonucleotide conjugate of this species is characterized below for its ability to deliver a quinone methide intermediate to the major groove of DNA as directed by formation of a model triplex.

Results and Discussion

Reagent and target design

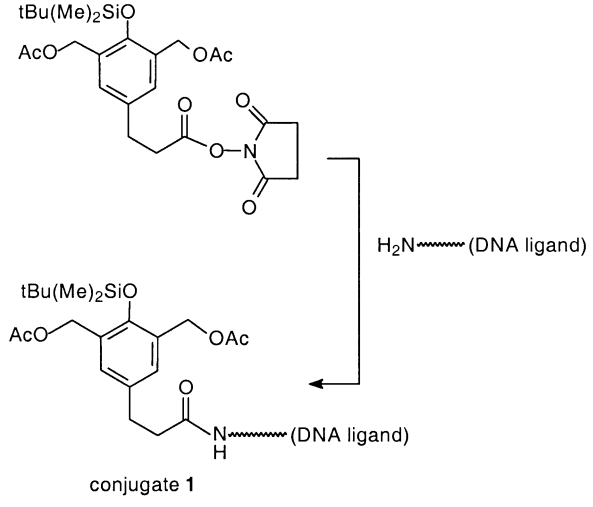
Initial manipulation of the bis(bromomethyl) derivative was complicated by its decomposition under a variety of synthetic conditions and hence was replaced with a less reactive bis(acetoxymethyl) derivative. Elaboration of this species to the illustrated *N*-hydroxylsuccinimide ester was designed for coupling to commercially available oligonucleotides modified at their 5′ terminus with a hexamethyleneamino linker (Scheme 2) (Pande et al., in preparation). Of course, the generality of this coupling procedure will also allow for direct comparisons of target alkylation using other recognition elements currently under investigation. The target sequence of DNA was selected from among many potential sites due to its presence in the plasmid pUC18. This will facilitate future studies with targets of higher molecular weight and complexity. In addition, the triplex-forming strand contains a minimum number of cytosine residues that require protonation in order to pair with guanine in the purine-rich target sequence of duplex DNA (Scheme 3).[47] Finally, a CGCG sequence is directly adjacent to the recognition site and represents the preferred site for cross-linking by the non-conjugated bis(bromomethyl) derivative.[46]



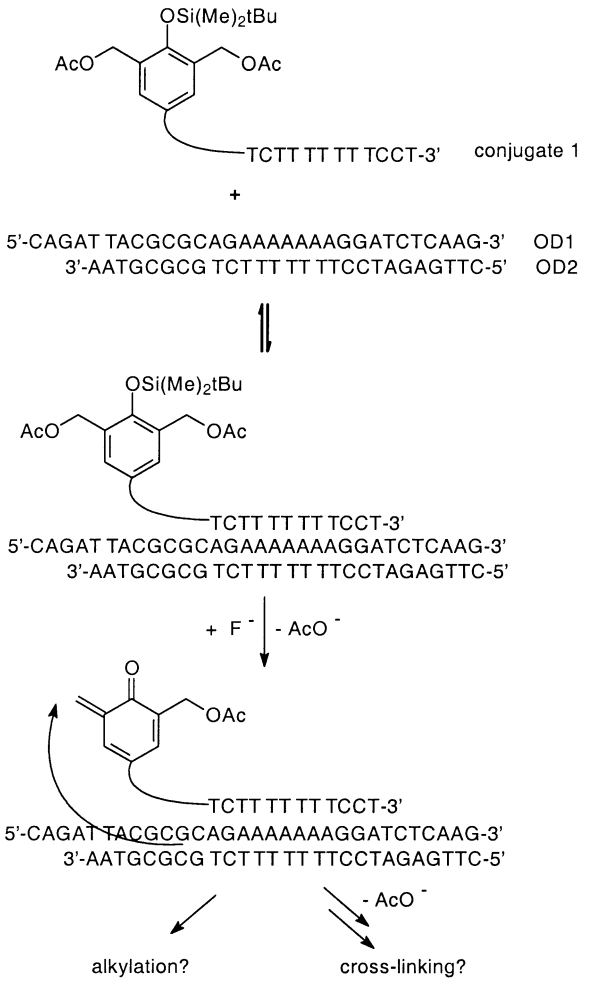
Scheme 1. DNA cross-linking through tandem quinone methide generation induced by fluoride.

