VOLUME 120, NUMBER 38
SEPTEMBER 30, 1998

JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Triplex-Directed Interstrand DNA Cross-Linking by Diaziridinylquinone−Oligonucleotide Conjugates

Michael W. Reed,* Ansel Wald, and Rich B. Meyer

Contribution from Epoch Pharmaceuticals Incorporated, 1725 220th Street SE, #104, Bothell, Washington 98021

Received November 6, 1997

Abstract: Triplex-forming oligonucleotides (TFOs) bind in the major groove to specific double-helical DNA sequences and have been shown to inhibit the function of targeted genes. Diaziridinylquinones are bifunctional alkylating agents that can form interstrand cross-links in DNA at 5′-GNC sites. To demonstrate the feasibility of targeted interstrand cross-linking, a diaziridinylquinone−TFO conjugate was prepared from a diaziridinylquinone intermediate bearing an activated ester linker. The first demonstration of triplex-directed interstrand DNA cross-linking by a single targeted bifunctional alkylating agent is described. Up to 38% interstrand cross-linking was observed at pH 6.2.

Introduction

Triplex-forming oligonucleotides (TFOs) bind in the major groove to specific double-stranded DNA (dsDNA) sequences and have been shown to inhibit function of targeted genes.[1] DNA alkylating agents have been attached to TFOs to stabilize the triple-stranded complex,[2] and photoactivated DNA cross-linking[3] by psoralen-TFO conjugates has been shown to induce sequence specific mutations in plasmid DNA models.[4] Interstrand cross-links are believed to be especially cytotoxic, but the mutagenic effects of these lesions are not clear.[5] Although alkylation of *one* strand of dsDNA has been demonstrated using a variety of TFO-directed electrophilic agents, this report is the first case of targeted DNA interstrand cross-linking by a single bifunctional alkylating agent.[6]

Diaziridinylquinones are bifunctional alkylating agents that form interstrand cross-links in DNA.[7] A variety of symmetrically substituted diaziridinylbenzoquinone analogues have been examined for DNA cross-linking properties.[8] In general,

* Corresponding author. Telephone: 425-485-8566. Fax: 425-486-8336. E-mail: mreed@epochpharm.com.

(1) For reviews on TFOs, see: (a) Thuong, N. T.; Helene, C. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 666−690. (b) Mirkin, S. M.; Frank-Kamenetskii, M. D. *Annu. Rev. Biophys. Biomol. Struct.* **1994**, *23*, 541−576. (c) Sun, J. S.; Garestier, T.; Helene, C. *Curr. Opin. Struct. Biol.* **1996**, *6*, 327−333.

(2) (a) Vlassov, V. V.; Gaidamakov, S. A.; Zarytova, V. F.; Knorre, D. G.; Levina, A. S.; Nikonova, A. A.; Podust, L. M.; Federova, O. S. *Gene* **1988**, *72*, 313−322. (b) Povsic, T. J.; Dervan, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 9428−9430. (c) Takasugi, M.; Guendouz, A.; Chassignol, M.; Decout, J. L.; Lhoumme, J.; Thuong, N. T.; Helene, C. *Proc. Natl. Acad. Sci. U.S.A* **1991**, *88*, 5602−5606. (d) Shaw, J.-P.; Milligan, J. F.; Krawczyk, S. H.; Matteucci, M. *J. Am. Chem. Soc.* **1991**, *113*, 7765−7766. (e) Gruff, E. S.; Orgel, L. E. *Nucleic Acids Res.* **1991**, *19*, 6849−6854. (f) Kutyavin, I. V.; Gamper, H. B.; Gall, A. A.; Meyer, R. B. Jr. *J. Am. Chem. Soc.* **1993**, *115*, 9303−9304. (g) Colombier, C.; Lippert, B.; Leng, M. *Nucleic Acids Res.* **1996**, *24*, 4519−4524.

(3) The term "cross-linking" as used here refers to interstrand alkylation of the targeted dsDNA. Alkylation of a single strand in the dsDNA target is defined as "monoalkylation".

(4) (a) Havre, P. A.; Gunther, E. J.; Gasparro, F. P.; Glazer, P. M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7879−7883. (b) Wang, G.; Glazer, P. M. *J. Biol. Chem.* **1995**, *270*, 22595−22601. (c) Raha, M.; Wang, G.; Seidman, M. M.; Glazer, P. M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2941−2946.

(5) Sanderson, J. S.; Shield, A. J. *Mutat. Res.* **1996**, *355*, 41−57 and references therein.

(6) Interstrand DNA cross-linking by a 3′,5′-bischlorambucil TFO was described in ref 2f.

(7) (a) Szmigiero, L.; Kohn, K. W. *Cancer Res.* **1984**, *44*, 4453−4457. (b) Szmigiero, L.; Erickson, L. C.; Ewig, R. A. G.; Kohn, K. W. *Cancer Res.* **1984**, *44*, 4447−4452.

S0002-7863(97)03819-5 CCC: $15.00
Published on Web 09/11/1998

the cross-linking efficiency of these agents is increased by reduction of the quinone to the hydroquinone or by lowering pH. Substituents can alter electron density in the benzoquinone ring and reactivity of the aziridines. For example, 2,5-diaziridinyl-1,4-benzoquinone (DZQ) can cross-link DNA at pH 7.2, whereas the 3,6-dimethyl-substituted analogue (MeDZQ) required enzymatic reduction at the same pH.[8b] Reductive activation can also alter the sequence selectivity of these agents. For example, the hydroquinone form of DZQ cross-links preferentially at 5′-GC or 5′-GNNC sites, whereas the hydroquinone of MeDZQ prefers 5′-GNC sites.[8c] These sequence preferences have been rationalized by molecular modeling.[8d]

