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Covalent cross-linking of duplex DNA using 4-thio-2′-deoxyuridine as a readily modifiable platform for introduction of reactive functionality into oligonucleotides

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ABSTRACT

Full details of the template-directed covalent cross-linking of duplex oligodeoxynucleotides are presented. 4-Thio-2′-deoxyuridine was incorporated synthetically into a 17mer oligodeoxynucleotide, and the thiocarbonyl group of the modified base was alkylated with a variety of α-bromoacetyl-derivatized diamines. Covalent cross-linking was initiated by annealing the electrophilic probe oligomers with their complementary sequences, where a dG base was targeted at the position complementary to the modified 4-thio-2′-deoxyuridine. The sequence selectivity of cross-link formation as a function of tether topology and rigidity was examined, and the thermal stability of the modified duplexes was measured by UV melting experiments.

INTRODUCTION

In 1967, Grineva and co-workers published a communication describing a novel method for the covalent modification of oligonucleotides, using what they described as ‘complementary addressed modification’ (1). In this protocol, a chemically reactive group is covalently linked to the end of a synthetic oligonucleotide, which upon duplex formation induces modification of the complementary strand. In the intervening 30 years, this basic protocol has been expanded upon by a large number of workers, for a variety of purposes (2).

A number of workers have used the Grineva concept to effect covalent cross-linking or modification of DNA. Particularly pertinent examples include those of Vlassov using a 3′ or 5′-tethered *N*-(2-chloroethyl)arylamines (3–5), Summerton and Bartlett (6) with a tethered α-bromoketone, Matteucci (7) with an *N*,*N*-ethanocytosine, Dervan (8–10) with an α-haloacetamido group within a triple-helical complex, and Ohtsuka (11) and Tabone and co-workers (12) using an α-haloacetamide system within duplex DNA. These workers have achieved varying degrees of success in effecting covalent cross-linking, ranging from 1–2% to essentially quantitative. In addition, there are a number of reports from Helénè (13), among several others, wherein intercalating or photochemically active groups have been tethered to oligonucleotides. The principal chemical feature differentiating these studies is whether the reactive group is tethered to the end of the oligonucleotide or is appended via the heterocyclic bases.

As part of these developments, and in conjunction with studies on the molecular mechanism of carcinogen action and biophysical studies on oligonucleotide structure, a diverse and extensive array of non-natural nucleosides and nucleoside surrogates have been incorporated into synthetic strands of DNA and RNA (14–17). Of direct relevance to our studies, purine and pyrimidine-based nucleic acids containing thiocarbonyl groups have been incorporated synthetically into oligonucleotides (18–26). The thiocarbonyl group gives these oligomers unique properties by virtue of the enhanced nucleophilicity of sulfur compared to the oxygen and nitrogen nucleophiles normally present in DNA and RNA. We have used this reactivity to incorporate tethers site-specifically onto 4-thio-2′-deoxyuridine (27,28) and 6-thio-2′-deoxyinosine bases (29) via chemoselective S-alkylation of the thiocarbonyl groups.

We have developed a protocol for covalent cross-linking of DNA that is based on introduction of chemically reactive functionality into probe oligonucleotides by post-synthetic S-alkylation of thionucleosides. We have reported our preliminary studies (30) on the template-directed cross-linking of duplex DNA using a 4-thio-2′-deoxyuridine ($d^{S4}U$) base to append an electrophilic cross-linking tether. In these studies, we demonstrated a method to form covalent lesions at the N7 position of complementary 2′-deoxyguanosine residues. We have more thoroughly explored this cross-linking reaction, and now we provide full details of our studies on the covalent cross-linking of duplex DNA using oligonucleotide probes to direct chemically reactive functionality to targeted sequences of nucleic acids.

MATERIALS AND METHODS

DNA synthesis and post-synthetic modification

Oligonucleotides were prepared as previously described using an Applied Biosystems 380B DNA Synthesizer with commercially available reagents and protocols. For the incorporation of 4-thio-2′-deoxyuridine, our *S*-cyanoethyl phosphoramidite (31) was coupled at the desired position. Synthetic oligonucleotides and