Time dependence of target alkylation

Incubation of the ligand-directed, fluoride-dependent alkylating agent, conjugate **1**, with its duplex oligonucleotide target (OD1 + OD2) resulted in time-dependent formation of high molecular weight products consistent with DNA alkylation (Fig. 1). The major product containing radiolabeled OD1 formed in approximately 28% yield relative to the initial duplex target and accounted for over 80% of all detectable products (Fig. 1A). Reaction was more than 80% complete within 24 h and was strongly dependent on the presence of fluoride. Without fluoride, the major product formed in a yield of only 1.6% after 60 h. This low residual activity may derive from a slow spontaneous or DNA-dependent cleavage of the O–Si bond that is otherwise quite effectively promoted by fluoride.[48] Substitution of the silyl group for a methyl blocked all activity and suggested that deprotection of the phenol and subsequence generation of the quinone methide intermediate is required for alkylation. Initial investigations additionally suggest that the rate of the overall process is controlled by formation of the quinone methide rather than from loss of the silyl group.[48] However, the efficiency of reaction appears limited by assembly of the triplex since target modification



Scheme 2. Conjugation of recognition and reactive elements to form a ligand-directed, fluoride-inducible alkylating agent.



Scheme 3. Triplex recognition and selective alkylation.

exhibited saturation in the presence of a 200-fold or greater excess of conjugate **1** relative to OD1 + OD2.

The length of the two target strands of DNA (OD1, OD2) were set differently in order to distinguish products that derived from alkylation of either OD1 or OD2 with those derived from cross-linking OD1 and OD2 together. The latter process would generate a species exhibiting the same electrophoretic behavior regardless of which strand was radiolabeled since it would contain OD1, OD2 and a derivative of conjugate **1**. In contrast, individual species were observed with unique electrophoretic behavior and consistent with alkylation of a single target strand (Fig. 1). No cross-linking of OD1 and OD2 was detected despite the ability of a non-conjugate bis(bromomethyl) derivative to cross-link duplex DNA in yields of up to 7.5% in the presence of fluoride.[46] The kinetics of alkylation were similar for OD1 and OD2, although the efficiency of reaction was greatly reduced for OD2 versus OD1 (Fig. 1). The major product of OD2 also did not dominate its reaction profile to the extent exhibited by that derived from OD1. Finally, the lack of reaction detected with radiolabeled OD2 in the absence of fluoride likely reflected the weak reactivity of OD2 and the minimal spontaneous reaction of conjugate **1**.

Selective reaction depends on triplex formation

The multiple products formed by OD1, OD2, and conjugate **1** likely differ in their site of alkylation since electrophoretic mobility depends on both molecular shape and weight.[49,50] To determine which product was dependent on triplex association, the fluoride-dependent reaction was repeated in the lone presence of either OD1 or OD2 (Fig. 2). Both conditions generated a series of new derivatives that differed from those formed by the

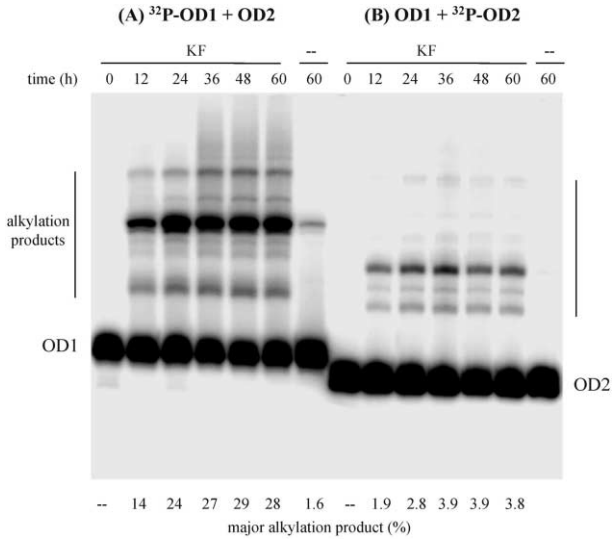


Figure 1. Alkylation of duplex DNA (OD1 + OD2) containing (A) 5′-[^{32}P]-labeled OD1 and (B) 5′-[^{32}P]-labeled OD2 with conjugate **1** under standard conditions. Reactions were quenched at the indicated times, and the major alkylation product of each oligonucleotide was quantified relative to the total radiolabel.