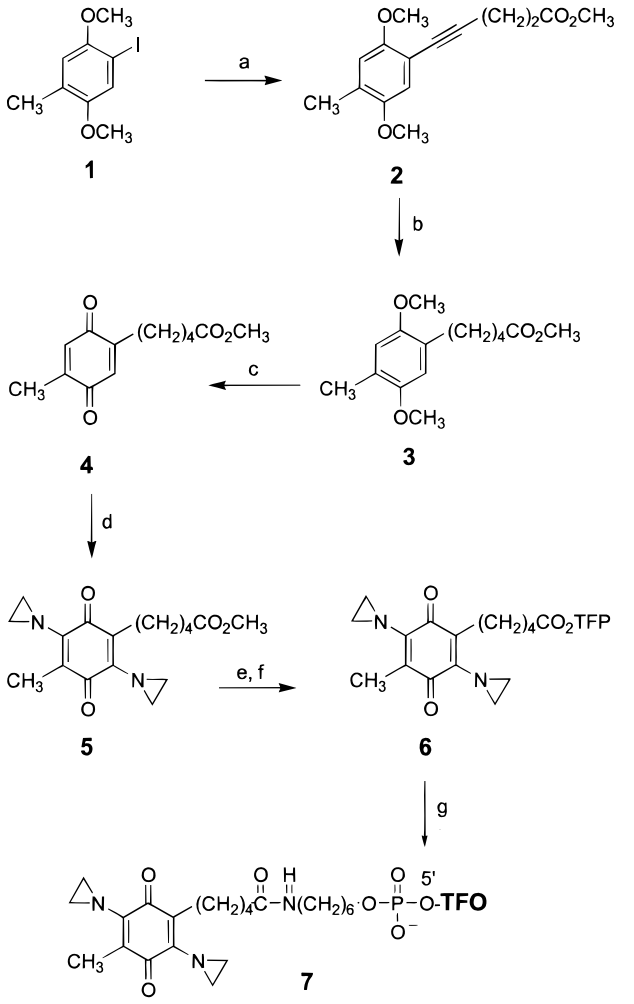
Diaziridinylquinone cross-links DNA in the major groove between the N-7 atoms of dG residues on opposite strands of the duplex,[9] an ideal location for targeted delivery by TFOs. To demonstrate the feasibility of targeted interstrand cross-linking, we prepared a diaziridinylquinone−TFO conjugate (**7**) from an activated ester intermediate (**6**) as shown in Scheme 1.

Results and Discussion

Synthesis. We have developed methods to selectively couple alkylating agents bearing activated ester linkers to amine-modified oligonucleotides.[10,11] Anhydrous conditions allow selective acylation of the oligo without competing side reactions of the DNA reactive agent. A suitable ester linker arm was first introduced into the diaziridinylquinone precursor (**2**) by palladium-catalyzed coupling[12] of 4-iodo-2,5-dimethoxytoluene (**1**) and methyl 4-pentynoate followed by hydrogenation of the alkyne to give the aliphatic linker arm in **3**. Ceric ammonium nitrate (CAN) oxidation[13] gave an intermediate quinone (**4**) that was reacted with excess ethyleneimine to give the diaziridinylquinone methyl ester (**5**). Careful saponification and treatment with 2,3,5,6-tetrafluorophenyl trifluoroacetate[14] (TFP-TFA) gave the desired diaziridinylquinone TFP ester (**6**). As shown in Figure 1, reaction of the triethylammonium salt of a 5′-hexylamine-modified TFO with excess **6** in DMSO gave clean conversion to diaziridinylquinone−TFO (**7**). Reverse phase HPLC purification and precipitation gave 56% recovery of conjugate **5** (97% pure by HPLC).

Triplex Model System. For the DNA alkylation studies described here, a 21-mer G/A motif TFO was chosen which targets a homopurine run in an endogenous human gene as shown in Figure 2. We used an analogous nitrogen mustard−TFO conjugate to alkylate this specific sequence in genomic DNA with 90% efficiency at a concentration of 0.5 μM.[15] In earlier work,[16] we showed that G/A motif TFOs formed more stable triplexes than the C/T or G/T motif for this G-rich target,

Scheme 1[a]



[a] (a) Methyl pentynoate, $Pd(PPh_3)_4$, CuI, $EtN(iPr_2)$, DMF; (b) H_2, Pd-C, triethylammonium formate (pH 6.5), THF, EtOH; (c) $(NH_4)_2Ce(NO_3)_6$, CH_3CN; (d) ethyleneimine, $Cu(OAc)_2$, MeOH; (e) LiOH (aq), MeOH; (f) 2,3,5,6-tetrafluorophenyl trifluoroacetate, Et_3N, CH_2Cl_2; (g) TFO-NH_2, Et_3N, DMSO.

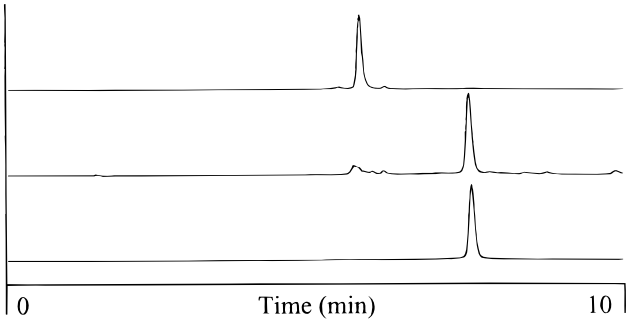


Figure 1. Reaction of 5′-hexylamine-modified TFO with TFP ester **6**. Progress of the reaction was monitored by C_{18} HPLC as described in the Experimental Procedures. The upper chromatogram shows starting hexylamine-modified TFO. The middle chromatogram shows the reaction mixture after 1 h. The lower chromatogram shows HPLC-purified diaziridinylquinone−TFO (**7**).

(8) (a) Hartley, J. A.; Berardini, M.; Ponti, M.; Gibson, N. W.; Thompson, A. S.; Thurston, D. E.; Hoey, B. M.; Butler, J. *Biochemistry* **1991**, *30*, 11719−11724. (b) Lee, C.-S.; Hartley, J. A.; Berardini, M. D.; Butler, J., Siegel, D.; Ross, D.; Gibson, N. W. *Biochemistry* **1992**, *31*, 3019−3025. (c) Berardini, M. D.; Souhami, R. L.; Lee, C. S.; Gibson, N. W.; Butler, J.; Hartley, J. A. *Biochemistry* **1993**, *32*, 3306−3312. (d) Haworth, I. S.; Lee, C.-S.; Yuki, M.; Gibson, N. W. *Biochemistry* **1993**, *32*, 12857−12863.

