

Interstrand Disulfide Cross-Linking of Internal Sugar Residues in Duplex RNA

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Abstract—Disulfide cross-linking is being used increasingly more to study the structure and dynamics of nucleic acids. We have previously developed a procedure for the formation of disulfide cross-links through the sugar-phosphate backbone of nucleic acids. Here we report the preparation and characterization of an RNA duplex containing a disulfide interstrand cross-link. A self-complementary oligoribonucleotide duplex containing an interstrand cross-link was prepared from the corresponding 2'-amino modified oligomer. Selective modification of the 2'-amino group with an aliphatic isocyanate, containing a protected disulfide, gave the corresponding 2'-urea derivative in excellent yield. An RNA duplex containing an intrahelical, interstrand disulfide cross-link was subsequently prepared by a thiol disulfide exchange reaction in nearly quantitative yield as judged by denaturing polyacrylamide gel electrophoresis (DPAGE). The cross-linked RNA was further characterized by enzymatic digestion and the structure of the cross-link lesion was verified by comparison to an authentic sample, prepared by chemical synthesis. The effect of the chemical modifications on duplex stability was determined by UV thermal denaturation experiments. The intrahelical cross-link stabilized the duplex considerably: the disulfide cross-linked oligomer had a melting temperature that was ca. 40 °C higher than that of the non-cross-linked oligomer. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

A major tenant in molecular biology is that understanding of the function of biomolecules is based on structure. Given the importance of RNA in cellular processes, it is not surprising that a large effort has been devoted to structural studies of RNA. In comparison to the study of tertiary structures of proteins, X-ray crystallography and NMR spectroscopy have made minimal impact on structural determination of complex RNA molecules. For example, only four complex RNA structures have been solved by X-ray crystallography, that of t-RNA,^{1,2} the hammerhead ribozyme,^{3,4} the P4-P6 domain of the *Tetrahymena* group I ribozyme^{5,6} and the hepatitis delta virus ribozyme.⁷ Therefore, other techniques have been extensively used for the study of RNA structure, such as fluorescence resonance energy transfer measurements (FRET),^{8–10} electric birefringence,¹¹ native gel electrophoresis and nucleotide analogue interference mapping,^{12,13} to name a few.

In recent years, chemical cross-linking has been used to study both structure and dynamics of RNA.^{14–30} A range of chemical and enzymatic methods have been developed to incorporate cross-links into the helical regions of DNA and RNA,¹⁴ usually through the nucleotide bases. Examples include disulfide cross-linking using the convertible nucleoside approach^{15–17} or other mercaptoalkyl modifications,^{18–23} photocross-linking with 4-thiouridine,²⁴ 5-bromouridine,²⁵ 5-methyleneaminouridine,²⁶ or 8-azidoadenosine.²⁷

We have previously developed a strategy for incorporation of site-specific disulfide cross-links in RNA, utilizing the efficient and selective modification of 2'-amino groups with either aromatic isothiocyanates²⁸ or aliphatic isocyanates.^{31,32} The advantages of this approach are that base-pairing is not perturbed, as might be the case for cross-linking through the base-moiety, and the 2'-position is located close to the exterior of the helix, oriented for an intermolecular or interhelical interactions.³³ This strategy was subsequently used to study global conformations of the hammerhead ribozyme,²⁸ for building a model of the tertiary structure of the hairpin ribozyme²⁹ and to study the internal dynamics of a group I ribozyme.³⁰

We report here the application of the aforementioned disulfide cross-linking method for stabilization of RNA

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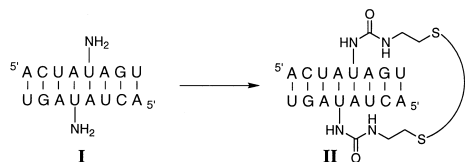
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duplex **I** by the formation of the interstrand, intrahelical disulfide cross-linked RNA **II** (Scheme 1). The structure of the cross-link lesion, obtained from enzymatic digests of the cross-linked RNA, was determined by comparison to an authentic sample prepared by chemical synthesis. The presence of the cross-link significantly stabilized the duplex structure as judged by a large increase in the melting temperature of the RNA duplex.

