Factors Influencing the Extent and Regiospecificity of Cross-Link Formation between Single-Stranded DNA and Reactive Complementary Oligodeoxynucleotides†

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Received August 6, 1993; Revised Manuscript Received October 25, 1993®

ABSTRACT: Cross-link formation, within the duplex, by oligodeoxynucleotides containing a 5-[ω-(ω-haloacylamido)alkyl]-2′-deoxyuridine to a complementary oligodeoxynucleotide was investigated under conditions approximating the physiologic environment. The site and extent of crosslinking to the target strand were determined for several electrophilic haloacylamidoalkyl structures. The regiospecificity of alkylation was primarily determined by the length of the electrophilic haloacylamidoalkyl group, while the extent of reaction was dependent upon both the structure of the acylamidoalkyl group and the reactivity of the electrophile. Cross-linking was additionally modulated by the sequence of the duplex in the vicinity of the alkylation site. The exact placement of the electrophile adjacent to the targeted nucleophile, an N-7 group on a specific guanine base in the target strand, was the most important factor in determining the rate of reaction. With the optimal haloacylamidoalkyl structure and duplex sequence, the most rapid rate obtained was $t_{1/2}$ = 1.3 h at 37 °C.

A limitation to the clinical utilization of antisense oligodeoxynucleotides (ODNs)[1] is insufficient sequence-specific potency. One approach to improve their effectiveness has been to design modified ODNs that are capable of chemically cross-linking with, and therefore irreversibly inhibiting the expression of, complementary target nucleic acid. Knorre and Vlassov (1985) have demonstrated sequence-directed cross-linking ("complementary addressed modification"), using an *N*-(2-chloroethyl)-*N*-methylaniline attached to either the 3′- or 5′-position of ODNs. All four bases of DNA are alkylated to differing extents. The base that is alkylated is dependent on whether chloroethylamine attachment is to the 3′- or 5′-end of the ODN. Summerton and Bartlett (1978) have shown that an eight-atom chain, attached to a cytosine N-4 and terminating with a highly reactive bromomethyl ketone, can cross-link to the N-7 of the guanine to which it is paired. Webb and Matteucci (1986) have prepared ODNs containing a 5-methyl-*N*,*N*-ethanocytosine and have shown that it is capable of cross-linking, maximal $t_{1/2}$ = 30 h, with strands that are complementary except for a mismatch at the site of alkylation. In a conceptually related alkylation via a linker arm within a DNA hybrid, Iverson and Dervan (1988) have shown opposite strand methylation, triggered by cyanogen bromide activation of a methylthio ether, predominantly on a guanine two base pairs from the base bearing the linker. More recently, ODNs modified with a binuclear platinum complex (Gruff & Orgel, 1991), a reductively activated naphthoquinone derivative (Chatterjee & Rokita, 1991), or a 2-[(*N*-iodoacetylaminoethyl)thio]adenine residue (Kido et al., 1992) have been shown to chemically cross-link to complementary DNA sequences.

Considerable effort has also been expended in the development of photochemically cross-linking ODNs (Gamper et al., 1987; Kean et al., 1988; Pieles et al., 1989). Introduction of a psoralen tethered onto an ODN permits rapid and efficient cross-linkage to complementary DNA and RNA targets upon irradiation with near-ultraviolet light. The use of psoralenated methylphosphonate ODNs as antisense agents in model cell culture systems is notable in that the cross-linking event increases potency by 30–50-fold (Chang et al., 1991). The necessity for ultraviolet activation, however, severely limits the utility of these reagents *in vivo*.

We reported (Meyer et al., 1989) cross-linking with an oligodeoxynucleotide containing a 5-(3-iodoacetamidopropyl)-2′-deoxyuridine. The mechanism of alkylation for this cross-linker is based on hybridization of the electrophilic ODN followed by nucleophilic attack on the iodoacetamide moiety. The cross-link was formed to a single guanine with a $t_{1/2}$ = 12 h at 37 °C. Povsic and Dervan (1990) have observed alkylation of a guanine in a DNA double helix by a similar side chain attached to an ODN bound within a triplex.

The design of our original electrophilic haloacylamidoalkyl group was inspired by Brookes and Lawley (1961), who hypothesized that bis(2-chloroethyl)amines formed N-7 to N-7 cross-links between adjacent opposing-strand guanines. A computer-generated model of a hybrid showed that an analogous C-5 to N-7 cross-link bridged by a five- or six-atom arm could be formed between a pyrimidine in one strand and an adjacent interstrand guanine. Instead, we found (Meyer et al., 1989) that an interstrand cross-link formed between the 5-(3-iodoacetamidopropyl)-2′-deoxyuridine containing ODN and the N-7 of the guanine two base pairs removed on the complementary strand, and we predicted that the cross-link formed by the anticancer nitrogen mustards similarly skips a base pair, instead of cross-linking adjacent pairs as had been widely believed. Ojwang et al. (1989) and Millard et al. (1990) have since found that to be the case: bis(2-

† This work was supported by NIH Grants CA 40336, CA 45905, and AI25959 and by DOD Contract DMAD17-88-C-8201.

® Abstract published in *Advance ACS Abstracts,* December 15, 1993.

[1] Abbreviations: ODN, oligodeoxynucleotide; DIAD, diisopropyl azodicarboxylate; Fmoc, fluorenylmethoxycarbonyl; Fmoc-Cl, fluorenylmethoxycarbonyl chloride; NHS, *N*-hydroxysuccinimide; SDS, sodium dodecyl sulfate; DEAD, diethyl azodicarboxylate; DMT, dimethoxytrityl; DMTr-Cl, dimethoxytrityl chloride; DMAP, 4-(dimethylamino)pyridine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; TBE, Tris-borate EDTA.

0006-2960/94/0433-375$04.50/0

chloroethyl)amine also forms an interstrand N-7 to N-7 crosslink that spans three base pairs.

We report here our investigations on specificity and extent of crosslink formation within an oligomeric duplex as a function of 5-[ω-(ω-haloacylamido)alkyl]-2′-deoxyuridine arm geometry, electrophilic substituent, and oligodeoxynucleotide sequence. The primary target ODN in this study has three consecutive guanines available for alkylation. These guanines should accommodate the potential reach of different electrophilic haloacylamidoalkyl groups.

MATERIALS AND METHODS

N-Hydroxysuccimidyl bromoacetate and iodoacetate were obtained from Sigma Chemical Co. (St. Louis, MO). *N*-Hydroxysuccinimidyl bromobutyrate and chloroacetate were prepared according to the method of Anderson et al. (1964). Tetrakis(triphenylphosphine)palladium(0) and copper iodide were purchased from Morton Thiokol Inc. (Danvers, MA).