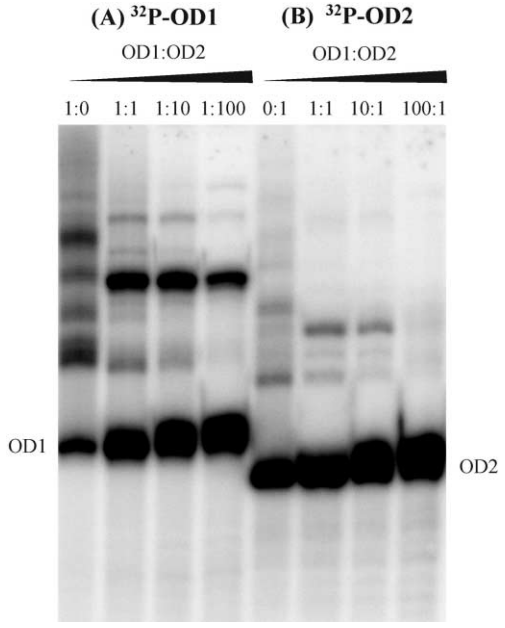


Figure 2. The product profiles of conjugate **1** with (A) 5′-[^{32}P]-labeled OD1 and (B) 5′-[^{32}P]-labeled OD2 were examined in the absence and presence of varying amounts of their complementary oligonucleotides.

duplex OD1 + OD2. The purine rich strand OD1 was again modified to a much greater extent than OD2, although its major product was not equivalent to that formed by the duplex target. At least for the single strand experiments, the greater reactivity of OD1 may be explained by its ability to associate with conjugate **1** through formation of a parallel helix of the purine- and pyrimidine-rich sequences.[51,52]

Excess oligonucleotide complementary to the radiolabeled strand was also added to reaction with conjugate **1** in order to maximize the extent to which the radiolabeled strand remained in its duplex form during alkylation. This complementary strand could additionally compete with the duplex DNA for nonspecific reaction of conjugate **1**. The yield of the major product formed by OD1 in the presence of OD2 and conjugate **1** remained unaffected by a 10-fold excess of OD2 and exhibited only a 40% decrease after the concentration of OD2 was increased 100-fold (Fig. 2A). The dominance of this species in the product profile also increased from 80% to over 95% under these conditions. Similar trends were observed for radiolabeled OD2 in the presence of excess unlabeled OD1 (Fig. 2B). However, the relatively high reactivity of OD1 effectively competed with alkylation of OD2 in its duplex form after the single strand OD1 was added in a 100-fold excess. The major alkylation products of OD1 + OD2 consequently seem to originate from the triplex structure formed with the sequence-directed conjugate **1** since these derivatives are only formed in the presence of all three oligonucleotide strands and are least susceptible to the presence of excess single-stranded DNA.

The involvement of triplex in selective alkylation was further confirmed by its characteristic dependence on pH. Triplex structures are typically stabilized by acidic conditions,[53] and consequently reactions that depend on this structure should demonstrate a similar response to pH. Alkylation of OD1 + OD2 by conjugate **1** was therefore repeated under standard conditions except the pH was varied from 5.00 to 7.25. The major alkylation product detected by radiolabeled OD1 was generated in greatest yield over a pH range of 5.75–6.00 (Fig. 3). Formation of this product decreased substantially below pH 5.25 and above pH 6.50. This pH optimum is most consistent with the characteristics of triplex formation since neither the stability of the duplex OD1 + OD2 nor the generation of the quinone methide from conjugate **1** require low pH. In contrast, quinone methide generation is facilitated by deprotonation of its immediate precursor, the unprotected phenol derivative of conjugate **1**. The resulting pH profile of alkylation then likely reflects the narrow range of pH conditions in which both a triplex structure and quinone methide intermediate may form.

