Cleavage properties of an oligonucleotide containing a bridged internucleotide 5'-phosphorothioate RNA linkage

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ABSTRACT

An oligonucleotide has been synthesized that contains a single bridging 5'-phosphorothioate at an RNA linkage (5'-ApCpGpGpTpCpTprCpsApCpGpApGpC-3'). This new phosphodiester linkage is found to be particularly susceptible to cleavage when compared with the corresponding oxo, deoxy and thiooxy derivatives. Divalent metal cations were observed to dramatically increase the cleavage rate. The products of the cleavage under a variety of conditions are a 5'-thiol-containing fragment (6mer) and a 2',3'-cyclic phosphate-containing fragment (8mer). The pseudo-first order rate constant, k_{obs} , for cleavage at pH 7.5 (50 mM Tris-HCl) in the presence of 5 mM EDTA is $1.5 \times 10^{-4}/\text{min}$. In the presence of 5 mM metal dichloride and 50 mM Tris-HCl, pH 7.5, the relative cleavage rate enhancements are 10, 24, 71, 98, 370 and 3400 for Mg^{2+} , Ca^{2+} , Mn^{2+} , Co^{2+} , Zn^{2+} and Cd^{2+} respectively. The rate enhancements correlate well with Pearson's HSAB principle, suggesting that cleavage is mediated in part by coordination of the metal to the 5'-mercapto leaving group. RNA linkages containing bridging 5'-phosphorothioates should prove valuable for studying the mechanistic details of a variety of RNA cleaving agents, such as ribozymes.

INTRODUCTION

Phosphorothioate esters, phosphate analogs in which an oxygen atom has been replaced by sulfur, have proven to be valuable tools for studying a wide variety of biochemical processes (1). Replacement of one of the non-bridging oxygens of the pro-chiral phosphodiester with sulfur generates two diastereomers about phosphorus. The presence of two diastereomers (*R_p* and *S_p*) about the phosphorothioate and the differences in the properties of the 'hard' oxygen ligand versus the 'soft' sulfur ligand has permitted the identification of metal coordination sites in a number of nucleotide processing enzymes (2). Phosphorothioate diesters have been incorporated into pre-selected sites in DNA, replacing one (3,4) or both (5,6) of the non-bridging oxygen atoms with sulfur. In addition to their value in studying biochemical processes, the non-bridging phosphorothioate diester (or dithioate) is a nucleophilic center that can be exploited

for the introduction of reporter groups into specific sites on the backbone of the DNA sequence (7,8). Alkylation of phosphorothioates by iodoethanol provides a route to site-specific strand cleavage and permits sequencing of DNA (9). More recently the synthesis of derivatives that contain sulfur in the bridging positions (3' or 5') of the phosphodiester linkage in DNA have been reported (10–14). Studies with both types of linkages have contributed to the understanding of the cleavage mechanisms of nucleases (15,16) and the *Tetrahymena* ribozyme (17–21).

The synthesis and properties of RNA oligomers containing non-bridging phosphorothioates has also been reported. Phosphorothioate interference assays have identified possible metal coordination sites in the hammerhead ribozyme (22) and introducing each of the diastereomeric phosphorothioate diesters into the cleavage site for the hammerhead ribozyme has permitted identification of an in-line $\text{S}_{\text{N}}2$ -type cleavage mechanism (17,19). Alkylation of non-bridging RNA phosphorothioate diesters produces extremely labile triesters which rapidly result in chain cleavage and permit the sequencing of RNA (9). The related oligomeric derivatives containing the bridging 3'- or 5'-phosphorothioates have not yet been described. RNA phosphodiester linkages are generally much more labile toward hydrolysis than DNA and there is additionally the potential for transesterification reactions involving the 2'-hydroxyl (23). Bridging phosphorothioate RNA linkages could suffer from a marked propensity to undergo cleavage at the modified site due to both the presence of the adjacent nucleophile (the 2'-OH) and the enhanced leaving group ability of the mercapto versus oxo phosphorus substituent. We wish to report the first synthesis of an oligonucleotide (a 14mer) containing a *bridged* phosphorothioate at an RNA linkage. The susceptibility of this linkage to cleavage has been examined under varying pH conditions and in the presence of a series of divalent metals. After the completion of this work a report appeared describing the solution-phase synthesis of a uridine dinucleotide monophosphate (UpU) containing a similar linkage (24).