Melting points were determined on a Mel-Temp melting point apparatus in open capillary tubes and are uncorrected. Nuclear magnetic resonance (NMR) spectra were obtained at 300 MHz on a Varian VXR-300 spectrometer or at 200 MHz on a Gemini 200 spectrometer. The chemical shift values are expressed in δ values (parts per million) relative to tetramethylsilane as an internal standard. Infrared spectra (IR) were recorded on a Perkin-Elmer 783 spectrophotometer. Ultraviolet spectra (UV) were recorded on a Beckman DU-40 spectrophotometer. Elemental analyses were performed by Robertson Laboratories (Madison, NJ) or Quantitative Technologies Inc. (Boundbrook, NJ).

Thin-layer chromatography was run on silica gel 60 F-254 (EM Reagents) aluminum-backed plates. EM Reagents silica gel (230–400 mesh) was used for flash column chromatography (Still et al., 1978). Components were detected on TLC by UV light and by charring by exposure to H_2SO_4 in MeOH spray followed by heating. Electrophiles were detected by spraying with 1% *p*-nitrobenzylpyridine in acetone followed by heating with 1 N NaOH (Baker et al., 1966).

Molecular graphics were constructed using Insight, and molecular mechanics energy minimizations were done with Discover (both from Biosym Technologies, San Diego) using the AMBER minimization algorithm and parameters on a Silicon Graphics Personal Iris workstation.

Unmodified ODNs. Oligodeoxynucleotide synthesis was done on a 1 μM scale on an Applied Biosystems Model 380B Synthesizer (Foster City, CA) controlled by Software System version 1.34 or on a 1 μM scale on a Milligen 7500 synthesizer (Novato, CA) controlled by DNA Express version 1.9. DNA synthesis reagents were purchased from Glenn Research (Herndon, VA) or Milligen.

HPLC analyses and semipreparative purifications were performed with a Rainin Gradient HPLC system (Emeryville, CA) equipped with 10 mL/min pump heads and a Gilson (Worthington, OH) Model 116 dual-UV wavelength detector. Analytical HPLC used a Rainin Dynamax 300A C_{18}, 4.6 × 250 mm, column eluted with a linear gradient of 9–13.5% acetonitrile in 0.1 M triethylammonium acetate (pH 7.5) over 30 min monitored at 260 nm. Semipreparative HPLC was performed with an identical column, equipment, and buffers using a linear gradient of 9–12% over 30 min. Centrifugal ultrafiltrations were done with a Centricon-3 concentrator from Amicon Inc. (Beverly, MA).

ODNs were labeled with [γ-^{32}P]ATP (specific activity >6000 Ci/mmol) from DuPont (NEN Research Products; Boston, MA) using T4 polynucleotide kinase from United States Biochemical (Cleveland, OH). The ^{32}P-labeled product was purified using DuPont Nensorb 20 columns (Wilmington, DE). Cerenkov counting was done on a Beckman LS 5000TD from Beckman Instruments, Inc. (Fullerton, CA). ODN concentrations were calculated using published extinction coefficients (Fasman, 1976) together with a value of 8.8 mL/μmol for the modified 2′-deoxyuridines. Melting temperatures of oligonucleotides were calculated with OLIGO 3.3 from National Biosciences (Hamel, MN) using the concentration of the strand in excess. The 20% polyacrylamide–7 M urea gels used to resolve labeled ODNs had dimensions of 0.4 × 200 × 400 mm with 4-mm square slots. The gels were run at 50–55 °C in 45 mM Tris-borate (pH 8.3) buffer containing 1 mM EDTA (TBE) at 55 W until the xylene cyanol marker dye had traveled 7.5 cm.

4-Phthalimido-1-butyne (**2b**). To a solution of triphenylphosphine (26 g, 100 mmol) in dioxane (200 mL) in an ice bath was added diisopropyl azodicarboxylate (20 mL, 90 mmol). After addition was complete, a solid formed. A slurry of phthalimide (14.7 g, 0.1 mol) and 3-butyn-1-ol (7 g, 100 mmol) in dioxane (50 mL) was poured in and stirred overnight. The butyne precipitated from CH_2Cl_2 as long prismatic needles (8.25 g, 42%): mp 134–138 °C. Anal. Calcd for $C_{13}H_9NO_2$: C, 72.35; H, 4.55; N, 7.03. Found: C, 72.20; H, 4.55; N, 7.08.

3-Phthalimido-1-propyne (**2a**). Compound **2a** was prepared according to the procedure for **2b**. The compound had previously been reported by Gibson and Benkovic (1987).

5-(3-Phthalimido-1-propynyl)-2′-deoxyuridine (**4a**). The title compound has been reported by Gibson and Benkovic (1987) and was prepared according to an adaptation of the method described by Hobbs (1989). A mixture of 5-iodo-2′-deoxyuridine (2 g, 5.65 mmol) and copper iodide (228 mg, 1.2 mmol) was flushed with argon and suspended in DMF (28 mL). Et_3N (1.6 mL, 11.3 mmol) and **2a** (2.09 g, 11.3 mmol) were added to form a blue solution. The reaction was flushed with argon, and tetrakis(triphenylphosphine)palladium(0) (652 mg, 0.565 mmol) was added. The reaction was warmed to 50 °C for 2 h and stirred at room temperature overnight. The solution was evaporated to an oil, which was dissolved in CH_2Cl_2 and applied onto silica gel. The adsorbed material was purified by chromatography on a 15 × 41 mm bed of silica gel using 5% methanol in dichloromethane. Removal of solvent from appropriate fractions gave 1.17 g (50.4%) of **4a** as a fine white powder: mp 188–190 °C (lit. 161–163 °C). Anal. Calcd for $C_{20}H_{17}N_3O_7$: C, 58.40; H, 4.17; N, 10.21. Found: C, 58.26; H, 4.22; N, 9.88.

5-(4-Phthalimido-1-butynyl)-2′-deoxyuridine (**4b**). Compound **4b** was prepared with **2b** according to the procedure for **4a**. After reaction was complete, the solvent was evaporated and the yellow gum dissolved in CH_2Cl_2. The material was recrystallized from 95% EtOH to give 335 mg (78%) of **4b** as needles: mp 175–178 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.56 (br s, 1, NH), 8.07 (s, 1, H6), 7.91–7.83 (m, 4, phenyl), 6.091 (t, 1, J = 6.6 Hz, 1, H-4′), 3.794–3.729 (m, 4, NCH_2 and H-3′), 3.473 (d, J = 3.9 Hz, 1, H-5′), 2.733 (t, J = 7.2 Hz, 2, propargyl CH_2), 2.104 (t, J = 4.8 Hz, 2, H-2′). Anal. Calcd for $C_{21}H_{19}N_3O_7 \cdot 0.8H_2O$: C, 57.33; H, 4.73; N, 9.55. Found: C, 57.33; H, 4.88; N, 9.46.

5-(3-Phthalimidopropyl)-2′-deoxyuridine (**5a**). A solution of **4a** (1.85 g, 4.67 mmol) in absolute EtOH and cyclohexadiene (25 mL) was refluxed with palladium hydroxide on carbon (500 mg) for 4 h. The hot reaction was filtered through a glass fiber pad. The filtrate was cooled and gave 1.09 g (58%)

of **5a** as sharp white needles: mp 204–205 °C (lit. 197–199 °C (Gibson & Benkovic, 1987). Anal. Calcd for $C_{20}H_{21}N_3O_7$: C, 57.83; H, 5.10; N, 10.12. Found: C, 58.26; H, 4.76; N, 9.79.