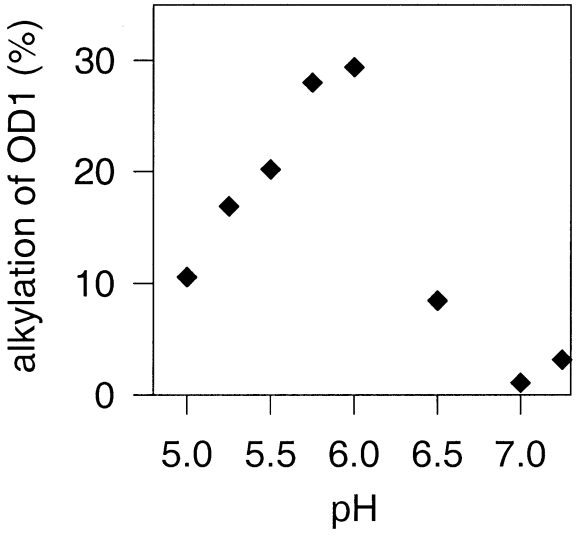


Figure 3. Alkylation of duplex DNA (OD1 + OD2) containing 5′-[^{32}P]-labeled OD1 with conjugate **1** was performed under standard conditions and the indicated pH (MES). Reactions were repeated twice and the average yield of the major product is presented relative to the total radiolabel.

Site and nature of modification

The oligonucleotide-directed conjugate **1** was originally designed to provide sequence selectivity to a bifunctional derivative capable of sequential generation of two quinone methide intermediates for DNA cross-linking.[46] This derivative had previously exhibited an intrinsic specificity for 5′A...CG... that was reminiscent of that expressed by mitomycin.[28] However, mitomycin primarily alkylates the 2-amino group of guanine which resides in the minor groove of duplex DNA and is stable to heat and piperidine treatment. The cross-linked product formed at 5′A...CG... by the bifunctional quinone methide was labile to piperidine treatment and induced strand scission at G that is considered diagnostic of alkylation at its N7 position.[54] This alternative site resides in the major groove of duplex DNA and was the expected target of conjugate **1** since triplex association would direct this reagent to the major groove. Previous model studies demonstrated that the 2-amino, N1, and N7 groups of guanine are all susceptible to alkylation by a simple monofunctional quinone methide although reaction at the 2-amino group is dominant.[45]

Piperidine treatment subsequent to reaction of conjugate **1** with OD2 and radiolabeled OD1 consumed the major alkylation product and induced strand scission of OD1 that in turn suggested G_{11} as the site of reaction (Fig. 4A, lane 3). No such strand scission was evident in the absence of alkylation (Fig. 4A, lane 1), and none was evident for the nonspecific products of alkylation. G_{11} represents the guanine that is nearest to the triplex-forming polypurine sequence of OD1, and the piperidine lability of its alkylation product indicates a linkage to its N7 position as shown possible by reactions of non-conjugated derivatives.[45,46] The nucleotide sequence surrounding this reactive site was next varied systematically in order to explore the determinants that control reaction within the region adjacent to the triplex. Substituting an A/T base pair for the C/G pair in OD1 + OD2 that separates the reactive G_{11} of OD1 and the triplex region decreased the efficiency of modifying G11 by approximately 50% and increased the side product that migrates somewhat faster than the G11 product (Fig. 4B). Otherwise, no other changes were

observed. Substituting the reactive G_{11} and its complement with an A/T pair removed the primary site of modification, and no alternative sites on the OD1 derivative appeared in the absence of G_{11} (Fig. 4C). Sequential substitution of the C/G and G/C base pairs on the 5′ side of G_{11} and its complement had no significant effect on the specificity or yield of modification on the OD1 family of sequences (Fig. 4D and 4E). In each case, G_{11} remained the major site of alkylation.