MATERIALS AND METHODS

General

Flash chromatography refers to column chromatography with Merk silica gel 60F (230–400 mesh). Reagents were from Sigma (St Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI) and

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were used as supplied. HPLC grade acetonitrile and methanol were from Fisher Scientific (Fair Lawn, NJ). Nucleoside phosphoramidites and solid supports were from CPG Inc. (Lincoln Park, NJ). The 1-(2-chloroethoxy)ethyl (Cee)-protected cytidine phosphoramidite was a gift of Glen Research (Sterling, VA). Enzymes were from Boehringer (Mannheim, Germany) or New England Biolabs (Beverly, MA). [γ - 32 P]ATP was from NEN/DuPont (Wilmington, DE). ^1H and ^{31}P NMR spectra were obtained on a Varian XL-300 at 300 or 121 MHz respectively. Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. Fast protein liquid chromatography (FPLC) was performed with a Pharmacia FPLC system using a Mono-Q HR 10/10 strong anion exchanger column. High performance liquid chromatography (HPLC) was performed with a Beckman HPLC system using C-18 reversed-phase columns (ODS-Hypersil, 5 μm particle size, 120 $\text{\AA}$ pore, 4.6×250 mm). Chromatograms were recorded and quantitated with a Shimadzu C-R3A Chromatopac integrator. Electrophoresis gels were analyzed with a Molecular Dynamics PhosphorImager 425 and Image Quant v3.3 software.

5'-O-(4-Toluenesulfonyl)-N6,3'-O-dibenzoyl-2'-deoxyadenosine [2]

5'-O-(4,4'-Dimethoxytrityl)-N6-benzoyl-2'-deoxyadenosine (1) (3.0 g, 4.56 mmol) was treated with benzoylchloride (2.56 g, 18.24 mmol) in anhydrous pyridine at 0°C overnight. The reaction was terminated by adding ~ 1 ml H_2O followed by removal of the pyridine under reduced pressure. The residue was dissolved in CH_2Cl_2 , washed with dilute NaHCO_3 , dried over Na_2SO_4 and concentrated to a foam. Traces of pyridine were removed by repeated co-evaporations with toluene. CH_2Cl_2 (100 ml) was added, the solution was cooled to 0°C and dichloroacetic acid/ CH_2Cl_2 (1:1, 10 ml) was added dropwise. After 4 h the reaction mixture was extracted with concentrated NaHCO_3 and concentrated to a foam. The foam was dissolved in MeOH (100 ml), cooled to 0°C and concentrated NH_4OH (8 ml) was added. After 3 h the reaction was concentrated under reduced pressure and purified by silica gel flash chromatography (1–2.5% MeOH in CHCl_3) to give a white foam. This 5'-hydroxy nucleoside was co-evaporated with anhydrous pyridine, dissolved in pyridine (75 ml), cooled to 0°C and *p*-toluenesulfonylchloride (869 mg, 9.12 mmol) was added. After 18 h the reaction was quenched by adding ~ 1 ml H_2O followed by removal of the pyridine under reduced pressure. The residue was dissolved in CH_2Cl_2 , washed with dilute NaHCO_3 , dried over Na_2SO_4 , concentrated to a foam and purified by silica gel flash chromatography (CHCl_3) to give a white foam (2.48 g, 89% yield from 1). ^1H NMR (CDCl_3): δ 8.93 (s, 1H, NH), 8.72 (s, 1H, H₈), 8.30 (s, 1H, H₂), 8.1–7.2 (m, 14H, Ar), 6.60 (dd, 1H, H_{1'}), 5.62 (dd, 1H, H_{3'}), 4.45 (m, 1H, H_{4'}), 4.39 (ddd, 2H, H_{5'}), 3.00 (m, 1H, H_{2'}), 2.78 (ddd, 1H, H_{2''}), 2.38 (s, 3H, Me).

5'-(S-Triphenylmethyl)-mercapto-N6,3'-O-dibenzoyl-2',5'-dideoxyadenosine [3]

NaH (60% dispersion in mineral oil, 200 mg, 5.0 mmol) was added to anhydrous DMF (5 ml), the mixture was cooled to 0°C and triphenylmethylmercaptan (1.38 g, 5.0 mmol) in DMF (15 ml) was added. The reaction mixture was removed from the ice bath and stirred for 15 min at ambient temperature before

cooling again to 0°C . The 5'-tosylnucleoside 2 (2.48 g, 4.04 mmol) was co-evaporated with anhydrous DMF, dissolved in DMF (10 ml) and added dropwise to the stirred sodium triphenylmethylmercaptide solution. After 12 h the DMF was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 , washed with dilute NaHCO_3 , dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (0–2% MeOH in CHCl_3) to give a white foam (2.76 g, 93% yield). ^1H NMR (CDCl_3): δ 9.08 (s, 1H, NH), 8.72 (s, 1H, H₈), 8.23 (s, 1H, H₂), 8.1–7.2 (m, 25H, Ar), 6.44 (dd, 1H, H_{1'}), 5.40 (m, 1H, H_{3'}), 4.15 (ddd, 1H, H_{4'}), 3.71 (ddd, 2H, H_{5'}), 3.00 (m, 1H, H_{2'}), 2.70 (m, 1H, H_{2''}).