(9) (a) Alley, S. C.; Brameld, K. A.; Hopkins, P. B. *J. Am. Chem. Soc.* **1994**, *116*, 2734−2741. (b) Alley, S. C.; Hopkins, P. B. *Chem. Res. Toxicol.* **1994**, *7*, 666−672.

(10) Reed, M. W.; Lukhtanov, E. A.; Gorn, V.; Kutyavin, I.; Gall, A.; Wald, A.; Meyer, R. B., Jr. *Bioconjugate Chem.* **1998**, *9*, 64−71.

(11) Lukhtanov, E. A.; Kutyavin, I. V.; Gorn, V. V.; Reed, M. W.; Adams, A. D.; Lucas, D. D.; Meyer, R. B. *J. Am. Chem. Soc.* **1997**, *119*, 6214−6225.

(12) Hobbs, F. W., Jr. *J. Org. Chem.* **1989**, *54*, 3420−3422.

(13) Jacob, P., III; Callery, P. S.; Shulgin, A. T.; Castagnoli, N. *J. Org. Chem.* **1976**, *41*, 3627−3629.

(14) Gamper, H. B.; Reed, M. W.; Cox, T.; Virosco, J. S.; Adams, A. D.; Gall, A. A.; Scholler, J. K.; Meyer, R. B., Jr. *Nucleic Acids Res.* **1993**, *21*, 145−150.

(15) Belousov, E. S.; Afonina, I. A.; Podyminogin, M. A.; Gamper, H. B.; Reed, M. W.; Meyer, R. B. *Nucleic Acids Res.* **1997**, *25*, 3440−3444.

(16) Gamper, H. B.; Kutyavin, I. V.; Rhinehart, R. L.; Lokhov, S. G.; Reed, M. W.; Meyer, R. B. *Biochemistry* **1997**, *36*, 14816−14826. As described in ref 15, an incorrect sequence recorded in GenBank was used for these initial studies. The correct dsDNA sequence is used for the work described here.

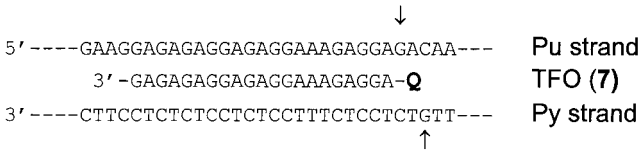


Figure 2. Design of model triplex system for interstrand cross-linking. The diaziridinylquinone linker structure (**Q**) in TFO **7** is shown in Scheme 1. Only the hybridization region is shown for the 65-bp dsDNA target. The alkylated dG residues in the purine-rich strand (Pu) and pyrimidine-rich strand (Py) are indicated by arrows. Since cell culture experiments were ultimately planned, a 3′-hexanol modification was incorporated into TFO **7** to provide resistance to nuclease degradation.

especially in the presence of the triplex-specific intercalating agent coralyne.[17] There is a potential 5′-GAC cross-linking site just downstream of the homopurine run in the dsDNA target that we hoped to alkylate with the diaziridinylquinone conjugate group. Despite the preferred[18] 5′-GXC mustard cross-linking sequence just downstream of the homopurine run, only monoalkylation products were observed for the mustard−TFO.[19] Under the conditions used here (100:1 TFO:dsDNA, 10 μM coralyne), no more than 48% monoalkylation was observed for the mustard−TFO analogue of TFO **7**.[10]

DNA Alkylation Assay. Sequence-specific alkylation by TFO **7** of a 65-mer dsDNA fragment containing the target site was examined by denaturing polyacrylamide gel electrophoresis (PAGE). The time course of DNA alkylation at 37 °C was determined for each 5′-^{32}P-labeled target strand at pH 6.2 as shown in Figure 3. Three bands in each of the imaged gels were observed which correspond to an interstrand cross-linked species, a monoalkylated species, and the unmodified labeled single-stranded target. At longer time points, a band due to spontaneous thermal depurination and cleavage of alkylated products appeared.

For each labeled DNA target, the slow-moving cross-linked species increased to a maximum at 6 h, then slowly degraded. As discussed below, the labeled Pu strand showed an intermediate monoalkylated product, whereas the labeled Py strand showed only a trace of monoalkylation. Lane CL for the Py-labeled target shows the short (15-mer) DNA fragments that result from heat/piperidine strand cleavage after 24 h of alkylation. A single major band appeared, indicative of the specificity of alkylation. This band (63% of the labeled Py strand) represents the sum of interstrand cross-linked products that had formed (and/or degraded) over 24 h. A control (lane MeDZQ) with 10 μM of Me-DZQ showed no interstrand DNA cross-linking, thus demonstrating the increased DNA reactivity of the diaziridinylquinone upon targeting with the TFO.

The dynamics of the interstrand cross-linking process is shown in Figure 4. The upper panel shows how monoalkylated product builds rapidly in the Pu strand, then slows as some of these products are converted to cross-links. For the labeled Py strand (lower panel), the cross-linked product forms at the same rate as for the Pu strand, but only a trace of monoalkylated product is observed. Apparently the N-7 dG target in the Py strand can react only after prior alkylation of the N-7 dG site in the Pu strand. Modeling studies show that alkylation of dG in the Pu strand (by the less hindered aziridine next to the methyl group) allows the remaining aziridine (next to the linker) to stretch across the major groove and react with dG in the Py strand. The TFO is held to the Pu strand (by reverse Hoogsteen H bonds) which may facilitate the initial alkylation event. Degradation of the cross-linked DNA presumably occurs by initial depurination of the alkylated Py strand (since no monoalkylation products appear in the labeled Py strand). Finally, depurination of the (more stable) alkylated Pu strand occurs to give the short cleaved fragments apparent at 24 h.