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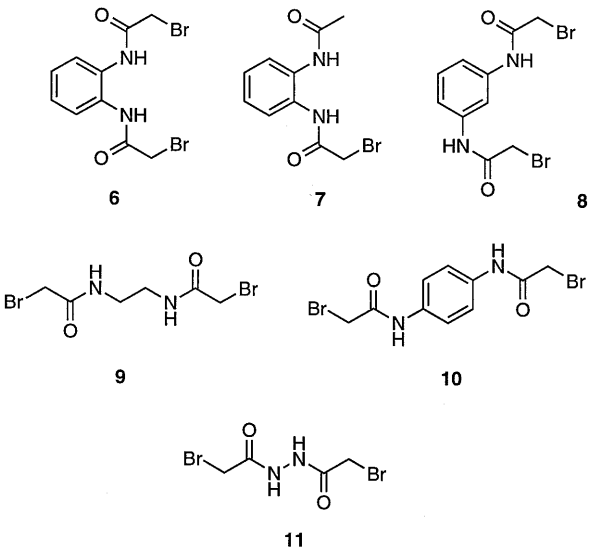


Scheme 1.

postsynthetically modified oligomers were characterized as described previously, by enzymatic digestion and chromatographic analysis (32). All oligonucleotides were purified by reverse-phase HPLC on a Hamilton PRP-1 column (4.6 mm × 25 cm).

Synthesis of electrophilic tethers

Electrophilic tethers (**6–11**, Scheme 1) were prepared by acylation of the corresponding commercially available diamines with bromoacetyl bromide (Et_3N, THF). The 'half-electrophile' **7** was prepared in a similar manner by first monoacylating *ortho*-phenylenediamine with acetic anhydride, then acylating the remaining amino group with bromoacetyl bromide. The design of tethers **6–11** incorporated variations in spacing between electrophilic sites and conformational flexibility of the intervening atoms.

N,N′-bis-bromoacetyl-1,2-diaminobenzene (6)

^{1}H NMR (300 MHz, acetone-d_6) δ 9.72 (s, 2H, CONH), 7.52 (dd, J = 6.0, 3.6 Hz, 2H, ArH), 7.22 (dd, J = 6.0, 3.6 Hz, 2H, ArH), 4.12 (s, 2H, $COCH_2Br$); ^{13}C NMR (100 MHz, acetone-d_6) δ 165.7, 130.8, 126.1, 125.4, 30.7; HRMS, *m*/z calcd for $C_{10}H_{10}N_2O_2{}^{79}Br_2$: 347.9109; found: 347.9104.

N-acetyl-*N′*-bromoacetyl-1,2-diaminobenzene (7)

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.62 (s, 1H, CONH), 9.43 (s, 1H, CONH), 7.60–7.45 (m, 2H, ArH), 7.20–7.12 (m, 2H, ArH), 4.13 (s, 2H, $COCH_2Br$) 2.08 (s, 3H, $COCH_3$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.7, 165.0, 130.7, 130.0, 125.3, 124.9, 124.7, 30.3, 23.5;. HRMS, calcd for $C_{10}H_{11}N_2O_2{}^{79}Br$: 270.0004; found: 270.0005.

N,N′-bis-bromoacetyl-1,3-diaminobenzene (8)

^{1}H NMR (200 MHz, DMSO-d_6) δ 10.42 (s, 2H, CONH), 7.94 (m, 1H, ArH), 7.29 (m, 3H, ArH), 4.02 (s, 4H, $COCH_2Br$); ^{13}C NMR (60 MHz, DMSO d_6) δ 164.8, 138.9, 129.1, 114.7, 110.7, 30.3; HRMS, *m*/z calcd for $C_{10}H_{10}N_2O_2{}^{79}Br_2$: 347.9109; found: 347.9114.

N,N′-bis-bromoacetyl-1,2-diaminoethane (9)

^{1}H NMR (200 MHz, $CDCl_3$) δ 6.96 (br s, 2H, CONH), 3.90 (s, 4H, $COCH_2Br$), 3.48 (m, 4H, NCH_2); ^{13}C NMR (60 MHz, DMSO d_6) δ 166.6, 40.1, 28.7; HRMS, *m*/z calcd for $C_6H_{10}N_2O_2{}^{79}Br_2$: 300.9010; found: 300.9016.

N,N′-bis-bromoacetyl-1,4-diaminobenzene (10)

^{1}H NMR (250 MHz, DMSO-d_6) δ 10.35 (s, 2H, CONH), 7.57 (s, 4H, ArH), 4.03 (s, 4H, $COCH_2Br$); ^{13}C NMR (60 MHz, acetone-d_6) δ 164.7, 134.7, 119.9, 30.5.; HRMS, *m*/z calcd for $C_{10}H_{10}N_2O_2{}^{79}Br_2$: 347.9109; found: 347.9108.