5'-(S-Triphenylmethyl)-mercapto-N6-benzoyl-2',5'-dideoxyadenosine [4]

The protected nucleoside 3 (2.76 g, 3.76 mmol) was dissolved in a small amount of CH_2Cl_2 and MeOH (90 ml) was added, followed by concentrated NH_4OH (25 ml). After 24 h at ambient temperature the solvents were removed under reduced pressure and the residue was dissolved in CH_2Cl_2 , washed with H_2O and dried over Na_2SO_4 . The resulting foam was dried in a vacuum oven overnight (50°C at 2 Torr) and co-evaporated from anhydrous pyridine. Pyridine (50 ml) was added, the solution was cooled to 0°C and chlorotrimethylsilane (3.0 ml, 23.64 mmol) was added dropwise. After 30 min benzoylchloride (3.0 ml, 25.84 mmol) was added dropwise and the reaction was allowed to warm to ambient temperature. After 2 h the reaction was cooled to 0°C and H_2O (9 ml) was added dropwise. After 15 min concentrated NH_4OH (9 ml) was added and the reaction was stirred for an additional 30 min and then concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 , washed with NaHCO_3 and dried over Na_2SO_4 . An additional brief ammonia treatment was needed to completely remove the desired benzoyl group. The material was purified by flash chromatography (2.5–5% MeOH in CHCl_3) to give a white foam (1.69 g, 71% yield). ^1H NMR (CDCl_3): δ 8.98 (s, 1H, NH), 8.73 (s, 1H, H₈), 8.08 (s, 1H, H₂), 8.00 (d, 2H, Ar), 7.6–7.2 (m, 18H, Ar), 6.35 (dd, 1H, H_{1'}), 4.33 (m, 1H, H_{3'}), 3.74 (dt, 1H, H_{4'}), 2.85–2.54 (m, 3H, H_{5'}+H_{2'}), 2.42 (ddd, 1H, H_{2''}), 1.92 (d, 1H, H_{3'-OH}).

5'-(S-Triphenylmethyl)-mercapto-N6-benzoyl-2',5'-dideoxyadenosine 3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) [5]

The 3'-hydroxy nucleoside 4 (1.0 g, 1.59 mmol) was dissolved in anhydrous CH_2Cl_2 (15 ml) and diisopropylammonium tetrazolide (272 mg, 1.59 mmol) was added followed by β -cyanoethylbis(diisopropylamino)phosphoramidite (600 mg, 1.99 mmol). After 4 h the reaction mixture was diluted with CH_2Cl_2 , extracted with dilute NaHCO_3 , dried over Na_2SO_4 and concentrated to an oil. The residue was purified by flash chromatography (5% MeOH in CHCl_3 containing 0.5% triethylamine), concentrated, dissolved in a small amount of EtOAc and precipitated into cold hexanes (300 ml) to give a white powder after filtration (1.04 g, 79% yield). ^{31}P NMR (CDCl_3): δ 150.2, 150.3.

Oligonucleotide synthesis and purification

The oligomers were synthesized at 1 μmol scale using conventional β -cyanoethylphosphoramidite methodology and long chain alkylamine controlled-pore glass supports. The 5'-terminal

dimethoxytrityl group was removed on the instrument (trityl 'off'). Detritylations were performed with 2% dichloroacetic acid in anhydrous CH_2Cl_2 . All oxidations were performed with the standard aqueous iodine/pyridine/THF mixture. The coupling time for the modified phosphoramidites was increased from 30 s to 9 min. After coupling, oxidizing and capping the 5'-thionucleoside, the synthesis cartridge containing the support-bound oligonucleotide was removed from the instrument, washed with H_2O and treated with AgNO_3 (50 mM, 3 ml) for 30 min in the dark to remove the triphenylmethyl group protecting the thiol. The cartridge was then thoroughly rinsed with H_2O , treated with dithiothreitol (50 mM, 3 ml) for 15 min, rinsed again with H_2O followed by acetonitrile and finally dried in a vacuum desiccator for ~1 h. The cartridge, now containing a 5'-terminal thiol, was re-attached to the synthesis instrument and the next phosphoramidite monomer was added, increasing the coupling time to 9 min. Subsequent phosphoramidite monomers were added in the conventional manner. After complete assembly of the oligonucleotide the solid support was treated with concentrated NH_4OH (5 ml) at 50°C for 12 h to release the oligomer from the support and remove the protecting groups. Sterile conditions were used from this point on when handling the oligomers. Ammonia was removed under reduced pressure and the oligomer was desalted with a Sephadex G-10 column (2×40 cm), eluted with H_2O and lyophilized.

