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Stabilization of double-stranded oligonucleotides using backbone-linked disulfide bridges

Hetian Gao*, Meihua Yang and Alan F. Cook

PharmaGenics Inc., 4 Pearl Court, Allendale, NJ 07401, USA

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ABSTRACT

A convenient, practical route to the synthesis of disulfide-bridged oligonucleotides has been developed. Aliphatic linkers with terminal thiol groups have been attached to the phosphodiester backbones of partially or fully complementary oligonucleotide sequences and oxidized to yield covalently closed oligonucleotides with disulfide bridges. This procedure has been used to prepare a duplex with disulfide bridges at both ends and stem–loop sequences with single disulfide bridges. Oxidation of a self-complementary duplex possessing terminal thiol groups produced both hairpin and duplex structures with disulfide bridges, the relative proportions of each being dependent upon the reaction conditions. These bridged hairpin and duplex structures were shown to be interconvertible by reduction and re-oxidation. The melting profiles of disulfide-bridged oligonucleotides were compared with the same sequences without bridges and with sequences possessing triethylene glycol bridges, and in all cases the introduction of disulfide bridges resulted in a considerable increase in thermal stability. *Eco*RI endonuclease was capable of cleaving a disulfide-bridged duplex possessing a recognition site for this enzyme, thus supporting a lack of distortion of the recognition site. The disulfide bridges could be cleaved using a large excess of DTT to regenerate the corresponding sulfhydryl compounds. A study of the serum stabilities of disulfide-bridged oligonucleotides showed that the bridged duplexes were much more stable than their unmodified counterparts, whereas the rate of degradation of the stem–loop structures was more dependent upon the size of the loop than the presence or absence of the disulfide bridge. In summary, we have described a novel methodology, employing commercially available reagents, for the stabilization of oligonucleotide duplexes or stem–loop structures by disulfide bridge formation.

INTRODUCTION

The introduction of non-nucleotide bridges or loops into DNA or RNA sequences can provide several advantages. (i) Short, double-stranded oligonucleotides with relatively low thermal dissociation temperatures can be greatly stabilized towards denaturation by the introduction of bridges. Such stabilization can be useful for oligonucleotides which bind to proteins, such as transcriptional factors, since relatively short recognition sequences can be locked into the double-stranded form. The transcriptional activity of a DNA binding protein has been inhibited using double-stranded oligonucleotides with nucleotide bridges, and the presence of bridges was shown not to interfere with binding (1). Oligonucleotides which bound to HIV-1 Tat protein have also been stabilized towards thermal denaturation by the introduction of glycol bridges (2). (ii) Introduction of non-nucleotide bridges is likely to reduce the extent of degradation by endo- and exonucleases. Endonuclease degradation of double-stranded oligodeoxynucleotides with nucleotide bridges has been demonstrated *in vitro* in the presence of cell extracts and serum and the sequence was shown to be preferentially degraded in the single-stranded bridge region (3). The introduction of non-nucleotide bridges would be expected to reduce the extent of degradation by single-stranded endonucleases, since non-nucleotide bridges are less likely to be substrates for these enzymes. 3′-Exonucleases in serum have been shown to degrade unmodified, single-stranded oligonucleotides (4), and attachment of bridges to the ends of sequences of this type would be expected to block exonuclease degradation. (iii) The stabilization of short oligonucleotide sequences by bridging allows for detailed conformational studies by nmr spectroscopy or other methods, since a reduction in the number of possible structures renders the spectra more amenable to interpretation.

Several types of non-nucleotide bridging groups have previously been used for the synthesis of hairpin molecules. Of these, glycol bridges have been studied most extensively (2,5–7), due in part to the ready availability of glycol-based phosphoramidites and/or their ease of synthesis. Other types of bridging group have been described, such as those with aromatic functionalities (8), bridges produced by platinum complexes (9), and bridges via the sulfur atoms of phosphorothioate groups (10). The synthesis of covalent-

* To whom correspondence should be addressed

ly closed glycol-bridged duplexes has been carried out by chemical ligation of a nicked, double-stranded precursor (11). Non-complementary, circular oligonucleotides with glycol bridges have also been synthesized using a second oligomer as a splint to facilitate ligation (12).

Several reports on disulfide-bridged oligonucleotide duplexes and hairpins have been described. In one approach, O^6-phenyl deoxyinosine or O^4-trimethylphenyl deoxyuridine monomers were converted into their phosphoramidites, introduced into oligonucleotides, and the *O*-phenyl groups were used as leaving groups in the attachment of disulfide-containing cross-linking agents (13,14). In a related approach, the disulfide bridges were connected via linker arms attached to the N3 positions of unpaired terminal thymine residues (15,16). The construction of bridges of this type required the synthesis of a solid support with a modified thymidine moiety together with an N3-substituted thymidine phosphoramidite. After synthesis and deprotection of the oligonucleotide, the sulfhydryl groups attached to the thymine residues at the ends of the oligonucleotide were oxidized to produce a disulfide-bridged hairpin or duplex, depending upon the nature of the sequence and the oxidation conditions employed.