5-(4-Phthalimidobutyl)-2′-deoxyuridine (***5b***). A solution of **4b** (1 g, 2.4 mmol) in 95% EtOH was refluxed with Raney nickel (3 g). After 48 h, the absorption peak had shifted from 290 to 263 nm. Catalyst was carefully removed by filtration. The filtrate was evaporated to dryness and the solid was recrystallized from MeOH/H_2O to give 960 mg (97%) of **5b**: mp 180–181 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.35 (br s, 1, NH), 7.89–7.82 (m, 4, Ph), 7.69 (s, 1, H-6), 6.16 (t, J = 6.6 Hz, 1, H-1′), 5.24 (d, 1, 3′-OH), 3.6–3.53 (m, 4, NCH_2 and H-5′), 2.22 (t, J = 6.3 Hz, 2, UCH_2), 2.09 (t, J = 6.0 Hz, 2, H-2′), 1.58 (m, 2, CH_2), 1.44 (m, 2, CH_2). Anal. Calcd for $C_{21}H_{23}N_3O_7$: C, 58.74; H, 5.40; N, 9.79. Found: C, 58.58; H, 5.39; N, 9.76.

3′,5′-Di-O-acetylthymidine (*7*). This preparation was adapted from the synthesis for 3′,5′-di-*O*-acetyl-2′-deoxyuridine (Robbins et al., 1986).

5-[[N-(9-Fluorenylmethoxycarbonyl)amino]methyl]-2′-deoxyuridine (***8***). 5-(Bromomethyl)-3′,5′-di-*O*-acetyl-2′-deoxyuridine was prepared according to the method of Barwolff and Langen (1975) from **7**. The brominated 5-methyl-2′-deoxyuridine was obtained as an amorphous mass after evaporation of the reaction and gave a positive spot with (*p*-nitrobenzyl)pyridine on TLC. This material was placed in a NH_3 bomb (200 mL) and stirred at room temperature. After 24 h, the bomb was cracked open and vented overnight. The brown gum was dissolved in 10% sodium carbonate (100 mL) and dioxane (60 mL). FMOC-Cl (4 × 1.1 g, 17 mmol) was added. After 24 h, the reaction was acidified to pH 6 with 1 N HCl and extracted with EtOAc (5 × 100 mL). The organic layer was dried over $MgSO_4$ and evaporated. The brittle foam was flash chromatographed with 5% MeOH/CH_2Cl_2. Appropriate fractions were combined to give 450 mg (6.25% yield) of **8** as a brittle foam.

Preparation of 5′-(Dimethoxytrityl) 3′-(Cyanoethyl N,N-diisopropylphosphoramidite)s. The nucleosides **5a**, **5b**, and **8** were converted to the 5′-(dimethoxytrityl) 3′-(cyanoethyl *N,N*-diisopropylphosphoramidite) derivatives **6a**, **6b**, and **9**, respectively, by standard methods (Atkinson & Smith, 1984).

Modified Oligodeoxynucleotide Synthesis. The Fmoc-protected oligonucleotide was synthesized on a 1-μmol scale on an Applied Biosystems Model 380B synthesizer. All other modified oligonucleotide synthesis was done on a 1-μmol scale on a Milligen 7500 synthesizer. The oligonucleotides were prepared by standard solid-phase synthesis and isolated by adaptations of standard methods (Van Ness et al., 1991).

Electrophilic Oligodeoxynucleotide Synthesis. One hundred micrograms (22 nmol) of each of the amine-modified ODNs was purified using the semipreparative HPLC conditions described earlier. The product peak, eluting at 14 min, was collected and evaporated to dryness. This material was dissolved in 80 μL of H_2O and 10 μL of 1 M sodium borate (pH 8.3) and mixed with 10 μL of 0.22 M of the NHS esters **10a–d** in acetonitrile. The reaction was followed by analytical HPLC (described above), and starting ODN was consumed within 1 h. The product was isolated by method A or method B described below.

Method A. Isolation of Electrophilic Oligodeoxynucleotide by Precipitation. The reaction mixture from above was treated with 1.1 mL of 1-butanol, cooled to −20 °C, and centrifuged (14 000 rpm) for 15 min at 4 °C. The pellet that formed was suspended in 100 μL of H_2O and reprecipitated with 1.1 mL of 1-butanol. Yield determinations were based on the A_{260} of the recovered material dissolved in distilled H_2O and ranged from 50 to 80% on the basis of unpurified starting material. The purity of the bromoacetamidomethyl (**Ia**) and bromoacetamidopropyl (**Id**) ODNs was greater than 80% and the purity of the other electrophilic ODNs was greater than 90% as determined by HPLC.

Method B. Isolation of Electrophilic Oligodeoxynucleotide by Centrifugal Ultrafiltration. To the reaction mixture from above was added 400 μL of H_2O. The solution was transferred to a Centricon-3 concentrator and centrifuged at 5000*g* in a fixed-angle rotor for 1 h. The retentate was washed with two additional portions of H_2O (500 μL). The recovered retentate had a volume between 35 and 45 μL. The yield of product based on A_{260} of starting material ranged from 40 to 75%, and the purity was greater than 95% on the basis of HPLC analysis.

Formation of Sodium Thiophosphate Adduct. In addition to direct HPLC analysis of the electrophilic ODNs, adduct formation with sodium thiophosphate was used to indicate integrity of the electrophile. For example, a 100-μL aliquot of 120 μM **Id** was combined with 10 μL of 1 M sodium borate (pH 8.3) and 10 μL of 12 mM sodium thiophosphate and heated at 37 °C. After 2 h, analytical HPLC showed that greater than 80% of the starting electrophilic ODN was converted to the thiophosphate adduct.

Cross-Link Formation Analysis. Each hybridization mixture contained 110 pmol of cross-linking ODN and 11 pmol of ^{32}P-end-labeled target ODN (400 000 cpm) in 100 μL of 130 mM NaCl and 10 mM phosphate (pH 7.2) at 37 °C. Aliquots of 10 μL were removed at 1, 3, 6, 12, 18, 24, 36, 48, 96, and 192 h and added to 90 μL of 1.1 M piperidine in H_2O, where they were heated to 90 °C. After 20 min, each sample was cooled in ice water and evaporated under vacuum overnight. Dried samples were stored for up to 8 days at −20 °C and then dissolved into 10 μL of 0.1% SDS followed by mixing with 10 μL of electrophoresis running buffer containing 0.02% bromophenol blue and xylene cyanol in 7 M urea and TBE.

Aliquots of 5 μL from the above solutions were loaded into every other well of a 20% polyacrylamide–7 M urea gel. The bands corresponding to unreacted target and resolved cleaved products or combined cleaved products were excised, and Cerenkov counts were measured. The amount of cross-linkage per time point was expressed as a percentage of the cleaved target counts over the total counts for the lane.