N,N′-bis-bromoacetylhydrazine (11)

^{1}H NMR (250 MHz, DMSO-d_6) δ 10.6 (s, 2H, CONH), 3.92 (s, 4H, $COCH_2Br$); ^{13}C NMR (60 MHz, DMSO d_6) δ 164.4, 26.9; HRMS, *m*/z calcd for $C_4H_6N_2O_2{}^{79}Br_2$: 271.8796; found: 271.8802.

Covalent modification of $d^{S4}U$-containing oligomers

DNA (5 µl, 60 OU_{260}/ml, 0.3 OU_{260} total) was diluted to 95 µl with 0.1 M K_2HPO_4/KH_2PO_4, pH 8.0. A solution of the electrophile was prepared (~1 mg in 30 µl of DMF) and this was added to the solution of DNA. The sample was allowed to sit at room temperature for 1.5 h. The reaction was terminated by passing the sample through a small spun column (0.3 ml of Sephadex G-25) and precipitating with n-butanol. The reaction was dried under vacuum briefly before further use. All electrophilic ODNs were used immediately after preparation.

ΔT_m Measurements

DNA helix–coil transitions were measured on a Beckman DU-660 spectrophotometer equipped with a Peltier melting apparatus, using 10 mm path-length cuvettes and the Beckman software program. A stock solution containing an unmodified oligodeoxynucleotide (ODN) and the complimentary modified or unmodified 4-thio-2′-deoxyuridine containing ODN in equimolar amounts was made. Aliquots were removed from this stock solution and a series of dilutions was made to generate samples ranging in concentration from 6.0×10^{-7} to 9.4×10^{-6} M in total strand concentration. All melting samples had a final volume of 200 µl and were buffered to pH 7.0 using 0.1 M K_2HPO_4/KH_2PO_4 containing 0.1 M NaCl. These samples were warmed at 95°C for 5 min and allowed to cool slowly back to room temperature prior to melting experiments. Samples were loaded into cuvettes and allowed to equilibrate at 15°C for 5 min prior to melting. Melting was performed from 15 to 75°C, with a gradient of 0.5°C/min. Measurements were recorded every 0.2°C. All experiments were done in triplicate, and experimental T_m values varied ≤1°C between runs. In all cases, the shape of the melting curves clearly indicated a two-state system.

Cross-linking studies

DNA complimentary to the modified 4-thio-2′-deoxyuridine containing probe strand [1.2 OU_{260} in 20 µl of distilled, deionized (dd) H_2O] was 5′ end-labeled using polynucleotide kinase (1 µl, 10 U) and [γ-^{32}P]ATP (1 µl, 10 mCi/ml, 6000 Ci/mmol). After 1 h at 37°C, the labeling reaction was warmed at 65°C for 5 min. The reaction was diluted to 100 µl with dd H_2O and the unincorporated label was removed by passing the solution through a spun column

(0.8 ml, Sephadex G-25). The 4-thio-2′-deoxyuridine containing probe DNA (0.2 OU_{260}) was dissolved in 4 µl of 0.1 M K_2HPO_4/KH_2PO_4 (pH 8.0) and 2 µl of the complimentary radiolabeled DNA was added. The solution was incubated at 25°C and was sampled at specified time intervals (0.25–16 h) by removing an aliquot that was mixed with an equal volume of denaturing gel loading buffer. Unless otherwise indicated, cross-linking reactions were run for 16 h at 25°C. Samples were analyzed on a 20% polyacrylamide gel containing 20% formamide and 7 M urea (denaturing PAGE). Sites of cross-linking were determined by treating an aliquot of the reaction (3 µl) with 100 µl of 10% aqueous piperidine and heating for 25 min at 95°C. The solution was lyophilized to dryness and evaporated from 100 µl of H_2O (3×) to remove residual piperidine. This sample was run on a denaturing 20% PAGE gel next to a standard Maxam–Gilbert G-sequencing lane (33). Gels were scanned using a PhosphorImager (Molecular Dynamics) and the bands were quantitated using the ImageQuant™ software provided by the manufacturer. Figures 2–4 were produced by scanning autoradiogram films at 400 d.p.i. on an Apple One Scanner into a PICT file, increasing the brightness and contrast of the resulting image to improve legibility using Adobe Photoshop v. 3.0, and printing the figures as TIFF files on a high-resolution laser printer. The ratio of image intensity to signal over the entire range was linear, and was not effected by electronic processing.