We have investigated an alternate procedure to produce disulfide-bridged oligonucleotides in which the disulfide groups are connected to the phosphodiester backbone via aliphatic linkers. This procedure can be carried out using commercially available reagents without the need to prepare any modified monomers, and does not involve disruption of the terminal base pairs of the duplex.

MATERIALS AND METHODS

Oligonucleotides were synthesized using conventional phosphoramidite chemistry on an Applied Biosystems Model 394 DNA synthesizer. 1-*O*-Dimethoxytritylhexyl disulfide, 1′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]phosphoramidite (**1**, 5′-thiol modifier-C_6 S-S) and dimethoxytrityl triethylene glycol phosphoramidite were purchased from Glen Research (Sterling, VA). Trityl-on HPLC separations were performed using a Waters RCM (8 mm × 10 cm) C_4 column for analytical purposes and a C_4 RCM (25 mm × 10 cm) column for preparative use. A gradient of 0.1 M triethylammonium acetate buffer, pH 7.0 (TEAA, solvent A)/acetonitrile (solvent B) was used for all separations. Polyacrylamide gel electrophoresis (PAGE) was carried out in tris–borate buffer, pH 8, using a 15% gel in the presence of 7 M urea. Oligonucleotides were converted into the sodium form by passage through a column of Bio-Rad AG50W-X8 resin, sodium form.

Synthesis of 3′,5′-dithiol oligonucleotides 2 and 9

For each oligonucleotide, four 1 μmol scale syntheses were carried out on a DNA synthesizer. For introduction of the thiol at the 3′-terminus, the thiol modifier **1** (0.1 M in acetonitrile) was directly coupled to a thymidine residue attached to a solid support, with the coupling time being extended to 15 min in this coupling cycle and the following two regular nucleoside phosphoramidite coupling cycles. Conventional nucleoside phosphoramidites were then incorporated at the appropriate positions and the thiol modifier was introduced at the 5′-end of the oligonucleotide using the amidite **1** with a 15 min coupling time. A 0.02 M iodine solution was used for all of the oxidation steps. Coupling efficiencies of ~98% were obtained for standard nucleoside phosphoramidites, and 90–95% for the amidite **1**, as determined by trityl assay. After synthesis and cleavage from the solid support, the crude oligonucleotide mixture in concentrated aqueous ammonia solution was stored overnight at 55°C, concentrated to a small volume, and the tritylated material (168 OD_{260} for **2**, 239 OD_{260} for **9**) was purified by HPLC on a preparative C_4 column using a linear gradient of 2–40% B over 40 min. The product peak (78 OD_{260} for **2**, 50 OD_{260} for **9**) was concentrated to a small volume, redissolved in sodium phosphate buffer (0.02 M, pH 8.0) and after addition of DTT (1 mg/OD_{260}), the mixture was stirred at room temperature for 4 h to cleave the disulfide bonds and liberate the free thiol groups at the termini of the oligonucleotide. Cleavage of the disulfide bonds was monitored by HPLC on a C_4 analytical column using a gradient of 2–40% B over 40 min. The by-products were removed either by extraction or HPLC purification depending on the purity of the intermediate sulfhydryl oligonucleotide. For relatively pure reaction mixtures the material was extracted using ethyl acetate (3×) followed by ether (2×), and then used directly for the oxidation reaction. If the reaction mixture contained more impurities, further purification was carried out using HPLC on a preparative C_4 column using a gradient of 2–20% B over 40 min. The oligonucleotides were used directly for the oxidation reaction as described below.

Disulfide-bridged oligonucleotides 3, 4 and 6

HPLC-purified sulfhydryl oligonucleotide **2** (52 OD_{260}) in 0.1 M sodium phosphate (5 ml, pH 8) containing 0.3 M NaCl was oxidized by stirring in air at 4°C for 3 days. Analysis of an aliquot with Ellman's reagent showed that no free thiol groups were present and HPLC analysis on a C_4 analytical column, with a gradient of 2–15% B over 5 min then 15–40% over 30 min, showed that no starting oligonucleotide remained and that two major peaks (elution times 14.3 and 14.8 min) were produced in a 3:1 ratio. The mixture was separated using reverse phase HPLC on a preparative C_4 column using a gradient of 2–32% B over 35 min, and the peaks were separately collected and evaporated to dryness. The residues were precipitated from ethanol and converted to the sodium form to give **3** (21.2 OD_{260}) and **4** (5.2 OD_{260}). Analysis by PAGE showed that each product migrated as a single band. A similar reaction at 37°C yielded the hairpin sequence **4** as the major product.

For **6**, the intermediate sulfhydryl oligonucleotide was synthesized using the standard method, except that the triethylene glycol bridge was introduced using the triethylene glycol phosphoramidite. The product (29 OD_{260}) was oxidized as described for **3** and purified by HPLC followed by conversion to the sodium salt to give **6** (12.6 OD_{260} units).