The crude oligonucleotides were initially isolated and purified by anion exchange chromatography using a FPLC system (10 mM KH_2PO_4 , pH 6.35, 30% acetonitrile with a KCl gradient). The optimum salt gradient for separation was 10–300 mM KCl over 13 min, followed by 300–400 mM KCl over 22 min at a flow rate of 1.5 ml/min. The full-length 14mer (5'-ACGGTCTrCsACGAGC-3') eluted at 360 mM KCl (25 min), the 12mer disulfide (5'-CGAGCAs-sACGAGC-3') eluted at 320 mM KCl (17 min) and the 8mer fragment (5'-ACGGTCTrC-3') eluted at 270 mM KCl (12 min). The appropriate fractions were collected, reduced to a low volume under reduced pressure, desalted on a Sephadex G-10 column as described above and lyophilized. The phosphorothioate oligomers were additionally purified by denaturing gel electrophoresis using 20% acrylamide, 7 M urea and $1 \times$ TBE, pH 8.4, running buffer. Oligonucleotides were recovered by stirring the excised, crushed gel pieces in 300 mM NaOAc (pH 5.5) at 4°C for 12 h. Buffers were removed by size exclusion chromatography with a Sephadex G-10 column. Some degradation of oligomer **9** (~25%) occurred during electrophoresis at pH 8.4, so an additional FPLC purification was performed.

Oligonucleotide characterization

Approximately 0.1 A_{260} units of each oligomer were incubated overnight at 37°C with snake venom phosphodiesterase (2 U) and alkaline phosphatase (2 U) in a total volume of 50 μl containing 100 mM Tris-HCl, pH 8.0, and 20 mM MgCl_2 . A portion of this mixture was analyzed directly by reversed-phase HPLC using a 60 min linear gradient of 0–70% methanol in 20 mM KH_2PO_4 , pH 5.5. Detection was at 260 nm. The following correction factors were applied to the integrated areas of the nucleoside peaks to normalize the extinction coefficients: rC, dC, 7.8; dG, 12.5; dT, 8.4; dA, dsA, 13.1; dAssA, 26. Retention times (min) were as follows: rC, 4.0; dC, 5.7; dG, 10.7; dT, 11.7; dA, 15.3; dsA, 22.0; dAssA, 34.4. Specific RNase T_2 cleavage was

performed by treating the radiolabeled oligomer with 1 U RNase T_2 at pH 4.5 (50 mM KH_2PO_4) for several hours.

Trimer deprotection

The trimer 5'-dTrCdA-3' was synthesized, treated with NH_4OH and desalted as described above and then purified by reversed-phase HPLC with a 60 min linear gradient of 0–35% acetonitrile in 50 mM triethylammonium acetate, pH 7.0. Base composition analysis revealed peaks at 11.7 and 15.1 min, corresponding to dT and dA, and two additional peaks at 17.4 and 17.8, corresponding to the diastereomeric Cee-protected rC nucleosides. No peak was detected near 4.0 min, the position of the deprotected rC nucleoside. The crude trimer (prior to HPLC purification) also contained no detectable deprotected rC. The trimer was treated with 0.01 N HCl, pH 2.0, to remove the Cee protecting group and the reaction progress was monitored by reversed-phase HPLC with the same acetonitrile gradient described above. The protected trimer (a pair of diastereomers) eluted as a single peak at 15.4 min, whereas the deprotected product eluted at 12.6 min. The Cee-protected trimer was also exposed to the FPLC buffer, pH 6.35, for up to 3 days at 37°C , but no deprotection could be detected.

Oligonucleotide labeling

The oligonucleotides (~200 pmol) were each end-labeled with [γ - ^{32}P]ATP (15 μCi , 25 pmol) and T4 polynucleotide kinase (30 U). Conventional conditions were used for the non-phosphorothioate oligomers (i.e. 60 min at 37°C , pH 7.6, 10 mM MgCl_2 , 10 mM 2-mercaptoethanol). The phosphorothioate oligomers were end-labeled at 27°C in the presence of 50 mM PIPES, pH 6.6, 1 mM MgCl_2 , 1 mM 2-mercaptoethanol; after 15 min the reactions were quenched with a $10 \times$ volume of 50 mM PIPES, pH 6.6, and 10 mM EDTA. All the labeling reaction mixtures were applied to Sep-Pak C-18 columns, washed with 4% MeOH in H_2O (20 ml), eluted with $\text{CH}_3\text{CN}/\text{MeOH}/\text{H}_2\text{O}$ (35:35:30, 4 ml) and lyophilized to dryness.