A G-specific chemical sequencing reaction lane was used as a marker for identifying the site of cross-linkage and was prepared by a modification of the published procedure (Maxam & Gilbert, 1977). A solution of 10 ng of 5′-^{32}P-labeled target oligonucleotide (1 × 10^6 cpm) and 1 μL of dimethyl sulfate in 100 μL of 10 mM Tris (pH 8) and 1 mM EDTA was incubated at room temperature. After 30 min, 1 μL of 1 mg/mL salmon sperm DNA and 1.1 mL of 1-butanol were added and the mixture was cooled on ice for 15 min and then centrifuged for 15 min at 4 °C. The supernatant was removed and the pellet suspended in 110 μL of 1 M piperidine in H_2O. After 20 min at 90 °C, the piperidine solution was cooled on ice, vacuum dried for at least 6 h, and dissolved in 10 μL to give a 10 000 cpm/μL solution.

RESULTS

Synthesis of Modified 2′-Deoxyuridines. The strategy for the synthesis of the electrophilic ODNs was to introduce 5-(ω-aminoalkyl)-2′-deoxyuridines into the ODNs via conventional DNA synthesis and subsequently couple the amine-modified ODN with the activated ester of a haloalkanoic acid. Synthesis

of the 5-(ω-aminoalkyl)-2′-deoxyuridines (**5a** and **5b**) commenced with palladium-catalyzed coupling of phthalimidoalkynes (Gibson & Benkovic, 1987; Hobbs, 1989; Haralambidis et al., 1987; Shiau et al., 1980) (**2a** and **2b**) to 5-iodo-2′-deoxyuridine (**3**) (see Scheme 1). The phthalimide protecting group has been used by Benkovic and Gibson (1987) in a palladium-mediated coupling and in conventional DNA synthesis. Preparation of **2a** and **2b** by direct conversion from alkynols (**1a** and **1b**) using the DEAD methodology (Mitsunobu, 1981) was employed because the starting materials are readily available. The procedure of Hobbs (1989) was adapted to couple the phthalimidoalkynes to **3** using tetrakis(triphenylphosphine)palladium(0) with copper iodide to give **4a** and **4b** in 50 and 78% yields, respectively. Reduction of **4a** and **4b** by catalytic transfer hydrogenation with cyclohexadiene in the presence of palladium hydroxide on carbon (Hannessian et al., 1981) gave the nucleosides **5a** and **5b**.

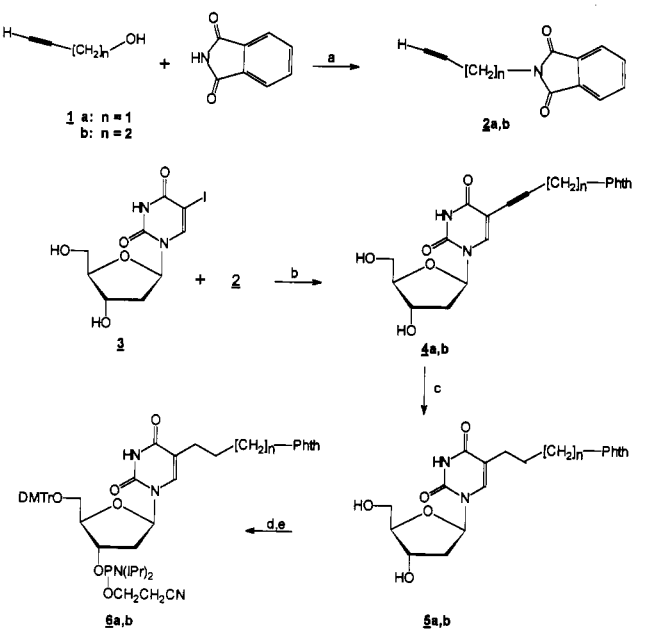
Preparation of 5-[[*N*-(9-fluorenylmethoxycarbonyl)amino]-methyl]-2′-deoxyuridine (**8**) (see Scheme 2) was accomplished in 5% overall yield from thymidine with no attempt made to improve yields. Synthesis started with preparation of 3′,5′-di-*O*-acetylthymidine (**7**) by adapting a procedure for 3′,5′-di-*O*-acetyl-2′-deoxyuridine (Robbins et al., 1986). The protected thymidine **7** was brominated as described by Barwolff and Langen (1975), giving a mixture of starting material, 5-(bromomethyl)- and 5,5-bis(bromomethyl)-3′,5′-di-*O*-acetyl-2′-deoxyuridine. This step accounted for most of the product loss. The reaction mixture was treated with ammonium hydroxide in a bomb at room temperature for 16 h. Treatment of the crude reaction containing 5-(aminomethyl)-2′-deoxyuridine with Fmoc-Cl in a 10% sodium bicarbonate solution gave **8**.

The protected 5-(ω-aminoalkyl)-2′-deoxyuridines **5a,b** and **8** were converted to the corresponding 5′-(dimethoxytrityl) 3′-*O*-cyanoethyl diisopropylphosphoramidites by standard methods (Jones, 1984). The 5-(Fmoc-aminomethyl)-2′-deoxyuridine **8** was protected with dimethoxytrityl chloride in the presence of 4-(dimethylamino)pyridine to avoid base-mediated removal of the Fmoc by triethylamine.

Synthesis of Modified Oligodeoxynucleotides. Amidites of the modified 2′-deoxyuridine derivatives (**6a,b** and **9**) were used in an automated synthesizer using standard 1-μmol scale protocols. The amidites **6a** and **6b** were used on a Milligen 7500 synthesizer, and the Fmoc amidite **9** was used on an ABI 380B synthesizer, which does not use triethylamine in the standard system washes. On either instrument, the coupling yields based on trityl release for all of the modified nucleosides were equivalent to the commercial thymidine amidite (>99%). The Fmoc and phthalimide groups were removed during routine ammonia treatment (45 °C for 16 h). The ammonia solutions containing the DMT ODN were directly purified on a polystyrene (PRP-1) column (Germann et al., 1987). After detritylation, the ODNs were purified further by C_{18} HPLC.