Chemical ligation approach to the synthesis of 3 from 5

This procedure employed the synthesis of an open chain, self-complementary oligonucleotide followed by chemical ligation to produce the closed, disulfide-bridged duplex. For the first cycle of the synthesis, (2-cyanoethoxy)-2-(2′-*O*-dimethoxytritylloxyethyl)ethoxy-*N*,*N*-diisopropylaminophosphine (17) was coupled to a thymidine residue attached to a solid support (2 × 1 μmol scale). Unmodified nucleotides were then added using standard reagents and the two disulfide bridges were introduced at the appropriate positions using amidite **1** as previously described. After cleavage from the support and treatment overnight with ammonium hydroxide at 55°C, the solutions were combined and evaporated to dryness. The residue of crude, tritylated oligonucleotide was dissolved in 0.02 M triethylammonium bicarbonate, pH 7.5, filtered through a 0.45 μm filter and isolated by HPLC using

a semi-preparative reversed phase C_{18} column (Whatman; 9.4×250 mm), flow rate 3 ml/min, with a gradient of 5–20% B over 8 min, then 20–40% B over 40 min, followed by 40–80% B over 10 min. The tritylated oligonucleotide, which eluted between 26 and 32 min, was collected, lyophilized, detritylated in 0.1 M acetic acid (2 ml) for 40 min at room temperature, and extracted with ethyl acetate (3×) followed by ether (6×). After lyophilization, the oligonucleotide was precipitated from 70% ethanol with 0.3 M sodium acetate and converted to the sodium form to give the 3′-phosphorylated oligonucleotide **5** (32 OD_{260}), which migrated as a single band on PAGE. This material was used directly in the ligation step.

The oligonucleotide **5** (6 OD_{260}) was ligated using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride according to a previously described method (11) and isolated to give **3** (1.5 OD_{260}). PAGE showed a single band with mobility identical to a sample prepared by the oxidative procedure as described above.

Interconversion of bridged duplex and hairpin sequences 3 and 4

DTT (1 mg) was added to a solution of **3** or **4** (1 OD_{260}) in sodium phosphate buffer (0.1 M, 100 µl, pH 8.0) and the resulting mixture was stirred at room temperature overnight. After removal of DTT by extraction with ethyl acetate followed by ether, the sulfhydryl oligonucleotide was re-oxidized by stirring overnight in air. Oxidation at room temperature followed by HPLC analysis gave two peaks of equal intensity, corresponding to **3** and **4**, which were separated by HPLC as described above. Oxidation at 37°C gave **3** and **4** in a 1:4 ratio, whereas oxidation at 4°C gave **3** and **4** in a 4:1 ratio.

Hairpin loop sequence 8

Method A. The oligonucleotide dithiol **9**, prepared as described above (36 OD_{260}), was oxidized by stirring overnight at room temperature in 0.02 M sodium phosphate buffer containing potassium chloride (50 mM, 2 ml, pH 8). No thiol groups were detected using Ellman's reagent and HPLC showed two major peaks (14.5 and 15.3 min) in a ratio of 5:1. Preparative HPLC as described above, followed by conversion to the sodium form yielded **8** (20 OD_{260}) and a second product with much slower mobility on PAGE, tentatively identified as a dimeric species (4 OD_{260}). Both migrated as single bands on PAGE.

Method B. The crude, ammonia deprotected trityl-on oligonucleotide (30 OD_{260}) in sodium phosphate buffer (0.02 M, 2 ml) was treated with DTT (30 mg) with stirring at 4°C overnight. HPLC analysis showed that no starting material remained after this time. The reaction mixture was extracted with ethyl acetate (4×) followed by ether (2×) and the aqueous solution was diluted to 5 ml with sodium phosphate buffer (0.02 M) and stirred in air at room temperature overnight. Both HPLC and 20% PAGE showed that no reduced oligonucleotide remained. A portion of this mixture was purified by 12% PAGE and the isolated bands were frozen, crushed and extracted with 2 mM EDTA, filtered through a cellulose acetate filter (0.45 µm) and precipitated with ethanol.

Thermal denaturation experiments

Oligonucleotides were dissolved in 5 mM Na_2HPO_4, pH 7, heated at 100°C for 5 min, and allowed to cool slowly to room temperature before data collection. The changes in absorbance at 260 nm versus temperature were measured using a Gilford Response II temperature-controlled spectrometer, with a heating rate of 0.8°C/min from 20–100°C. Transition temperatures were obtained from the first-order derivative plot of absorbance versus temperature.

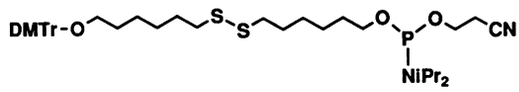

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Figure 1. Phosphoramidite **1**, which was used for the introduction of sulfhydryl groups into oligonucleotides.

Serum stability

Freshly drawn human blood was coagulated by storage at room temperature for 30 min. Fibrin clots and cells were spun down and the supernatant was filtered through a 0.45 µm filter. Sera from five individuals were pooled and stored at –20°C prior to use. Samples of the oligonucleotide (0.15 OD each) were treated with human serum to give a concentration of 0.5 µM, incubated at 37°C for the appropriate period of time, and injected directly onto an ion exchange HPLC column (Dionex NucleoPac PA-100; 4 × 250 mm). The column was eluted with a linear gradient of 0.1–0.75 M ammonium chloride in 25 mM Tris–HCl, pH 8 containing 0.5% acetonitrile over 20 min, with a flow rate of 1.5 ml/min. A plot of amount of full-length oligonucleotide versus time was employed to calculate the half-life.