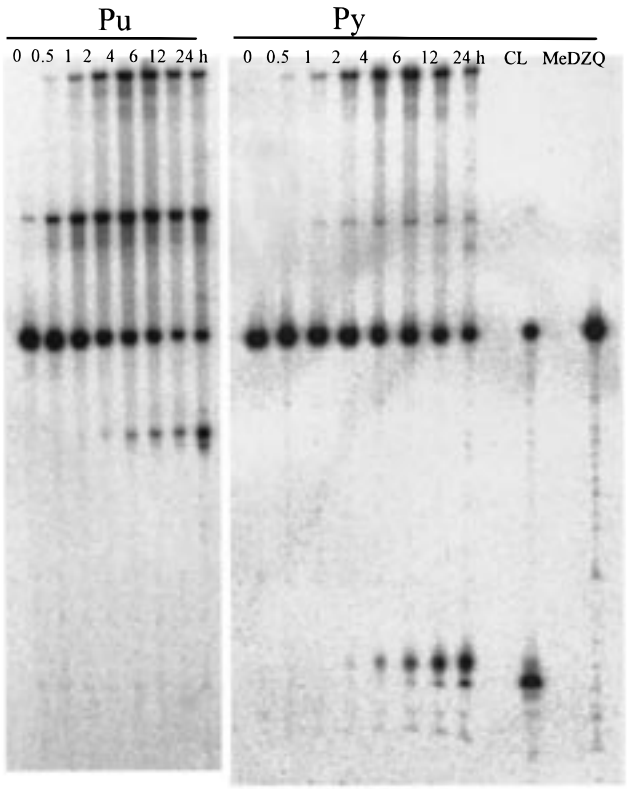


Figure 3. Alkylation of dsDNA target by TFO **7** at pH 6.2. Preformed duplex (20 nM labeled strand and 40 nM unlabeled strand) was incubated for 24 h at 37 °C with TFO **7** (2 μM) in 20 mM PIPES (pH 6.2), 140 mM KCl, 10 mM $MgCl_2$, 1 mM spermine, and 10 μM coralyne chloride. Denaturing gel electrophoresis analysis of the products from each ^{32}P-labeled strand vs time is shown. Upper bands are interstrand cross-linked products, intermediate bands are monoalkylation products, and lower bands are unmodified labeled strands. The lowest bands (at long time points) correspond to cleavage of alkylated products. The CL lane shows strand cleavage by heat/piperidine treatment of the 24-h sample (63% cleavage). MeDZQ lane shows 24-h incubation of the labeled duplex with 10 μM MeDZQ (no coralyne).

Effect of pH on Interstrand Cross-Linking Efficiency. Since the mechanism of DNA alkylation by (nonreduced) aziridinylquinones requires protonation of the aziridine, it was expected that alkylation by TFO **7** would be slower at neutral pH. As shown in Figure 5, this was the case. DNA alkylation by TFO **7** was measured over time at pHs 6.2, 6.7, and 7.2 using carefully prepared PIPES buffers. Only the labeled Pu strand was examined since we were also interested in monoalkylation rates. At pH 7.2, the diaziridinylquinone−TFO gave up to 17% interstrand cross-linking under the assay conditions but required extended reaction time with a plateau at 24 h. Monoalkylation at pH 7.2 was 47% at 24 h (data not shown). At pH 6.7, the interstrand cross-linking reached a maximum 25% at 720 min (42% monoalkylation). At pH 6.2, a plateau of 38% interstrand cross-linking was reached at 360 min (36% monoalkylation). It makes sense that alkylation rates are faster at lower pH, but it is not clear why the maximum percent

(17) Lee, J. S.; Latimer, L. J. P.; Hampel, K. J. *Biochemistry* **1993**, *32*, 5591−5597. This reference describes stabilization of C/T motif triplexes by coralyne.

(18) (a) Ojwang, J. O.; Grueneberg, D. A.; Loechler, E. L. *Cancer Res.* **1989**, *49*, 6529−6537. (b) Millard, J. T.; Raucher, S.; Hopkins, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 2459−2460.

(19) The mustard−TFO analogue of **7** alkylated primarily the dG residue in the purine-rich strand.

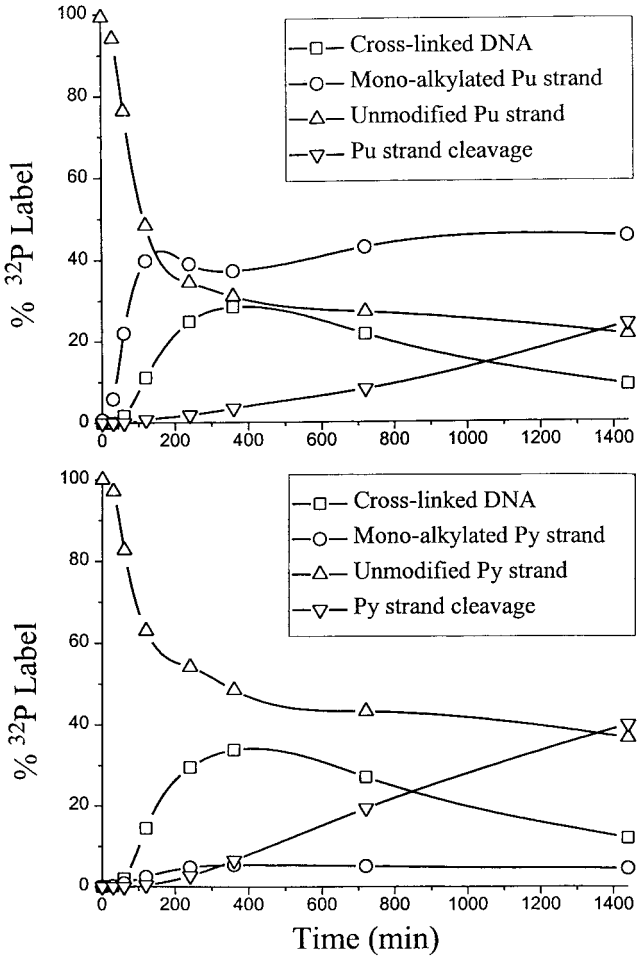


Figure 4. Dynamics of DNA alkylation by TFO **7** at pH 6.2. Results of the PAGE assay shown in Figure 3 were quantitated by phosphorimaging. The upper panel shows the fate of the labeled Pu strand, and the lower panel shows the fate of the labeled Py strand.