The dependence of alkylation on nucleobase versus nucleotide position was finally examined by inverting the entire sequence adjacent to the triplex region so that the reactive G_{11} was replaced by a C and two adjacent cytosines were replaced by guanines G_{10} and G_{12}. The triplex-dependent reaction of this new target shifted almost exclusively to G_{12} (Fig. 4F). No competing modification was detected at G_{10}. Alkylation of C_{11} may also have occurred but with an efficiency of no more than 5% of that for G_{10} if the persistent piperidine stable material that comigrated with the major product is assigned to cytosine alkylation (Fig. 4F). Previous model studies with a simple, nonconjugated quinone methide demonstrated that cytosine was more reactive than either adenosine or guanine at the nucleoside level. However, modification of cytosine is dramatically suppressed in duplex DNA and becomes minor in comparison to reaction of guanine.[44] Overall, the hierarchy of reaction between the purine-rich target strand and conjugate **1** reflects a primary specificity for guanine and a secondary preference for nucleotide position.

Modification of the complementary strand within the duplex target by conjugate **1** was investigated concurrently with the 5′ radiolabeled derivative of OD2 and its analogues. Initial study of the parent OD2 revealed three alkylation products, and the one formed in greatest yield appeared most associated with a triplex-specific event (Fig. 2B). None of these products was labile to piperidine, and therefore their sites of modification were not evident from strand scission (Fig. 5A, lanes 2 and 3). However, the triplex-specific product was not formed when its G_{20} of the G/C base pair adjacent to the triplex region was substituted by a T/A base pair (Fig. 5B). This suggests that the triplex-specific reaction involved G_{20} although not at its N7 position since the linkage