Cleavage studies

The pH stability experiments were carried out by treating a small amount of the radiolabeled oligomer with 5 mM EDTA and 50 mM Tris-HCl at pH 7.5, 8.0, 8.5 or 9.0 at ambient temperature and under sterile conditions. Aliquots of 5 μl were removed at appropriate intervals and quenched with 10 μl gel loading buffer containing the following: 20% glycerol, 0.01% bromophenol blue, 0.01% xylene cyanol, 7 M urea, 80 mM Tris, 40 mM NaOAc, 100 mM EDTA, pH 6.8. Denaturing PAGE (20% acrylamide, 2% cross-linking, 7 M urea) was performed at pH 6.8 with a running buffer of 40 mM Tris, 20 mM NaOAc, 1 mM EDTA; the buffer was re-circulated between the upper and lower chambers to maintain a constant pH. The gels were pre-electrophoresed for 2 h prior to the run, which was conducted at 10 mA for 8 h. The metal-assisted cleavage experiments were similarly carried out by treating a small amount of the radiolabeled oligomer with 50 mM Tris-HCl, pH 7.5, and 5 mM metal dichloride. Aliquots were removed, quenched and analyzed as described above; control experiments verified that no oligomer degradation occurred during the brief storage in the gel loading buffer. Gels were run as described above and exposed to a Kodak S0230 phosphor storage screen (Molecular Dynamics) for 12–24 h.

Scheme 1. Reaction conditions: (i) benzoylchloride, pyridine; (ii) 5% dichloroacetic acid dichloromethane, 0°C; (iii) 8% ammonium hydroxide, methanol, 0°C; (iv) *p*-toluenesulfonylchloride, pyridine; (v) triphenylmethylmercaptan, sodium hydride, dimethylformamide; (vi) 8% ammonium hydroxide, methanol, 0°C; (vii) β-cyanoethyl-bis(diisopropylamino)phosphoramidite, diisopropylammonium tetrazolide, dichloromethane.

Data was corrected to account for the small amount of cleavage at time $t = 0$ (generally <8%). Pseudo-first order rate constants were determined from 5–8 data points at <40–50% completion using the relationship $k_{\text{obs}} = \ln 2 (t_{1/2})^{-1}$.

Characterization of the cleavage products

Cleavage products were separated by anion exchange FPLC using the conditions described above. The fragments were then subjected to quantitative nucleoside composition analysis. The cytidine 2',3'-cyclic phosphate was identified by analysis of the digestion mixture on an anion exchange HPLC column (APS-Hypersil, 5 μm particle size, 120 Å pore, 4.6 × 250 mm) with a 60 min linear gradient of 5–250 mM KH₂PO₄, pH 6.3, 5% CH₃CN. Under these conditions the cytidine 2',3'-cyclic phosphate eluted at 7.0 min (the 2'- and 3'-monophosphate standards eluted at 11.8 and 12.7 min respectively).

The three electrophoresis standards (5'-fragment, ACGGTCTrC) were prepared as follows. The 2',3'-dihydroxy rC oligonucleotide was chemically synthesized on a solid support employing 2'-OTBDMS protection. Deprotection was with NH₄OH/EtOH followed by triethylamine trihydrofluoride. The 3'-phosphate rC oligonucleotide was prepared by digesting 5'-³²P-labeled 8 with ribonuclease T₂ in 50 mM KH₂PO₄, pH 4.5. The 2',3'-cyclic

Figure 1. Sequences 6–9 differing only in the nature of the phosphodiester linkage between residues C8 and A9.

phosphate rC oligonucleotide was prepared by digesting 5'-³²P-labeled 8 with an appropriate hammerhead ribozyme (25,26). The standards were purified by denaturing PAGE.

Radiolabeled 9 was treated under each of the conditions shown in Table 1 for an amount of time sufficient to cause >80% cleavage at the phosphorothioate linkage. Reactions were terminated by adding EDTA to a final concentration of 10 mM, reducing the volume and adding 20 μl formamide. Analytical electrophoresis (20% acrylamide, 7 M urea, 1× TBE) was performed on a 27 × 80 cm × 0.4 mm gel run at 9 mA constant current for 12 h. Gels were dried and analyzed by phosphorimaging, as described above.

RESULTS AND DISCUSSION

Oligonucleotide design, synthesis and characterization

Four oligonucleotides (see Fig. 1) were synthesized by conventional solid phase β-cyanoethylphosphoramidite methodology (27) and differed only in the nature of the linkage between residues C8 and A9. Sequences 6 and 7 are composed entirely of 2'-deoxynucleotides, while 8 and 9 each contain a single cytidine ribonucleotide linkage at position eight. The bridging 5'-phosphorothioate linkage was incorporated into 7 and 9 at position nine using the previously described building block 5'-(*S*-triphenylmethyl)mercapto-*N*6-benzoyl-2',5'-dideoxyadenosine 3'-*O*-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite) [5] (10,28). Our own synthetic route to phosphoramidite [5], shown in Scheme 1, retains the displacement of a 5'-arylsulfonyl group with sodium triphenylmethylmercaptide as the key transformation, but employs an alternative protecting group strategy. After addition of the thio analog to the oligomer the 5'-triphenylmethyl protecting group was removed by treatment with aqueous silver nitrate (29). Subsequent couplings were made in the usual way; all oxidations were performed with a conventional aqueous iodine solution and concentrated ammonium hydroxide was used for deprotection.