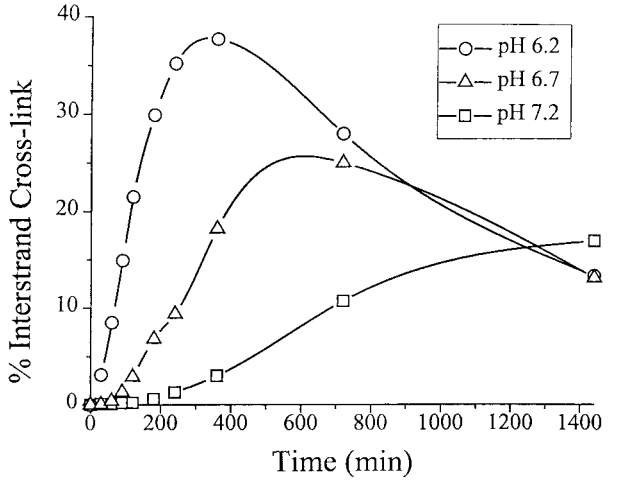


Figure 5. Effect of pH on interstrand DNA cross-linking by TFO **7**. The pH 6.2 PAGE assay described in Figure 3 was repeated at pH 6.7 and pH 7.2. The percent interstrand cross-link was quantitated for each time point by phosphorimaging.

interstrand cross-linking decreases at higher pH. Competing side reactions (such as alkylation of spermine in the triplex buffer by the aziridine groups in **7**) or less efficient triplex formation at neutral pH may be possible explanations.

Aqueous Stability/Self-Alkylation of TFO 7. The aqueous stability of diaziridinylquinone−TFO conjugates may be an important factor for cross-linking efficiency. The electrophilic aziridine rings can react with other nucleophiles or water to give degradation products that compete with intact TFO and prevent targeted cross-linking. The G-rich nature of TFO **7** is also a potential liability since self-alkylation is possible. For example, a G/A motif mustard−TFO in a related target was found to self-associate as parallel stranded duplexes and form alkylated products.[20] A reverse phase HPLC assay was used to determine the aqueous half-life of TFO **7** at pH 6.2 and 7.2 (37 °C). As shown in Figure 6, **7** decomposes at pH 6.2 to a complex mixture of faster eluting products.[21] At pH 6.2, only 11% intact **7** remained after 24 h ($t_{1/2} = 6$ h). After 24 h at pH 7.2, 64% intact TFO **7** remained.

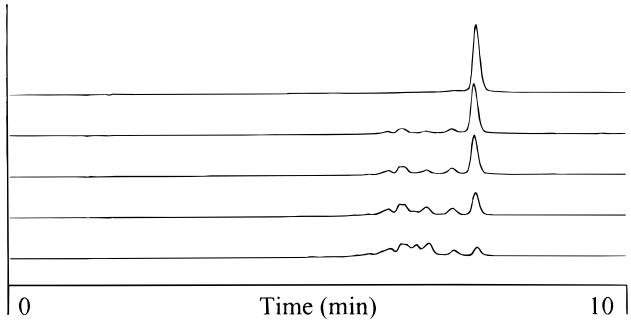


Figure 6. Aqueous stability of TFO **7** at pH 6.2 (37 °C). Degradation was monitored by C_{18} HPLC as described in the Experimental Procedures. Starting **7** (top chromatogram) was analyzed at 3, 6, 12, and 24 h (bottom chromatogram).

Conclusion

The interstrand DNA cross-linking observed for the 3,6-dialkyl-substituted diaziridinylquinone when linked to the TFO shows the advantage of sequence-specific targeting. The analogous dimethyl-substituted compound (MeDZQ) required enzymatic reduction for DNA cross-linking.[8c] This hybridization-driven reactivity is presumably due to the higher effective local concentration of alkylating agent at the targeted site, but other interactions in the major groove may contribute. Molecular modeling studies show that interstrand cross-linking by diaziridinylquinones at the 5′-GAC flanking sequence can be accomplished with little distortion of the B-DNA structure. This is in contrast with nitrogen mustard-mediated cross-linking that requires a severe reduction in helical twist at the preferred cross-link site.[18]

The linker system in TFO **7** has not been optimized, and self-alkylation with the G/A motif is a competing side reaction. We are currently exploring structural modifications of the diaziridinylbenzoquinone cross-linkers and determining the sequence specificity of DNA cross-link formation in other triplex systems. An understanding of the geometric constraints for cross-link formation should allow us to optimize the TFO linker structure for various genetic targets. By analogy with MeDZQ, reduction of the diaziridinylquinone to the hydroquinone should increase DNA reactivity.

Diaziridinylquinone−TFOs may have applications in gene therapy if cellular (chromatinized) dsDNA can be modified. They may also be used as rare-cutting DNA cleavage agents. We showed up to 63% targeted DNA cleavage by heat/

(20) Lampe, J. N.; Kutyavin, I. V.; Rhinehart, R.; Reed, M. W.; Meyer, R. B.; Gamper, H. B. *Nucleic Acids Res.* **1997**, *25*, 4123−4131.

(21) Similar mixtures of degradation products were observed for decomposition of mustard−TFOs (see ref 10).

piperidine treatment. As shown by others,[22] N-7 alkylation sites in the DNA target give dsDNA fragments with residual phosphates that can be used as substrates for ligation enzymes. In this sense, these agents are the first example of synthetic "restriction enzymes". In addition, the diaziridinylquinone activated ester **6** is suitable for conjugation to a wide variety of amine-containing targeting ligands for evaluation as possible anticancer agents.

Experimental Procedures

^{1}H NMR spectra were run on a Varian Gemini 300-MHz spectrometer. Elemental analyses were performed by Quantitative Techonologies Inc. (Boundbrook, NJ). Melting points were determined on a Mel-Temp melting point apparatus in open capillary tubes and are uncorrected. All air- and water-sensitive reactions were carried out under a slight positive pressure of argon. Flash chromatography was performed on 230−400 mesh silica gel. Analytical thin-layer chromatography was carried out on EM Science F_{254} aluminum-backed, fluorescent indicator plates. Triethylammonium bicarbonate (TEAB) (0.1 M) was prepared by sparging an aqueous mixture of the appropriate amine with CO_2 until the organic layer disappeared. MeDZQ was prepared by treatment of 2,5-dimethyl-*p*-benzoquinone (Acros Organics) with excess ethyleneimine and was characterized by ^{1}H NMR.