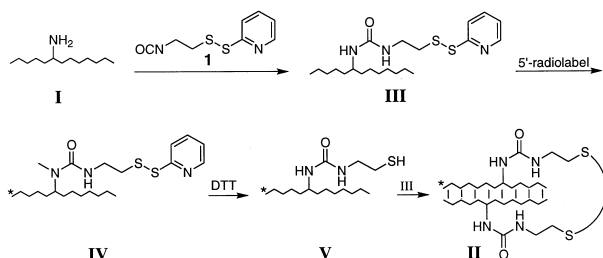
Results and Discussion

Our approach to the synthesis of the disulfide cross-linked oligoribonucleotide **II**, by linking two 2'-positions on opposite strands of the duplex, is an extension of earlier reports from our laboratory.^{28,31,32} Self-complementary oligoribonucleotide **I**, containing a 2'-amino nucleotide, was prepared by standard solid-phase synthesis.³⁴ The 2'-amino group is more nucleophilic than the exocyclic amines of the bases and can, therefore, be selectively reacted with isocyanate **1** in high yield to give the 2'-urea modified oligomer **III** (Scheme 2).³² Oligoribonucleotide **III** was 5'-radiolabeled and the disulfide reduced by DTT to yield the thiol-containing oligomer **V** that was subsequently incubated with oligomer **III** in an attempt to prepare oligoribonucleotide **II** by a disulfide exchange reaction. DPAGE analysis of the cross-linking reaction (Fig. 1) showed that oligoribonucleotide **IV** (Lane 1) was converted to a new product in approximately 95% yield (Lane 2) which had electrophoretic mobility similar to that expected for oligoribonucleotide **II**. Incubation of this new product with DTT reduced the cross-link to yield single stranded RNA (Lane 3), providing further evidence for the identity of oligoribonucleotide **II**.

To provide enough material for further structural and thermodynamic characterization of oligoribonucleotide **II**, a large-scale synthesis was performed. Oligoribonucleotide **III** was prepared as before and subjected to conditions of duplex formation and treated with DTT.



Scheme 1. Preparation of cross-linked oligoribonucleotide **II**.



Scheme 2. Preparation of interstrand disulfide cross-linked duplex RNA **II** via a disulfide exchange reaction.

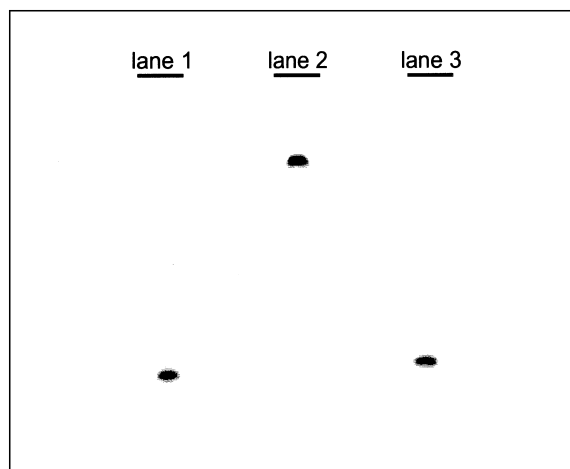


Figure 1. Analysis of cross-linking reaction by DPAGE. Lane 1, oligoribonucleotide **III**. Lane 2, after cross-linking reaction (oligoribonucleotide **II**). Lane 3, reduction of cross-linked oligoribonucleotide **II** with DTT, followed by reaction with *N*-ethylmaleimide⁴⁰ to prevent oxidative formation of cross-links during analysis by DPAGE.

Exposure to oxygen gave oligoribonucleotide **II** in 90% yield, as analysed by HPLC (data not shown).

For structural characterization of the cross-link lesion, oligoribonucleotides **III** and **II** were enzymatically digested and the digests analysed by HPLC (Fig. 2). For oligoribonucleotide **III** (Fig. 2A), a strongly retained compound was observed which had previously shown to be compound **2**.³² On the other hand, HPLC analysis of digested oligoribonucleotide **II** (Fig. 2B) yielded a new compound, which had retention time between that of adenosine and compound **2**, and was tentatively assigned structure **3**. To provide direct evidence for this structure, compound **3** was synthesized, by treatment of compound **2** with 0.5 equiv of DTT, and characterised by ¹H NMR and HRMS. Synthetic **3** and compound **3** found in the enzymatic digest were shown to be the same compound by co-injection on HPLC (data not shown).