RESULTS

Electrophilic oligonucleotide probes

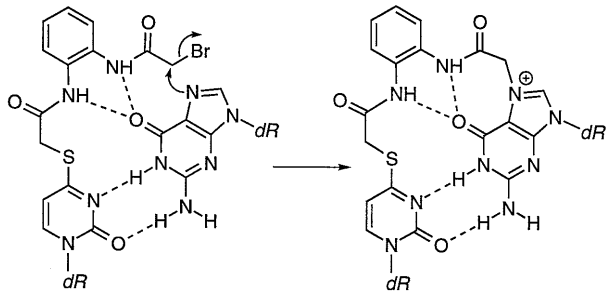
The electrophilic tethers were designed based on our initially successful *ortho*-phenylenediamine system (30). Design criteria included: (i) electrophile reactivity; (ii) length of tether; (iii) topology of electrophile placement; (iv) conformational mobility of tether. We were most successful with α-bromoacetamide electrophiles, using a system where diamine platforms were used to position the electrophile with the desired topology, and this basic protocol provided us with a sufficient diversity of diamine-based tethers with which to explore cross-linking. Increasing tether length was less than effective at achieving reasonable rates and yields of cross-links, and the studies reported herein are limited to simple phenylenediamine and ethylenediamine derivatives. Propylenediamine and its higher homologs were poor tethers in our protocol. Topolology of electrophilic site placement and the tether rigidity played a major role both in effecting the yield and sequence selectivity of cross-link formation, and rather dramatic examples of this can be seen in a comparison of the *ortho*- and *meta*-phenylenediamines, and in the *ortho*-phenylenediamine versus ethylenediamine systems.

Cross-linking

Experiments on covalent cross-linking were performed with the following 17mer duplexes (Scheme 2), where X = 4-thio-2′-deoxyuridine ($d^{S4}U$). In these three systems (**1**·**4**, **2**·**4** and **3**·**4**), the $d^{S4}U$ base was positioned opposite to a 2′-deoxyguanosine (dG) within a sequence derived from the T7 RNA polymerase promoter. We had used this system in the development of our cross-linking protocol, and the presence of three dG residues on the target strand proved optimal for the examination of sequence selectivity of cross-link formation.

Within the duplex **2**·**4**, we observed essentially quantitative cross-link formation with the *ortho*-phenylenediamine system **6**, presumably according to the following mechanism (Scheme 3).

Scheme 2.



Scheme 3.

This cross-linking reaction between duplexes **2** and **4** using the *ortho*-phenylenediamine tether **6** was completely selective for the complementary dG N7, and the reaction occurred in >95% yield over 12 h at 25°C. It was from this highly successful result that we embarked on the studies detailed herein.

Kinetics of cross-link formation

The time course of cross-linking reactions were monitored by both HPLC and denaturing PAGE. The derivatization of the $d^{S4}U$ containing strands **1**, **2** and **3** with bis-electrophiles **6–11** proceeded to completion over the course of 1.5 h, as demonstrated by HPLC.

Upon addition of complimentary strand **5b** to *ortho*-phenylenediamine **6**-derivatized **5a** (Scheme 4) under conditions conducive to hybridization, a new product with HPLC mobility intermediate between that of the two starting strands was formed. This is shown in Figure 1 for duplex **5a**·**5b** with the *ortho*-phenylenediamine tether **6**. After incubation of these two strands at 25°C overnight (data shown), the new peak corresponding to cross-linked material was the major product present.

Isolation of this material by HPLC, 5′ end-labeling with ^{32}P phosphate, and analysis by denaturing PAGE indicated that this new product was the cross-linked duplex. Alternatively, the cross-linked duplex could be purified using denaturing PAGE and then isolated by extraction from the appropriate gel slice. Maxam–Gilbert sequence analysis indicated that the alkylated base was at the complimentary dG.

In the course of optimizing cross-linking reactions, we examined the stability of the electrophilic strands to the reaction conditions. Strand **1** was alkylated with bis-electrophile **6**, the S-alkylated product was isolated, and it was incubated under the conditions used in the cross-linking reaction. Aliquots were removed from the reaction mixture and the amount of active electrophile present was determined by derivatization with

5′ A A T T G G C C T T C X G T A T A 3′ **5a**
3′ T T A A C C G G A A G G C A T A T 5′ **5b**

Scheme 4.