Incubation with *Eco*RI endonuclease

Samples of oligonucleotides **3** and **4** (0.5 OD_{260}) were each dissolved in buffer (50 mM NaCl, 100 mM Tris–HCl, 10 mM $MgCl_2$, 0.025% Triton X-100, pH 7.5, 270 µl) and treated with *Eco*RI restriction endonuclease (30 µl, 20 U/µl; New England BioLabs, Beverly, MA). Each solution was incubated at 37°C overnight, quenched by addition of 0.5 M EDTA (15 µl) and heated at 75°C for 15 min to destroy the enzyme. The solution was extracted with phenol/chloroform (1:1, pH 8), followed by chloroform and ether, concentrated to dryness and precipitated from ethanol. The products were examined by 20% PAGE.

RESULTS AND DISCUSSION

Synthesis of the self-complementary, bridged dodecamers 3 and 4

Our approach to the synthesis of disulfide-bridged oligonucleotides involved the synthesis of a precursor oligonucleotide with thiol groups at both termini, followed by oxidation of the thiols to produce the disulfide-bridged species. Several approaches to the introduction of thiols onto the 5′- or 3′-termini of oligonucleotides have been reported (18–20), although these approaches were not directly applicable for the synthesis of oligonucleotides with thiols at both ends of the sequence. The introduction of thiols at both ends could be most easily accomplished using the commercially available disulfide-bridged phosphoramidite **1** (Fig. 1) for the first and last cycles of addition on the DNA synthesizer. After trityl-on purification of the crude oligonucleo-

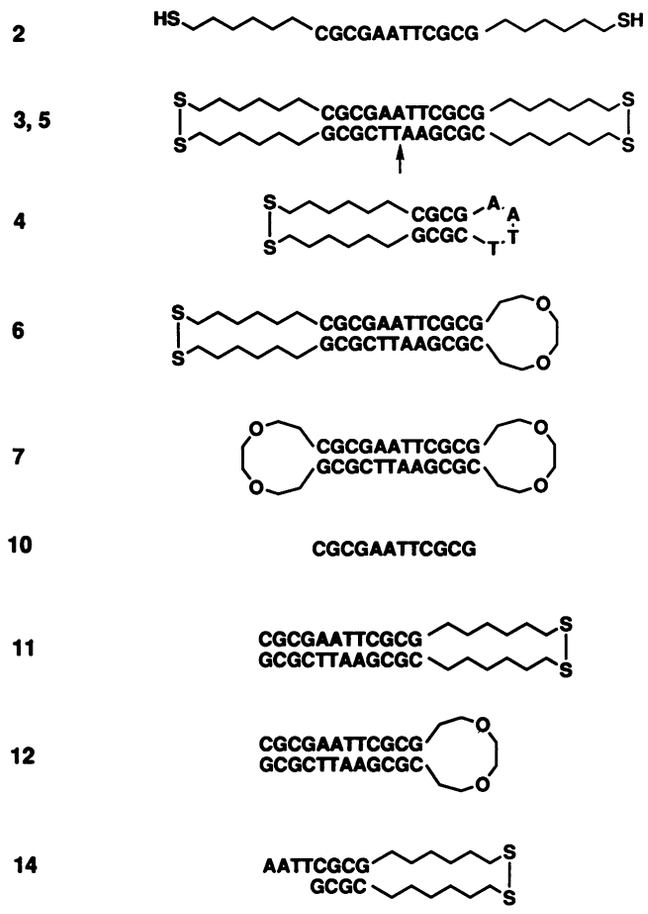

Figure 2. Structures of bridged oligonucleotides derived from the self-complementary dodecamer **10**. Oligonucleotide **3** is the closed, circular sequence and **5** is its nicked precursor, with the arrow indicating the site of the nick. The upper strand in all sequences reads in the 5′→3′ direction.

tide, the disulfide bridges were cleaved with DTT to produce the required intermediate possessing thiol groups at both ends of the sequence, attached via six-carbon linkers. This dithiol intermediate could then be cyclized to a disulfide-bridged oligonucleotide by oxidation in air. The process of cyclization was facilitated by duplex formation of nucleotides near the ends of the sequence so that the thiol groups were in relatively close proximity to each other.

The synthesis of the disulfhydryl oligonucleotide **2** (Fig. 2) was carried out by conventional methodology on a DNA synthesizer, using the disulfide-bridged phosphoramidite **1** for the first and last cycles of the synthesis. The oxidation of **2** was performed by stirring a solution of the oligonucleotide in air, and the disulfide-bridged oligonucleotide **3** was isolated in high yield. PAGE could be used for the purification of small amounts of material, since the bridged sequences were well resolved from their corresponding open chain intermediates. A second product from the oxidation of the disulfhydryl oligonucleotide **2** was also obtained in relatively high yield under certain conditions, and on the basis of its mobility on PAGE was determined to be the bridged hairpin structure **4**.