To determine the effects of the chemical modifications on the duplex stability, the thermal denaturation curves of oligoribonucleotides **I**, **III** and **II** were obtained. The melting curves for each sample demonstrated a single cooperative and reversible melting transition. While modification of the 2'-amino group in **I** with isocyanate **1** did not appreciably change the melting temperature of the duplex, the presence of the cross-link had a significant effect on the duplex stability (Table 1). In fact, the melting temperature of the cross-linked duplex is over 40°C higher than for the uncross-linked sample. The increased thermal stability of the cross-linked duplex, relative to the non-cross-linked duplexes, is presumably due to a favorable entropy term for the free energy of duplex formation.³⁵

There are three major advantages to the cross-linking approach described here. First, the thiols are introduced by post-synthetic modification of 2'-amino nucleotides

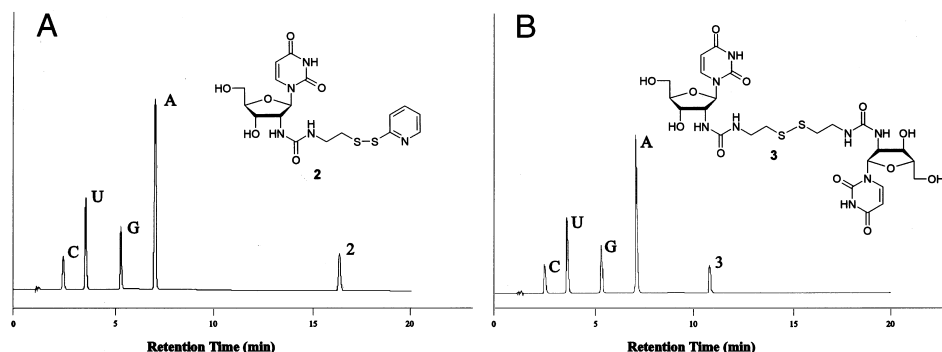


Figure 2. HPLC analyses of the products from enzymatic digestion of oligoribonucleotide **III** (A) and oligoribonucleotide **II** (B).

Table 1. Melting temperatures of oligoribonucleotides **I**, **III** and **II**

Oligoribonucleotide duplex	Melting temperature (°C)
I	18.2
III	17.6
II	61.4

that can be incorporated into the oligomers using commercially available phosphoramidites. This reduces the synthetic effort and enables the length of the linker to be easily varied. Second, the 2'-amino nucleotides can be incorporated at internal positions in the nucleic acid. Thus, the position of the cross-links can be altered and is not restricted to the oligomer termini. Third, the cross-links are formed between sugar residues and therefore, interference with base-pairing is minimized.

In conclusion, we have shown that interstrand disulfide cross-links can be efficiently formed between the sugar phosphate backbones of the two strands in duplex RNA by either a disulfide exchange reaction or by oxidation with oxygen. The structure of the cross-link lesion was determined by comparison to an authentic sample, prepared by chemical synthesis. The disulfide cross-link was shown to considerably increase the thermal stability of the duplex RNA. This approach should find use in the structural engineering of nucleic acids and will complement similar approaches that utilise disulfide cross-linking through the base-moiety, either at the duplex termini^{18,20–22} or at internal positions.^{15,16,19,23,35}

Materials and Methods

General

Reactions of compound **1** with the 2'-amino group was carried out under an argon atmosphere. Flash column chromatography was performed on silica gel 60 (Merck, Darmstadt, Germany) with particle size less than 0.063 mm. ¹H and ¹³C NMR spectra were recorded in DMSO-*d*₆ on a Bruker AM 360L instrument at 360.13 and 90.55 MHz. Chemical shifts are reported in ppm, relative to DMSO-*d*₅ as internal standard at δ 2.50 ppm in ¹H and δ 39.5 ppm in ¹³C NMR. Coupling constants (*J*) are reported in Hertz. High resolution, accurate mass spectra (HRMS) were recorded on a VG analytical

Autospec-T tandem mass spectrometer using electron impact ionization.