Reaction of the amine-modified ODNs at a concentration of 1–1.5 mg/mL with a 100-fold molar excess of *N*-hydroxysuccinimidyl haloalkanoate (**10**) (see Scheme 3) in 0.1 M sodium borate (pH 8.3) at room temperature caused complete disappearance of starting material within 1 h. Electrophilic ODN formation in the reaction was followed by HPLC and indicated by consumption of the amine-modified ODN starting material and appearance of a product peak (see Figure 1). The reactivity of the electrophilic moiety on the ODN was indicated by its conversion to a new product peak after treatment with sodium thiophosphate. Initially, the electrophilic ODNs were purified by chromatography. Both gel

Scheme 1:[a] Synthesis of Protected 5-(3-Aminopropyl)- and 5-(4-Aminobutyl)-2′-deoxyuridine Phosphoramidites

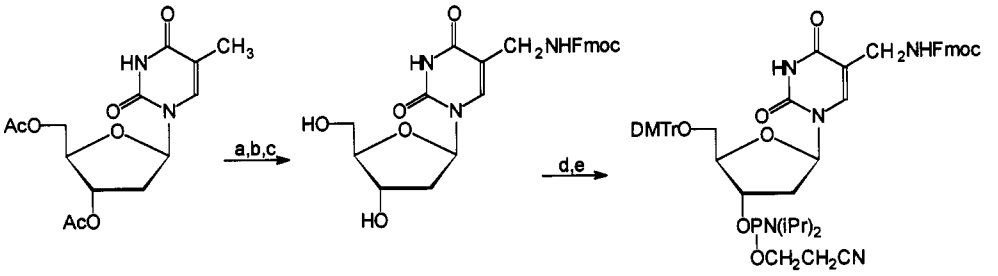


[a] Reagents: (a) Ph_3P, DIAD; (b) $(Ph_3P)_4Pd(0)$, CuI, Et_3N; (c) $Pd(OH)_2$/carbon, cyclohexadiene; (d) DMTr-Cl, DMAP; (e) CNEt-P-$(N(iPr)_2)_2$.

filtration and reversed-phase HPLC resolved the reaction mixture; however, the electrophilic ODNs degraded upon lyophilization, and the ODNs purified by HPLC were too dilute to be used in hybridization experiments. Precipitation of the electrophilic ODNs from the reaction mixture provided reactive ODNs that were >90% pure and adequate for hybridization experiments. The purity of the electrophilic ODNs was improved further to >95% by isolation of the product from the reaction mixture by centrifugal ultrafiltration with a Centricon-3 microconcentrator. The precipitated or centrifugally ultrafiltered haloacetamidoalkyl ODNs were diluted with deionized water to 0.5–1.5 mg/mL and stored at −20 °C for up to 6 months with no loss of activity. The 4-bromobutyramidoalkyl ODNs, which could undergo intramolecular cyclization, were significantly less stable upon storage.

Analysis of Cross-Link Formation. The nucleotide sequence within the vicinity of the potential cross-linking site and both the structure and electrophilicity of the haloacylamidoalkyl group were assessed as factors in the cross-link reaction. Figure 2 summarizes the ODN sequences, and Figure 3 summarizes the haloacylamidoalkyl structures used in this study. The electrophilic ODNs **Ia–j** listed in Table 1 were hybridized to the complementary target **V** and contained, respectively, 5-(aminomethyl)-, 5-[3-(aminopropyl)]-, or 5-[4-(aminobutyl)]-2′-deoxyuridines linked to chloro-, bromo-, and iodoalkanamido groups at position **U**. A second set of electrophilic ODNs was designed for studying the effect of a mismatch on cross-linking. In this group, summarized in Table 2, the electrophilic ODN **Id** was hybridized to a series of target ODNs (**VI, IX–XII**), each of which contained a single mismatch. Table 3 summarizes a third set of electrophilic ODNs (**I–IVd**), each containing a different nucleotide at position **Y** and hybridized to complementary targets (**V–VIII**) to examine how cross-linking was affected by the identity of the spanned base pair. In these latter two studies the electrophilic ODN **Id** contained 5-[(3-bromoacetamido)-propyl]-2′-deoxyuridine at position **U**.

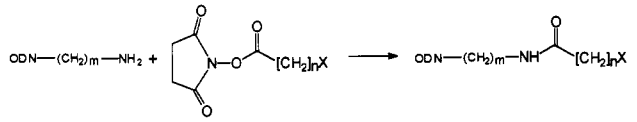
Scheme 2:[a] Synthesis of Protected 5-(Fmoc-aminomethyl)-2′-deoxyuridine Phosphoramidite



7 8 9

[a] Reagents: (a) Br_2, hv; (b) NH_3 bomb; (c) Fmoc-Cl, 10% Na_2CO_3; (d) DMTr-Cl, DMAP; (e) CNEt-P-$(N(iPr)_2)_2$.

Scheme 3: Synthesis of Electrophilic ODNs



Amine-modified ODNs 10 a: X = Cl, n = 1; b: X = Br, n = 1; c: X = I, n = 1; d: X = Br, n = 3 I-IV

The analysis of sequence specificity and extent of cross-link formation by the electrophilic ODNs **I–IV** was assessed by adapting procedures used in the Maxam–Gilbert method of sequencing DNA (Wilkins, 1985). The cross-linking ODNs prepared for this study were targeted at the N-7 group of guanines on the target strand, and the base on which the cross-linking alkylation had occurred was determined by comparing the target strand fragments resulting from crosslinkage and subsequent cleavage to the G-lane of the Maxam–Gilbert method.

The hybridization experiments were conducted at 37 °C with a target ODN concentration of 1.1 μM and a 10-fold molar excess of electrophilic ODN in 130 mM saline buffered with 10 mM phosphate (pH 7.2). The T_m for the hybrids formed under these conditions was calculated to be near 47 °C, and no secondary structure or self-hybridization for any of the ODNs was predicted by computer analysis (see Materials and Methods).

The events that follow the hybridization of a cross-linking ODN to its complement ultimately result in the cleavage of the target ODN at the site of cross-linkage. The alkylated guanine on the ^{32}P-kinased ODN target undergoes depurination, followed by strand scission of the target. In our previous paper (Meyer et al., 1989), we showed the course of this reaction by PAGE analysis of the reaction mixture run at various temperatures. In this work, the reactions were driven to strand scission at cross-linked sites by treatment of the hybrids with 1 M piperidine at 90 °C for 20 min. This treatment, which simplifies identification of cross-link formation, concomitantly inactivates unreacted electrophilic ODNs. In the absence of an electrophilic ODN, treatment of the target ODNs with piperidine did not cause strand scission (data not shown). The products from piperidine treatment were stored until an entire series of time points had been collected for analysis by PAGE. The ratio of radiolabeled bands corresponding to fragmented vs intact target ODN gave the extent of reaction.

The rate of cross-link formation was determined by plotting the amount of cross-linked product as a percentage of the total target ODN vs time. The amount of target ODN was determined by measurement of Cerenkov counts of bands or lanes excised from the polyacrylamide gel. The site specificity of cross-link formation was determined by comparison of the electrophoretic pattern of cross-link fragments to the pattern produced using dimethyl sulfate alkylation.