4-Iodo-2,5-dimethoxytoluene (1). To a stirred solution of 7.19 g (47 mmol) of 2,5 dimethoxytoluene (Aldrich) in 50 mL of ether was added a solution of 7.66 g (47 mmol) of iodine monochloride in 20 mL of chloroform over 30 min. Stirring was continued for another 3 h. The mixture was partitioned between 250 mL of ether and 200 mL of 10% sodium thiosulfate with 6 g of sodium bicarbonate. The decolorized organic layer was washed again with a similar portion of the thiosulfate solution and dried over sodium sulfate. Removal of solvents gave a white solid residue that was recrystallized from 100 mL of methanol to yield 9.28 g (71%) of **1** as a white solid: mp 81−82 °C; TLC (methylene chloride) $R_f = 0.87$ (0.83 for starting material); ^{1}H NMR ($CDCl_3$) δ 7.18 (s, 1H), 6.68 (s, 1H), 3.82 (s, 3H), 3.78 (s, 3H), 2.19 (s, 3H). Anal. Calcd for $C_9H_{11}O_2I$: C, 38.87; H, 3.99; I, 45.63. Found: C, 39.20, H, 3.76, I, 45.33.

4-Pentynoic Acid, 5-(2,5-Dimethoxy-4-methylphenyl), Methyl Ester (2). To 3.88 g (14.0 mmol) of aryl iodide **1** and 1.88 g of methyl pentynoate (16.7 mmol) in 50 mL of dry DMF were added 0.277 g (1.45 mmol) of cuprous iodide and 0.80 g (0.69 mmol) of tetrakis-((triphenylphosphine)palladium (0)) (Lancaster). The flask was flushed with argon and sealed with a septum, and 3.6 mL of *N*,*N*-diisopropylethylamine was added. After the mixture was stirred overnight, TLC showed a trace of unreacted starting material. The solution was evaporated, and the residue was purified by flash chromatography (5 × 40 cm silica) using a gradient of 1:1 hexanes−methylene chloride to 100% methylene chloride. Evaporation gave a dark brown solid. Despite good NMR purity, a second silica gel column was run using 4:1 hexanes−ethyl acetate to eliminate color. Appropriate fractions were evaporated to give 1.88 g (51% yield) of the desired product as an off-white solid: mp = 66−67 °C; TLC (2:1 hexanes−ethyl acetate)-$R_f = 0.63$; ^{1}H NMR ($CDCl_3$) δ 6.77 (s, 1 H), 6.62 (s, 1 H), 3.78 (s, 3 H), 3.73 (s, 3 H), 3.67 (s, 3 H), 2.75 (t, 2 H, $J = 6.3$ Hz), 2.63 (t, 2 H, $J = 6.3$ Hz), 2.16 (s, 3 H). Anal. Calcd for $C_{15}H_{18}O_4$: C, 68.69; H, 6.92. Found: C, 68.59; H, 6.53.

Benzenepentanoic Acid, 2,5-Dimethoxy-4-methyl-, Methyl Ester (3). To 186 mg of 10% palladium on carbon were added 3.7 mL of ethanol and 3 drops of formic acid. The mixture was warmed enough to product slight hydrogen evolution. After 30 min, this mixture was flushed with hydrogen and left under a balloon of hydrogen. In a separate flask, 3.7 mL of 4 M aqueous triethylammonium formate (pH 6.5) was mixed with an equal volume of ethanol, flushed with hydrogen, then added to the activated catalyst. A solution of 1.12 g (4.27 mmol) of alkyne **2** in 7.5 mL of dry THF and 3.7 mL of ethanol was introduced into the hydrogenation flask. After the mixture was stirred at room temperature for 19 h, TLC showed no residual starting material. The mixture was filtered through diatomaceous earth and washed with ethanol. The filtrate was concentrated in vacuo, and the residue was partitioned between 3% sodium bicarbonate and ethyl acetate. The organic layer was evaporated to dryness to give 1.08 g (96%) of **3** as a colorless liquid: TLC (2:1 hexanes−ethyl acetate) $R_f = 0.80$; ^{1}H NMR ($CDCl_3$) δ 6.67 (s, 1 H), 6.64 (s, 1 H), 3.80 (s, 3 H), 3.77 (s, 3 H), 3.67 (s, 3 H), 2.60 (t, 2 H, $J = 7.7$ Hz), 2.36 (t, 2 H, $J = 7.1$ Hz), 1.8−1.6 (m, 4 H). Anal. Calcd for $C_{15}H_{22}O_4$: C, 67.65; H, 8.33. Found: C, 67.39; H, 8.10.

1,4-Cyclohexadiene-1-pentanoic Acid, 4-Methyl-3,6-dioxo-, Methyl Ester (4). To a solution of 1.08 g (4.07 mmol) of **3** in 20 mL of acetonitrile was added a solution of 4.69 g (8.56 mmol) of ceric ammonium nitrate in 10 mL of water. The aqueous solution was added to the stirred organic solution, dropwise, with stirring, until the transient green color that occurs during addition is no longer visible (about 2 equiv of CAN were used). After the mixture was stirred for 45 min, the solvents were removed and the residue was partitioned between water and ethyl acetate. The organic layer was dried over sodium sulfate and evaporated to dryness. The residue was purified by flash chromatography (5 × 50 cm silica) using 4:1 hexanes−ethyl acetate. The yellow band was collected and evaporated, and the residue was recrystallized from aqueous methanol solid to give 0.69 g (71% yield) of the quinone **4** as yellow crystals: mp = 46−48 °C; TLC (4:1 hexanes−ethyl acetate) $R_f = 0.45$; ^{1}H NMR ($CDCl_3$) δ 6.59 (s, 1 H), 6.55 (s, 1 H), 3.67 (s, 3 H), 2.43 (t, 2 H, $J = 7.4$ Hz), 2.35 (t, 2 H, $J = 7.1$ Hz), 2.04 (s, 3 H), 1.69 (m, 2 H), 1.54 (m, 2 H). Anal. Calcd for $C_{13}H_{16}O_4$: C, 66.09; H, 6.83. Found: C, 66.12; H, 6.75.