In order to confirm the structure of **3**, this material was also prepared by an alternate route in which the two disulfide bridges were incorporated directly into the oligonucleotide. This procedure closely paralleled that previously employed for the synthesis of a triethylene glycol-bridged duplex (11) and involved the preparation of a self-complementary nicked intermediate **5** possessing two disulfide bridges and a 3′ phosphomonoester group. The intermediate **5** was then chemically ligated using a carbodiimide coupling agent to produce the closed circular duplex **3**. Ligation could be monitored either by the appearance of a faster running species on PAGE, or by HPLC. The material obtained by this procedure was shown to migrate identically to a sample of **3** prepared by the oxidation method as described above.

The bridged duplex **6**, possessing both disulfide and triethylene glycol bridges, was prepared by the synthesis of a 24 base oligonucleotide with a central glycol bridge and terminal disulfide groups, followed by cleavage with DTT and oxidation as previously described. The corresponding duplex **7** possessing two triethylene glycol bridges was also prepared as previously described (11) and used for comparative studies.

Interconversion of dumbbell and hairpin structures 3 and 4

Previous studies have shown that the self-complementary, unmodified dodecamer **10** undergoes a biphasic thermal dissociation profile as a result of an initial transition from a duplex to a hairpin structure followed by a second transition to the single-stranded species (21); thus the relative proportions of hairpin and duplex are strongly temperature dependent. The formation of the corresponding disulfide-bridged oligonucleotides from the thiol precursor **2** was therefore studied under a variety of temperature and salt conditions to evaluate the influence of these factors on the relative proportions of the duplex and hairpin species **3** and **4** formed in these reactions. Reaction of **2** at 37°C under dilute oligonucleotide concentration and low salt conditions produced a new species with higher mobility on PAGE which was determined to be the hairpin **4** on the basis of gel mobility and resistance to *Eco*RI endonuclease digestion (see below). The process could be reversed by treatment of **4** with DTT followed by re-oxidation under high salt conditions at 4°C. When either **3** or **4** was treated with DTT followed by re-oxidation at room temperature, a mixture of **3** and **4** were produced in an almost equal ratio. Thus **3** and **4** can be interconverted depending upon the reaction conditions employed. It is of interest that **4** was formed to a significant extent at room temperature, even though the duplex would be expected to be the predominant species at this temperature.

Disulfide-bridged stem–loop oligonucleotide 8

Oligonucleotides with specific shapes or motifs are of interest as agents which can bind to, and exert an effect upon, proteins of biological interest. Unmodified oligonucleotide sequences, however, are more susceptible to degradation by exonucleases, since the termini are not protected. We have therefore investigated the feasibility of stabilizing motifs of this type by introducing disulfide bridges onto the ends of a model stem–loop oligonucleotide (**8**, Fig. 3). Two approaches were evaluated for the preparation of **8**. The steps involved in the first method, which was more suitable for obtaining larger quantities, are illustrated

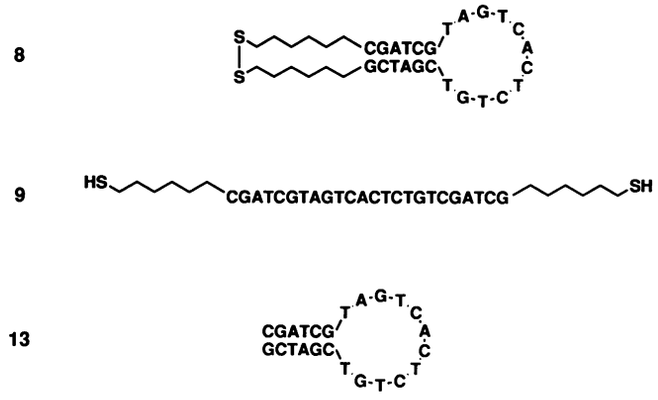

Figure 3. Structures of disulfide-bridged, stem–loop oligonucleotides.

in Figure 4. The crude, tritylated oligonucleotide from the DNA synthesizer was deprotected with ammonia and purified by reversed phase HPLC (Fig. 4A). The HPLC-purified material (Fig. 4B) contained a small amount of an impurity eluting just before the main peak. The HPLC-purified material was treated with DTT and the reaction mixture from this reaction is shown in Figure 4C. The main peak eluted much faster than the tritylated starting material, as would be expected for a detritylated species, and was assigned as the dithiol oligonucleotide **9**. After removal of the reducing agent, **9** was oxidized to the disulfide-bridged species **8** and the reaction mixture from this reaction is shown in Figure 4D. The oxidation reaction is quite efficient, since virtually all starting material was consumed and the main, faster eluting peak accounted for ~85% of the total, as determined by the areas under the peaks. A final HPLC purification gave pure material (Fig. 4E). Aliquots of these intermediates and the final product were also examined by PAGE, as shown in Figure 5.