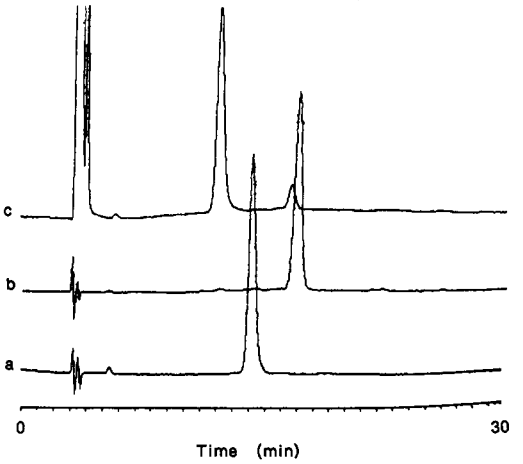


FIGURE 1: Analytical HPLC traces were performed as described under Materials and Methods. (a) Purified 5-(3-aminopropyl)-2′-deoxyuridine containing oligonucleotide; (b) purified 5-(3-iodoacetamidopropyl)-2′-deoxyuridine containing oligonucleotide **Ie**; (c) adduct formation of the iodoacetamido oligonucleotide **Ie** with sodium thiophosphate.

```
                                15   10    5   1
Electrophilic ODNs I-IV:          3'-ATT GAT CCY TTG GGT-5'
Target ODNs V-XII:  5'-C TCT GGC TAA CTA GGZ AAC CCA CTG CTT AA-3'
                    1     5     10    15    20    25    30
```

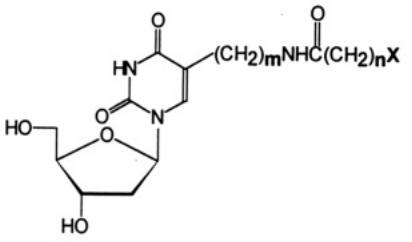
U = 5-(ω-(ω-haloacylamido)alkyl)-2′-deoxyuridine at position 6 in electrophilic ODNs I-IV.

Electrophilic ODN	Y (position 7)
I	C
II	T
III	G
IV	A

Target ODN	Z (position 16)	Other sequence substitutions
V	G	none
VI	A	none
VII	C	none
VIII	T	none
IX	G	A for G at position 14
X	G	A for G at position 15
XI	G	G for A at position 17
XII	G	T for A at position 17

FIGURE 2: Oligodeoxynucleotides used to study cross-link formation.

Relationship of Haloacylamidoalkyl Structure to Regiospecificity and Extent of the Cross-Linking Reaction. Both



Haloacylamidoalkyl Designation	Haloacylamidoalkyl Composition		
	m	n	X
a	1	1	Br
b	1	3	Br
c	3	1	Cl
d	3	1	Br
e	3	1	I
f	3	3	Br
g	4	1	Cl
h	4	1	Br
i	4	1	I
j	4	3	Br

FIGURE 3: 5-[ω-(ω-Haloacylamido)alkyl]-2′-deoxyuridines used to study cross-link formation.

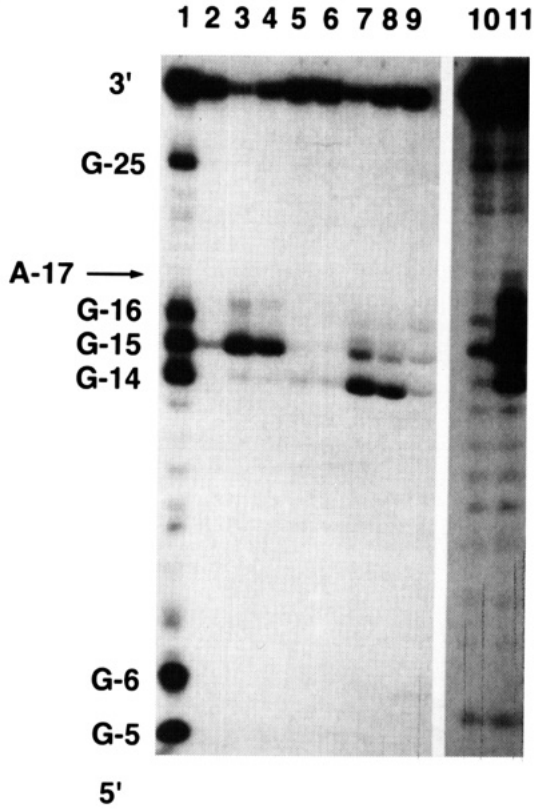


FIGURE 4: Electrophoretic analysis of cleavage products formed after 24 h at 37 °C from the reaction of electrophilic ODNs with a complementary ^{32}P-labeled target (**V**). Lane 1 is a G-specific sequencing lane for ODN **V**; in the hybrid A-17 is hydrogen bonded to U-6, and the haloacetamide tail of that base is positioned in the vicinity of G-14 through G-16. The electrophilic haloacylamidoalkyl group of the cross-linking ODN was varied as follows: lane 2, 3-(chloroacetamido)propyl (**Ic**); lane 3, 3-(bromoacetamido)propyl (**Id**); lane 4, 3-(iodoacetamido)propyl (**Ie**); lane 5, 3-(4-bromobutyramido)propyl (**If**); lane 6, 4-(chloroacetamido)butyl (**Ig**); lane 7, 4-(bromoacetamido)butyl (**Ih**); lane 8, 4-(iodoacetamido)butyl (**Ii**); lane 9, 4-(4-bromobutyramido)butyl (**Ij**); lane 10, bromoacetamidomethyl (**Ia**); lane 11, 4-(bromobutyramido)methyl (**Ib**). Lanes 10 and 11 are overexposed; on the basis of band counting, the reaction in lane 10 is insignificant, while the reaction in lane 11 is over 75% G-15 specific.

the specificity and the extent of intramolecular cross-link formation in the oligomeric duplex were dependent on the structure of the electrophilic haloacylamidoalkyl group and on the nature of the leaving group. The sequence of the target ODN **V** was chosen so that there were three guanines on the target strand immediately 5′ to the A which is hydrogen bonded to the modified U in the cross-linking oligomer. In this system, a suitable target site (the highly nucleophilic N-7 of guanine) was available at three different positions, and the optimum site of cross-linking could be determined for different haloacylamidoalkyl groups. Figure 4 shows the results of how the structure of the haloacylamidoalkyl group modulates the ability of the electrophilic ODN to cross-link within a hybrid to a guanine on the target strand. Both the position and extent of cross-link formation as a function of haloacylamidoalkyl length, haloacylamidoalkyl composition, and electrophile type were evaluated using ODNs **Ia–j**, which have the same sequence and only vary in the structure of the haloacylamidoalkyl group attached to the 5-position of U-6. The influence of haloacylamidoalkyl structure on cross-link formation was determined by measuring the amount of cross-linked product present after 24 h at 37 °C; the results are summarized in Table 1.

The haloacylamidoalkyl groups that were examined contain four to nine atoms between the uracil base and the halogen and are listed in Table 1. ODN **Id**, with a six-atom 3-(bromoacetamido)propyl group, cross-linked almost exclusively to **V** at G-15, the complementary site two base pairs removed from U-6. Another type of six-atom haloacylamidoalkyl group, the (4-bromobutyramido)methyl **Ib**, cross-linked predominantly at this position, although not with quite the remarkable specificity of **Id**. For the seven-atom haloacylamidoalkyl group, the 4-(bromoacetamido)butyl **Ih**, the predominant site of cross-link formation shifted to the guanine (G-14) three base pairs removed from U-6, with minor amounts of cross-link formed to G-15. The longest haloacylamidoalkyl group tested, the nine-atom 4-(4-bromobutyramido)butyl group in **Ij**, formed minor amounts of cross-linked product at all three guanines.