1,4-Cyclohexadiene-1-pentanoic Acid, 2,5-Bis(1-aziridinyl)-4-methyl-3,6-dioxo-, Methyl Ester (3). To a stirred solution of 0.686 g (2.90 mmol) of quinone **4** in 18.8 mL of methanol was added 105 mg (0.594 mmol) of cupric acetate. Ethyleneimine[23] (1.53 mL, 29.0 mmol) was added, and an air-filled balloon was placed over the neck of the flask to provide a reservoir of oxygen while controlling evaporation of ethyleneimine. After the mixture was rapidly stirred overnight, TLC showed nearly complete reaction. The mixture was evaporated, and the residue was purified by flash chromatography (5 × 50 cm silica) using 2:1 hexanes−ethyl acetate (2% triethylamine). A red band was collected and evaporated to give 827 mg (90% yield) of **5** as orange-red crystals: mp = 97−99 °C; TLC (2:1 hexanes−ethyl acetate) $R_f = 0.33$; ^{1}H NMR ($CDCl_3$) δ 3.66 (s, 3 H), 2.53 (t, 2 H, $J = 7.7$ Hz), 2.35 (t, 2 H, $J = 7.4$ Hz), 2.29 (s, 4 H), 2.27 (s, 4 H), 1.71 (m, 2 H), 1.53 m, 2 H). Anal. Calcd for $C_{17}H_{22}N_2O_4$: C, 64.13; H, 6.96; N, 8.80; O, 20.10. Found: C, 64.04; H, 6.85; N, 8.61.

1,4-Cyclohexadiene-1-pentanoic Acid, 2,5-Bis(1-aziridinyl)-4-methyl-3,6-dioxo-, 2,3,5,6-Tetrafluorophenyl Ester (6). To a solution of 135 mg (0.424 mmol) of methyl ester **5** in 2 mL of methanol was added 0.5 mL of 1 N LiOH. The mixture was stirred at 50−60 °C for 4 h, cooled to room temperature, and concentrated in vacuo. The residue was purified by flash chromatography (2 × 25 cm silica) using 19:1 methylene chloride−methanol (2% triethylamine). The product eluted with a gradient up to 10% methanol. The major red band was collected and evaporated to give 187 mg (109% yield) of the product as a dark red syrup: ^{1}H NMR showed residual triethylamine; TLC (98:2 ethanol−triethylamine) $R_f = 0.28$; ^{1}H NMR ($CDCl_3$) δ 2.53 (t, 2 H, $J = 8.0$ Hz), 2.31 (t, 2 H, $J = 5$ Hz), 2.27 (s, 4 H), 2.26 (s, 4 H), 1.99 (s, 3 H), 1.71 (m, 2 H), 1.50 (m, 2 H).

The intermediate acid was dissolved in 10 mL of acetonitrile, and 0.3 mL of dry triethylamine was added (1.03 mmol). The flask was flushed with argon and cooled in an ice bath. A solution of 315 μL (1.67 mmol) of 2,3,5,6-tetrafluorophenyl trifluoroacetate[14] in 10 mL of acetonitrile was added dropwise to the flask. After 30 min, the solvents were evaporated and the product was purified by flash chromatography (2 × 25 cm silica) using 4:1 hexanes−ethyl acetate. The orange band was collected and evaporated to give 50 mg (26% yield, based on methyl ester **5**) of the desired product **6** as a red solid: mp = 110−114 °C; TLC (2:1 hexanes−ethyl acetate) $R_f = 0.56$; ^{1}H NMR ($CDCl_3$) δ 7.01 (m, 1 H), 2.75 (t, 2 H, $J = 7.2$ Hz), 2.61 (t, 2 H, $J = 8.1$ Hz), 2.33 (s, 4 H), 2.30 (s, 4 H), 2.03 (s, 3 H), 1.89 (m, 2 H),

(22) Povsic, T. J.; Strobel, S. A.; Dervan, P. B. *J. Am. Chem. Soc.* **1992**, *114*, 5934−5941 and references therein.

(23) Allen, C. F. W.; Spangler, F. W.; Webster, E. R *Org. Synth., Coll. Vol. IV* **1963**, 433−435.

1.59 (m, 2 H). Anal. Calcd for $C_{22}H_{20}F_4N_2O_4$: C, 58.41; H, 4.46; N, 6.19. Found: C, 58.47; H, 4.86; N, 5.88.

Synthesis and Purification of Oligodeoxynucleotides. Oligonucleotides were prepared on an Applied Biosystems Model 394 synthesizer using the 1-μmol protocols supplied by the manufacturer. Protected β-cyanoethyl phosphoramidites, CPG supports, deblocking solutions, cap reagents, oxidizing solutions, and tetrazole solutions were purchased from Glen Research (Sterling, VA). A 3′-hexanol modification was introduced into the TFO using a hexanol-modified CPG support,[24] and a 5′-aminohexyl linker was introduced using an *N*-monomethoxytritylhexanolamine phosphoramidite (Glen Research). Preparative HPLC purification, detritylation, and butanol precipitation of the oligos were carried out as usual. Aliquots (0.2 mg) of the 65-mer oligos for triplex cross-linking experiments were further purified by preparative gel electrophoresis. The concentrations of all oligos were determined from the UV absorbance at 260 nm in phosphate-buffered saline (pH 7.2). An extinction coefficient for each oligo was estimated using a nearest-neighbor model.[25] For the TFO used in these studies, a value of 29.1 μg/mL per A_{260} unit was used.