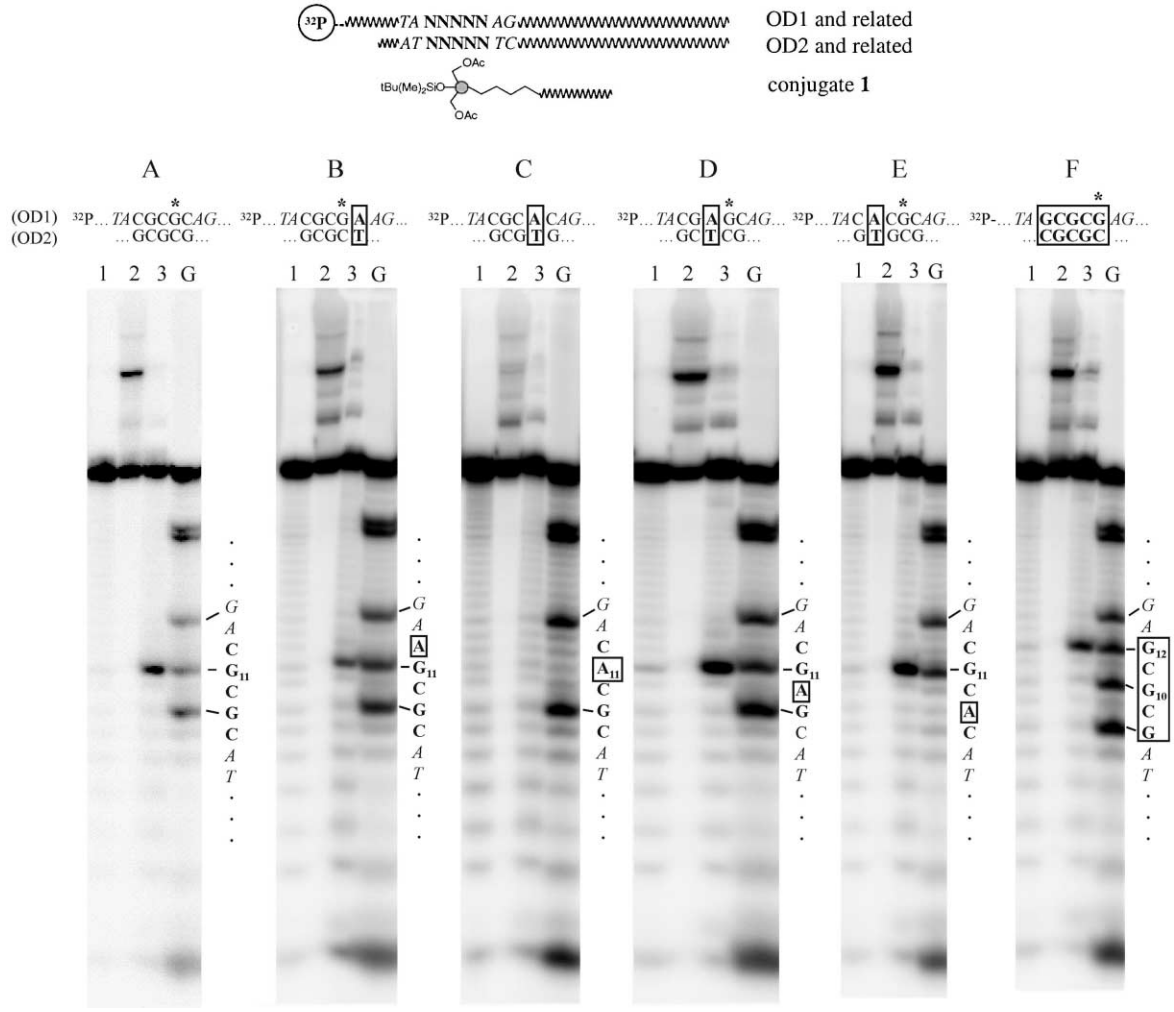


Figure 4. Specificity of alkylation on the purine-rich strand by conjugate **1** was determined using the parent duplex OD1 + OD2 and a series of sequence variants. Product profiles were detected by uniquely labeling the 5′-termini of the purine-rich strands (e.g., OD1) with [^{32}P]-phosphate. Target duplexes were incubated with conjugate **1** in the absence (lanes 1) and presence of KF (lanes 2 and 3) under standard conditions. Products were further treated with hot piperidine (lanes 1 and 3) and compared to a standard G-sequencing lane prepared by dimethylsulfate (lane G).[54]