Interestingly, the ODN with the shortest haloacylamidoalkyl group (**Ia**), a four-atom bromoacetamidomethyl group, reacted poorly with the target even though the bromoacetamide was the most reactive group used in this study. By contrast, the less reactive 4-bromobutyramide (**Ib**) was an effective crosslinker, demonstrating that the correct geometry of the acylamidoalkyl group is critical and that the bromoacylamides do not significantly alkylate DNA nucleophiles unless they are positioned correctly for an intramolecular reaction. Other unreactive groups tested were the eight-atom sidearm (**If**) and the chloroacetamide sidearms (**Ic** and **Ig**), which did not form detectable amounts of cross-linked products. Both iodoacetamides **Ie** and **Ii** reacted about half as fast as their bromine counterparts. A similar relative rate of alkylation for a bromoacetamide and an iodoacetamide has also been reported by Baker and Dervan (1989). The reaction rates for the more reactive ODNs were determined by conducting time courses and ranked as follows: 3-(bromoacetamido)propyl (**Id**) > 4-(bromoacetamido)butyl (**Ih**) > 3-(iodoacetamido)propyl (**Ie**) > 4-(iodoacetamido)butyl (**Ii**).

Table 1: Effect of Composition of the 5-[ω-(ω-Haloacylamido)alkyl]-2′-deoxyuridine on the Extent of Cross-Link Formation in a Duplex Formed by Electrophilic ODN I and Target ODN V[a]

haloacylamidoalkyl designation	haloacylamidoalkyl composition			% reaction in 24 h
	m	*n*	X	
Ia	1	1	Br	<1
Ib	1	3	Br	31
Ic	3	1	Cl	<5
Id	3	1	Br	80
Ie	3	1	I	55
If	3	3	Br	<1
Ig	4	1	Cl	<1
Ih	4	1	Br	68
Ii	4	1	I	44
Ij	4	3	Br	<5

[a] These results include reaction with all 3 targeted G's using electrophilic ODNs isolated by method A. The electrophilic ODNs were purified by butanol precipitation (see Materials and Methods). The cross-linking regions of the hybrid used for these studies were as follows: 3′-TCCC*U*, positions 10 through 6 of the electrophilic ODNs Ia–j oriented in the 3′ to 5′ direction. 5′-AGGGA, positions 13 through 17 of the target ODN V oriented in the 5′ to 3′ direction. The complete ODN sequences are listed in Figure 2. The structure of U is listed in Figure 3.

Table 2: Effect of a Single Mismatch on the Extent of Cross-Link Formation in a Duplex Formed by Electrophilic ODN **Id** and Target ODNs VI and IX–XII[a]

target ODN	reactive region of hybrid	% reaction in 5 h
V	3′-TCCCU 5′-AGGGA	36
IX	3′-TcCCU 5′-AaGGA	4
X	3′-TCcCU 5′-AGaGA	2
VI	3′-TCCcU 5′-AGGaA	7
XI	3′-TCCCu 5′-AGGGg	15
XII	3′-TCCCu 5′-AGGGt	10

[a] The mismatched bases are in lower case. ODN **Id** was purified by butanol precipitation (see Materials and Methods). Top sequence: Positions 10 through 6 of the electrophilic ODN **Id** oriented in the 3′ to 5′ direction. Bottom sequence: Positions 13 through 17 of the target ODNs oriented in the 5′ to 3′ direction. The complete ODN sequences are listed in Figure 2. The composition of the haloacylamidoalkyl group in electrophilic ODN **Id** is $m = 3$; $n = 1$; X = Br. See Figure 3 for the deoxyuridine core structure.

Effect of Mismatches on Extent of Cross-Link Formation. The effect of a single mismatch on the efficiency of cross-linking was examined by introducing a mismatched base in target ODN V at positions 14–17 and is summarized in Table 2. In all cases, the efficiency of cross-link formation relative to the perfect match was significantly reduced when using electrophilic ODN **Id**. The extent of cross-link information in each of the modified sequences after a 5-h incubation is shown in Table 2. A single guanine to adenine substitution, creating an A:C mismatch in the duplex, was introduced at position 14, 15, or 16 in ODNs **IX**, **X**, and **VI**, respectively. Two substitutions, a guanine or a thymine for the adenine at position 17 that is normally paired with the cross-linking U in ODN **Id**, were also evaluated. These mismatches (**XI** and **XII**) had less impact than the others examined, giving a 2.4–3.6-fold reduction in cross-link formation.

Effect of Sequence on Rate of Cross-Link Formation. Molecular modeling studies (see Figure 5) in our laboratories showed that the amide linkage that occurs in all of the haloacylamidoalkyl groups we examined has the potential of hydrogen bonding to the first base pair proximal to the modified U (3′-side on the cross-linking ODN, 5′-side on the target ODN). Furthermore, these modeling studies predicted that the hydrogen bond formed by the 3-(bromoacetamido)propyl group in **Id** using our initial test sequence (5′-UCCC-3′) held the electrophilic center of the acetamide furthest away from the guanine we were targeting when compared to the sequences containing the three other possible base pairs at the same intervening position. Our modeling studies predicted that if the 5′-UGCC-3′ sequence was used in the cross-linking ODN (with appropriate complementarity in the target), the H-bonding to the intermediate base pair would facilitate the cross-linking reaction relative to **Id** by holding the electrophilic center closer to the targeted G.

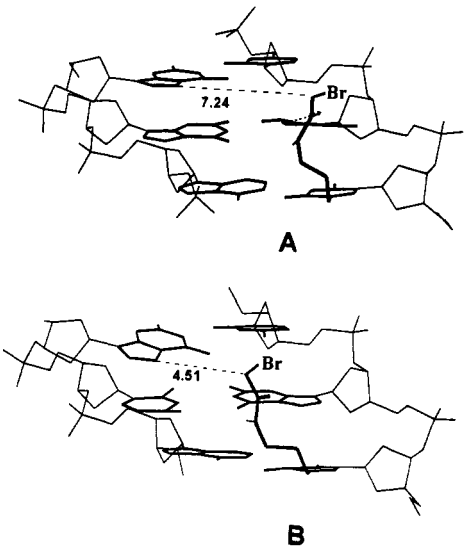


FIGURE 5: Effect of hydrogen bonding on the distance (shown in angstroms) between the electrophilic site of the side chain and the N-7 of the targeted guanine. The bromoacetamidopropyl side chain is H-bonded to the base pair between the substituted deoxyuridine and the site of alkylation. (A) Portion of minimized structure of duplex with a 5′-GGA sequence in the target strand (**V**, on left) and a 5′-(5-(3-bromoacetamido)propyl)UCC sequence in the cross-linking strand (**Id**, on right). (B) Portion of minimized structure of duplex with a 5′-GCA sequence in the target strand (**VII**, on left) and a 5′-(5-(3-bromoacetamido)propyl)UGC sequence in the cross-linking strand (**IVd**, on right).