5′ T A A T A C G A X C C A C T A T A 3′ **1**
3′ A T T A T G C T G G G T G A T A T 5′ **4**

Scheme 5.

benzyl mercaptan. HPLC analysis allowed quantitation of the reacted and unreacted DNA, and this was used to calculate the rate at which the electrophilic strand was deactivated under our standard reaction conditions. These experiments indicate that the electrophilic strand generated using **6** had a half-life of ~72 h, and allowed us to feel confident that the lack of quantitative cross-link formation was not a result of non-competent electrophile. Stability studies were not performed with tethers other than **6**.

Additional control studies were performed on the modified strand **1** derivatized with tether **6** in order to determine if a significant amount of intrastrand alkylation occurred. In these studies, oligomer **1** was 5′ end-labeled and alkylated with **6**, and the resulting product was incubated in the absence of complementary DNA under the standard reaction conditions. The sample was treated with piperidine (90°C) and analyzed by denaturing PAGE. No cleavage products were observed, indicating that self-alkylation did not occur at detectable levels.

Sequence selectivity

The sequence selectivity of the cross-link formation was shown to depend on both the duplex sequence in which the $d^{S4}U$ was incorporated and on the identity of the bis-electrophilic tether used in the cross-linking experiment. Cross-linking experiments were performed using duplex **1·4** (Scheme 5) where the $d^{S4}U$ is positioned complementary to the 3′ end of the run of three dG residues (Fig. 2). In the case of tether **6**, in addition to alkylation at the complimentary dG, significant reaction takes place at the dG two bases to the 5′ side (lane 5). Alkylation at these two sites occurs in an ~1:1 ratio in >90% combined yield.

Experiments were conducted with the same sequences using the ethylenediamine-derived bis-electrophile **9**. With the sequence **1·4**, good yields of cross-linking were observed following alkylation of the $d^{S4}U$ of **2** with **9** (60%), and Maxam–Gilbert sequence analysis demonstrated that the reaction occurs almost totally at the dG two bases in the 5′ direction, to the near exclusion of reaction at the dG complimentary to the derivatized $d^{S4}U$ (lane 4). Lengthening the tether as the *meta*-phenylenediamine derivative **8** abolished alkylation at the three dG residues closest to the $d^{S4}U$ residue, but permitted alkylation to occur in moderate yields at the dG four bases to the 5′ side (lane 3). Not surprisingly, the *para*-phenylenediamine based tether **10** was ineffective in the cross-linking of **1·4** (data not shown).

The result obtained with *meta*-phenylenediamine tether **8** within duplex **1·4** was unexpected and was not readily explained by examination of a computer generated model of this system. From such models, it was clear that the tether **8** was too short to form the experimentally observed cross-link without significantly distorting the B-DNA double helix. In practice, however, this system prefers this mode of alkylation over the proximal and apparently more accessible dG residues.

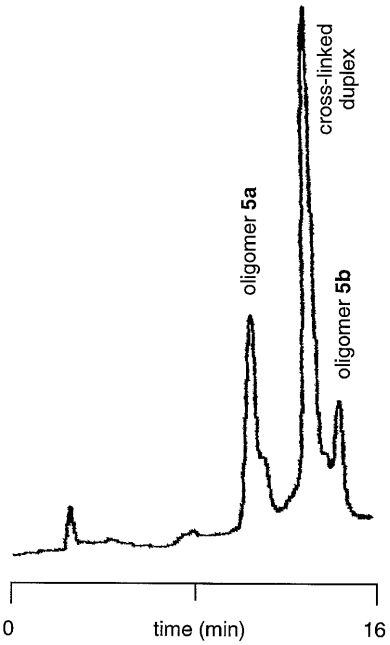


Figure 1. HPLC trace of cross-linking reaction between **6**-derivatized oligomer **5a** and target strand **5b** after 12 h at 25°C.

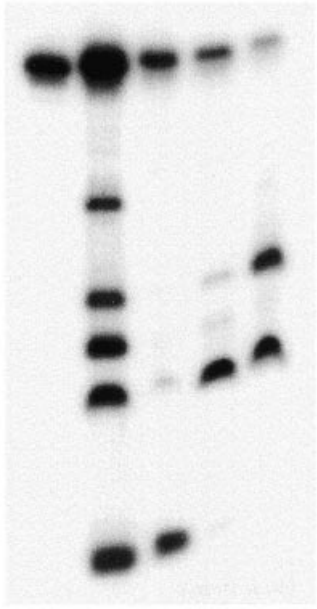


Figure 2. Denaturing PAGE of the cross-linking reaction between derivatized oligomer **1** and ^{32}P end-labeled **4**, after treatment with 1 M piperidine (95°C, 20 min). Lane 1: ^{32}P 5′ end-labeled target strand **4**; lane 2: Maxam–Gilbert G-sequencing reaction; lane 3: cross-linking reaction with *meta*-phenylenediamine tether **8**; lane 4: cross-linking reaction with ethylenediamine tether **9**: lane 5: cross-linking reaction with *ortho*-phenylenediamine tether **6**.