A crucial issue in the successful synthesis of sequence 9 was the choice of protecting group for the 2'-hydroxyl group adjacent to the bridging 5'-phosphorothioate. This protecting group must be stable under the conditions of sequence assembly, but must still be removable under conditions that will ensure survival of the bridging 5'-phosphorothioate diester. The commonly used *t*-butyldimethylsilyl ether (TBDMS) group (30) fails on this latter

Figure 2. Analysis of the cleavage reaction. (a) Autoradiogram showing cleavage of oligomer 9 as a function of time (h) at pH 8.5 and 9.0. (b) Autoradiogram showing cleavage of oligomer 9 as a function of time (min) in the presence of Mn^{2+} or Co^{2+} . (c) Graph showing the fraction of uncleaved 9 versus time in 50 mM Tris-HCl, pH 7.5, and 5 mM of the following: ●, EDTA; ○, Mg^{2+} ; ×, Ca^{2+} ; ■, Mn^{2+} ; ▲, Co^{2+} ; □, Zn^{2+} .

criterion; 9 degraded substantially upon treatment with triethylamine trihydrofluoride (31,32) or tetrabutylammonium fluoride. Deprotection of the recently developed 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) group (33,34) requires prolonged treatment with aqueous acid at pH 2.0, conditions under which deoxynucleotides, particularly purines, are susceptible to depurination (35). The tetrahydropyranyl and 4-methoxytetrahydropyran-4-yl (Thp and Mthp) groups (36), successfully used for solution-phase oligoribonucleotide synthesis and in the recent synthesis of a simple UpU dinucleotide containing a 5'-bridging phosphorothioate linkage (24), are not entirely suitable for solid-phase methods. However, the Cee group (37–39) appears to possess the necessary properties; the acetal remains intact during sequence assembly, but subsequently can be readily hydrolyzed in aqueous acid.

To evaluate the appropriateness of the Cee group for our application and to establish the mildest possible hydrolysis conditions we prepared a simple trinucleoside diphosphate containing a single rC linkage, dTprCpdA, with the 2'-hydroxyl of rC protected as the Cee derivative. The Cee group could be readily cleaved from the trimer at pH 2.0 (0.01 N HCl) with a half-life of ~100 min, in agreement with the previously reported values for uridine diribonucleoside monophosphates (37–39). With the 14mers used in the present work (8 and 9) we anticipated applying these same conditions to remove the single Cee group. However, after treatment of sequences 8 and 9 with aqueous ammonia followed by size exclusion chromatography and lyophilization the single Cee protecting group was unexpectedly lost, to yield the completely deprotected oligonucleotide. An acid deprotection step was unnecessary in these cases. The unusual lability of the single Cee group was not observed in the shorter sequence dTprCpdA described above. Whether or not intramolecular, general acid-catalyzed hydrolysis of the single Cee acetal occurs in the longer sequences, due to the proximity of additional phosphate moieties or other functionality, remains to be determined. The susceptibility of native, unprotected RNA linkages towards cleavage via base-promoted transesterification or hydrolysis is well known and this present study shows an even

more pronounced base lability of a bridging phosphorothioate (*vide infra*). Therefore, it is clear that the Cee group was not lost during the ammonium hydroxide treatment, but at a subsequent step in the isolation scheme.

The oligonucleotides 6–9 were purified to homogeneity under sterile conditions by anion exchange chromatography followed by denaturing PAGE. Complete Cee deprotection was verified by both quantitative nucleoside composition analysis and by complete and specific enzymatic hydrolysis at the ribonucleotide position by ribonuclease T₂. The nucleoside composition analysis of each oligomer gave the expected results and the bridging phosphorothioate linkage of 7 and 9 was readily hydrolyzed by snake venom phosphodiesterase. After nuclease digestion and dephosphorylation the 5'-thioadenosine nucleoside eluted as the disulfide on a C-18 reversed-phase column (retention time 34 min), but could be easily reduced to the free thiol monomer (retention time 22 min) by a brief treatment with tris(carboxyethyl)phosphine (40) or dithiothreitol. Each oligonucleotide was end-labeled with T4 polynucleotide kinase and [γ - ^{32}P]ATP. To minimize degradation oligonucleotide 9 was specially labeled at 27°C, pH 6.6, with one tenth the usual concentration of Mg^{2+} and 2-mercaptoethanol. Conventional denaturing PAGE employing 1× TBE, pH 8.4, could be used for each of the oligomers except 9. In this case the running buffer was 40 mM Tris, 20 mM NaOAc, 1 mM EDTA, pH 6.8. The buffer was re-circulated between the upper and lower chambers to maintain a constant pH. When analyzed in this way radiolabeled 9 showed <6% cleavage at the phosphorothioate linkage.