Table 3 shows the effect of sequential substitution of each of the four possible base pairs into the position between the modified U-6 in the ODN **Id** and the site of cross-linking at G-15 on the target ODN. The electrophilic ODNs contained 5-(3-bromoacetamidopropyl)-2′-deoxyuridine at position U-6 and each of the possible other bases (5′-U[C,A,T,G]CC-3′) at position **Y**. The site of cross-linking in the complementary target ODN was found to be the same with each substitution. When the electrophilic ODN contained G instead of C at position **Y** in its sequence, the cross-linking half-life was reduced from 4.9 to 1.3 h. The molecular modeling studies described above provide a possible explanation for the enhanced reaction rate.

DISCUSSION

Our interest in the design of oligodeoxynucleotides bearing chemically cross-linking functionalities has been prompted by their potential application as exceptionally active antisense and antigene drugs. Traditionally, the ability to form a covalently linked drug–receptor complex leads to enhanced therapeutic potency. In the case of antisense ODNs, covalent

Table 3: Effect of Nucleotide Sequence in the Reactive Region of the Hybrid on the Rate of Cross-Link Formation by Electrophilic ODNs I–IVd[a]

ODN hybrid			
electrophilic ODN	target ODN	reactive region of hybrid	$T_{1/2}$ (h)
Id	**V**	3′-TCCcU 5′-AGGgA	4.9
IId	**VIII**	3′-TCCaU 5′-AGGtA	3.3
IIId	**VI**	3′-TCCtU 5′-AGGaA	2.0
IVd	**VII**	3′-TCCgU 5′-AGGcA	1.3

[a] The site of base pair substitution is in lower case. The electrophilic ODNs were purified by centrifugal ultrafiltration (see Materials and Methods). Top sequence: positions 10 through 6 of the electrophilic ODN oriented in the 3′ to 5′ direction. Bottom sequence: positions 13 through 17 of the target ODNs oriented in the 5′ to 3′ direction. The complete ODN sequences are listed in Figure 2. The composition of the haloacylamidoalkyl group in electrophilic ODNs **I–IVd** is $m = 3$; $n = 1$; X = Br. See Figure 3 for the deoxyuridine core structure.

linkage of the DNA–RNA heteroduplex would be expected to provide an RNase H independent pathway for the blockage of translation. This could expand the utility of methyl phosphonate and α-anomeric ODNs, which form RNase H resistant heteroduplexes (Inoue et al., 1987; Gagnor et al., 1987); it could also facilitate the targeting of translated sequences, which up to now has been problematic due to the displacement of antisense ODNs by actively translating ribosomes. Similarly, cross-linkage of the triple-stranded complexes formed by antigene ODNs with duplex DNA should create blocks to transcription and replication (Young et al., 1991; Duval-Valentin et al., 1992).

To realize these goals, the modified ODNs must meet certain strict criteria. First, the chemical cross-linker should be fairly nonreactive so as to minimize spurious reactions with nontarget biomolecules, including self-alkylation. Second, both the hybridization event and the cross-linking reaction should be perfectly sequence-specific. And third, proper positioning of the cross-linking group within the hybrid should lead to a half-life of reaction on the order of minutes (for antisense applications) or hours (for antigene applications). An additional practical requirement is that the ODNs be reasonably simple to prepare. In general, chemical cross-linkers coupled to antisense ODNs to date fail to meet one or more of these criteria. The modifications described here, while not optimal, represent a partial solution to each of the requirements listed above for chemically cross-linking antisense ODNs.

In the present study, we have shown that the rate of crosslink formation of an oligodeoxynucleotide containing certain types of 5-[ω-(ω-haloacylamido)alkyl]-2′-deoxyuridine is highly dependent on the structure and reactivity of this group and the nucleotide sequence in the vicinity of the modified deoxyuridine. These and perhaps additional parameters may allow further optimization of the reaction rate.

The 5-(ω-aminoalkyl)-2′-deoxyuridines with aminomethyl, -propyl, and -butyl groups were prepared, protected, and converted to phosphoramidites for standard automated DNA synthesis. After deprotection, the aminoalkyl ODNs, when treated with a 100-fold mole excess of activated ester of a haloacyl moiety, quantitatively formed the electrophilic ODN. These reagents could be purified and were sufficiently stable when stored frozen in solution to keep for several weeks. The reactivity of samples stored for extended periods was routinely verified by direct HPLC analysis or by reaction with sodium thiophosphate. A detailed study of the self-alkylation of these haloacetamido-modified ODNs and their reaction with biologically relevant nucleophiles will be reported elsewhere.

The length of the flexible haloacylamidoalkyl group impacts the rate and regiospecificity of cross-linking. Under the conditions used, haloacetamide ODNs with six-atom side chains produced cross-links faster than the corresponding ODNs with seven-atom side chains. Within each set of ODNs, the extent of reaction observed with the Cl, Br, and I acetamides probably reflects their inherent reactivities, although in the case of iodine its bulk may hinder reaction. A similar halogen electrophile reactivity pattern has been observed by Baker and Dervan (1989).

On the basis of molecular modeling studies (Figure 5A), a hydrogen bond appears to be formed between the amide carbonyl of the side-chain acetamide in ODN **Id** and the N-4 of the adjacent intrastrand deoxycytidine. Changing the 5′-UCCC-3′ sequence of **Id** to the 5′-UGCC-3′ sequence of **IVd** (and altering the target accordingly to maintain complementarity) (Figure 5B) allowed us to determine whether hydrogen bond formation with the oxygen of the 6-position of the adjacent intrastrand deoxyguanosine could be used to our advantage, by moving the electrophilic site closer to the nucleophilic N-7 position of the target guanine residue. As shown in Table 3, this was indeed the case, with a 4-fold increase in the rate of cross-linking being obtained. Figure 5 is a view of the minimized conformation of the bromoacetamidopropyl group in the two hybrids, showing that the electrophilic carbon in the **IVd/VII** hybrid is significantly closer to the target nucleophilic site than in the **Id/V** hybrid. It is possible that differences in the immediate environment of the helix major groove may also account for some of the difference in the rates of reaction.

Using complementary DNA targets, the best reaction half-life achieved in the current study is 1.3 h. Molecular modeling studies employing RNA targets (data not shown) suggest that cross-linkage in heteroduplexes should proceed at approximately the same rate since positioning of the 3-(bromoacetamido)propyl group in the major groove is very similar for both duplexes. While such a cross-linkage rate would be suboptimal for an antisense ODN, there is reason to expect that more rapidly cross-linking ODNs could be developed by applying the systematic approach described here to other conjugated electrophiles.

ACKNOWLEDGMENT

We thank Theresa Martinez for preparation of oligonucleotides, A. David Adams for preparation of phosphoramidites, and Drs. Michael Reed and Charles Petrie for helpful discussions.

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