The second method for isolation of **8** was intended to be a rapid procedure, more suitable for obtaining small amounts of bridged sequences, and which could be used for the simultaneous processing of a large number of oligonucleotides such as for use in binding studies. In this procedure, the crude trityl-on mixture was deprotected with ammonia (Fig. 5, lane 2), directly treated with DTT to give the oligonucleotide dithiol **9** (lane 4) followed by oxidation in air. Lane 7 shows the reaction mixture from the oxidation reaction, and illustrates that the faster band, which corresponds to the bridged material **8**, was produced less efficiently from the crude material but nevertheless provided sufficient quantities of **8** for isolation by standard procedures. The required band was cut out from the gel and extracted by a conventional procedure to give **8**. Lane 9 shows the mobility of an unmodified oligonucleotide of the same sequence as a control. A minor, slower migrating band was produced in these oxidation reactions and isolated by extraction from the gel. This material (lane 10) was tentatively regarded as a dimeric species formed by bridging two separate molecules of **9**, although no further characterization was carried out. Thus the simplified procedure is well suited for the rapid isolation of small amounts of bridged oligonucleotides. These results suggest that disulfide bridges can be introduced into a variety of oligonucleotides provided that the ends of the molecule can be held together by base pairing.

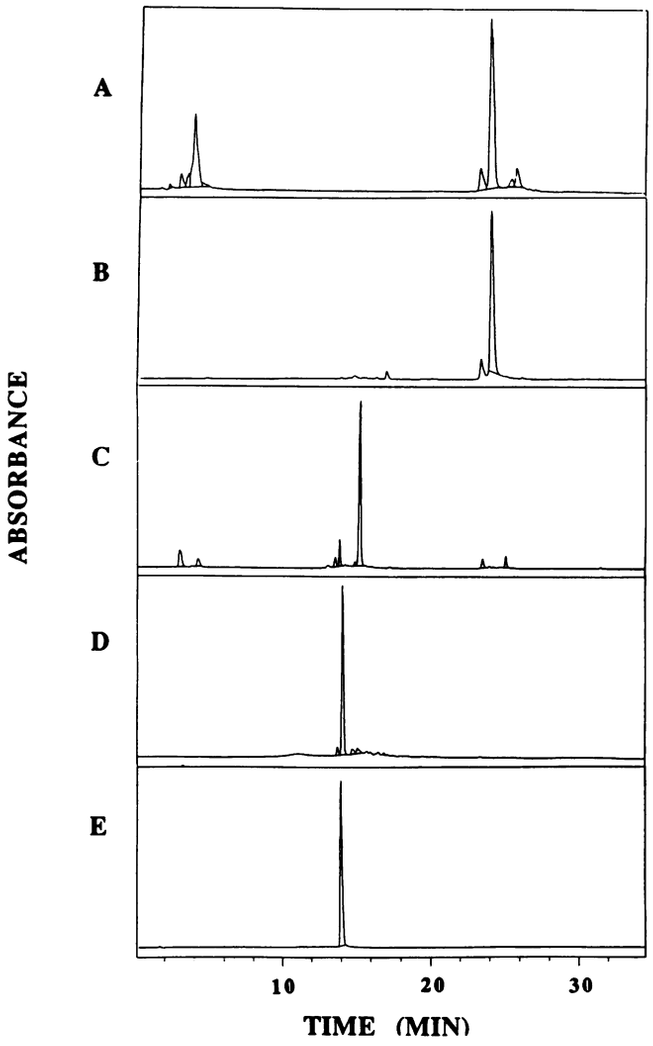

Figure 4. HPLC profiles of intermediates in the synthesis of the bridged stem–loop sequence **8**. (**A**) crude, tritylated precursor; (**B**) HPLC-purified, tritylated precursor; (**C**) reaction mixture from the treatment of material from (B) with DTT; (**D**) reaction mixture from the oxidation of material from (C); (**E**) purified **8**.

Gel electrophoresis of bridged sequences

The bridged oligonucleotides prepared in this study were examined by gel electrophoresis (Fig. 6). The three bridged duplexes **3**, **6** and **7** displayed similar mobilities (lanes 5–7 respectively), with the disulfide-bridged sequence migrating slightly slower than the other two. The disulfide-bridged hairpin sequence **4** (lane 4) migrated faster than an unmodified 12 base sequence (lane 2), and similar to the self-complementary unmodified dodecamer **10** (lane 3). It appears likely that **10** exists as a hairpin structure, even though the gel was run under denaturing conditions. The transitions of this oligonucleotide have been well documented, although its behavior on a gel was not reported (21). The bridged sequence with the large loop (**8**, lane 8) migrated faster than an unmodified oligonucleotide with the same sequence (**13**, lane 9). The by-product from the oxidation of **9** (lane 10) was tentatively assigned as a dimeric

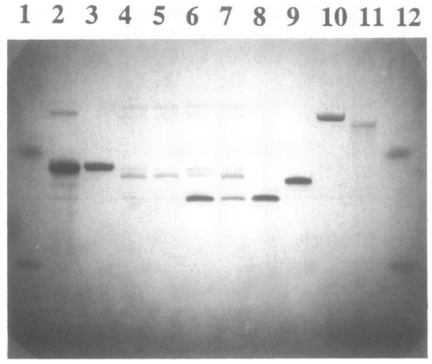

Figure 5. Polyacrylamide gel of the steps involved in the isolation of the bridged stem–loop structure **8**. Lanes 1 and 12, bromophenol blue and xylene cyanol markers; lane 2, crude, tritylated material after ammonia deprotection; lane 3, HPLC purified, tritylated material; lane 4, oligonucleotide dithiol **9**, obtained by the reduction of material from lane 2; lane 5, sample of **9** obtained by the reduction of HPLC-purified material from lane 3; lanes 6 and 7, treatment of material from lanes 5 and 4 respectively with air; lane 8, purified **8**; lane 9, unmodified oligomer with the same sequence as **8**; lane 10, dimeric by-product from the oxidation of **9**; lane 11, unmodified 48mer control.