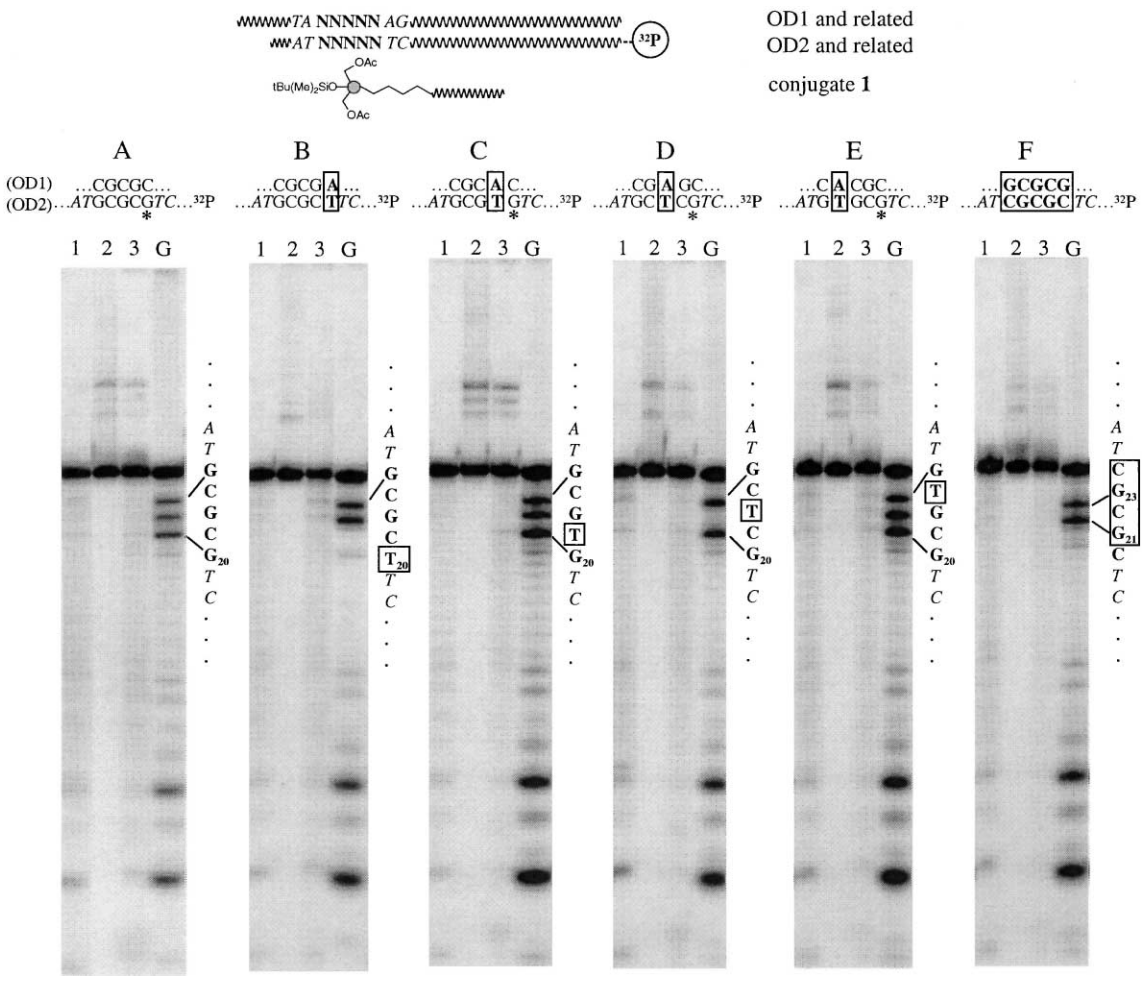


Figure 5. Specificity of alkylation on the pyrimidine-rich strand by conjugate **1** was determined using the parent duplex OD1 + OD2 and a series of sequence variants. Product profiles were detected by uniquely labeling the 5′-termini of pyrimidine-rich strands (e.g., OD2) with [^{32}P]-phosphate. Target duplexes were incubated with conjugate **1** in the absence (lanes 1) and presence of KF (lanes 2 and 3) under standard conditions. Products were further treated with hot piperidine (lane 1 and 3) and compared to a standard G-sequencing lane prepared by dimethylsulfate (lane G).[54]

resisted degradation in the presence of piperidine. Alternative substitution of the following C/G base pair with T/A restored the formation of the triplex-specific product at G_{20} (Fig. 5C). Lastly, substitution of the next G/C and C/G base pairs also did not affect the triplex-specific product (Fig. 5D and E).

The origins of the additional high molecular products formed by OD2 and its analogues are not as readily apparent. The fastest migrating species was generated without regard to the sequence substitutions above whereas the product of intermediate migration seemed to depend on the presence of G_{20} and G_{22} (Fig. 5). Even an inversion of the sequence immediately adjacent to the triplex binding region did not effect the fastest migrating species (Fig. 5F). However, the triplex-specific reaction was not observed for this inverted sequence. This highlights once again the contrasting reactivity of OD2, the polypyrimidine-containing strand, and that of OD1, the polypurine-containing strand. First, alkylation of OD2 is much less efficient than of OD1. Second, the triplex-specific site of reaction on OD2 appears to be limited to G_{20}, the guanine directly adjacent to triplex recognition, and reaction is not supported by a guanine that is only a single residue away from the triplex. In addition, equivalent sequences within OD1 and OD2 do not behave equivalently (Figs 4A vs. 5F; Figs 4F vs. 5A).

The nature of the products formed by OD1 and OD2 also differed in their sites of alkylation as illustrated by their contrasting response to sequence variation and piperidine treatment. The anticipated piperidine-labile product resulting from guanine N7 alkylation in the major groove was evident for OD1 but not for OD2. All of the high molecular weight products of OD2 were stable to piperidine, but most importantly, the triplex-specific reaction of OD2 still involved a proximal guanine. Alternative reaction at the 2-amino or N1 position of guanine is known to form with simple, non-conjugated quinone methide intermediates[29,44] and might explain the stability of the specific OD2 product formed with conjugate **1**. However, these alternatives would be unusual, although not unprecedented, for conjugates that bind to the major groove. Small changes in linker length connecting acridine and a nitrogen mustard had previously been able to suppress reaction of guanine N7 in the major groove and allow for reac-

tion in the minor groove.[55] Of equal interest, this acridine conjugate also supported reaction at adenine N1 which is typically rendered inert due to Watson–Crick base pairing with its complementary thymine. Perhaps the linker joining the quinone methide precursor and the triplex-forming oligonucleotide also diverted reaction of OD2 to its minor groove or sufficiently disrupt the adjacent G/C pair to allow for reaction at guanine N1.