5′ T A A T A C G A C X C A C T A T A 3′ **2**
3′ A T T A T G C T G G G T G A T A T 5′ **4**

Scheme 6.

5′ T A A T A C G A C C X A C T A T A 3′ **3**
3′ A T T A T G C T G G G T G A T A T 5′ **4**

Scheme 7.

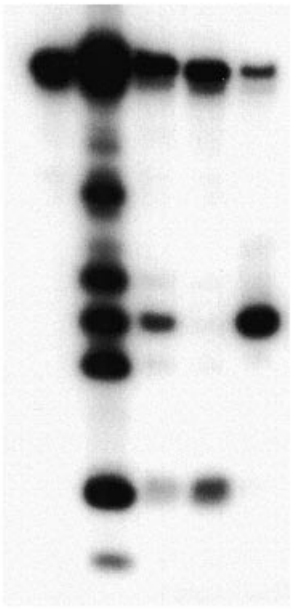


Figure 3. Denaturing PAGE of the cross-linking reaction between derivatized oligomer **2** and ^{32}P end-labeled **4**, after treatment with 1 M piperidine (95°C, 20 min). Lane 1: ^{32}P 5′ end-labeled target strand **4**; lane 2: Maxam–Gilbert G-sequencing reaction; lane 3: cross-linking reaction with ethylenediamine tether **9**; lane 4: cross-linking reaction with *meta*-phenylenediamine tether **8**: lane 5: cross-linking reaction with *ortho*-phenylenediamine tether **6**.

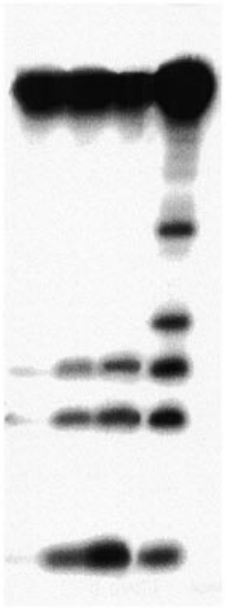


Figure 4. Denaturing PAGE of the cross-linking reaction between derivatized oligomer **3** and ^{32}P end-labeled **4**, after treatment with 1 M piperidine (95°C, 20 min). Lane 1: cross-linking reaction with *meta*-phenylenediamine tether **8**; lane 2: cross-linking reaction with ethylenediamine tether **9**; lane 3: cross-linking reaction with *ortho*-phenylenediamine tether **6**; lane 4: Maxam–Gilbert G-sequencing reaction.

To further explore the topological features of the tether (Scheme 6) that are important for efficient cross-linking, we examined cross-linking in sequence **2·4** where the d^{S4}U is positioned opposite the middle dG residue of the three-base run of dGs (Fig. 3). Sequence analysis of the cross-linking with *ortho*-phenylenediamine tether **6** demonstrated that this tether exclusively alkylated the complementary dG in excellent yields (lane 5). In contrast to duplex **2·4**, ethylenediamine-based tether **9** alkylated the complementary dG in lower yield, and a small amount of alkylation was observed three bases in the 5′ direction (lane 3). With the *meta*-phenylenediamine tether **8**, a moderate yield of cross-linking was observed three bases in the 5′ direction (lane 4). Experiments with **10** using the sequence **2·4** showed only very small amounts of cross-linking, which occurred exclusively at the dG three bases in the 5′ direction. Additional experiments conducted with the very short hydrazine-derived tether **11** also showed low levels of cross-linking. Sequence analysis demonstrated that this reaction took place entirely at the complimentary dG.

Cross-linking in sequence **3·4** (Scheme 7) where the d^{S4}U is positioned opposite the 3′ end dG residue of the three-base run of dGs (Fig. 4) was significantly less effective, and poorly sequence selective. This was the only sequence where we observed cross-linking in the 3′ direction. The *meta*-phenylenediamine tether **8** gave very low yields of cross-linking (<5%), and the ethylenediamine (9) and *ortho*-phenylenediamine (6) tethers effected cross-linking in <20 and 35%, respectively. This compares with the typical yields for cross-linking of 80–95% in the previously discussed sequences.