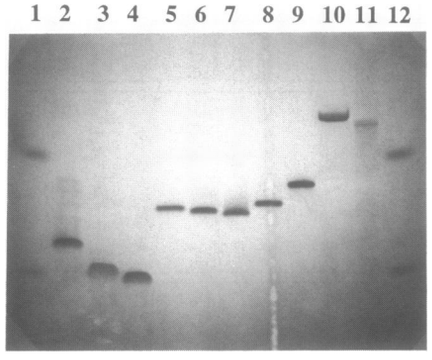

Figure 6. Polyacrylamide gel of disulfide-bridged duplexes and hairpins. Lanes 1 and 12, bromophenol blue and xylene cyanol markers; lane 2, unmodified 12mer control; lane 3, unmodified self-complementary sequence **10**; lane 4, bridged hairpin **4**; lanes 5–7, bridged duplexes **3**, **6** and **7** respectively; lane 8, bridged sequence with large loop (**8**); lane 9, unmodified 24mer control (**13**); lane 10, putative dimer from the oxidation of **9**; lane 11, unmodified 48mer control.

species formed by bridging two molecules via a disulfide linkage, since it migrated at approximately the same rate as an unmodified oligonucleotide of the same size (lane 11).

Thermal dissociation studies

The melting behaviors of the compounds described above were studied in comparison with their unmodified counterparts, and with the corresponding triethylene glycol-bridged oligonucleotides (Table 1). As previously reported (21), the unmodified duplex **10** melted in a biphasic manner at a relatively low temperature, with transitions from duplex to hairpin at 38.5°C and hairpin to single-stranded form at 60°C, whereas the bridged duplex **3** was exceptionally stable and melting was not complete at 95°C (data not shown). It was not possible to draw any precise conclusions concerning the relative thermal stabilities of the disulfide-bridged and triethylene glycol-bridged sequences **3**, **6** and **7**, since for two of these sequences the melting curves had not reached a plateau at the conclusion of the run. The relative stability of disulfide bridges versus triethylene glycol bridges was more evident in a comparison of the hairpin sequences **11** and **12**. In this experiment the disulfide-bridged oligonucleotide **11** was clearly more stable (T_m 79°C) than the glycol-bridged oligonucleotide **12** (T_m 73.5°C). A previous nmr investigation of a triethylene glycol-bridged duplex showed evidence for distortion at the terminal base pairs (11), which may provide an explanation for the lower melting temperature of the glycol-bridged oligonucleotide **12** as compared with the disulfide-bridged material **11**. HPLC analysis of **3** and **4** after thermal denaturation experiments showed that no breakdown of the oligonucleotide bridges had occurred during this time. The closed hairpin **4** was particularly stable towards thermal denaturation and no melting was observed. This result is consistent with previous reports which showed that an oligonucleotide hairpin which had been bridged through the terminal bases was also found to be very stable towards thermal denaturation (15,16).

Table 1. Thermal dissociation temperatures of bridged oligonucleotides in 5 mM sodium phosphate, pH 7

Oligonucleotide	Motif	Type of bridge	Dissociation temperature (°C)
3	Duplex	Disulfide	>95
6	Duplex	Disulfide, glycol	>95
7	Duplex	Glycol	92
11	Stem–loop	Disulfide	79
12	Stem–loop	Glycol	73.5
4	Stem–loop	Disulfide	>95
10	None	None	38.5, 60

Serum stability

Pooled human serum was incubated with several bridged oligonucleotides to examine their resistance to degradation and the results of these experiments are displayed in Figure 7. The disulfide-bridged duplex **3** was the most stable compound examined, with a half-life of 31 h, as compared with the unbridged sequence **10** with a half-life of 1.9 h. It is not clear whether **10** exists as a duplex or hairpin under these conditions, although the former is the most likely species. Both bridged and unbridged sequences with putative large loops (**8** and **13**) were more readily degraded (half-lives of 3.9 and 1.4 h respectively) than the bridged duplex, although the addition of a disulfide bridge did produce an almost 3-fold increase in stability as compared with the unbridged species. The bridged hairpin **4**, with a much smaller loop, was more stable with a half-life of 11.2 h. This result suggests that the single-stranded regions of the loops are the primary sites of degradation. All sequences were more stable than a standard, single-stranded oligonucleotide which was degraded with a half-life of ~15 min (data not shown).