Oligoribonucleotides were prepared by automated chemical synthesis using phosphoramidites from MilliGen/Bioscience, except for the incorporation of the trifluoroacetyl-amino-5'-dimethoxytrityl uridine-3'-cyanoethyl phosphoramidite.³⁶ Work up of oligoribonucleotides as well as conditions for DPAGE was as described by Tuschl et al.³⁷ Concentrations of the oligoribonucleotides were calculated using a molar extinction coefficient of 6600 M⁻¹ cm⁻¹/nucleotide.

HPLC was carried out on a Waters Associates system with a model 6000A pump, a model 680 automated gradient controller, a model 730 data module and a model 481 LC spectrophotometer. Separations were performed on reverse phase material 5 μm ODS Hyper-sil (MZ Analysetechnik, Mainz, Germany), in a column of dimension 250×4 mm. Solvent gradients for analytical HPLC (enzymatic digestion) were run at 2 mL/min. Elution was performed with a linear gradient of 100 mM triethylammonium acetate, pH 7.0, containing from 0 to 16% acetonitrile over 15 min, followed by an increase to 70% acetonitrile over 5 min, which was then maintained for 10 min, with a subsequent return to the starting conditions (0% acetonitrile). For preparative HPLC purification of modified oligoribonucleotides, the following modifications to the analytical conditions were made: flow-rate 4 mL/min, 100 mM triethylammonium bicarbonate, pH 7.0, was used instead of 100 mM triethylammonium acetate, pH 7.0, and during the elution the conditions were left at 16% acetonitrile for 5 min. The 2'-amino-modified oligoribonucleotide **II** was 5'-³²P-labelled using T4 polynucleotide kinase and [γ-³²P] ATP.³⁸ Radioactivity in gels was quantified by a Fuji Bio-Imaging Analyzer BAS-2000.

Synthesis of compound 3. A solution of substance **2**³² (30 mg, 66 μmol) in DMSO (100 μL), Tris-HCl (50 mM aq, pH 8.0, 100 μL) and water (400 μL) was treated with DTT (3.9 mg, 25.3 μmol, 0.385 equiv). After stirring for 4.5 h at room temperature the solvent was removed in vacuo. The residue was dissolved in CH₂Cl₂ and the product purified by flash-column chromatography using CH₂Cl₂ with a 0–30% gradient of MeOH, giving **3** as colorless powder (12.1 mg, 40%). ¹H NMR (DMSO-*d*₆):

δ 2.64 (2H, t, $J=6.6$, SCH₂), 3.20 (2H, m, NHCH₂), 3.56 (2H, m, H5'), 3.90 (1H, m, H4'), 4.03 (1H, m, H3'), 4.29 (1H, m, H2'), 5.18 (1H, t, $J=5.0$, 5'-OH), 5.66 (1H, d, $J=8.1$, H5), 5.80 (1H, d, $J=8.6$, H1'), 5.85 (1H, d, $J=4.8$, 3'-OH), 6.12 (1H, d, $J=8.7$, 2'-NH), 6.54 (1H, t, $J=5.8$, CH₂NH), 7.87 (1H, d, $J=8.2$, H6), 11.18 (1H, s, H3). ¹³C NMR (DMSO-*d*₆): δ 38.0, 38.4, 55.0, 61.7, 70.7, 86.2, 86.6, 101.9, 140.9, 150.9, 157.3, 163.1. HRMS: 690.7104 (calcd 690.7109 for C₂₄H₃₄N₈O₁₂S₂).

Reaction of oligoribonucleotides with isocyanates. Reactions of (2-isocyanato) 2-pyridyl disulfide **1**³¹ with oligoribonucleotide 5'-ACUA(2'-NH₂U)AGU-3' (**I**) were performed essentially as described earlier.³² A solution of oligoribonucleotide **I** (2 mM in 233 μ L 70 mM borate buffer, pH 8.6) was treated with **1** in DMF (30 mM, 233 μ L) in an ice/salt bath at -8°C . The reaction was incubated for 4 h, extracted with dichloromethane (3 $\times$ 150 μ L) to remove the organic material, and oligoribonucleotide **II** was purified by preparative HPLC. The conversion of oligomer **I** (retention time 18.84 min) to oligomer **II** (retention time 14.11 min) occurred in 82% yield as monitored by analytical HPLC.