Synthesis of Diaziridinylquinone−TFO (7). HPLC-purified TFO−NH_2 was detritylated and precipitated as usual. The dried pellet was dissolved in 0.5 mL of water and injected on a 300 × 7 mm PRP-1 column (Hamilton, Reno, NV) that was equilibrated with 0.1 M TEAB (pH 7.2). The TEA salt of the ODN was eluted from the column using a gradient of 0−60% acetonitrile/30 min. The desired peak (∼15 min) was collected and dried in vacuo on a centrifugal evaporator. The residue was dissolved in 0.5 mL of water, and the concentration was determined. A 705-μg aliquot (0.1 μmol) was redried in a 1.7-mL Eppendorf tube, and the residue was dissolved in 0.1 mL of DMSO (Aldrich sure-seal) with 10 μL of triethylamine. A solution of 1.4 mg (3.1 μmol) of TFP ester **4** in 70 μL of DMSO was prepared and added to the ODN. After shaking for 1 h, C_{18} HPLC analysis showed complete reaction. After 1.5 h, the ODN products were precipitated by adding to 6 mL of 2% $NaClO_4$/acetone in a 14-mL polypropylene tube. The mix was centrifuged at 3000 rpm for 5 min, and the pellet was sonicated (2 min) with 1 mL of acetone and recentrifuged. The pellet was dried in vacuo for 15 min, dissolved in 0.2 mL of water, and stored frozen. Purification by C_{18} HPLC used a 4.6 × 250 mm Rainin Dynamax C_{18} column (10-μm particle size, 300-Å pore size) and eluted using a gradient of 5−65% acetonitrile in 0.1 M triethylammonium acetate (pH 7.5) over 30 min (flow rate = 1 mL/min). The peak eluting at 13 min (collected in ∼1 mL of TEAA/acetonitrile) was concentrated to ∼0.2 mL by adding ∼3 mL of cold butanol. The lower aqueous layer was removed, and the purified conjugate (**7**) was precipitated by adding 100 μL of 3 M sodium acetate and 6 mL of absolute ethanol. The mixture was centrifuged at 3000 rpm for 5 min, and the pellet was sonicated with 2 mL of ethanol and recentrifuged. The pellet was dried in vacuo for 15 min, and the purified product was dissolved in 0.20 mL of water. A 5-μL aliquot was removed for C_{18} HPLC analysis, and another 5-μL aliquot was removed for concentration determination. The bulk solution was immediately stored at −20 °C for future use. HPLC analysis showed 97% purity. A_{260} analysis showed a concentration of 1.97 mg/mL (56% recovery based on starting TFO−NH_2).

Reverse Phase HPLC Analysis of TFO 7. Purity of the starting TFO−NH_2, extent of reaction, and purity of TFO **7** were analyzed by C_{18} HPLC (see Figure 1). Analyses were performed using a Rainin Gradient HPLC system (Emeryville, CA) equipped with 5-mL pump heads and a Rainin Dynamax PDA-1 photodiode array detector. The samples were injected on a 4.6 × 150 mm Rainin Microsorb C_{18} column and eluted using a gradient of 5−65% acetonitrile in 0.1 M triethylammonium acetate (pH 7.5) over 20 min (flow rate = 1 mL/min). Oligonucleotide products were detected by UV absorbance at 260 nm, and data were integrated and analyzed using Rainin Dynamax software. The UV spectrum of **7** showed a characteristic absorbance at 320 nm due to the aziridinylquinone conjugate group.

Electrophoresis Assay for DNA Alkylation by TFO 7. The duplex used in these studies was 65 bp's long and contained a 33-bp homopurine−homopyrimidine run.[26] Either the purine-rich strand (Pu) or the pyrimidine-rich strand (Py) of the duplex was 5′-end-labeled by treatment with T4 polynucleotide kinase and [γ-^{32}P]ATP under standard conditions. Labeled DNA was purified using a Nensorb column (NEN Research Products) and had a specific activity of ∼6000 dpm/fmol. Duplexes were formed by annealing 20 nM of the Pu strand with 40 nM of the complementary Py strand in 20 mM of PIPES buffer (pH 6.2) with 140 mM KCl, 10 mM $MgCl_2$, and 1 mM spermine using an incubation profile of 1 min at 95 °C and 30 min at 37 °C. After annealing, coralyne chloride (Sigma) was added to give a final concentration of 10 μM. Triplexes were formed in capped and siliconized polypropylene microcentrifuge tubes (1.7 mL) at 37 °C in a final volume of 25 μL. The labeled duplex (22.5 μL) was combined with 2.5 μL of TFO **7**, and the solutions were incubated at 37 °C. The concentration of labeled duplex was 20 nM, and the concentration of TFO was 2 μM. Aliquots (2.5 μL) were removed at 0, 30, 60, 120, 240, 360, 720, and 1440 min and stored frozen in 4 μL of loading buffer (80% formamide, 0.01% xylene cyanol, and bromphenol blue). The aliquots were thawed, and cross-linked products were electrophoretically resolved in a denaturing 8% polyacrylamide gel. The labeled bands were visualized by autoradiography (see Figure 3) and quantified using a Bio-Rad GS-250 Phosphorimager. The percent of each ^{32}P-labeled band was plotted vs time (see Figure 4).

Effect of pH on DNA Alkylation by TFO 7. A 100 μM solution of PIPES buffer was prepared with 700 μM KCl, 50 mM MgCl, and 5 mM spermine hydrochloride. These solutions were carefully titrated with 3 M HCl to provide solutions of pH 6.2, 6.7, or 7.2 after 5-fold dilutions. These solutions were used for preparation of Pu-labeled duplexes and triplexes as described above. Kinetics of DNA alkylation were determined by PAGE, and the results are plotted in Figure 5.

Aqueous Stability of TFO 7. A 50 μM solution of **7** (100 μL) was prepared in 20 mM appropriate PIPES buffer (pH 6.2 or 7.2) with 140 mM KCl and 10 mM $MgCl_2$. The 0.1 mM stock solution was immediately aliquoted to Eppendorf tubes and heated in a 37 °C oven. Aliquots were frozen at 3, 6, 12, and 24 h and immediately thawed just prior to HPLC analysis as described above. Stacked chromatograms for pH 6.2 are shown in Figure 6.

Acknowledgment. This work was supported in part by NIH Contract N01-AR-6-2230.

JA973819U

(24) Petrie, C. R.; Reed, M. W.; Adams, A. D.; Meyer, R. B. *Bioconjugate Chem.* **1992**, *3*, 85−87.

(25) Cantor, C. R.; Warshaw, M. M.; Shapiro, H. *Biopolymers* **1970**, *9*, 1059−1077.

(26) The full sequence of the 65-mer dsDNA target can be found in ref 10.