Cleavage studies

The effect of pH on the stability of the various linkages present in 6–9 was examined by incubating each of the radiolabeled oligomers in 50 mM Tris-HCl at pH values of 7.5, 8.0, 8.5 and 9.0 in the presence of 5 mM EDTA to sequester potential contaminating metals. Aliquots were removed from these solutions at appropriate time intervals and re-buffered to pH 6.8. Cleavage products were separated by denaturing PAGE and quantified by

phosphorimaging. The effects of various divalent metal cations on the cleavage rate were similarly investigated by treating the sequences with 5 mM metal dichloride in 50 mM Tris-HCl, pH 7.5. These latter reactions were terminated by re-buffering to pH 6.8 in the presence of 100 mM EDTA. Of the four sequences we examined only the bridged 5'-phosphorothioate **9** exhibited noticeable cleavage under the conditions employed and the time periods examined. The results for cleavage of sequence **9** are summarized in Table 1 and representative autoradiograms are shown in Figure 2a and b.

We anticipated that a bridged 5'-phosphorothioate at a ribonucleotide site would be more susceptible to cleavage by transesterification than the corresponding oxo analog, due to the significantly improved leaving group ability of the 5'-mercapto substituent (the pK_a of methanol is 15.5 versus 10.0 for methanethiol). The observed cleavage rate of 1.5×10^{-4} /min for sequence **9** at pH 7.5 is $\sim 1\,000\,000$ -fold greater than the estimated rates for cleavage of natural (5'-oxo) RNA linkages in diribonucleoside monophosphates (41,42) and supports this expectation. In contrast, sequence **7**, which contains the same bridging phosphorothioate linkage but lacks the internal nucleophilic 2'-hydroxyl, did not undergo any detectable cleavage after 24 h at pH 9.0 (cf. 58% for **9**). This observation indicates that the likely mode of cleavage of oligomer **9** is one involving nucleophilic displacement of the mercapto substituent by the adjacent 2'-hydroxyl of C8, as would be expected.

To experimentally establish the mode of cleavage we isolated and identified the products of the cleavage reaction. The two cleavage fragments were separated by anion exchange chromatography and were then subjected to nuclease digestion and nucleoside composition analysis via HPLC to directly identify the terminal residues at the site of cleavage between C8 and A9. The 3'-fragment (6mer) contained a 5'-terminal thiol (identified as the disulfide). The 5'-fragment (8mer) contained a 3'-terminal 2',3'-cyclic CMP moiety. Both of these cleavage products are consistent with a transesterification pathway. We also electrophoretically analyzed, by comparison with authentic standards, the 5'-cleavage fragment of radiolabeled **9** generated under a variety of conditions. To serve as standards three 8mers with the sequence 5'-ACGGTCTrC-3' were prepared that differed only in the identity of the 3'-terminal cytidine ribonucleotide: 2',3'-dihydroxy, 3'-phosphate, or 2',3'-cyclic phosphate. These species represent the possible cleavage products of the 5'-fragment and the mixture can be separated on an appropriate analytical electrophoresis gel. The results, shown in Figure 3, reveal that the 2',3'-cyclic phosphate is formed in each case examined except for zinc-promoted cleavage. The reaction mixture containing zinc (Fig. 3, lane 8) shows substantial 3'-phosphate. However, control experiments showed that this metal is capable of hydrolyzing the 2',3'-cyclic phosphate standard to the extent shown in Figure 3. Therefore, it is likely that in the case of zinc, as with the other metals, the product is still a 2',3'-cyclic phosphate fragment which is subsequently hydrolyzed to the 3'- (or 2'-) monophosphate. Even complexed zinc atoms are known to efficiently hydrolyze 2',3'-cyclic phosphates to the corresponding 2'- and 3'-monophosphates (43). These fragment analysis experiments provide direct evidence that transesterification is the mode of cleavage of the bridged 5'-phosphorothioate RNA linkage under a variety of conditions.