Cross-linking of 2'-amino-modified oligoribonucleotides. Cross-linking of the RNA-oligonucleotide **III** on an analytical scale were using the following procedures. A solution of oligomer **III** (3.5 μ M) was 5'-³²P-labelled with γ -³²P-ATP (0.5 mCi) and T4 polynucleotide kinase in a volume of 10 μ L. This reaction mixture was diluted with water (20 μ L) and treated with DTT (5 μ L, 250 mM in water) for 1 h to obtain oligoribonucleotide **V**. Excess DTT was removed by extracting the aqueous phase with ethyl acetate (3 $\times$ 100 μ L). After the solvent was removed in vacuo, oligoribonucleotide **V** (0.5 pmol) was mixed with a solution of oligoribonucleotide **III** (1.25 pmol) in water (8 μ L), boric buffer (12 μ L, pH 8.6), MgCl₂ (500 mM, 3 μ L) and NaCl (1 M, 2 μ L) for subsequent annealing and cross-linking. Annealing was performed by heating the solution to 56 $^\circ\text{C}$ for 5 min followed by slow cooling to 25 $^\circ\text{C}$ over a period of 2.5 h. The cross-linking reaction was performed by further incubation overnight at 25 $^\circ\text{C}$ and DPAGE analysis revealed 95% conversion to oligoribonucleotide **II**. The product was precipitated by addition of cold absolute ethanol (1 mL) followed by centrifugation (14,000 rpm, 4 $^\circ\text{C}$, 30 min). The product was dissolved in water (25 μ L) and stored at -80°C .

Large-scale preparations of cross-linked RNA-oligonucleotide **II** was performed with minor modifications of the procedure described by Sigurdsson et al.²⁸ A solution of oligoribonucleotide **III** (1 mM) in Tris-HCl (50 mM, pH 8.0, 8 μ L) and NaCl (1 M, 1 μ L) was heated to 56 $^\circ\text{C}$ for 5 min followed by slow cooling to 25 $^\circ\text{C}$ over a period of 2.5 h. DTT (50 mM aq, 1 μ L) was added to the solution containing oligoribonucleotide **IV** and incubated for 1 h at 37 $^\circ\text{C}$. The resulting solution was extracted with ethyl acetate (2 $\times$ 200 μ L) and precipitated at -20°C for 4 h after addition of ethanol (1 mL). After centrifugation (14,000 rpm, 4 $^\circ\text{C}$, 30 min) the RNA pellet was collected and dissolved in a Tris-HCl buffer (50 mM Tris-HCl, pH 8.0, 18 μ L) and NaCl (1 M NaCl,

2 μ L) and incubated for 30 h at 25 $^\circ\text{C}$ under an atmosphere of oxygen. The cross-linking reaction was monitored by analytical HPLC, precipitated by addition of cold ethanol after 90% conversion to the desired product and purification by preparative HPLC yielded oligoribonucleotide **II** (0.6 mM, 60%).

Enzymatic digestion of oligoribonucleotides III and II. Enzymatic digestion was performed as described by Connolly.³⁹ A solution of the oligoribonucleotide (0.3 mM) in Tris-HCl buffer (54 μ L, 50 mM, pH 8.0) containing MgCl₂ (10 mM) at 37 $^\circ\text{C}$ was treated sequentially with snake venom phosphodiesterase (6 μ L, 0.003 U/ μ L, incubation for 18 h) and alkaline phosphatase (6 μ L, 1 U/ μ L, further incubation at 37 $^\circ\text{C}$ for 30 min), followed by HPLC analysis.

Determination of duplex melting temperatures (T_m). Thermal denaturation curves were acquired on a Varian/Model Cary 1 UV/vis-spectrophotometer equipped with a thermoelectrically controlled cell holder and interfaced to a computer. Oligoribonucleotides (5–15 μ M in duplex concentration) in sodium HEPES buffer (10 mM, pH 7.5) containing 140 mM NaCl were heated from 10–85 $^\circ\text{C}$ at 0.5 $^\circ\text{C}/\text{min}$. The values for T_m were determined by calculating the first derivative of the melting curve.

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