Conclusion

Conjugation of a sequence-specific recognition element and a quinone methide precursor has allowed for selective delivery of a highly reactive intermediate to duplex DNA. Use of fluoride as the signaling reagent to unmask this intermediate provided a convenient and orthogonal condition to the common alternatives based on reductive, oxidative or photochemical signals. Both oligonucleotides in the target duplex were selectively modified by conjugate **1** within two residues of the triplex-forming region. Although cross-linking to both oligonucleotides was theoretically made possible by the bis(acetoxylmethyl) groups of conjugate **1**, none of the nucleotide sequences examined in this investigation generated such a product. Instead, oligonucleotides within the target duplex were alkylated individually. This result may once again be the consequence of the linker used to couple the recognition and reactive elements. Even highly flexible linkers can strongly influence the efficiency of olignucleotide-directed alkylation to extent that a dependable cross-linking system based on a nitrogen mustard was similarly restricted to single alkylation events.[56] Future efforts will consequently focus on linker design as well as new structure-directing elements that could be used to react with a variety of nucleic acids and their protein complexes.

Experimental

Materials

All solvents and reagents were purchased from Fisher Scientific (Pittsburgh, PA) and used without purification. Oligonucleotide were synthesized by Gibco BRL Life Technologies (Rockville, MD) and purified by polyacrylamide gel electrophoresis under standard conditions.[57] Oligonucleotides were labeled at their 5′ terminus with 5′-[γ-^{32}P]-ATP (Amersham Pharmacia, Piscataway, NJ) and T4 kinase (New England Biolabs, Beverly, MA) as directed by the supplier (New England Biolabs).

Conjugate 1. A solution of the appropriate oligonucleotide containing a 5′-hexamethyleneamino linker (20 nmol) in 3-(*N*-morpholino)propanesulfonate (MOPS, 250 mM pH 7.5, 400 μL) was mixed with an equal volume of *N*-succinimidyl-3-(4′-*tert*-butyldimethylsiloxy-3′,5′-bis(acetoxymethyl)phenyl)propionate (Pande et al., in preparation) in acetonitrile/DMF (2:1) and incubated under ambient conditions for 5 h. The desired conjugate **1** was then isolated in 70% yield (14 nmol) after purification using a reverse-phase C-18 column (Varian Microsorb-MV, 300 Å pore, 250 mm, Woburn, MA) and gradient of 10–55% acetonitrile in an aqueous solution of triethylammonium acetate (50 mM, pH 6) over 35 min (1 mL/min) as controlled by a Jasco PU-980 HPLC system (Easton, MD). The homogeneity of this material was confirmed by reanalysis on HPLC under the same conditions described above.

DNA alkylation

Complementary oligonucleotides (100 nM each and 1.4 μCi of the 5′-[^{32}P]-labeled derivative as specified) was annealed in 20 mM $MgCl_2$, 300 mM NaCl and 40 mM 4-morpholineethanesulfonate (MES, pH 6) by heating the solution to 90 °C and then allowing it to cool to room temperature over more than 3 h. Conjugate **1** was then added to the mixture, and its reaction was initiated by addition of aqueous KF. Final concentrations were 50 nM duplex DNA, 10 μM conjugate **1**, 100 mM KF, 10 mM $MgCl_2$, 150 mM NaCl, 20 mM MES pH 6. Reaction was maintained at 4 °C and quenched at the indicated times by addition of an equal volume of formamide. After salts were removed by dialysis (1000 MW cutoff, 3 h) and electrophoresis loading solution (8 μL, 0.05% xylene cyanole and 0.05% bromphenol blue in formamide) was added, samples were concentrated to 10 μL under reduced pressure. These procedures were also repeated with the specified changes in the ratio of complementary oligonucleotides (Fig. 2) and pH of annealing and reaction (Fig. 3).

Product analysis

Reaction mixtures (50 nCi/lane) described above were separated by denaturing polyacrylamide gel electrophoresis (20%, 7 M urea) and detected by PhosphoImage analysis (Molecular Dynamics, Sunnyvale, CA). Product profiles were quantified using ImageQuant software provided by the supplier. As indicated, certain samples were treated with 10% aqueous piperidine at 90 °C for 30 min after desalting.

Acknowledgements

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