Figure 3. 5'-Cleavage fragment analysis. Autoradiogram showing PAGE analysis of the 8mer 5'-cleavage fragments of sequence **9** under a variety of conditions

Divalent metal cations were observed to dramatically increase the rate of cleavage of the bridged 5'-phosphorothioate RNA linkage. Relative rate enhancements for the cleavage of sequence **9** at pH 7.5 in the presence of 5 mM Mg^{2+} , Ca^{2+} , Mn^{2+} , Co^{2+} , Zn^{2+} and Cd^{2+} , were 10, 24, 71, 98, 370 and 3400 respectively (see Fig. 2c and Table 1). By comparison, sequences **6–8** in the presence of each metal cation from this series did not undergo any detectable cleavage during kinetic evaluation of the cleavage of sequence **9** (where the extent of cleavage varied from 30 to 60%). Exceptionally soft metals such as mercury and silver salts, known to cause rapid cleavage of bridging 3'- and 5'-phosphorothioates in DNA (10,44), were not kinetically characterized in this study, although we did confirm that these metals promote the cleavage of both **7** and **9**.

Table 1. Pseudo-first order rate constants, k_{obs} , for the cleavage of phosphorothioate **9** at several pHs and in the presence of various divalent metal cations

Metal ^a	pH	k_{obs} (per min)	k_{rel}	pK_a^b	radius ($\text{\AA}$) ^c
None ^d	7.5	1.5×10^{-4}	1.0		
None ^d	8.0	3.8×10^{-4}	2.5		
None ^d	8.5	4.7×10^{-4}	3.1		
None ^d	9.0	7.5×10^{-4}	4.8		
Mg^{2+}	7.5	1.5×10^{-3}	10	11.4	0.65
Ca^{2+}	7.5	3.7×10^{-3}	24	12.8	0.99
Mn^{2+}	7.5	1.1×10^{-2}	71	10.6	0.80
Co^{2+}	7.5	1.5×10^{-2}	98	9.9	0.74
Zn^{2+}	7.5	5.7×10^{-2}	370	9.0	0.74
Cd^{2+}	7.5	5.3×10^{-1}	3400	9.0	0.97

^aConditions: 5 mM metal or EDTA, 50 mM Tris-HCl, ambient temperature.

^bAcid dissociation constants for hydrated metal cations at 25°C, from Dean (45).

^cIonic radius, from Dean (45).

^d5 mM EDTA.

The relative rates of cleavage observed here do not completely correlate with the acid dissociation constants (pK_a values) (45) of

the hydrated metal cations tabulated in Table 1. This observation argues against the metal hydrate acting solely as a general base (i.e. $[M(OH)]^+$) to facilitate deprotonation of the attacking 2'-hydroxyl group, as is thought to occur in specific Pb^{2+} -promoted cleavage of tRNA (46–49) and with the hammerhead ribozyme (21). For example, the pK_a values of hydrated Zn^{2+} and Cd^{2+} are quite similar, but the Cd^{2+} -catalyzed cleavage of the bridging phosphorothioate **9** is an order of magnitude faster than that of Zn^{2+} . Table 1 also reveals that there is no direct relationship between the rate of cleavage and ionic radius (45) of the metal cation. The ionic radius could be critical if the nucleic acid is obligated to provide specific coordinating ligands to precisely position the metal for catalysis, but this does not seem to be the case. However, if Pearson's HSAB principle (50–52) is invoked there is a direct correlation between the rate of cleavage and the 'softness' of the divalent metal. This relationship is consistent with an interaction of the metal with the 'soft' mercapto leaving group or the PO_2 moiety (via symbiosis) increasing along the series Mg^{2+} , Ca^{2+} , Mn^{2+} , Co^{2+} , Zn^{2+} and Cd^{2+} . Such interactions could enhance the cleavage rate in a variety of ways: activation of the phosphorus center toward nucleophilic attack, stabilization of the transition state or improvement in the leaving group ability of the mercapto substituent (53).

CONCLUSIONS

Oligonucleotides containing a bridged internucleotide 5'-phosphorothioate at an RNA linkage are particularly susceptible to cleavage via transesterification. Moreover, the cleavage rate is dramatically accelerated by metal ions. Metal cations play an important role in enzymatic cleavage of nucleic acids (54,55). Catalytic RNAs (ribozymes) promote specific cleavage of RNA via transesterification in the presence of a divalent metal(s) (56,57). Ribonuclease-catalyzed cleavage of RNA also employs a transesterification reaction, but generally has no metal requirement. Several metals, most notably Cu^{2+} , Zn^{2+} , Pb^{2+} and assorted lanthanides, are capable of facilitating non-specific cleavage of RNA linkages in the absence of protein or higher order nucleic acid structure (41,58,59). Metal cations have additionally been shown to catalyze hydrolysis of small phosphoester model compounds by different mechanisms (60–66). RNA linkages containing bridging 5'-phosphorothioates should prove valuable for studying a variety of RNA cleaving agents by exploiting the 'thio effect' (67,68).

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