The stability of different forms of hairpin and dumbbell DNA in human serum has previously been studied by Chu and Orgel (3). These workers showed that single-stranded loops of stem–loop oligonucleotides were degraded faster than the double-stranded stems, and concluded that human serum contains highly

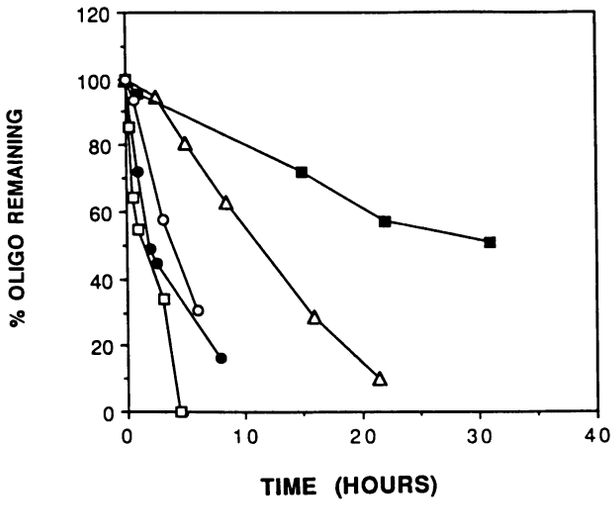

Figure 7. Time course of degradation of disulfide-bridged duplex 3 (■), unmodifed sequence **10** (●), and hairpins **4** (△), **8** (○) and **13** (□) in serum.

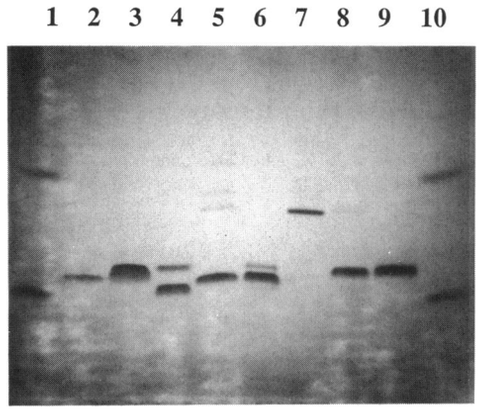

Figure 8. Polyacrylamide gel of the products from *Eco*RI digestion. Lanes 1 and 10, bromophenol blue and xylene cyanol markers; lane 2, 8mer control; lane 3, unmodified dodecamer **10**; lane 4, **10** + *Eco*RI; lane 5, hairpin **4**; lane 6, **4** + *Eco*RI; lane 7, bridged duplex **3**; lane 8, **3** + *Eco*RI; lane 9, hairpin **14**.

active single-stranded endonucleases and less active double-stranded exonucleases. The results obtained in this work are consistent with these previous findings.

*Eco*RI cleavage

Disulfide-bridged oligonucleotides were treated with the restriction endonuclease *Eco*RI, which cleaves double-stranded DNA between the G and A residues of the recognition site GAATTC, to examine the effects of the bridges on recognition and cleavage. The results of these experiments are shown in Figure 8. The unmodified control sequence **10** (lane 3) was cleaved by *Eco*RI to give a new species (lane 4), as expected. The ligated duplex **3** was readily degraded by *Eco*RI (lane 8) to give the 12mer hairpin **14** produced by cleavage of both strands between the G and A residues of the recognition site GAATTC. The structure of the oligonucleotide **14** was confirmed by an independent synthesis and demonstration that its PAGE mobility (lane 9) was identical to the material produced by *Eco*RI digestion of **3**. Thus the presence of disulfide bridges presumably does not distort the double helix to a significant extent, since the enzyme was capable of recognizing the bridged duplex as a substrate. As expected, incubation of the disulfide-bridged hairpin **4** under the same conditions (lane 6) did not produce any significant cleavage, as shown by the absence of faster running bands, since it does not possess a recognition site. The appearance of a new band in lane 6 is thought to be due to the cleavage of dimeric impurities present in **4**. Previous work with the triethylene glycol-bridged dodecamer **7** showed that cleavage with *Eco*RI was relatively slow, which could be due to the existence of distortion of the ends of the duplex, or to steric hindrance by the bridges (11).

Conclusions

Oligonucleotides with backbone-linked disulfide bridges attached via aliphatic six-carbon linkers have been synthesized and their properties have been studied. The precursor oligonucleotides could be readily prepared on a DNA synthesizer using commercially available reagents and purified by conventional trityl-on reversed phase HPLC. Oligonucleotides with terminal thiol groups were generated by treatment of their tritylated precursors with DTT, and oxidation of the thiol groups was efficiently accomplished by treatment with air to produce disulfide-bridged oligonucleotides in high yield. For a fully self-complementary sequence, the relative proportions of duplex and stem–loop structures could be altered by changes in the oligonucleotide concentration, salt conditions and reaction temperature. The bridges were stable at elevated temperatures, but could be cleaved by exposure to a large excess of DTT. Thermal dissociation profiles of disulfide-bridged oligonucleotides showed that these sequences were slightly more stable than the same sequences with triethylene glycol bridges and much more stable than the corresponding unbridged sequences. Incubation in human serum showed that the stability of a duplex was substantially increased by the introduction of disulfide bridges, whereas the stability of a stem–loop structure was primarily dependent upon the nature of the single-stranded loop region. Thus these procedures provide an attractive new approach to the stabilization of duplex and stem–loop oligonucleotide structures.

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