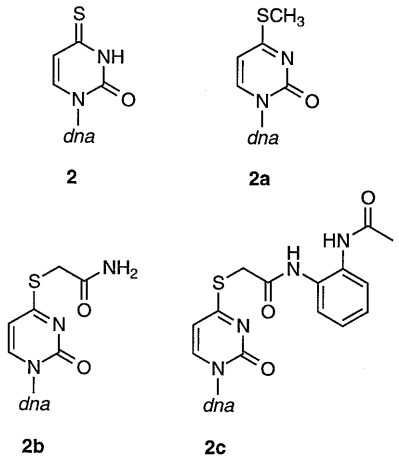
Kinetics

Of the sequences investigated, duplex **2·4** underwent cross-linking most rapidly, with a half-life for reaction of slightly <1 h, when **2** was derivatized with bis-electrophile **6**. Similarly, duplex **1·4** derivatized with **6** reacted rapidly to form cross-linked DNA. The half-life for this reaction was slightly >1.5 h. In contrast to this, sequence **3·4** derivatized with **6** reacted more slowly than the other sequences investigated. Kinetic analysis of the progress of this reaction demonstrated a half-life of almost 33 h. This implies that a higher degree of cross-linking could be obtained, compared to the 30% observed, by running the reaction for an extended period. However, a limitation of this is the half-life of the electrophilic strand in aqueous solution, which is just over two times the half-life for the reaction.

The rate of complimentary strand modification was measured with the sequence d(CXC)·(GGG) (duplex **1·4**). These experiments were done in the presence of an excess of probe strand **1** that had been derivatized by electrophile **6**, so that the reaction was pseudo-first order in **4**. Under these conditions, k_{obs} for the

5′ T A A T A C G A C X C A C T A T A 3′ **2**
3′ A T T A T G C T G G G T G A T A T 5′ **4**

Scheme 8.



2 **2a** **2b** **2c**

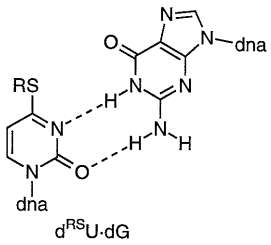
Scheme 9.

alkylation of **4** was found to be 1.1×10^{-4}/sec. Using this value, along with the recently determined second-order rate constant for alkylation of DNA by bromoacetamide (34), an effective molarity of 3.1 M was calculated for the bromoacetyl group of **6** within the **1·4** duplex.

Thermal stability

UV melting (helix–coil transition) experiments were performed to evaluate the thermodynamic consequences of incorporating 4-thio-2′-deoxyuridine and modified derivatives into duplex DNA. Cross-linking in these systems depends on three separate events: (i) effective duplex formation; (ii) appropriate tether topology and conformation; and (iii) complementary base nucleophilicity. We have examined factors (ii) and (iii) in the above cross-linking studies, but in order to understand the hybridization thermodynamics of the modified DNA and its complement, the stability of a series of duplex structures with various $d^{S4}U$ derivatives opposite a dG base were examined (Scheme 8). Non-electrophilic tethers (i.e., the desbromo systems) were appended by S-alkylation of $d^{S4}U$ within the 17mer sequences. In total, four thiouridine systems were examined in the complementary position to a dG base: the native $d^{S4}U$ system **2**, the *S*-methyl derivative **2a**, and the *S*-acetamido derivatives **2b** and **2c** (Scheme 9). This last system (**2c**) possesses all of the physical characteristics of the cross-linking system, but without the electrophilic α-bromoamide. This allowed us to estimate the thermodynamic destabilization caused by the presence of the bulky phenylenediamine system. In all cases, clear two-state melting curves were observed.

There was essentially no difference between the destabilization caused by 4-thio-2′-deoxyuridine (2), or its *S*-methyl (**2a**) or *S*-acetamido (**2b**) derivatives (Table 1). All non-natural bases destabilized the duplex by ~9–10°C compared to the C·G control system, although when the bulky *ortho*-phenylenediamine derivative **2c** was positioned opposite to a dG base, the destabilization was considerably greater. Concentration dependence experiments showed that the destabilization depended equally on enthalpic and entropic effects. The thermodynamic parameters derived from the concentration dependence of the T_m showed no obvious correlation between T_m of the duplexes and either ΔH or ΔS (35).



$d^{RS}U{\cdot}dG$

Scheme 10.

Table 1. Duplex melting temperatures (°C)

Duplex	Concentration of DNA ($\times 10^7$) (M)					
	6	12	24	36	47	72
2·4	44.5	45.9	47.7	49.1	49.7	50.9
2a·4	44.2	45.6	46.4	47.3	47.6	49.3
2b·4	44.1	45.1	46.7	47.4	48.3	49.5
2c·4	41.9	42.3	44.6	44.9	45.4	47.1
C·G	–	–	56.9	–	–	–

The UV melting studies demonstrated that the $d^{S4}U{\cdot}G$ (Scheme 10) mismatched base pair caused a decrease in the stability of the 17mer duplex of ~9°C in T_m. Reduced H-bonding coupled with the steric bulk of the R-groups could explain the similarly depressed T_m values for $d^{RS}U{\cdot}dG$ containing duplexes.

In an attempt to determine the degree to which the interstrand cross-link stabilized the duplex, the cross-linked material was isolated by HPLC and UV melting studies were performed. Unfortunately, thermal depurination at the site of alkylation was rapid at temperatures >60°C, preventing the determination of T_m.

DISCUSSION

Effective covalent cross-linking of duplex DNA has been demonstrated by appending electrophilic tethers to 4-thio-2′-deoxyuridine ($d^{S4}U$) contained within oligonucleotides; annealing these probes to their complementary strand induces covalent cross-link formation in a process driven by duplex formation. We have used a series of *bis*-bromoacetyldiamines as tethers, and we have demonstrated high yielding cross-link formation to deoxyguanosine bases on the complementary strand. We observed a significant sequence dependence of cross-link formation that was highly sensitive to tether topology and conformational rigidity. Under optimal conditions within the sequence d(CXC)·(GGG), where X = $d^{S4}U$ with an *ortho*-phenylenediamine based tether, we observed essentially quantitative cross-link formation (>95%) at the complementary dG N7 position. In other cases where the $d^{S4}U$ base was positioned opposite to other dG residues in the same sequence, we observed moderate to high yields of cross-link formation (40–80%), and sequence selectivity that was dependent on the tethering moiety.

Our observations of the selectivity of alkylation of dG residues is in general accord with literature data on the sequence preferences of alkylation at the N7 position of dG residues (36–38). It is well Structure Block 10.established both experimentally and theoretically that a dG base 5′ to another dG base intrinsically has enhanced nucleophilic character, supposedly due to an overlap of the filled HOMO with empty LUMO orbitals. In the present system, this implies that the central and 5′ dG bases of the target sequence d(GGG) should be most readily alkylated, in the absence of overriding steric constraints.

In the sequence d(XCC)·(GGG) (duplex **1·4**), the optimal *ortho*-phenylenediamine tether **6** was effective at formation of cross-links, although it was poorly sequence selective. Alkylation occurred at both the complementary dG and the dG two bases to the 5′ side in approximately equal proportions. Modeling results indicated that both of these alkylation events could proceed without a significant distortion of either the tether or duplex DNA. The ethylenediamine tether **9** underwent alkylation almost exclusively at the dG two bases to the 5′ side in good yield, but the *meta*-phenylenediamine tether **8** preferentially alkylated the distal dG four bases to the 5′ side in good yield. This result was also observed with duplex **2·4** (vida infra).

In the sequence d(CXC)·(GGG) (duplex **2·4**), we observed the most effective level of cross-link formation with *ortho*-phenylenediamine tether **6**. Under optimized conditions over 12–24 h at 25°C we observed ≥95% cross-link formation, which occurred exclusively at the N7 position of the complementary dG. With the more flexible ethylenediamine tether **9**, the amount of cross-linking was reduced, without a change in the sequence selectivity. With the *meta*-phenylenediamine tether **8**, which was intended to be incapable of alkylating the complementary dG, we saw distal alkylation three bases to the 5′ side. Computer generated models of this cross-link showed that tether **8** was just long enough to form this cross-link without distorting the DNA double helix.

In the sequence d(CCX)·(GGG) (duplex **3·4**), only modest levels of cross-linking were observed at the indicated complementary dG even with the most effective *ortho*-phenylenediamine tether **6**. This is partly due to the particular sequence in which the dG target is positioned, as we demonstrated previously that the C5-methyl group of the 5′ thymidine sterically blocks alkylation at the adjacent 3′ dG. Replacement of this thymine with a 2′-deoxyuridine residue was shown to result in enhanced cross-linking (30). In addition, based on electronic arguments this dG is significantly less nucleophilic than the other two dG residues.

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